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DL-METHIONINE: AN IMMUNOPOTENTIATOR IN *MYCOPLASMA GALLISEPTICUM* CHALLENGED BROILERS TREATED WITH PULMOTIL *AC*®

by

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AN ABSTRACT OF THE THESIS OF

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The thesis experiment was performed to determine the effect of 20% dietary DL-Methionine (Met) in excess of modern breeder requirement level compared to Tilmicosin (Pulmotil *AC*®); on the immune response and growth performance of male Ross 308 broilers challenged with *Mycoplasma gallisepticum* (MG). The experiment is divided into 3 part studies. Study A constituted a preliminary trial for evaluating three separate MG field isolates on inducing clinical signs and symptoms of challenged Ross 308 broilers. Study B aimed at molecularly identifying the strain of each evaluated field isolate adopted in Study A via MG *pvpA* gene based conventional PCR amplification and sequencing. Study C comprised a 2x2x2 factorial arrangement of treatments with interactions in a complete randomized design. The main factors were *Mycoplasma gallisepticum* a) challenged vs. b) unchallenged; Methionine: a) adequate vs. b) excess, and Antibiotic a) Pulmotil *AC*® treated vs. b) Non-treated. A total of 1200-day old male Ross 308 broilers were randomly divided into 8 treatments with 3 replicates (pen) of 50 birds each. Starter, grower and finisher diets were formulated to meet 100 and 120% of Met requirements in the adequate and excess Met groups, respectively. Feed and water were offered *ad libitium* for a period of 35 days. Results of Study A and B revealed that all MG isolates were of the F-strain and MG2 isolate resulted in higher frequency of disease signs in comparison to the other groups; this strain was retained in challenging birds in Study C. Results of Study C demonstrated that 20% excess Methionine significantly increased average body weight (BW) at 10 days of age; with no significant differences in BW and feed conversion ratio (FCR) at 17 and 35 days of age. Pulmotil *AC*® significantly reduced BW at 10 days of age; however, it did not affect BW and FCR at 17 and 35 days of age. In MG-challenged birds, the 20% excess Met treatment significantly increased IgG titers (3170) in comparison to adequate Met level (1843) along with coefficient of variation (CV) of 14.84 and 66.38%, respectively. Excess Met significantly increased bursal indices in MG-challenged broilers at 35 days of age. In addition, treatment of 20% excess Met augmented hematological parameters with a significant increase of hematocrit (HCT%) compared to Pulmotil that significantly decreased HCT%. Further, excess 20% Met significantly decreased the percentage of birds with severe tracheatis caused by MG-infection from 40 to 10% at 17 to 35 days of age. Pulmotil *AC*® was efficient in clearing tracheal MG colonization of challenged birds at 35 days of age and the 20% excess Methionine treatment was proven successful as immune enhancer against MG-infection in broilers.

Keywords: Immunology, DL-Methionine, *Mycoplasma gallispeticum*, Pathology, Pulmotil *AC*®, Broiler.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percent
6/85 strain	Mycoplasma gallisepticum 6/85 strain
A2m(1)	Allotype of Immunoglobulin A subclass 2
AA	Amino acid
AAFCO	Association of American Feed Control Officials
Ab	Antibody
Ab/Ag	Antibody/Antigen complex
ADCC	Antibody-dependent cell-mediated cytotoxicity
AF	Abdominal fat
AFP	Abdominal fat pad
AGP	Alpha (□)-1 acid glycoprotein
AIV	Avian Influenza virus
AME	Apparent metabolizable energy
AP- PCR	Arbitrary primed polymerase chain reaction
ApoA-I	Apolipoprotein A1
Arg	Arginine
B-cell	B-lymphocyte (Bursa of Fabricius)
B. bronchiseptica	*Bordetella bronchiseptica*
BALT	Bronchial-associated lymphoid tissue
BCR	B-cell receptor
BHMT	Betaine-homocysteine methyltransferase
BSDCs	Bursal secretory dendritic-like cells
BWG	Body weight gain
C	Constant region
C. glutamicum	Corynebacterium glutamicum
CAC	Citric Acid Cycle
CALT	Conjunctival-associated lymphoid tissue
CAM	Chorioallantoic membrane
CAS	Chemical Abstracts Service
CBH	Cutaneous basophilic hypersensitivity
CD3	Custer of differentiation 3
CD4	Custer of differentiation 4
CD8	Custer of differentiation 8
CEAA	Conditionally essential amino acid
CFR	Code of Federal Regulations
ChIFN-γ	*Chicken interferon gamma*
CIR	Cell immune response
CMI	Cell mediated immunity
ConA	Concanamycin A
CP	Crude protein

CRD	Chronic respiratory disease
CrmA	Cytadherence-related molecule A
CT	Caecal tonsils
Ct	Cycle threshold
Cys	Cysteine
D	Diversity
D-form	D enantiomer
DF-1	Chicken fibroblast cell line
DH	Diversity Heavy chain
DL-	Racemate
DLM	D, L-Methionine
DM	Dry matter
E	Efficiency
e	Embryogenesis
E. coli	Escherichia coli
E.C. 2.1.1	Methyltransferase
E.C.2.1.1.5	Betaine-homocysteine S-methyltransferase
EAA	Essential amino acid
EC	Enzyme commission number
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked-immunosorbent Assay
EM	Electron microscopy
EP	Egg production
F-strain	Mycoplasma gallisepticum F-strain
FA	Fatty acids
FAA	Functional amino acid
FAE	Follicle-associated epithelium
FC	Feed conversion
FcR	Fc receptors
FCR	Feed conversion ratio
FDA	Food and Drug Administration
FI	Feed intake
g	Gram (s)
GALT	Gut associated lymphoid tissues
GapA	Cytadhesin gene
Gly	Lycine
GMO	Genetically modified organisms
GSH-Px	Glutathione peroxidase
H-chain	Heavy chain
H-L	Heavy-light chain heterodimer
HALT	Head-associated lymphoid tissue
HCT	Hematocrit
HCys	Homocysteine
HEV	High endothelial venules

HGB	Hemoglobin
HI	heat increment
HI test	Hemagglutination inhibition test
His	Histidine
Hlp3	HMW3-like protein
IB	Infectious bronchitis
IBDV	Infectious bursal disease virus
IFE	Interfollicular epithelium
IFE	interfollicular epithelium
IFNγ	interferon gamma
IgA	Immunoglobulin A
IgA1	Immunoglobulin A subclass 1
IgA2	Immunoglobulin A subclass 2
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
IgY1	Immunoglobulin Y subclass 1
IgY2	Immunoglobulin Y subclass 2
IgY3	Immunoglobulin Y subclass 3
IL	Interleukin
IP	Ideal protein
Iso	Isoleucine
J	Joining
J-chain	Joining chain
JH	Joining Heavy chain
JL	Joining Light chain
Jκ	Joining Kappa light chain)
Jλ	Joining Lambda light chain
κ	kappa
kg	Kilogram(s)
l	Liter
L-chain	Light chain
L-form	L enantiomer
LAMP	lipid-associated membrane proteins
Leu	Leucine
LMI	Leucocyte migration inhibition
Log	Logarithm
Lpd	Dihydrolipoamide dehydrogenase
LPS	Lipopolysaccharide
Lys	Lysine
m	Meter (s)
MAT I	Methionine adenosyltransferase I
MAT II	Methionine adenosyltransferase II

MCH	Mean corpuscular hemoglobin
MDA	Malondialdehyde
ME	Metabolic energy
ME:CP	Metabolic energy to crude protein ratio
Met	Methionine
mg	Milligram
MG	Mycoplasma gallisepticum
MGA_0676	putative lipoprotein
MHA-FA	D,L-methionine hydroxy analog-free acid
MHAC	DL-methionine hydroxyl analog calcium
MHC	Major histocompatibility complex
min	Minute (s)
ml	milliliter
mm	Millimeter (s)
MslA	Polynucleotide binding protein
N	Nitrogen
NAD	Nicotinamide adenine dinucleotide
NDV	Newcastle disease virus
NEAA	Nonessential amino acid
Neu5Ac	N-acetylneuraminic acid
NK	Natural killer cells
NPIP	National Poultry Improvement Plan
NRC	National Research Council
ºC	Degrees Celsius
OsmC	Osmotically induced gene
Ovo	Transovarian
P	Probability value
P. aeruginosa	*Pseudomonas aeruginosa*
p67	67 kDa glycoptrotein
PALS	Peri-arteriolar lymphatic sheath
PALS	Periarteriolar lymphoid sheaths
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PCV	Packed cell volume
PELS	Periellipsoid lymphoid sheaths
Pen G	Penicillin G
pH	Potential hydrogen
PHA	Phytohaemagglutinin
Phe	Phenylalanine
PlpA	Pneumoniae-like protein
pMGA	Hemagglutinin protein A
PP	Peyer's patches
PPLO	Pleuropneumonia-Like Organisms
Pro	Proline
pVPA	Phase variable putative adhesion protein A

PWP	Peri-ellipsoidal white pulp
q-PCR	Real-time polymerase chain reaction
R-strain	Mycoplasma gallisepticum R-strain
R^2	Coefficient of determination
RAPD	Random amplification of polymorphic DNA
RBC	Red Blood Cell
RFLP	restriction fragment length polymorphism analysis
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RSSs	recombination signal sequences
S protein	Secretory protein
S. enetrica	*Salmonella enterica*
SAA	Sulfur amino acid
SAH	S-adenosylhomocysteine
SAMe	S-adenosylmethionine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	Second (s)
SEM	Standard error of mean
SNc	Staphylococcal nuclease
SOD	Superoxide dismutase
SPA	Serum Plate Agglutination
SQ	Starting quantity
SRBC	Sheep red blood cell
SucCoA	Succinyl-CoA
T-cell	T-lymphocyte (Thymus)
Tc	Cytotoxic/Cytolytic T-cell
TCA	Tricarboxylic acid
TCR	T-cell receptor
TCR- αβ1+	T-cell receptor-alpha beta 1
TCR- αβ2+	T-cell receptor-alpha beta 2
TCR-αβ+CD4+	T-helper lymphocytes
TCR-αβ+CD8+	T-cytotoxic lymphocytes
TCR-γδ+	T-cell receptor gamma delta
TCR2	T-cell receptor 2
TCR3	T-cell receptor 3
T_D	Delayed hypersensitivity T-cells
TD	Thymic/T-cell dependent
TEC	tracheal epithelial cells
Th	Helper T-cell
Th1	Helper T-cell 1/I
Th2	Helper T-cell 2/II
Thr	Threonine
TI	Thymic/ T-cell independent
TI-I Ags	T-cell independent antigen-I
TI-II Ags	T-cell independent antigen-II

T_R	Regulatory T-cell
TRBC	Total red blood cells
tRNA	Transfer ribonucleic acid
Try	Tryptophan
Ts	Suppressor T-cells
ts-11	Mycoplasma gallisepticum temperature sensitive-11 strain
TSAA	Total sulfur amino acids
ug	microgram
UGA	Stop codon
ul	microliter (s)
UNII	Unlicensed National Information Infrastructure
V	Variable region
Val	Valine
VH	Variable Heavy chain
VL	Variable Light chain
VlhA	Variable lipoprotein hemagglutinin A
Vβ1	T-cell receptor 2 Vβ gene (β chain variable)
Vβ2	T-cell receptor 3 Vβ gene (β chain variable)
Vκ	Variable Kappa light chain
Vλ	Variable Lambda light chain
WA SEM	Weighted average of standard error of mean
WBRT	Whole blood recalcification time
WT	Weight
α chain	Alpha chain
γ-chain	Gamma chain
μ-chain	Mu heavy chain
λ	lambda
μ	mu

CHAPTER 1

INTRODUCTION

With poultry meat and egg production showing a significant increase worldwide since 1970s (Scanes, 2007[a]) poultry industry is the fastest producing animal sector today (Scanes, 2007[b]), providing important source of animal protein (FAO 1999), a tool for poverty alleviation (Permin et al., 2001) and food security. Along with this rapid growth of global poultry production, advancements in fields of nutrition, breeding, disease control and management (Bell and Weaver, 1990; Leeson and Summers; 2001; Leeson and Summers, 2009; Ewing, 1951; Morgan and Lewis, 1962) have been integrated under a system that suggest a cooperative effort for a successful poultry production taking into consideration the health status of the bird (National Chicken Council, 2012). Good understanding and sound application of principles of health management is essential for excellent performance of poultry and productivity (Sugiharto, 2014).

Factors potentially affecting the immune system of avian species are known as immune stresses (McNab, et al., 2002). These include nutritional deficiencies, vaccination, medications (Perry, 2006), transportation, contamination (Taylor-Pickard and Spring, 2008; Lesson and Summers, 2001), ammonia gas (Taylor et al. 2009), temperature, humidity, noise, feed density, pathogens and diseases (McNab, et al., 2002)

Mycoplasmas are pathogenic species known to be the smallest self-replicating prokaryotes enclosed solely by plasma membrane and thus are cell wall vacant (Kleven, 1997). They have the capability of causing disease in humans, various animals, insects and plants (USGS, 1999). *Mycoplasma gallisepticum* (**MG**) is the primary etiologic agent of chronic respiratory disease (**CRD**) in chickens and infectious sinusitis in turkeys. *Mycoplasma gallisepticum* infects wide range of avian species including pigeons, passerine birds, finches and game birds (Hennigan et al. 2012, Ley, 2008). The infection is spread horizontally from one fowl to another and it can be egg-transmitted vertically. In chickens, *M. gallispeticum* is symptomatic causing severe inflammation of the trachea and lungs, airsaculitis, conjunctivitis, rales, oral and nasal mucosal discharge. *Mycoplasma gallisepticum* is characterized by complex antigenic variation, high frequency phase variability and high degree of plasticity. In addition, the presence of modulins and superantigens are few of the contrivances adopted by MG to evade the lymphatic system. It causes chronic colonization of the primary airways by interacting with respiratory epithelium, thickening epithelial layer and causing loss of cilia. The capability of penetrating erythrocytes, chicken embryo fibroblasts and HeLa cells (Winner et al. 2000; Vogl et al. 2008) in addition to causing devastating exacerbations of pulmonary infections are reasons why MG is the leading cause of morbidity and mortality in chickens with CRD (Levisohn and Kleven, 2000; Nascimento et al., 2005). The condition is exacerbated as the result of concurrent infections with *E. coli*, Avian Influenza, Newcastle disease virus, Infectious Bronchitis, etc. In commercial settings, the mortality caused by airsacculitis following MG-infection ranges from 10 to 30%; and if accompanied with *E. coli* and/or

other respiratory pathogens it results in higher mortality and higher downgrading at processing (Yoder, 1991).

Global poultry industry incurs significant losses as MG accompanies emerging problem for commercial flocks and endemic outbreaks in multiple-age commercial layers causing significant economic deficit despite the absence of clinical signs (Levisohn and Kleven, 2000). Death, condemnations of carcasses, reduced feed efficiency, reduced egg production and egg quality, decrease in hatchability, insufficient growth, exasperation of other disease agents and escalation in medication, vaccinations and control programs prepare MG as the costliest disease facing poultry production (Ley, 2008; Levisohn and Kleven, 2000; Bradbury et al., 1993; Wieslander et al. 1992; Mohammed et al. 1987; Carpenter et al., 1981; Parker et al., 2002; Bradbury and Kleven, 2008; Herrero et al., 2009).

Eradication in combination with extensive biosecurity and surveillance is a well control MG-program. In the United States, an extensive National Poultry Improvement Plan has been adopted by hatcheries and poultry breeders with success in increasing MG-free flock. Yet, MG outbreak persists in many countries and various measures have been enforced with outcomes demonstrated far from satisfactory (Bradbury and Kleven, 2008 Ley, 2008; Whithear, 1996; Evans and Hafez, 1992; OIE, 2008).

In addition to food safety and security, environmental impacts of disease and economic perspective regarding the market demand for egg and meat production, the welfare of the animal during production process is eminently valued and is the main impetus driving this scientific research.

As an essential nutrient and a first limiting amino acid in corn-soybean meal-based poultry diets, methionine (**Met**) is of rich nutritional value and physiological functions (Rubin et al., 2017; Avila et al., 2000). Multiple functions are dependent on Methionine, however its notable roles include 1) protein synthesis and feather development 2) precursor for glutathione; a tripeptide that curtails reactive oxygen species (**ROS**) safeguarding cells from oxidative stress; 3) methionine is required for the synthesis of spermine and spermidine which are polyamines that engage in nucleus and cell division; and 4) methionine is a vital methyl donor, methylating the reaction of DNA as well as several distinct molecules (Kidd, 2004; Rubin et al., 2007, Wu et al., 2006; Ugarte et al., 2013; Baker, 2009; Fanatico, 2010).

Methionine is suggested to play an influential role in both humoral and cell-mediated immune responses. Its immune functions include stimulation of phagocytic activity of leukocyte (Elmada et al., 2016, Shuai and Zhou, 2006), higher leucocyte migration inhibition (Bhanja and Mandal, 2007), detoxification (Kim et al., 2006), triggering serum lysozyme activity (Chen et al., 2010), and resistance for coccidium infection (Rama Rao et al., 2003).

The NRC (1994) requirements for methionine are 0.50, 0.38 and 0.32% for starter (0-3 weeks of age), grower (3-6 weeks of age) and finisher (6-8 weeks of age) broiler phases respectively for optimum growth and feed efficiency. Yet, higher level of Met is suggested to be required to stimulate immune responses in various animals (Ruan et al. 2007) including poultry (Rama Rao et al., 2003; Zhang et al., 2008; Konashi et al., 2000), carp (Elmada et al., 2016), catfish (Shuai and Zhou, 2006), largemouth bass (Chen et al., 2010)

and humans (Grimble, 2006). However, it is curial to note that this is dependent upon the inclusion rate of Met, the pathogen in question, the breed and species. Particularly, for the reason that excessive Met can provoke negative turn out as it is the most toxic member of the amino acid family (Zhang et al. 2012; Kim et al., 2015; Wu et al., 2009; Chaturvedi et al., 2016; Duan et al., 2003; Ruan et al.2017; Selhub et al., 2016).

The lymphatic system prospers immensely from adequate nutrition, consisting of a balanced diet with supplementation of certain essential nutrients. The following also indirectly equip the body for periods of stress by reducing the unfavourable effects and reinforcing speedy recovery. Thus, the valuable benefits of sufficient nutrition on the immune system cannot be over emphasized and incorporating particular nutrients above required levels, without being excessive have confirmed to increase immune responses during stress cycles and disease outbreaks in poultry and other animals.

No insight of the immune aspect of methionine on *Mycoplasma gallisepticum* is reported in literature. Consequently, the aim of this work is to investigate the immunopotentiating role of incorporated 20% dietary methionine above the modern broiler recommended level on *Mycoplasma gallisepticum* challenged broilers, treated with or without Pulmotil *AC*®; measuring growth performance, haematological parameters, relative organ weight and immunological response criteria.

CHAPTER 2

LITERATURE REVIEW

2.1 AVIAN LYMPHATIC SYSTEM OVERVIEW

Much- anticipated insight in immunology has been increasing due to its ability to provide deeper understanding of the anatomy and function of lymphatic system dredging into combinations of unique yet similar characteristics between different species. Knowledge of the structure and the organization of the avian lymphatic system is essential in understanding how the system operates. The immune system depends on its specialized peripheralization in order to function properly. Full comprehension of the avian lymphatic system craves an enlightenment of its anatomical compartments and articulation. This section presents knowledge regarding the gross anatomy as well as the histology of the avian lymphatic system specifically that of chicken.

2.1.1 Anatomy of Lymphoid Organs

Lymphoid organs are eminently compartmentalized. Each anatomical domain has its specialized microenvironment of lymphoid and non-lymphoid cells, with distinguishable B and T-cells (Aughey et al., 2001). The primary central lymphoid organs in avian species are the bursa of Fabricius and the thymus (Aughey et al., 2001). While, secondary lymphoid organs in avian species include the spleen, bone marrow, Harderian gland, gut associated lymphoid tissues (**GALT**); where oesophageal tonsils, pyloric tonsils, Peyer's patches (**PP**),

caecal tonsils (**CT**) and Meckel's diverticulum are present, bronchial-associated lymphoid tissue (**BALT**), conjunctival-associated lymphoid tissue (**CALT**), nasal and genital associated lymphoid tissues, as well as skin and pineal-associated lymphoid tissues and lymph node like- structures (Kriere, 2001; Pastoret et al., 1998).

B and T cells in these lymphoid organs employ different zones, referred to as B- cell and T-cell dependent areas (Aughey et al., 2001). The different types of non-lymphoid cells create the specific microhabitat in each compartment. In different regions antigen submission to T- cells by non- lymphoid cells occurs, where T-cells interact with B-cells and production of immunoglobulin is triggered (Davison et al. 2008). It is therefore crucial to realize that information generated from in vitro studies on the possible interactions between cells is not thoroughly exhaustive because such interactions are obviated in vivo due to compartmentalization (Kaiser et al., 2014).

Lymphatic cells are ordinarily introduced into the circulation to conquer lymphoid organs (Davison et al., 2008). Lymphocytes occurs through high endothelial venules (**HEV**) (Kaiser et al., 2014). These occupy the T- dependent zones of lymphoid organs (Kaiser et al., 2014). In the avian spleen, the T-cell dependent zone; called peri-arteriolar lymphatic sheath (**PALS**) engulfs the splenic central artery, whereas in the other lymphoid organs this is not as well defined; and the interfollicular region is regarded as the T-cell dependent region (Davison et al., 2008). Germinal centers and the peri-ellipsoidal white pulp (**PWP**) of the spleen are known to be B- dependent areas (Davison et al., 2008; Kreier, 2001). Germinal Centers which consist of nodular lymphoid tissue and lymphoid nodules are round to oval and composed of a mixed population of large immature lymphocytes and small to medium mature lymphocytes (Kreier, 2001; Pastoret et al., 1998). The mature

lymphocytes are the more intensely stained lymphocytes because they deeply stain with hematoxylin and eosin, in contrast to the mature lymphocytes which are pale in colour. These germinal centers are distinguishable from the surrounding tissue by a fine connective tissue capsule (Pastoret et al., 1998). Plasma cells and cells of germinal centers are originated from the bursa; these are called bursa-dependent tissue. Other assortments of lymphocytes are referred to as thymus dependent (Pastoret et al., 1998).

The lymphoid tissue is described to be a 'three dimensional meshwork' (Rose, 1981) composed of fixed cells (which are reticular cells and macrophages) and reticular fibers (Pastoret et. al, 1998).

2.1.1.1 Bursa of Fabricius

Uniquely, birds are characterized by possessing a bursa of Fabricius. The bursa of Fabricius is a major primary lymphoid organ in avian species (Perry, 2006). It was with the discovery of the bursa by Hiero Fabricius that the concept of T and B cells was introduced to immunological vocabulary. The bursa demonstrated by Hiero Fabricius as a blind sac located dorsal to the cloaca; in the caudal body cavity (Whitton, 2000). It is lymphoepithelial organ composed of a dorsal median diverticulum of the proctodeum (Pastoret et. al, 1998). The development of bursa of Fabricius begins during late embryogenesis (Taylor, R. L.et al. 2009). Although the first primordium of bursa is detectable on day 4 of embryo (**e**); the stem cells of yolk sac origin starts populating on day 7.5 e (Pastoret et. al, 1998). The bursa reaches a weight of 4g and a maximum size of 3cmx2 cmx 1cm at about ten weeks of age (King and McLelland, 1975) and then it undergoes involution (Pastoret et al., 1998).

Although bursa's follicles consist of more than ninety percent of B-cells (Kreier, 2001), up to nighty nine percent of them undergo apoptosis (Pastoret et al., 1998; Kreier, 2001). Its lumen is filled by 15 to 20 longitudinal folds or on average of twelve plicae of the mucous membrane of the bursal wall (Hodges,1974); each consisting of many follicles (Scannes, 2014). Such that the lymphatic tissue in the walls of the bursa are separated by connective tissue into lobules divided into a dark exterior cortex and pale inner medulla (King and McLelland, 1975). The epithelial buds have developed into the medullary portions of bursal follicles and the surrounding tunica propria become the cortex of the follicle. The cortex and the medulla, both possess a network of supporting of stellate look-a- like reticuloepithelial cells of meshes occupied with lymphoid cells and their cytoplasmic processes being joined by desmosomes (Hoskins, 1977). The network of cells in the cortex is continuous with the surrounding connective tissue (Davison et al., 2008). It consists of numerous small dense lymphocytes. large capillaries directly underlying a basement membrane separate the cortex from the medulla (Scannes, 2014). The exterior layer of the medulla close to this basement membrane possess undifferentiated epithelial cells that is continuous with the epithelium surface of the fold (Hoskins, 1977). These undifferentiated epithelial cells are characterized with round nuclei, low cuboidal and pale staining. The lymphoid cells of the medulla are composed of medium and small lymphocytes and greater number of lymphoblasts observed to be near the periphery (Kaiser et al., 2014).

The bursa is interlined with epithelium that cover numerous folds consisting of patches of specialized tissue that cover the bursal follicles (Davison et al., 2008). Each fold has a surface epithelium consisting of interfollicular epithelium (**IFE**) and follicle-associated epithelium (**FAE**) (Davison et al., 2008). Two types of follicles exist: one follicle is button-

like shaped; similarly leveled as the surrounding interfollicular tissue although apart by a 'crypt-like' concavity (Hoskins, 1977). A smooth uninterrupted epithelium lays over the button-like follicle. The second type of follicle extends from the bursal surface which possess rough surface epithelium and they do not consist of crypts. Each fold appears to have only one of the two types of follicles (Scannes, 2014. The wall of the bursa is composed of three layers: 1. Thin collagenous serosal layer, 2. Muscularis that is formed either of an exterior longitudinal layer and an inner circular layer or of circularly organized smooth muscle fibers (Hoshkins, 1977; Davison et al., 2008) or of two longitudinal layers with an interposed circular layer (Davison et al., 2008). 3. Mucosa, which dominates thickness portion of the wall. This is divided into three parts: the connective tissue framework, the lymphoid follicles, and surface epithelium (Kaiser et al., 2014; Davison et al., 2008). It consists of approximately twelve thick vertical folds and each of which greatly arranged mainly of polyhedral lymphoid follicles closely combined with few connective tissues separating the follicles (Kaiser et al., 2014; Davison et al., 2008).

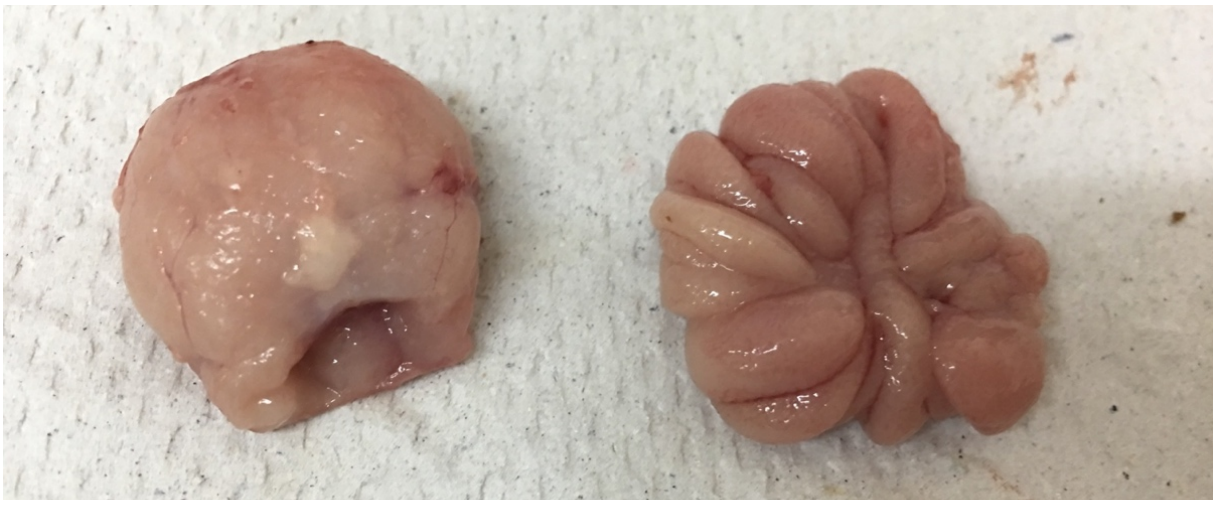

Figure 2-1. Bursa of Fabricius from a 35 day-old male Ross 308 broiler. The bursa on the right has been everted to show the plicae.

2.1.1.2 Thymus

The avian thymus has numerous lobes; 3 to 8 pale pink, on average of 7 (Pastoret et al., 1998) irregularly shaped, flattened lobes (King and McLellan, 1975); with lobes divided into lobules (Aughey et al., 2001) that are separated by connective tissue (King and McLellan, 1975). Each lobe is approximately 1 cm long, or 10- 12mm in diameter by the age of 3 to 4 months (Davison et al., 2008) secured along each side of the neck near the jugular vein (King and McLellan, 1975) and vagus nerves (Pastoret et al., 1998) extending from the third cervical vertebra to the upper thoracal segments (Davison et al., 2008). Onset of sexual maturity, the thymus reaches it maximal size and then it starts its involution (King and McLellan, 1975). Moreover, the lobes are engulfed with a fine fibrous connective tissue capsule and embedded into adipose tissue (Davison et al., 2008). Septae incompletely divide the lobes into lobules (Scannes, 2014). Thymus lobes consist of an outer dark cortex and an inner pale medulla of thymocytes (Aughey et al., 2001). The medulla is found in the center and is surrounded by the lobulated cortex. Both, the cortex and medulla are formed from a framework of scattered reticular cells and fibers containing population of small lymphocytes (King and McLellan, 1975). However, the fibers are much more densely arranged in the medulla in contrast to the cortex (King and McLellan, 1975).

The basal lamina isolates the surface of the lobules from the capsule and septae (Scannes, 2014). The thymus extends from anterior cervical regions into the thorax and parts of the thymus may be embedded into the thyroid gland (Pastoret et. al, 1998). It closely associates with the parathyroid and the ultimobranchial glands. The thymus is inverted during embryogenesis through a topographical distribution of fibers coming from the central nervous system (Pastoret et. al, 1998).

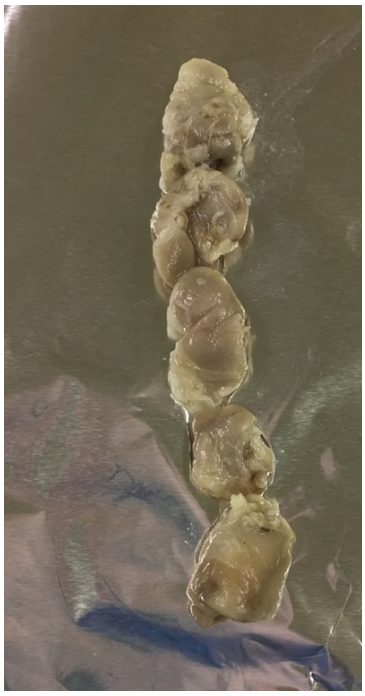

Figure 2-2. Thymus with 5 lobes from Ross 308 male broiler at 35 days of age.

2.1.1.3 Spleen

The spleen is largest secondary lymphoid organ in avian species (Kreier, 2001). It is located dorsally to the right of the proventriculus and right lobe of the liver (Pastoret et al., 1998; Whitton, 2000). It a reddish-brown (Whitton, 2000), nearly a spherical organ, approximately 2 m in diameter (King and McLellan, 1975) The splenic primordium first occurs in a 48 hour as a mass of mesenchymal cells (Kaiser et al., 2014). Onset of hatching, the spleen begins to provide the necessary microenvironment for interplay between lymphoid and non-lymphoid cells (Davison et al., 2008). In fact, as an entity, the contribution of the avian spleen to the lymphatic system could have more of a vital role than it does in mammals due to the of the poorly developed lymph nodes and vessels in Aves (Davison et al., 2008). The spleen is composed of bridges of reticular cells and

reticular fibers (King and McLellan, 1975). It consists of red and white pulp (Aughey et al., 2001). The red pulp is composed of venous sinuses that are disconnected by several cells that include macrophages, lymphocytes and components involved in blood circulation (King and McLellan, 1975). The white pulp, on the other hand, is formed of lymphatic tissue which surround the arteries (King and McLellan, 1975). However, the distinction between the splenic red and white pulp in avian species is less apparent, this is due to the fact that birds possess an open splenic circulation; where there is no direct vascular connection between the veins and arteries (King and McLellan, 1975).

2.1.1.4 Bronchial-Associated Lymphoid Tissue (BALT)

BALT is a key component of secondary, peripheral or seeding lymphoid organs which represent all the lymphoid structures and the collection of lymphoid cells that exist in the respiratory tract (Abdelmotti, 2015). BALT includes sub-mucosal lymphoid cell in the respiratory tracts as well as the bronchial epithelium of cells that are mainly non-ciliated squamous of which then develops with age to columnar ciliated cells (Abdelmotti, 2015; Kaiser et al., 2014). Moreover, the lymphoid nodules that are present in the lung are associated with the primary bronchi (Kaiser et al., 2014).

2.1.2 Function of The Lymphatic System of Avian

With the beauty of the architectural organization, Aves demonstrate a lymphatic system unlike other species. A much appreciation for such avian anatomical design is encrypt upon the understanding of its function. Novel insight into avian lymphoid organs has contributed

significantly to the understanding of their lymphatic system. With the absence of lymph nodes and a unique B-cell development organ, avian lymphatic system demonstrated exclusive characteristics distinguishable from that of other species. Recognizing the functions of avian lymphoid organs; each independently and in coordination with other organs; provides deep perception of the fundamental basis of the lymphatic system.

The lymphatic system developed as a barrier of protection in living organisms (Cooper, 2014); from several pathogenic microorganisms that potentially may cause diseases or death (Kreier, 2001). Its principle objective was to provide a safeguard and survival of the species through its reproductive age and life (McElwee, 2011). This is not limited to the protection and repair of organ damage (Cooper, 2014), the protection against assimilation and from parasitism and the regulation of species' integrity (McElwee, 2011).

Lymphoid tissue consists of two types of lymphocytes: In early avian embryonic life, the first developing aggregation of lymphocytes is contingent on the thymus for differentiation (Rose, 1979). These are known as T-lymphocytes which are cells developed only for the interactions with antigens and for limited direct lymphocyte-antigen interaction; a function of cell- mediated immunity which is responsible for the discrimination of self versus non-self and the expression of cell-mediated (Wakenell, 1999; Rose, 1979). While, B-lymphocytes, the second type of lymphocytes is responsible for the production of immunoglobulins and antibodies. This is dependent upon the bursa of Fabricius, which is a B-dependent system, known as humoral immunity (Cooper, 2014). Both, cell-mediated immunity and humoral immunity are specific responses that require a processed antigen to stimulate the response, thus their response is to devise a specific antibody for each antigen

(McNab, et al., 2002, Wakeness, 1999). Non-specific response in avian also exists. Such immunity is able to respond to all antigens available, and it is mainly associated with Macrophages, thrombocytes and heterophils (Wakenell, 1999).

The function of antibody production by B-cells aid in preventing the spread of pathogens by combining with the pathogen and neutralizing it; allowing easier digestion of the pathogen by phagocytic cells, as well as facilitating cell lysis and death (Scannes, 2014; Whittow, 2000). The majority of avian antibody responses are thymic dependent. These depend on the cooperation between T-cells B-cells and macrophages (Scannes, 2014). While, thymic independent antigens consists of thymic independent type I (such as pneumococcal polyssaccharide and Brucella) and thymic independent type II (such as ficoll and dextran) (Whittow, 2000; Scannes, 2014).

The lymphatic system functions could be subdivided into humoral immunity and cell-mediated immunity. Both, humoral and cell-mediated immunities have evolved to protect living organisms from external and internal threats and ensure their survival (Boehm et al, 2012). Humoral immunity describes B-cells that produce antibodies of variable classes which recognize antigens that spread in the lymph or blood (Boehm et al., 2007). While cell-mediated immunity (**CMI**) describes T-cells. T-cells classes include T-cytolytic cells (**Tc**), T-helper cells (**Th**), T-suppressors cells (**Ts**), T-regulatory ($\mathbf{T_R}$) and T-delayed hypersensitivity cells ($\mathbf{T_D}$). Also, it includes neutrophils, macrophages as well as killer cells (Takahama, 2006; Balogh, 2010; Gill et al., 2003). In primary lymphoid organs, T-cells develop in specialized thymopoietic tissues located in the pharynx, while the B-cells develop in general hematopoietic areas as the kidney or the bone marrow (Boehm et al., 2012). The secondary lymphoid organs such as the spleen is present in all vertebrates,

while lymph nodes are a particular advancement to mammals and some birds (Balogh, 2010).

Avian species have lymphocytes that arise in the yolk sac and either travel to the thymus or the bursa of Fabricius. Any immature lymphocytes that migrate to the thymus, develop and mature under the effect of hormones arriving from thymic epithelial cells. And, detectable T-cell markers leave the thymus (Aughey et al., 2001; Kreier, 2001; Pastoret et al., 1998). While immature B cells migrate to the bursa after being produced in the bone marrow where they mature and penetrate into the blood stream (Kreier, 2001). The bursa contains a diffuse of lymphoid cell infiltration of T-cells and B-cells (Aughey et al., 2001). Plasma cells and cells of germinal centers are originated from the bursa; these are called bursa-dependent tissue. Other assortments of lymphocytes are referred to as thymus dependent (Pastoret et al., 1998). Approximately 5% of bursa cells emigrate daily into the blood then into B-cell locations (Kreier, 2001) of the thymus, spleen and cecal tonsils. And about 1% of the blood B-cell collection is recouped by cell migration from the bursa each hour (Pastoret et al., 1998). Post-hatching the thymus consists 5-15% of B-cells and the spleen functions as antibody (**Ab**) producers and antigen processor (Pastoret et al., 1998). The Harderian gland in birds consists of plenty plasma cells that generate and secrete IgA chiefly as well as other immunoglobulins (Kardong, 2012; Pastoret et al., 1998). There is a continuous increase in plasma cells by the age of 2 weeks old bird. Of 1.5 weeks post-hatching there is small number of IgA positive cells, but this number increases by 46% by the age of 3.5 weeks old (Pastoret et al., 1998). B-cells in Haderian gland constitute of at least 80-90% of lymphoid cells (Pastoret et al., 1998). While the cecal tonsils are absent at hatching, its size is dependent at the rate of antigen provocation and its size increases up to

approximately 12 weeks old. The cecal tonsils contains bulk amount of GALT and contains both T and B cells (Boehm et al., 2007) of 35% and 45-55% respectively (Pastoret et al., 1998). They are also concerned with functions of cell-mediated immunity and antibody production (Pastoret et al., 1998).

In avian species, 60-70% of lymphocytes located in peripheral blood are T cells (Kreier, 2001) The bone marrow contains T-cells as well as B-cells; about of which 15-25% are mononuclear cells. In other lymphoid tissue, IgA-positive cells can be found in mucosa of the gall bladder and immunoglobulins can be illustrated in bile (Pastoret et al., 1998).

IgY is the most prevalent immunoglobulin in avian species. Some reports claim the presence of three subclasses of IgY: IgY1, IgY2, and IgY3. However, this has yet to be validated (Kreier, 2001).

2.1.2.1 Bursa of Fabricius

The bursa of Fabricius plays an important role in antibody formation (King and McLelland, 1975). As a central lymphoid organ, it possesses cells with the ability to generate differing antibody-forming elements. The development of bursa of Fabricius begins during late embryogenesis (Taylor, R. L.et al. 2009). Onset of hatching, the bursal duct opens to transport antigens from cloacal region to the bursal lumen and straight down into the lymphoid follicles (Bar-Shira et al., 2003). These bursal cells migrate to secondary lymphoid organs such as spleen and the gut-associated lymphoid tissues (Hoskins, 1977). B- lymphocytes originate from the bursa of Fabricius with the capacity to migrate to the circulation to transform into antibody-producing plasma cells when exposed to antigens. Thus, the bursa of Fabricius has a function of humoral immunity (Hoskins, 1977, Bar-Shira

et al., 2003). Bursa's follicles consist of more than ninety percent of B-cells (Kreier, 2001). However, these rapidly proliferating B-cells, up to nighty nine percent of them undergo apoptosis (Pastoret et al., 1998; Kreier, 2001). Such process illustrates a negative selective pressures on the self-reactivating B- cells (Kreier, 2001).

Introduction of antigens into the bursa results in an increase in B-cell responses; however, it does not encourage antibody production directly (McNab, et al., 2002; Spring et al., 2008). Prior and post to hatching, on the surface of bursa's lymphocytes, a slow consecutive occurrence of immunoglobulin M-, immunoglobulin G- and immunoglobulin A-determinants illustrates that immature precursor cells convert into immunoglobulin by a genetically antigen-independent transformation (Cooper et al., 1972; Hoskins, 1977). Function of the bursa also consists of two categories of stem cells; bursal and post-bursal stem cells (Mansikka et al., 1989). Although they are unable to transform into precursors of plasma cell without communicating with bursa; bursal cells which exist post- hatching approximately three weeks of age; are able to however develop germinal centers in the spleen and recover the morphology of the bursa in cyclophosphamide treated chickens (Hoskins, 1977; Baba and Okuno, 1976). On the other hand, post-bursal stem cells which originate at approximately two weeks of age later exist in the thymus, spleen and bone marrow; have the ability to continue maturing into antibody producing cells without the contact with bursa's microenvironment nor restoring the bursal morphology of the cyclophosphamide-treated chickens (Toivanen and Toivanen, 1973; Hoskins, 1977).

Since constantly within bursal follicles there is a direct contact between lymphoid cells and material actively taken up from the cloacal lumen, the bursa of Fabricius has an additional function as a peripheral lymphoid organ (Hoskins, 1977). This is embellished by the

presence of a pumping mechanism as the bursa of Fabricius contracts along with respiration by a rhythmic contraction created by the sphincter muscles. Today birds, specifically chickens are vaccinated for the laryngotracheitis infection due to such bursal operation, known as the vent method (Sorvari et al., 1975; Hoskins, 1977).

As mentioned earlier, lymphoid cells in bursa consists mainly of B-cells, an approximately 98%. But there is also few T-cells of about 2% along with macrophages in the cortex and medulla, as well as macrophage-like cells in the FAE (Scannes, 2014). FAE are able to gather elements and or antigen from the lumen (Whittow, 2000; Olah and Glick, 1978) and in the opposite direction gather products of the bursal secretory dendritic-like cells (**BSDC**s) (Scannes, 2014). BSDCs functions n instructing the B cells by administering the interaction they have with antigens (Scannes, 2014).

2.1.2.2 Thymus

T cells make up most of the thymus. Few scattering macrophages are present throughout the cortex (Whittow, 2000) with the subcapsular region being main T cell proliferating site (Scannes, 2014), the medulla, as well as the epithelial reticular cells. As maturation of T cell occur, the cells travel to the corticomedullary border. In this region, the thymic dendritic cells and macrophages pick up the thymocytes, prior to entering the medulla and later the circulation (Whittow, 2000). Avian thymus-dependent tissues are of cellular immunity with a graft versus host reactions (Cooper et al., 1966; Pastoret et al., 1998), responses of delayed hypersensitivity as well as homograft rejection (Cooper et al., 1966). It also functions for macrophages activation, cytotoxic responses and the development of helper and suppressor cells that adjust the production of antibodies (Pastoret et al., 1998).

To few antigens, such tissues may have less function in antibody response (Cooper et al., 1966). It has been demonstrated that thymectomized-irradiated chickens lack these responses and thus develop slower gradual growth rate. However, with the withstanding of their plasma cells, germinal centers, and the ability for immunoglobulin synthesis (Cooper et al., 1966). Through heterodimeric T cell receptor (**TCR**), T lymphocytes can track antigens that exist in the major histocompatibility complex (**MHC**) molecules (Whittow, 2000). Aves, specifically chicken (same as mammals) possess both αβ and γδ TCRs. Now in the thymus each TCR consist of two immunoglobulins and all TCRs signaling is executed by the CD3 complex (Scannes, 2014; Whittow, 2000) In chickens, **Vβ1** and **Vβ2** are the two **Vβ** (β chain variable) gene families. These are identified respectively by the following antibodies: **TCR2** and **TCR3** (Kaiser et al., 2014; Whittow, 2000). T lymphocytes asserting TCR of αβ are also subdivided into two additional surface molecules: CD4 and CD8. CD8 is a co-receptor for MHC class I while CD4 is a co-receptor for MHC class II (Whittow, 2000).

Furthermore, **TCR-αβ+CD4+** T lymphocytes are termed as T helper (Th) cells, while **TCR- αβ+CD8+** T lymphocytes are characterized as cytotoxic T (Tc) cells (Scannes, 2014; Whittow, 2000). By day 15 of bird's embryonic development, **TCR-γδ+** mature T cells travel to the spleen while by day 19 migration of **TCR- αβ1+** occurs. However, **TCR-αβ2+** cells do not occur in the spleen until post-hatching (Whittow, 2000; Kaiser et al., 2014).

2.1.2.3 Spleen

In general, the spleen has two functions: one is to aid in the supply of oxygen to the tissues

and second is to aid in disease resistance. Avian splenic operates by transporting oxygen to the tissues which plays role in removing abnormal or parasitized red blood cells from the circulation (John, 1994). In early development, erythropoiesis does occasionally take place in the avian spleen (John, 1994; Dieterlen-Lievre, 1988), however, it is normally restricted to bone marrow in mature birds (John, 1994). Unlike other organisms, the spleen in aves is disregarded as being a reservoir of blood that is able to expel morel erythrocytes into the circulation (John, 1994; Rose, 1981). The spleen is known as the largest lymphoid organ in aves. Since afferent and efferent lymph cells are absentee, the spleen may thus only attain antigen from the blood (Scannes, 2014). Bird spleen cells also possess the capacity to respond to tumor and their removal; and the spleen follows the same antigen trapping manner as described above in previous lymphoid organs (Chi and Sharma, 1990; John, 1994). The red and white pulp tissues are functionally devoted to disease resistance, with only the red pulp is directly concerned with conveying oxygen (John, 1994). The red pulp tissue in avian cover 40 to 45% population in the spleen and there exist capsule and trabeculae (Pastoret et al., 1998). The crucial development of bird spleen occurs post-hatching period once it is in presences of antigens. Thus, embryonic lymphopoiesis is not achieved by the spleen (John, 1994).

Since the spleen is a secondary lymphoid organ (Kreier, 2001) certainly lymphocyte differentiation takes place in the spleen; it is not however a central ground for antigen-independent differentiation and proliferation of lymphocytes (John, 1994).

As mentioned, the spleen plays an important role in blood cell development, it engages granulopoiesis and erythropoiesis during embryo stage. At hatching, the granulocytes emigrate the spleen and lymphocytes seize their site and this process is key during

incubation (Dieterlen-LiZevre, 1988; John, 1994). Besides, the spleen has a unique function in hemopoiesis system. This is because stem cell aggregation is augmented by the embryonic spleen prior to its formulation in the bone marrow (John, 1994). Also, precursors of B and T cells are present in the spleen of embryos of which are afterwards distributed before colonizing primary lymphoid organs (Houssaint et al., 1991; John, 1994). In young birds too, B cells persist to emigrate from the bursa prior almost to hatching until onset of sexual maturity, as bursal involution occur (John, 1994). Such B cells are mainly found in the blood and the spleen, with few migrating to the thymus, bone marrow, and gut-associated lymphoid tissues (John, 1994; Pastoret et al., 1998). Further, the importance of disease resistance role of the avian spleen in mature birds is stressed upon the meager system of lymphatic vessels and nodes (King and McLelland, 1984). The red pulp of the spleen contains lymphoid and non-lymphoid cells (Whittow, 2000). Sinuses are rich of CD8+TCRγδ+ T cells. However, CD4+TCRαβ1+ or CD4+TCRαβ2+ cells do also exist (Whittow, 2000). In mature Aves, most of the TCRγδ+ cells are CD8αβ+ (Scannes, 2014). Spleen plasma cells express three different immunoglobulin isotypes. These are found in the red pulp of the spleen close to the large blood vessels (Pastoret et al., 1998). Abouding macrophages are located in the red pulp characterized as strong acid- phosphatase positive and stained with monoclonal antibodies as well as few heterophils found all over their sinuses (Scannes, 2014; Kaiser et al. 2014). On a side note, the spleen interacts with the bone marrow periarteriolar macrophages in order to remove gold colloid particles while behaves in a hepatic process to take up elements from the blood of turkeys (John, 1994). In all, innate and adaptive immune responses are efficiently executed by the avian spleen. The Periarteriolar lymphoid sheaths (**PALS**) observed to be mainly occupied with adaptive

immune responses, whereas the Periellipsoid lymphoid sheaths (**PELS**) are involved in innate as well as adaptive immune responses (Scannes, 2014).

2.1.2.4 Mucosal Lymphoid Tissues

The total mucosal surfaces in avian species constitute the largest surface area which include those of the of the gut; gut-associated lymphoid tissue (**GALT**), bronchial; bronchial-associated lymphoid tissue (**BALT**), head; head-associated lymphoid tissue (**HALT**) and reproductive tracts (Davison et al., 2008). These face constant challenge from foreign elements and pathogenic microorganisms (Scannes, 2014). Entrance of these foreign objects through the mucosal tissues is obviated by the employment of protective means (Kreier, 2001). Such include barrier functions; for example, involving keratinized skin, ciliated cells in the trachea and/or mucus secretion (Whittow, 2000). As well as protection mechanisms associated with highly specialized lymphatic cells, as for instances the Langerhans cells in the skin and the highly organized lymphoid structures (Scannes, 2014). Furthermore, many lymphatic cells are found in lamina propria as intraepithelial and lamina propria lymphocytes, macrophages, as well as dendritic cells (Pastoret et al., 1998; Kaiser et al., 2014). The gut is composed of lymphocytes to greater extent than those found in secondary lymphoid tissues combined. This probably due to the fact that avian lack the presence of lymph nodes (Scannes, 2014). In these mucosal lymphoid tissues, T cell areas surround the B cell follicles (Whittow, 2000) and their epithelial cells are capable of administering specific lymphatic responses to various pathogens (Scannes, 2014).

2.1.3 Molecular Structure of Humoral Immune System Components

2.1.3.1 Immunoglobulins

Antibodies produced by clonal B-lymphocytes specifically respond to only one antigen. Such antibodies contribute to organism's immunity by their responsibility in guarding the organism's body through the prevention of pathogens entry by binding to these pathogens, process known as neutralization. A second function of antibodies include the stimulation of the discharging of pathogens by macrophages as well as by opsonization. While, lastly antibodies evoke the destruction of pathogens via the elicitation of other immune responses such that of the complement system (Tiwari and Sinha, 2008; Boundless, 2016). This section comprehensively discusses the molecular structure, function, methods and mechanisms involving the humoral immune system and its components.

There exist five primary classes of immunoglobulins. These are **IgG**, **IgM**, **IgA**, **IgD** and **IgE**. They differ from each other by their heavy chain found in the molecule. Immunoglobulin IgG possess heavy chains called g-chains; IgMs possess μ-chains; IgAs have a-chains; IgEs have e-chains; and IgDs consist of d-chains. What makes them function differently are these differences in heavy chain polypeptides which can result in different immune responses and at specific levels of the immune response (Elgert, 1998; Goldsby et al, 2002). The polypeptide protein sequences that influence this distinguishability are located predominately in the Fc fragment.

As five differing kinds of heavy chains exist, there are however only two types of light chains: these are known as kappa (κ) and lambda (λ). Further, the main reason why such

antibody classes differ in their valency is due to the differing numbers of Y-like units, known as monomers which couple together in order to form a complete protein. In humans for instance, immunoglobulin IgM have five Y-shaped units, known as pentamer, that consists of 10 heavy chains, 10 light chains, and 10 antigen-binding sites (Goldsby et al., 2002; Janeway et al., 2001; Elgert, 1998).

2.1.3.1.1 Immunoglobulin G (IgG)

Immunoglobulin G (**IgG**), a monomer, is the dominant class of immunoglobulin that exist in humans, making up for approximately 75% of total serum. Only immunoglobulin IgG is able to pass over the placenta in humans, and it mainly functions for the protection of the newborn in the first few months of life. Due to its affluence and targeted specificity toward antigens; immunoglobulin IgG is the primarily antibody applied in research as well as clinical studies.

2.1.3.1.2 Immunoglobulin M (IgM)

Immnunoglobulin M (**IgM**) is present in mammals as pentamer which is responsible in primary immune responses to most antigens and it has an important and efficient role in complement-fixing immunoglobulin. It can be found to be of approximately 10% of normal human serum and featured on the plasma membrane of the B lymphocytes as a monomer. These IgM monomers are joint together by disulfide bonds and a joining (**J**) chain. Each of the five monomers is composed of two light chains, which can be either kappa or lambda as well as two heavy chains. IgM ‘s heavy chain consists of one variable domain and four

constant domains with no hinge region. Agglutination could be a result IgM due to the detection of epitopes on invading pathogens. Such **Ab/Ag** complex is then attacked via complement fixation or receptor-mediated endocytosis aided by macrophages.

2.1.3.1.3 Immunoglobulin A (IgA)

Immunoglobulin A (**IgA**) on the other hand, is present in the serum in both monomeric and dimeric forms, found approximately 15% of the total immunoglobulin serum. As a dimer, the secretory IgA, can allow for primary defense mechanism against local infections due to its aggregation in membrane secretions such as in tears and saliva. Secretory IgA' s responsibility could not involve the attack of antigen directly; however, it is key in preventing the passage of foreign elements into the circulatory system.

2.1.3.1.4 Immunoglobulin D (IgD) and Immunoglobulin E (IgE)

In few scattering quantity, immunoglobulin D (**IgD**) and E (**IgE**) can be found in immunoglobulin serum. Immunoglobulin D is a membrane receptor for antigen that is located mainly on mature B-lymphocytes. While, immunoglobulin E functions as a defensive immunoglobulin against pathogenic invasion.

Several subclasses also exist in addition to the major immunoglobulin classes. These are also distinguishable due to minor differences in their heavy chain (Tiwari and Sinha, 2008; Elgert, 1998; Thermofisher, 2016; Goldsby et al. 2002; Albert et al. 1983).

For instance, humans have four subclasses of IgG these are IgG1, IgG2, IgG3 and IgG4 and these occur in the same following order of decreasing concentration found in serum and have having γ_1, γ_2, γ_3, andγ_4 heavy chains, respectively (Albert et al., 1983). Moreover, the

variance present between the differing subclasses is smaller than that of the variance between different classes. Meaning that IgG1 is much more associated to IgG2, IgG3 or IgG4 than it is to immunoglobulins IgA, IgM, IgD or IgE. Due to this reason, there occurs a cross-reactivity among these subclasses, while however little cross-reactivity occurring among differing immunoglobulins classes (Tiwari and Sinha, 2008; Elgert, 1998; Thermofisher, 2016; Goldsby et al. 2002; Albert et al. 1983).

2.1.3.2 Differences in Molecular Weight of Antibody Classes (Isotypes)

Antibodies consist of two identical polypeptides of approximately a molecular weight of 25,000, these are known as the light chains or noted by an (**L**), and also of two identical larger polypeptides of approximately a molecular weight of 50,000 and these are known as heavy chains or noted by an (**H**) (ThermoFisher, 2016; Lodish et al., 2000).
Noncovalent bonds link each light chain to a heavy chain by hydrogen bonds, hydrophobic bonds and salt linkages as well as they are linked via one covalent disulfide bridge in order to form a heterodimer; an **H-L** heterodimer. These two identical light chain-heavy chain combinations; H-L are also linked together by analogous noncovalent bonds and disulfide bridge in order to create the fundamental antibody structure of four chain $(H\text{-}L)_2$, making it a dimmer of dimmers. such interchain disulfide bonds vary in their exact quantity and precise positions in the company of antibody classes and subclasses (Tiwari and Sinha, 2008). Immunoglobulin IgG molecular weight is 150,000, with a heavy chain type (gamma) molecular weight of 53,000. The molecular weight of immunoglobulin IgM is 900,000 with a heavy chain type (μ) molecular weight of a 65,000. While the molecular

weight of immunoglobulin IgA is 320,000; this is of the secretory IgA. This includes a heavy chain type alpha, with a molecular weight of 55,000 (ThermoFisher, 2016).

2.1.3.3 Differences in Molecular Structure of Antibodies

Classes of antibody differ in valency due to the varying numbers of monomers of Y-like units that bind together building the complete protein. For instance, immunoglobulin IgM has five Y-shaped units; making it a pentamer (Janeway et al., 2001; Elgert, 1998).

Antibodies are large proteins; glycoproteins; shaped as Y form. Portions that constitutes the tips of the Y's arms, or variable (**V**) regions, differ largely from one antibody to the next, forming a pocket uniquely structured for the enfolding of a specific antigen epitope. The fork of the Y is known as the hinge region. The hinge region attaches the arms to the stem and permits them to swing. The stem of the Y or also known as the constant (**C**) region supports the linkage of the antibody to other components of the immune response. This stem is identical in all immunoglobulin classes (Elgert, 1998). The constant region of heavy chains is concerned with the attachment to carbohydrates (Tiwari and Sinha, 2008). Difference in the molecular structure of each antibody is described below:

Main IgG immunoglobulins properties are: IgG, a monomer, it has a serum concentration of 10 to 16mg/Ml, and it constitute a percentage of total immunoglobulin of approximately 75%. Its glycosylation (by weight) is about 3%. Its distribution accounts for intra- and extravascular, and it functions as a secondary response (ThermoFisher, 2016; Benjamini et al., 1991).

IgG molecules have γ chains. Its structural unit is composed of four polypeptide chains, two identical light (L) chains; each consist of approximately 220 amino acids; as well as two identical heavy (H) chains with each consisting of approximately 440 amino acids. Such four chains are joined together by a combinations of noncovalent bonds (such as hydrogen bonds) and covalent bonds (such as disulfide bonds). Immunoglobulin IgG constitutes two identical halves, each with identical antigen-binding site. The light and heavy chains collaborate together producing the antigen-binding surface. (Albert et al., 1983).

Properties of IgM immunoglobulin: IgM, a pentamer, has a serum concentration of 0.5 to 2mg/ml, constitutes a percentage of total immunoglobulin of approximately 10%. Its glycosylation (by weight) is 12%. Its distribution accounts for mostly intravascular, and it functions for primary response (Benjamini et al., 1991; Harlow et al., 1988).

IgM has μ heavy chains. These μ chains and surrogate light chains are necessary for the cell to develop where it creates bona fide light chains. The light chains join with the μ chains, replacing the surrogate light chains, building the four-chain IgM molecules; each with two μ chains and two light chains. These molecules later embed into the plasma membrane, where they behave as receptors for antigen. Once departing the bone marrow, the cell begins to develop cell-surface IgD molecules with the same antigen-binding site as the IgM. Such cell is known as mature naïve B cell which is has the capability to respond to foreign antigen in secondary lymphoid organs. (Albert et al., 1983).

IgM is composed of five (pentamer); four-chain units, making a total of 10 antigen-binding sites. Each pentamer is composed of one copy of another polypeptide chain, known as a J chain, or joining chain. The J chain is created by immunoglobulin IgM-secreting cells and

is covalently embedded between two adjacent tail regions (Albert et al., 1983; Elgert, 1998).

Properties of immunoglobulin IgA are: IgA is present in serum as monomeric and dimeric; it has a serum concentration of 1 to 4mg/ml. It constitutes a percentage of total immunoglobulin of approximately 15%. Its glycosylation (by weight) is 10%. Its distribution accounts for intravascular and secretions, and it functions for protecting mucus membranes (Harlow et al., 1988; Benjamen et al., 1991).

IgA molecules have α chains. As a monomer, it consists of a four-chain. However, it is an eight-chain dimer form in secretions (Albert et al., 1983).

2.1.3.4 Role of Secretory Component in IgA Isotype

Immunoglobulin IgA is found concentrated in body fluids in order to protect the entrances of pathogens into the organism's body. IgA is the predominant class of antibody in extravascular secretions. Immunoglobulin IgA exists in secretions such that of tears, saliva, nasal secretions, bronchial and digestive tract mucus, as well as mammary gland secretions in the form of secretory IgA (Elgert, 1998). Secretory IgA has crucial effector function on mucous membrane surfaces, guarding the main entry sites for many pathogens. Secretory IgA is polymeric and due to this reason it has the capability of cross-linking large antigens with multiple epitopes (Goldsby et al., 2002). When secretory IgA binds to either or bacterial and viral surface, the antigens prohibit the attachment of the pathogen to the mucosal cells, therefore inhibiting infections (Goldsby et al., 2002).

Secretory IgA has a J chain that is 15-kD polypeptide constituting of 129 amino acid residues and a single carbohydrate group. Plasma cells synthesize this J chain and then bind

to IgA (Elgert, 1998) prior or during secretion time. The J chain is linked to the carboxyl-terminal penultimate cysteine of the α or μ chain (Elgert, 1998).

While, dimeric IgA attaches via the Fc receptors to the blood section of the epithelial cells. Fc receptors are also known as secretory, or S, proteins. Once IgA is bound it becomes internalized and passes through the epithelial cells' cytoplasm. IgA then cleaves from the cell following the detachment of the S protein. The remainder of the peptide known as the secretory component joins the dimeric IgA. However, depending on the species, this secretory component can either be disulfide-linked to the IgA dimer or perhaps not. It can also provide resistance to the enzymatic cleavage during mucosal secretions. The α chain consists of a single V domain and three C domains. Immunoglobulin **IgA1** is the most common form found in serum, while immunoglobulin **IgA2** is little more common during secretions. Exclusively, IgA2 has allotypic determinants, and only the **A2m(1)** allotype particularly has no interchain disulfide bridges between light chains and heavy chains; rather the chains are joint to their own counterparts whereby one light chain is linked to the other light chain. Additional distinguishable appearance is between IgA allotypes in the size of their hinge regions. (Elgert, 1998; Janeway et al., 2001).

2.1.3.5 Isotypes in Primary and Secondary Responses to Same Antigen

A key feature of the immune response is its capability demonstrate memory; whereby the immune system remembers or recognizes the antigens it has previously encountered, and therefore responds much more effectively in the next encounter (Gutman, 2011).

There are main characteristics of immunological memory, illustrated in isotypes in primary and secondary responses. The complete graph in Figure 2-3 demonstrates the results of immunizing an animal with a typical antigen. The graph that resides on the left shows the results of a primary immunization; plotted on the X-axis is the time post-immunization in weeks, while the Y-axis represents a measure of the amount of antibody found in the serum that can be driven by any of the techniques and methods such as agglutination or Enzyme-Linked Immunosorbent Assay ELISA, etc. While the graph residing the right side, the results demonstrate secondary immunization, that is the post re-immunization of the same animal with the same antigen at another date. The differences represented between the two graphs illustrate the characteristics that classify immunological memory (Gutman, 2011). The first three characteristics in the table are due to the cellular events occurring in immune responses, while the last two characteristics showing in the table are associated with the physical properties of the antibodies produced. The key features of isotypes responding to same antigen in primary and secondary responses are as follows:

1) Speed. With a typical primary response, a lag of approximately 4 days exists, followed by a slow rise of antibody to a peak at approximately two weeks. In the secondary, the lag is however only two days, and antibody levels rise extremely rapidly to a peak between 4 to 6 days.

2) Antibody levels. The secondary response achieves peak antibody levels that are extremely higher than that found in primary response.

3) Duration. Primary responses occur much gradually, but they also vanish swiftly. Antibody titers in secondary responses stay at high levels for lengthier periods; for instances in humans it can may remain for many years.

4) Isotype. Immunoglobulin IgM is the first responder and it is the prevalent in primary response (Goldsman et al., 2002), with few immunoglobulins IgG occurring at a later period in the response. During a secondary response, IgG is the predominant antibody involved. Immunoglobulin IgM is also present during a secondary response, but at degrees and time similar to the primary response; thus, having higher levels of IgG. IgM does not possess immunological memory. The reason for this is because it does not occur more rapidly or at higher levels in the secondary responses, as well as it is not subjected to substantial affinity maturation.

5) Affinity. In the secondary response, the average affinity of antibody in a is observed to be higher than in the primary response. This is the case, despite amid the course of a certain secondary response. Such that the affinity of a late immunoglobulin is higher than that of an early immunoglobulin. Such continuous increase illustrated the average antibody's affinity is called the affinity maturation.

This concludes that the immunological memory is an immune response of enormous progressive efficiency and sufficiency. Whereby, antibody can be made much more rapidly, in higher quantity, endures longer, and it is made with higher affinity in order to bind more effectively to its target. The mechanisms involved relate to the clonal selection theory.

It is necessary to note that that immune responses vary immensely in their duration, intensity as well as persistence (Gutman, 2001).

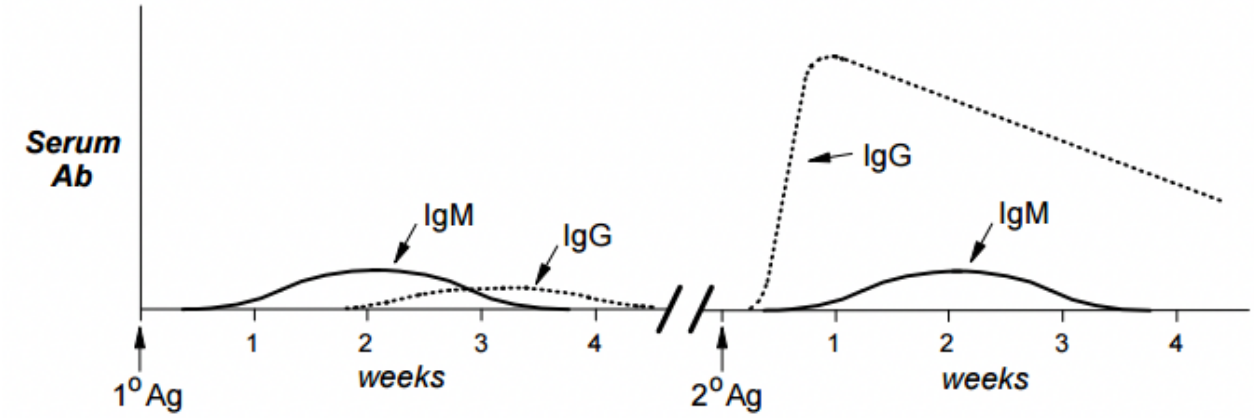

Figure 2-3. Primary and secondary humoral responses. Results demonstrating the results of immunizing an animal with a "typical" antigen. The X-axis is the time after immunization in weeks, while the Y-axis represents a measure of the amount of antibody in the serum. (Graph credit to Gutman, 2001).

While Figure 2-3 shows the antibody responses to a typical antigen in a typical species, any unique response may vary in many of its characteristics. A single antigen may cause much slower or a much faster response than another, or perhaps it does not have the capability to generate a secondary response (Gutman, 2011).

2.1.3.6 Isotypes Mechanism in Opsonization

Immunoglobulin IgG is involved in efficient opsonization of pathogens that induces the phagocytosis by macrophages and neutrophils (Tiwari and Sinha, 2008) and thus engulfing the pathogens. It is an essential factor in antibacterial defenses. Such opsonization mechanism allow for the activation of the complement system. Immunoglobulin IgA is on the other hand a much less potent opsonin and also allows a weak activation of the complement system (Janeway et al., 2001). This is due to the fact that IgG functions

predominately in the body tissues, in which accessory cells and molecules are present. While, immunoglobulin IgA functions mostly on epithelial surfaces in which the complement and phagocytes are absent; thus, playing a role mainly as a neutralizing antibody (Janeway et al., 2001).

Antibodies permit interaction of various types of cells with antigen–antibody complexes via the Fc receptors. Fc receptors (**FcR**) are protein molecules that exist on the surfaces of macrophages and neutrophiles. Due to their feature of immunoglobulin-like extracellular domains, Fc receptors classify to the Ig gene superfamily (Elgert, 1998). Numerous biologic functions can be triggered through the crosslinking of any Fc receptor classes. Macrophages allow for the enhancement of engulfing of antigen–antibody complexes through Fc receptors. The interaction among the Fc portions of the antibodies and the Fc receptor of phagocytic cells cause the binding of the pathogen to the phagocyte membrane. Such cross-linking of the Fc receptor by binding to a range of antibody Fc regions prompts a signal-transduction pathway that cause phagocytosis of the antigen-antibody complex (Tiwari and Sinha, 2008). Other types of cells expressing FcR (CD16) may use antibody-dependent cell-mediated cytotoxicity (**ADCC**), to lyse target cells coated with IgG. Particular immunoglobulin such as IgA, can be translocated to the lumens of mucosal-lined organs in order to contribute a mucosal immunity (Elgert, 1998).

2.1.3.7 Isotype Mechanism in Phagocytosis

Immunoglobulin IgG, four-chain monomer generated in mass in quantities in secondary immune responses has the capability of activating the complement system. In addition, IgG molecule's tail portion can bind to specific receptors on macrophages and

neutrophils. This mechanism is carried through by Fc receptors. The phagocytic cells attach, engulf, ingest, and then destroy harmful elements that have become coated through opsoninzation with the immunoglobulin IgG that is produced in response to the pathogenic infection (Alberts et al., 2002).

2.1.3.8 T-Dependent Antigens versus T-Independent Antigens

Antigens are categorized as either T-cell dependent antigens (**TD**) or T-cell independent antigens (**TI**); this depends on whether the T-lymphocyte support is required in order to induce an antibody response (Mohey et al., 2009).

For the majority of antigens, the production of antibodies by B-lymphocytes is dependent on the incentive of helper T-cells (Murphy, 2012).

T-cell independent antigens can be further categorized into T-cell independent antigen types: type I and II. The T-cell independent antigen-I, or **TI-I Ags** (or **TI-1 Ags**) such as LPS, are B cell mitogens (Mohey et al., 2009) as they induce several cell divisions. They are responsible for non-specifically or polyclonally activating majority of B-lymphocytes through Toll-like receptors (Mohey et al., 2009; Lesinski et al., 2001; Obukhanych et al., 2006). These are expressed on the surface of B-cells post BCR stimulation (Murphy, 2012). In higher concentrations, TI-1 Ags can attach to B-cell receptor (**BCR**) and T-cell receptor (**TCR**) of different clones of B- cells which influence the formation of multi-clonal antibodies. However, when the concentration of TI-1 Ags is low, it can only elicit only B-cells with particular binding of TI-1 on their BCR, and thus causes the formation of monoclonal antibodies (Murphy, 2012; Dintzis et al., 1976). This is crucial step particularly in immune response during early stages of infection by extracellular pathogens, due to the

fact that it is swiftly activated and there is no need for the support of T-cell or clonal maturation and expansion. (Murphy, 2012; Dintzis et al., 1976).

The **TI-II Ags** (or **TI-2 Ags**), for example as polysaccharides, of which are large molecules consisting of repeating epitopes that are capable of initiating the complement system, however they are unable to activate MHC-dependent T-cell support (Mond et al., 1995; Mohey et al., 2009). TI-2 antigens are able to evoke tough and enduring primary antibody responses; confer a long-term humoral protection (García de Vinuesa et al., 1999). Although they cannot evoke a recall response; that is, they are unable to increase antibody generation onset of secondary immunization, TI-2 can however react to secondary objection once adoptively moved into naive irradiated receiver; and with the injection of immune serum into naive receiver prior adoptive transfer cause response suppression. (Obukhanych et al., 2006). Thus, TI-2 Ags do not possess an intrinsic B-cell eliciting activity. Rather, the incitement of B-cells is led by the cross-linking of a critical number of B-cell receptors, which causes the aggregation of BCRs and thus the cross activation of such receptors. Such mechanism generates the proliferation and differentiation of B-cells and the production of antibodies (Murphy, 2012). TI-2 Ags are known to activate only mature B-cells, immature B-cells on the other hand are energized, thus they are unable to activate an immune response (Murphy, 2012). TD antigens are able to induce memory B-cells. These are produced in the TD germinal centers and can be recognized by somatic mutations in their immunoglobulin loci or either via surface expression of secondary immunoglobulin isotypes (Mohey et al., 2009; Obukhanych et al., 2006).

Contrary to TD immune responses against protein antigens, TI responses against polysaccharides illustrate long-lasting humoral immunity in the absence of recall responses and also are not known to generate memory B-cells (Obukhanych et al., 2006).

2.1.3.9 Class Switch of Isotypes

Idiotypes describe the antigen-binding specificities of immunoglobulins through depicting unique structural amino acid sequences in the variable regions. The variability in these determinants lie in the hypervariable regions surrounded by the framework residues. Along so, diversity as powerful aspect of the lymphatic system plays role in generating limitless array of various antibody molecules, each with its own antigenic specificity.

As immunoglobulin sequences compile, antibody molecules observed to be composed of unique amino acid sequence in the variable region yet only one of a definite quantity of invariant sequences located in the constant region. The fundamental genetic for such amalgamation in a single protein molecule is concentrated in the arrangement of the immunoglobulin genes (Goldsby et al, 2002; Albert et al, 2002).

The B cell development process is carefully regulated through first the maturation of a progenitor B cell progresses via an ordered sequence gene rearrangements of immunoglobulin that is added to changes in gene that lead to diversity in the complete product. As a result, a mature immunocompetent B cell will be composed of coding sequences for a single functional heavy chain variable domain and a single light chain variable domain. A B-cell is therefore antigenically devoted to a particular epitope. Post the induction of an antigen of a mature B cell in secondary lymphoid organs, further

rearrangement of the constant domain gene segments can produce modifications in the isotype being expressed, hence causing alterations in the functions of biological effector of an immunoglobulin without altering its specificity. Hence, a mature B cells consist of chromosomal DNA that is no longer identical to germline DNA. Still in the absence of an antigen induction, an organism is yet capable of producing 10^{12} varying immunoglobulin molecules, as its pre- immune collection of antibody. In addition, the antigen-binding sites of numerous immunoglobulins are capable of cross-reacting with various of related yet different antigenic determinants, causing the antibody defense force to be much more arduous. The pre-immune repertoire is large assuring the fitting of antigen- binding site to approximately any potential antigenic determinant, although possessing low affinity. Post the repeating stimulation by antigen, B cells can generate immunoglobulin that attach their antigen with much greater affinity. Here, the induction of antigen greatly increases the antibody defense (Goldsby et al, 2002; Albert et al, 2002; Tusculum College; 2016; DNA sequencing demonstrated the appearance of particular recombination signal sequences (**RSSs**) flanking each germline V, D, and J gene segment; (Variable, Diversity, Joining). One RSS is found at 3' to each V gene segment, and 5' to each J gene segment, and along on both sides of each D gene segment. Such sequences serve as signals for the recombination process that realigns the genes.

Individual RSS consists of a conserved palindromic heptamer and a conserved AT-rich nonamer sequence that disassociate by an intervening sequence of either 12 or 23 base pairs. The intervening 12- sequences relate to one turn of the DNA helix while the 23-bp relate to two turns of the DNA helix. This is why the sequences are known as one-turn

recombination signal sequences and two turn signal sequences. The **Vκ** (Variable Kappa light chain) signal sequence possess one-turn spacer whereas the **Jκ** (Joining Kappa light chain) signal sequence possess two-turn spacer.

In **λ** light-chain DNA, this order is reversed; that is, the **Vλ** (Variable Lambda light chain) signal sequence has a two-turn spacer, and the **Jλ** (Joining Lambda light chain) signal sequence has a one-turn. spacer. Within the heavy-chain DNA, the signal sequences of the **VH** (Variable Heavy chain) and **JH** (Joining Heavy chain) gene segments consist of two-turn spacers, the signals on either side of the **DH** (Diversity Heavy chain) gene segment consist of one- turn spacer. Signal sequences classifying a one-turn spacer can combine only with sequences classifying a two-turn spacer. This is known as one-turn/two-turn joining rule. This joining rule secures that for instance a **VL** (Variable Light chain) segment attaches to a **JL** (Joining Light chain) segment and not to another VL segment; this rule similarly also assures that V_H, D_H, and J_H segments combine in an appropriate order and that segments of the exact types do not combine with one another (Goldsby et al., 2002; Albert et al., 2002; Janeway et al., 2001; Chaplin, 2009; Tusculum College; 2016).

2.2 METHIONINE

2.2.1 Amino Acid and Methionine Overview

Amino acids **(AA)** are the building blocks of biological proteins. Over 500 AA exist in nature, though 20 of which are capable of incorporating into proteins by arranging in myriad of ways (i.e metabolic proteins, structural proteins, enzymes, as well as precursors of multiple body constituents) serving variety of functions (Applegate and Angel, 2008; Emmert et al., 1996). AA obtained from protein are utilized by avian species to fulfill a variety of function. Such that, proteins are underlying constituent of structural and protective tissues such as skin, feathers, ligaments, bone matrix, bone matrix, soft tissues, inclusive of organs as well as muscles (NRC, 1994).

The carbon skeleton of AA categorizes the dietary essentiality of AA (Wu, 2009). According to the National Research Council's **(NRC),** ten out of the twenty-two AA (arginine (**Arg**), histidine (**His**), isoleucine (**Iso**), leucine (**Leu**), lysine (**Lys**), Methionine (**Met**), phenylalanine (**Phe**), threonine (**Thr**), tryptophan (**Try**) and valine (**Val**) found in body proteins are classified as the essential AA (**EAA**) for the nutrient requirements of poultry (NRC, 1994); that is, they are indispensable as their carbon skeleton cannot be synthesized de novo by the avian species. In contrast, AA that can be synthesized by the animal are known as nonessential AA **(NEAA)** (Wu, 2009; Leeson and Summers, 2009; Lesson and Summers, 2001). It was assumed that animals do not require NEAA in their diets for maximal nutrition as they are capable of synthesizing adequate quantity of NEAA. Scientific research on animal as well as cell culture have nonetheless demonstrated

evidence of NEAA carrying critical roles in multiple signaling pathways, while further demonstrating the importance of considering AA functions beyond their protein synthesis as young animals are unable to synthesize adequate amounts of NEAA particularly during early phase development to support their growth as the rates of utilization are relatively greater than rates of synthesis; to poultry this is true for Arg and proline (**Pro**). Such AAs are referred to as conditionally EAA (**CEAA**) (Wu, 2009, Kim and Wu, 2004; Mateo et al., 2007; Shen, 2013).

Functional AAs **(FAA)** is rather a notion developed to further define those AAs that regulate and engage in key metabolic pathways to improve health, survival, growth, development, and the reproduction of organisms. An observed deficiency in FAA whether it be EAA or NEAA hinders both the synthesis of protein as well as the species' body homeostasis (Wu, 2010).

Methionine is an EAA as it cannot be biologically synthesized by the bird and due to its exceptional emphasis on poultry growth and production (Osti and Pandey, 2004). Met is also classified as a FAA. Animals ought to obtain EAA from the diet, however many feed ingredients lack some of the EAA. In a typical corn-soybean meal, birds' requirement for fulfilling certain EAA may fail; this is the case for lysine (**Lys**), methionine (**Met**), threonine (**Thr**), and tryptophan (**Trp**). Met is considered the first limiting AA, with lysine and threonine being the second and third limiting AA respectively in a practical corn-soybean poultry fed diet (Gill, 2003; Fernandez et al., 1994).

When formulating bird diet, sulfur amino acids (**SAA**) are crucial addition. Met and cysteine (**Cys**) are the two sulfur-containing AA (**SAA**); both are the principle providers of

organic sulfur within the avian body and ought to be considered together as total sulfur amino acids (**TSAA**) nutrient requirements (Aldrich, 2007; Knowles and Southern, 1998; Cole and Haresign, 2004; Novak et al., 2004).

All AA must be present in cell into order for correct protein synthesis to take place. Therefore, it is crucial for protein and EAA to be supplied by the diet, where formulating dietary requirements for both ensures the appropriate approach for physiologically required AA to be implemented (Leeson and Summers, 2001).

Met is ought to be available sufficiently in the diet to provide the building blocks of tissues, immune cells and to support the development of feathers (Baker, 2009; Fanatico, 2010, Leeson and Summers, 2009). Elevated levels of Met exist in eggs, sesame seeds, in addition to Brazilian nuts, fish meal, corn gluten meal, alfalfa meal, as well as sunflower seeds meal (Blair, 2008; Fanatico, 2010; Burley, 2012). Yet, Met is found to be limited in most natural sources of plant protein and it is therefore established as the first limiting AA for broilers and the second limiting AA for laying hens in a typical corn-soybean meal ration (Blair, 2008; Li, 2015).

2.2.2. Methionine Metabolism

Methionine possesses an empirical formula of C5H11NO2S, Chemical Abstracts Service (**CAS**) Registry Number (RN) 63-68-3; US Food and Drug Administration (**FDA**) Unlicensed National Information Infrastructure (**UNII**) with molecular structure demonstrated in Figure 1-1 (FDA, 2017; ChemIDPlus, 2017). The sulfur-containing EAA has an asymmetric form, constructing both L- and D-isomers and the molecular structure of racemethionine is illustrated in figure 1-2 below. The liver is the main site of Met

metabolism where Met is the first limiting AA in commercial poultry (Finkelstein, 1990). The metabolism of Met is vital for numerous physiological processes (Figure 2-3). Expressly, Met is principally involved in the aid of methyl transfer reactions via its conversion to its active form as S-adenosylmethionine (**SAMe**) which is achieved by the catalyzation of methionine adenosyltransferase I (**MAT I**) and methionine adenosyltransferase II (**MAT II**) (Mato et al., 1997; Horikawa and Tsukada, 1991). The fact that vertebrates are unable to synthesize methyl groups which are essential constituents in their diets (Kidd et al., 1997) proves Met methyl role a crucial one. Other than Met, potential dietary sources of methyl groups include choline, folic acid and betaine (Kidd et al., 1997); however, they differentiate in their methylation availability and reactions. In the case of Met; it is necessary for protein synthesis. While as for instance with choline; it is primarily utilized in cell membranes as well as neurotransmitter (Metzler-Zebeli et al., 2009; Stryer, 1988).

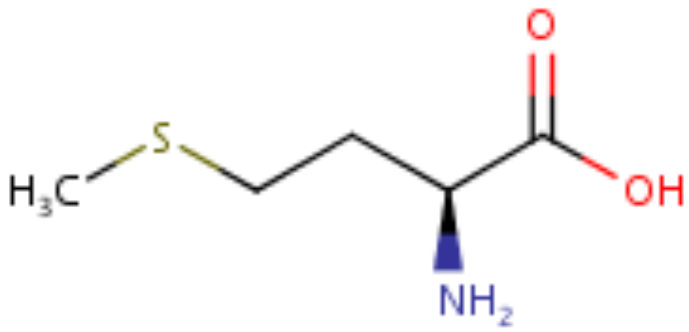

Figure 2-4. Molecular structure of methionine, CAS RN: 63-68-3; UNII: AE28F7PNPL (ChemIDplus, 2017; FDA, 2017) [Diagram credit to ChemIDplus, 2017].

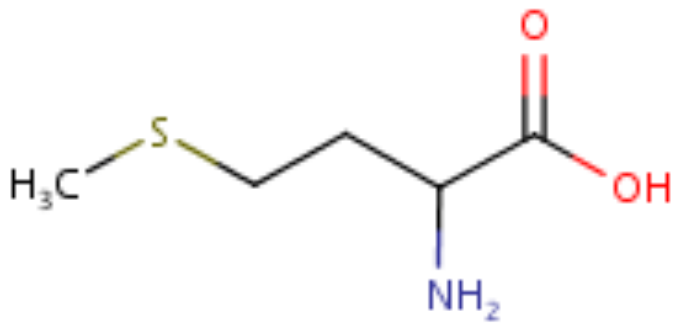

Figure 2-5. Molecular structure of racemethionine, CAS RN: 59-51-8; UNII: 73JWT2K6T3 (ChemIDplus, 2017; FDA, 2017) [Diagram credit to ChemIDplus, 2017].

Table 2.1. Chemical and Physical properties of Methionine[1].

Name	L-Methionine
IUPAC	L-2-amino-4-(methylthio)butyric acid, S-2-amino-4-(methylthio)butanoic acid, H-Met-OH
Formula	$C_5H_{11}NO_2S$
Molecular weight	149.208g/mol
Water solubility (20°C)	53g/L
Vapor Pressure	5.23E-07mm Hg (25°C)
Melting point	283 dec °C
***pKa*[1] (-COOH)**	2.28 (25°C)
***pKa*[2] (-NH3)**	9.21 (25°C)
Isolelectric point (*pI*)	5.74 (25°C)
Henry's Law constant	2.11E-11 atm-m3/mole (25°C)
pH (1% aqueous solution)	5.6-6.1

[1]Information and values credit to PubChem, 2017; ChemIDPlus, 2017.

Post its conversion in Met metabolism, SAMe is next capable of behaving as a methyl-donor by releasing its terminal methyl group (and is the predominant methyl ($-CH_3$) donor in Met metabolism) of which can occur in an assortment of methyltransferase reactions (**E.C. 2.1.1**) (Metzler-Zebeli et al., 2009; Abbexa, 2017). Such reactions permit the synthesis of choline, creatine, epinephrine, glutathionine, lipoic acid, DNA as well as several other necessary compounds (Li, 2015; Rack, 2008; Kanehisa Laboratories, 2017). Once SAMe has donated its methyl group, it is irreversibly converted to S-adenosylhomocysteine (**SAH**). Via the removal of an adenosine molecule, adenosylhomocysteinase converts SAH to homocysteine (**HCys**). Then there are two fates of HCys; it is either transsulfurated to Cys or remethylated to Met in Met metabolism (Finkelstein, 1990).

Governed by the activity of Cystathionine β-Synthetase, HCys merges with serine constructing a molecule of cystathionine. This is broken down via cystathionine-γ-lyase into a molecule of Cys and a molecule of ∝-ketobutyrate (Li, 2015; Metzler-Zebeli et al., 2009; Burley, 2012). In both of these reactions, a co-factor of vitamin B6 is obligatory in the form of pyridoxal phosphate (Brosnan and Brosnan, 2006). The ∝-ketobutyrate generated through this pathway is later converted to propionyl-CoA via multiple steps using ∝-ketoacid dehydrogenase. Succinyl-CoA (**SucCoA**), the end product metabolized from propionyl-CoA then enters the Citric Acid Cycle (**CAC**) (also known as tricarboxylic acid (**TCA**) cycle or the Krebs cycle) to generate energy (Finkelstein, 1990; Metzler-Zebeli et al., 2009). Thereby, two molecules of the regenerated cysteine will compose a single

molecule of cysteine, that is the structural component of keratin; the leading protein present in skin, hair, nails, and feathers (Li, 2015; Burley, 2012).

Alternatively using vitamin B12 as a cofactor; HCys can be remethylated to Met from N5-methyl-tetrahydrofolate by the route of methionine synthase (**MS**) (Finkelstein, 1990; Brosnan and Brosnan, 2006). In addition, post its development by the oxidation of choline; homocysteine can also be remethylated to Met from betaine via betaine-homocysteine methyltransferase (**BHMT, E.C.2.1.1.5**) (Finkelstein, 1990; Metzler-Zebeli et al., 2009; Škovierová et al., 2016).

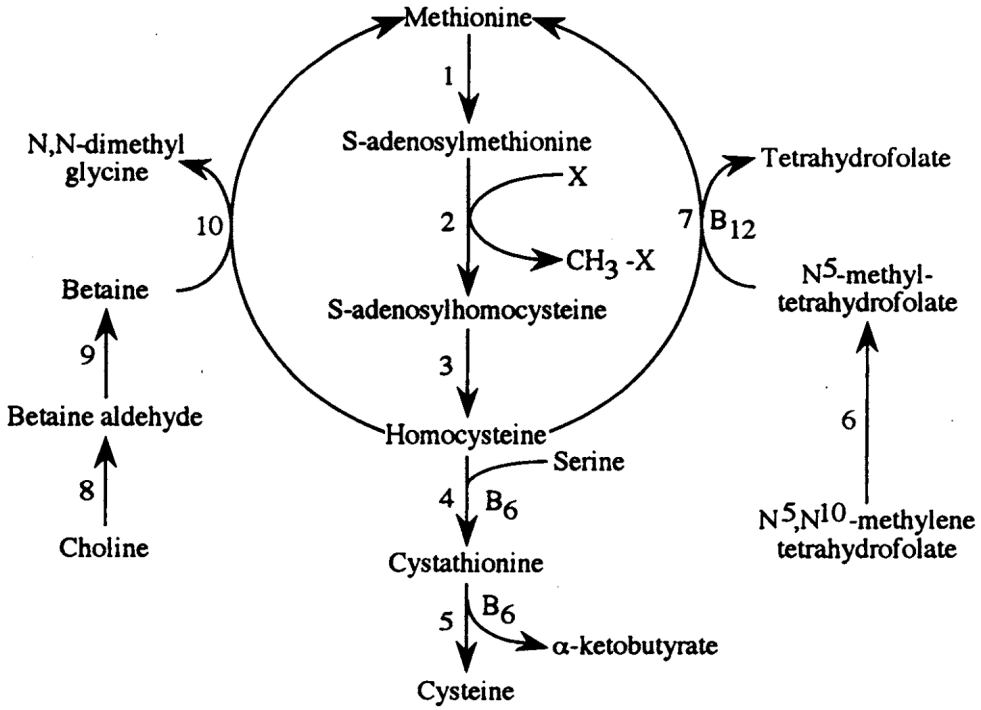

Figure 2-6. Methionine metabolism. Enzymes depicted are as followed (1) methionine adenosyltrasferase, (2) various enzymes, (3) S-adenosylhomocysteine hydrolase (EC 2.1.1), (4) cystahionine β-synthase, (5) cystathionine γ-lyase, (6) α-ketoacid dehydrogenase, (7) N^5, N^{10}- methylenetetrahydrofolate reductase, (8) methionine synthase, (9) choline dehydrogenase, (10) betaine aldehyde dehydrogenase, and (11) betaine-homocysteine methyltransferase (EC 2.1.1.5) (Illustration adopted from Emmert et al., 1996).

2.2.3 Choosing Between Cysteine and Methionine as the Ideal Sulfur Amino Acid in Diets

The reason for Met to be perceived as an ideal **SAA** than **Cys** is due mainly to the fact that Met is an EAA and Cys is not. In addition, TSAA requirement can be provided solely by the metabolism of Met. This is achieved via Met transsulfuration pathway where Met serves as a precursor of Cys. In contrast, Cys does not serve as the precursor of Met due to the irreversibility of Met transsulfuration pathway (Finkelstein, 1990; Wheeler and Latshaw, 1981; Finkelstein et al., 1988; Baker et al., 1996). Yet, studies on Met-sparing effect of Cys (Wheeler and Latshaw, 1981; Finkelstein et al., 1988; Baker et al., 1996) where Met utilized in transsulfuration pathway was replaced with Cys demonstrated to be inadequate. This is because a raise dietary Cys and a reduction in dietary Met consequently raise dietary organic sulfur at the same concentration of TSAA, which means Met-sparing experiments may alter the quantity of organic sulfur in TSAA (Chung and Baker, 1992; Adegboyega Fatufe, 2004).

2.2.4 Forms of Methionine

Mueller, in 1923 was the first to isolate Methionine from casein and since then much research has been towards the importance of Met in nutrition and animal feed (Mueller, 1923). AA, apart from glycine (**Gly**) (Wu, 2009, Baker, 2008) are available in two forms which are referred to as D- or L enantiomers. In nature, Met is predominantly present in the L-form. While, the D-enantiomer is biologically inactive. Poultry are capable of utilizing both D- and L- forms of Met (Jacob, 2013). Both forms can be metabolized by a DL-

racemase, which is needed for supplementing the chemically synthesized DL-methionine racemate as feed additive in livestock farming (Willke, 2014). Met usually exists as a white crystalline powder (Li, 2015). As a limiting AA, Met is supplemented in the form of dry D,L-methionine (**DLM**), racemic 2-amino-4-methylthiobutyric acid, with approximately 99% purity (Jacob, 2013) , or liquid as either D,L-methionine hydroxy analog-free acid (**MHA-FA**) with equivalency of 88% racemic 2-amino-4-methylthiobutyric acid or DL-methionine hydroxyl analog calcium (**MHAC**) with 97% racemic 2-amino-4-methylthibutyric acid and calcium salt activity post-conversion of the analog into the active biological form (Jacob, 2013, Li, 2015) according to the Association of American Feed Control Officials (**AAFCO**) Code of Federal Regulations (**CFR**), Title 21CFR 582.5477 (AAFCO, 2012; USDA, 2017). As α-keto acid analogs (the amine group replaced by a hydroxy group), MHA-FA and MHAC in avian species can be converted to the amino form via liver transamination applied by non-EAA (i.e. glutamic acid) (Leeson and Summers, 2009, Li, 2015). **DLM, MHA-FA, MHAC** are all recognized as safe by the Food and Drug Administration (FDA) according to Title 21CFR 582.5475 and Title 21CFR 582.5477 (FDA, 2017, USDA, 2017).

As mentioned earlier, it is not solely the level of methionine that is required but rather a balance of all EAA. Focusing only on methionine levels will generate AA imbalances and thus protein degeneration. Moreover, antagonistic relationships between certain AA have been present, an example of this exist between branched-chain AA (**BCAA**) leucine-isoleucine-valine, threonine and tryptophan and between arginine and lysine (Farran and Thomas, 1992; Jacob, 2013).

Met is commercially synthesized via acrolein and methyl mercapton condensation

(Goldfarb et al., 1981, Lüssling et al., 1981). This compressed compound is then reacted with ammonia and hydrogen cyanide to generate a racemic mixture of the D and L isomers of Met which is effectively 100% pure (Goldfarb et al., 1981). The resulting compound is 1:1 ratio of D-Met and L- Met; where the racemic mixture DL-Met is a of 50% D-Met and 50% L-Met (Lüssling et al., 1981; Willke, 2014).

Most of this manufactured Met is used for animal feed in livestock production amounting above 600,000 tons/year in 2013 according to the world market (FeedInfo, 2014; Willke, 2014). This does not include organic farming due to the ban or high limitation on synthetic Met usage (Fanatico, 2010; NOP, 2014). Met has also been shown to be synthesized by bacterial fermentation (Syldatk et al., 1999; May et al., 2000; Kim et al., 2008) for up to 5 g/L with unemployment of genetically modified organisms (**GMO**s) (Willke, 2014). The topmost concentration level of L-methionine via fermentation depicted at 35 g/L using a GMO of *Escherichia coli* (**E. *coli***) (Willke, 2014). Yet, to naturally synthesis Met, the process undergoes complex regulations including mutations, genetic modification, selection and optimization with only few strains of bacteria are capable of producing relevant amounts of Met, a major one being *Corynebacterium glutamicum* (**C. *glutamicum)*** (Lee and Hwang, 2003; Willke, 2014; Bolten et al., 2010).

As to whether which of L-, D-, or D-L- Methionine is more efficiently absorbed, statistical methodology and research trials to evaluate the precise bioefficacy for D-, L-, DL-Met have estimated to be comparable (Sauer et al. 2008; Katz and Baker, 1975; Tipton et al., 1966; Littell et al. 1997; Dilger and Baker, 2007).

2.2.5 Methionine Requirement

2.2.51 Nutritional Requirement

Birds eat to satisfy their metabolic energy (**ME**) requirement (Ewing, 1951; Leeson and Summers, 2001). As with all animals, poultry require energy (carbohydrates, and fats), protein, vitamins, minerals and water (Fisher and Boorman, 1985; Leeson and Summers, 2001). Formulation of diet must meet the EAA and these ought to be relative to the level of ME. Several factors can affect dietary AA requirement and their utilization efficiency (Bell and Weaver, 1990). These include age gender, genetics, reproductive state, stage of production, ambient temperature, housing system, immunological stressors, production aim as well as metabolic energy, the level of proteins, and the availabilities of vitamins and minerals in the diet (NRC, 1994, Leeson and Summers, 2009; Leeson and Summers, 2001). Poultry do not have specific requirements for crude protein (**CP**) per se; rather only for AA levels. Yet, CP should be present to meet the requirements of EAA, and enough nitrogen (**N**) to synthesize the non-EAA. Amino acid requirements are usually presented as percentages of the diet or may percentage of protein requirement. National Research Council's (**NRC**) *Nutrient Requirements of Poultry* is the fundamental reference for feed formulation. Dietary AA can be classified as both qualitative and quantitative (Wu, 2014). Qualitative requirements rest under questions of "*what*" are the AA needed for maintenance, optimal performance; including growth, lactation and reproduction and optimal health including prevention of abnormalities, resistance and the recovery to infectious diseases (Wu, 2009; Wu, 2014). Quantitative requirements follow questions of "*how much"* of an AA is needed for maintenance, optimal growth and health (Wu, 2014).

The nutritional requirements of broilers are applied for starter, grower, and finisher phases as the requirements change with age; generally with decrease in AA levels and an increase in ME. Overall CP contents of 23, 20, and 18% are applied for starter, grower, and finisher stages, respectively.

An unbalanced diet leads to poor poultry performance. In the case of deficient protein diet, i.e metabolic energy to crude protein (**ME:CP**) ratio; birds will overconsume energy to obtain sufficient protein (Fanatico, 2010). Increasing the level of Met in broiler diets significantly reduces the abdominal fat (**AF**) content, this was reported by Summers and Leeson (1985) whose work agreed with results by Mabray and Waldroup (1981) demonstrating decreased abdominal fat (AF) weights as the levels of dietary Lys and Met increased. Deficient EAA diets can increase FI. According to Cherry & Siegel (1981) pullets' diets equal in energy and contained 15% crude protein with only difference in levels of Met and SAA observed increase in FI to compensate for a marginal SAA deficiency, and that the SAA requirement for maximum feed conversion (**FC**) efficiency was higher than the requirement for egg production (**EP**).

Including ME and AA content in feed, feed intake (**FI)** could be influenced by nutritional value and toxicity of the feed, palatability, particle size and environmental temperature (Zhai et al., 2014; Leeson and Summers, 2001). With high metabolic energy diets, poultry FI decreases and AA intakes are thus restricted (Emmert and Baker, 1997; Leeson and Summers, 2009; Li, 2015).

During heat stress (high ambient temperature and humidity), FI decreases, evoking reduced growth and egg production, poor performance, leading to physiological and immunological

stress, susceptibility to disease, reduced welfare status and high mortality rates. This results in detrimental effects of reduced welfare status of birds and economic losses to poultry production (Dhagir, 2008).

In period of heat waves, it is important to implement a combination of methods to aid in alleviating heat stress, ranging from housing, management, and feeding practices (Daghir, 2008; Bell and Weaver, 1990). An additional method is nutritional manipulation. Administering high AA and high protein diets to birds during high ambient temperature demonstrated negative impact body weight gain (**BWG**), feed efficiency (**FE**), and carcass composition and yield (Cheng et al., 1997; Daghir, 2008; Osti et al., 2017; Abu Dieyeh, 2016.). Thus as agreed with Willemsen et al. (2011) and Zhai et al. (2014) AA supplementation may partially prevent growth depression of heat stressed flocks, yet it is supplementation of low- apparent metabolizable energy (**AME**) and high AA diets at high temperature that significantly decreases the bird's FI, BW, absolute carcass, breast, wing, front half, including back half weights (Fisher and Boorman, 1985; Leeson and Summers, 2001).

Universally recognized as the first limiting AA in broilers, methionine is also the second limiting AA for laying hens fed on practical corn-soybean meal diets (Leeson and Summers, 2001; Wen et al., 2014). NRC (1994) requirement for methionine are 0.50, 0.38 and 0.32% for starter (0-3 weeks of age), grower (3-6 weeks of age) and finisher (6-8 weeks of age) broiler phases respectively; with 90% dry matter (**DM**) basis of 0.90, 0.72, and 0.60% total TSAA requirement respectively.

If the NRC requirement is not met for any EAA, the efficiency of poultry production is reduced immensely with great losses in broiler growth and egg size in laying hens (Fanatico, 2010; Jankowski et al., 2014; Wen et al., 2014). Therefore, synthetic Met in form DLM, MHA-FA or MHAC are generally supplemented in poultry feed to meet their dietary requirements. It is crucial to adjust the concentration of all nutrients in diet in relation to the level of metabolic energy to provide a nutrient balanced diet. Thus, in the case of increased ME, Met requirement increases (Ewing, 1963), and it is recommended to prepare poultry feed with AA needs calculated as percentage of ME. Donaldson *et al*. (1956) observed that as wider the ratio of ME to protein tended to be, broiler consumed greater energy and deposited greater fat and less water in their carcasses. The present-day commercial bird is very distinct from commercial birds prior to 1991 (Havenstein *et al*., 1994; Applegate and Angel, 2014), some research suggests that AA requirement today differ greatly from those highlighted by the NRC (1994) as to reasons of genetic selection, management practice and feed-related alterations (Applegate and Angel, 2014). Other studies state that increasing levels of Met ought to be above the NRC (1994) recommendations (Wallis, 1999; Gorman and Balnave, 1995; Nadeem et al., 1999; Schutte and Pack,1995). Further, studies by Tsiagbe et al. (1987a); Shini et al., 2005; p;;vcjbhbSwain & Johri, 2000 report that methionine requirements for optimal immunity are higher than for optimal growth.

2.2.5.2 Fast-growing versus Slow-growing Broilers Requirements

Dietary AA concentrations ought to match the needs for both maintenance and skeletal muscles (Kidd et al., 2004). The requirement of AA of fast-growing broiler breeds may be

greater than slow-growing breeds. This is coherent with higher protein to fat ratio of fast-growing genotypes than slow-growing genotypes (Morris et al., 1992, Fanatico, 2010). Thus, higher AA to ME ratio is required for faster growing breeds (Gous, 2010). As expected, the rapid growth rate of broiler today requires increased contents of nutrients and ME daily, however these demands for different nutrients are not in the same proportions as previously stated (Morris and Njuru, 1990; Gous, 2010).

Met requirement of fast-feathering versus slow-feathering genotypes has been indicated to be the same of Met level of 0.46% and 0.46% respectively for optimized nitrogen retention and 0.50% and 0.50% respectively for optimized version (FC) during the grower phase (Kalinowski et al., 2003, Ajang et al., 1993). As a sulfur AA and compared to Met, Cys requirement for fast and slow growing strain illustrated to be lower, with 0.44% and 0.39% respectively (Kalinowski et al., 2003).

Methionine and total sulfur amino acid requirements for broilers with fast, medium, and slow growing genotypes have demonstrated to be analogous in the starter and grower phases, (Fanatico et al., 2006, Fanatico et al., 2009; Leeson and Summers, 2001). Increasing graded level of Met in basal diets has significantly increase BWG, but no interaction has been illustrated between the Met content and broiler genotype (Fanatico, 2010). Yet an interaction was evident taking into consideration the measurement of breast yield. Breast yield of fast-growing broilers responded to increasing dietary content of Met, breast yield of medium-growing breeds responded solely to the intermediate content of Met, and breast yield of the slow-growing breeds responded solely to the diets with higher content of Met (Fanatico et al., 2007, Fanatico, 2010).

2.2.6 Digestibility and Ideal Protein Ratio

An ideal protein (**IP**) is one that consists the explicit amounts of AA necessary for the animal without deficiencies or excesses (Glista et al., 1951; Dean and Scott, 1965). No particular assortment of AA requirements is assigned to any animal following conditions of age, gender, body composition and nutrition combined (Wu, 2014;) The concept of IP holds a mixture of EAA that explicitly match protein accretion and maintenance demand i.e meeting the animal's need for its specific growth stage or level of production, without under- or over-feeding of AA (Burley et al., 2016). In order to determine the ideal amino acid ratio, it is mandatory to know the digestible amino acid requirement of each EAA for the chicken and its relationship to lysine (Moore et al., 2001). IP ratios (AA-to-Lys ratios) are expressed as percentages of digestible Lys established on digestible AA requirements rather than TAA requirements (Vieira and Angel, 2012). This means that to consider Met requirement, it is crucial to focus on Met as well as a balanced profile of all the EAA (Applegate and Angel, 2008; Vieira and Angel, 2012). For this reason, the concept of IP is employed in diet formulation to aid in balancing and supplying with greater precision all the EAA in addition to the NEAA (such as glutamine, glutamate, proline, glycine and arginine which are influential in regulating gene expression, cell signaling, anti-oxidative responses, fertility, neurotransmission, and immunity) for ideal performance and increased profitability (Burley et al., 2016; Wu, 2014; Daghir, 2008).

Poultry possess very short digestive tract of which is particularly sensitive to pH alterations. Few natural crude proteins are gradually digested, thus the available AA that can be absorbed and deaminized prior to those that are gradually released are available for

absorption. The liver however is not capable of storing AA, this means that if the AA are not absorbed when necessary, they cannot be utilized for protein synthesis (Patrick and Schaible, 1980). Several factors may influence protein digestibility. Yet AA content and chemical analysis may demonstrate a complete essential AA profile; factors as solubility, structure and type of proteins can affect digestibility (Patrick and Schaible, 1980). Regular assays for requirements are not satisfactory. This is due to the factors affecting AA requirements: dietary ME or CP levels, age of birds, genetics and gender. Thus, it is impossible to address all these factors in one trial, taking into consideration individual AA. This is the reason for a need of ideal ratios with Lys used as reference AA. The justifications for using Lys as a reference for ideal protein is 1) Lys is the second limiting AA in poultry diet, and in fact supplementing a limiting AA (i.e. methionine and lysine) to poultry diets increases the efficiency of protein utilization, and in turn N excretion will be reduced; 2) Lys is easier to analyze than Met or Cys, 3) Lys is almost exclusively used for body protein and thus not complicated by pathways related to maintenance and feathering 4) Ample of data for the digestible Lys requirement of poultry are available, and 5) Lys requirement for several dietary, environmental, and body compositional circumstances are readily available (Emmert and Baker, 1997; Leveille et al., 1960; Fisher 1998; Leeson and Summers, 2001; Schutte and De Jong, 1999; Brown et al. 2006).

Yet, the NRC (1994) lists the TAA requirement as opposed to levels of digestible AA; and in ingredients of practical plant based diet, the content of AA is not equal to the available AA content for the presence of anti-nutritional factors (Schutte and De Jong, 1999; Baker et al., 2002). To determine the digestibility of feed ingredients, the below equation is used.

This classical method for evaluating feed ingredients where measurements of digestibility allow for determining the amount of a certain nutrient absorbed in gastrointestinal tract (GI) from a given quantity of food. For instance, the % digestibility of protein is calculated as:

$$\frac{Dry\ weight\ of\ diet\ eaten\ \times\ \%\ Protein\ in\ diet - Dry\ weight\ of\ feces\ voided\ \times\ \%\ Protein\ in\ feces}{Dry\ weight\ of\ diet\ eaten\ \times\ \%\ Protein\ in\ diet} \times (100)$$

The similar equation may be utilized to determine the percentage of digestibility of CP, fat, dry matter, energy or any other nutrient (Leeson and Summers, 2001).

The use of an ideal AA ratio may aid in decreasing feed costs. Diets formulated on a digestible basis have illustrated to provide augmented performance when compared to diets formulated on a total amino acid basis (Fernandez et al., 1995; Moore et al., 2001).

Note that proteins of those which supply solely the desired level of essential AA are preferred to those which provide a high excess of some essential with minimum level of another essential. This is due to the fact that large surplus of one type of AA can be antagonistic to another (Patrick and Schaible, 1980). This antagonism relationship among AA particularly occurs between or among AAs belonging to the same group, such as Lys and Arg, or branched chain AA (Leu, Ile and Val) (Farran and Thomas, 1990). In such case increasing the level of one AA above its requirement necessitates increasing the level of the other AA (Farran and Thomas, 1990). It is important to highlight that this experimental thesis is aware and considers the antagonist relationship between AA as well as the previous research done on high excess of methionine demonstrating its negative turn out (Wu et al., 2012; Jankowski et al., 2014; Wu and Meininger, 2002, Zhang et al., 2008).

This is why within this thesis experiment methionine is not supplemented in high excess but rather slightly above nutritional requirements for poultry.

It is also important to note that the utilization efficiencies of individual EAA are different (Batterham 1994; Baker, 2009).

2.2.7 Methionine Deficiency

Malnutrition and infections are major obstacles to survival, growth, reproduction and health (Calder &Yaqoob, 2004; Field et al., 2002). Dietary AA deficiency hinder concentrations of majority of AA found in plasma while damaging the lymphatic system (Woodward, 1998; Wu et al., 1999; Dasgupta, 2005).

According to Elwinger and Tausen (2009) reduced MET levels decreased feather cover and egg weight (EW), though egg production (EP) was not affected. Futher, they observed that FI increased as feather cover deteriorated, hence a reduction in feed efficient (FE) was apparent. Met deficiency in poultry is presented with reduced growth, performance, FI, BWG, FCR, breast meat yield, and increased abdominal fat pad (**AFP**) deposition (Corzo et al., 2006; Carew et al., 2003; Leclercq, 1983). Met-deficient diet induces reduced growth and performance as feed lacking adequate level of Met to accommodate for maintenance, growth and production of poultry prompts for poor growth rate, FCR, BW, FCR, egg size and production for layers and breeders (Sekiz et al., 1975; Fanatico, 2010, Li, 2015). As Met is a SAA and sulfur is a major constituent of feathers, Met-deficient diet is associated with poor feather development. Bird with met-deficiency is likely to feed on feathers in an

effort to satisfy a craving for Met and as such feather picking can lead to cannibalism circumstances in a flock or lead the bird to pick on its own feathers resulting in higher incidence and/or severity of bacterial infections (Dahiya et al., 2007; Li, 2015; Ashton, 2015). Further SAA execute antioxidant functions in the avian body preventing destruction of cells. Rubin et al., 2007 have reported that higher levels SAA may be beneficial to resilience to diseases. Swain and Johri, 2000 demonstrated Met incorporation of (0, 1.5, 2.0, and 4.5 g/kg diet) significantly augmented ($P < 0.05$) the cellular immune response. Avian met-deficiency can well lead to a flawed lymphatic system, high morbidity and mortality due to debilitated mechanisms of T and B lymphocytes (Tsiagbe et al., 1987) (Refer to section *Immunopotentiating Function of Methionine* of this chapter for exhaustive information).

Not incorporating Met to diet formulation leads to elevated levels of CP and unbalanced IP ratios, leaving excesses of AA behind unemployed for growth, production, etc. that ought to be metabolized and excreted. This therefore propagates incidence of kidney disorders and elevated heat increment (**HI**). Unable to cope with high HI, birds undergo HS particularly in warm regions establishing high mortality rates (Khalil et al. 1968; Daghir, 2008). On the other hand, the supplementation of Met aids in lowering the pH level of urine via excretion of sulfate anion and thus impeding the development of kidney stones, uroliths, or urologic syndromes (Wideman et al., 1989; Li, 2015).

2.2.8 Methionine Toxicity

High levels of Met have been indicated to be toxic for avian species, this is in accordance with Zhang and Guo, 2008; Wu et al., 2012; Hafez et al., 1978; Scherer and Baker, 2000; Acar et al., 2001. Xie et al. (2004) stated that the high excess of DLM or DL-HMB-FA Met supplementation reduced WG and FI of ducks significantly. Particularly growth depression was observed in broilers as well as turkeys when Met content in diet was supplemented above 1% (Katz and Baker, 1975a; Hafez et al., 1978; Harter and Baker, 1978; Edmonds and Baker, 1987; Han and Baker, 1993; Carew et al., 1998; Scherer and Baker, 2000; Acar et al., 2001). It is worth noting that high levels of plasma homocysteine (HCys) is suggested to be an index of overage dietary Met considering HCys is a precursor for Met synthesis and a metabolite of Met degradation (Finkelstein, 1990; Frontiera et al., 1994).

2.2.9 Immunopotentiating Function of Methionine

As an essential and first-limiting amino acid, Methionine is suggested to play an influential role in both humoral and cell-mediated immune responses. Amino acids are required for clonal proliferation of lymphocytes; the delivering of new bone marrow monocytes and heterocytes and synthesis of effector molecules (immunoglobulins, lysozyme, nitric oxide, complement); formation of bursa of Fabricius' germinative centers to perfect immunoglobulin affinity; and the development of communication molecules (such as cytokines and eicosanoids) (Rubin et al., 2007; Jankowski et al., 2014). Multiple functions are dependent upon Methionine, however its notable roles include 1) protein synthesis; 2) precursor for glutathione; a tripeptide that curtails reactive oxygen species (**ROS**)

safeguarding cells from oxidative stress; 3) methionine is required for the synthesis of spermine and spermidine which are polyamines that engage in nucleus and cell division; and 4) methionine is a vital methyl donor, methylating the reaction of DNA as well as several distinct molecules (Kidd, 2004; Rubin et al., 2007, Wu et al., 2006; Ugarte et al., 2013). Considerable scientific research studies have demonstrated methionine protagonistic interference in the immune system of poultry, leading to an enhancement in both humoral and cellular responses. One reason for such effect is employed by the intracellular glutathione and cysteine levels (Rubin et al., 2007). Immune cells proliferation is sensitive to intracellular disparities in glutathione and cysteine levels which are also involved in the metabolism of methionine (Shini et al., 2005). Glutathione, carrying multiple important activities is known as the most abundant intracellular antioxidant compound and is crucial for the protection against the emergence of oxidative stress occurring posts inflammatory processes (Le Floch et al., 2004, Rubin et al., 2007). For protein synthesis to occur in immune cells, sufficient dietary intake of both methionine and cysteine [sulfur-containing amino acids (SAA)] is important (Grimble, 2006).

A study by Takahashi et al. (1997) illustrated both Methionine and Cysteine (SAA) exerting beneficial aspect on immune and inflammatory responses to stress induced Escherichia *coli* lipopolysaccharide injection, and concanavalin A in male broiler chickens. Results indicated plasma α-1 acid glycoprotein (**AGP**) concentration and interleukin (**IL**)-l-like activity in chicks fed on the SAA- sufficient diet were higher following a single e injection of lipopolysaccharide (**LPS**) than those in chicks fed on the SAA- deficient diet (Takahashi et al., 1997).

Swain and Johri (2000) indicated that cellular immune response measured as leucocyte migration inhibition (**LMI**) increased significantly (P< 0·05) at supplemented concentrations of methionine in broiler diets at 21 d of age demonstrating enhanced immunity (Swain and Johri, 2000). Such observation was in conformity with study by Tsiagbe et al. (1987a), that revealed enhanced mitogen stimulation by Phaseolus vulgaris (PHA-P) as responses to phytohaemagglutinin and significant increase in total antibody immunoglobulin G (IgG) in chicks fed on corn-soybean diets supplemented with methionine (Tsiagbe et al. 1987a). Increased methionine is reported to be critical for the synthesis of the IgG necessary for Th cells function (Tsiagbe et al. 1987a).

Experiment carried out to study Methionine deficient diet in challenged chicks with infectious bursal disease demonstrated significant decrease (P<0.05) in monocyte ratio and blood triglyceride; in addition to protein efficiency ratio, body weight gain, and feed conversion ratio (Hashemi et al., 2014).

Moreover, dietary supplementation with Methionine or Cysteine was beneficial for the lymphatic system under various catabolic conditions. In chickens challenged Newcastle disease virus (**NDV**) increasing dietary levels of Methionine (from 0.4 to 0.6, 1.2 and 1.8% respectively) in diets noticeably augmented T-lymphocyte proliferation in response to mitogen stimulation as well as IgG plasma levels (Tsiagbe et al. 1987b). While, an increased level of dietary Cysteine (from 0.185 to 0.37%) has shown similar effects as Methionine. However, high supplemental levels of Methionine and Cysteine (1.8 and 0.37%, respectively) were inimical to the chickens' performance and immune responses. This can be explained by the excess production of highly toxic elements such as homocysteine and sulfuric acid (Wu and Meininger, 2002; Jankowski et al., 2014) and thus

a higher Cysteine content supplemented in poultry diet is considered to be toxic (Li et al., 2007, Jankowski et al., 2014). While, lower sulfur-containing amino acid levels have resulted in a severe lymphocyte depletion in the Peyer's patches and in the lamina propria (Swain & Johri, 2000; Rubin et al., 2007).

Hence, it is crucial to remark that neither the excess nor the deficiency of methionine in diets influence the generation of primary antibodies in chickens (Takahashi et al., 1993, Takahashi et al., 1994, and Swain & Johri, 2000; Jankowski et al., 2014). Moreover, according to Rubin et al., (2007) vaccines administered on 1-day of age can impair the bird's performance up to 21 days of age. Thus, it is recommended to carefully administer vaccines, considering the risk of mortality caused by disease as compared to mortality caused by vaccines. (Rubin et al., 2007b).

Majority of experimental studies conducted have focused on the effect of methionine deficiency on selected immune mechanisms in chickens (Swain and Johri 2000, Hashemi et al., 2014, Rubin et al. 2007, Deng et al. 2007), and only a few researches investigated the influence of methionine on lymphocytes in peripheral blood and lymphoid organs in broilers (Wu et al. 2012, Wu et al., 2013, Zhang and Guo 2008).

According to Wu et al., (2012) methionine deficient diet in broilers caused ultrastructural pathological changes in the thymus, decreased T-lymphocyte populations, reduced serum concentrations of interleukine-2 and T-lymphocyte proliferation via increase in the percentage of apoptotic cells. In another study, Wu et al., 2013 cited relative decrease in weights of the thymus and bursa of Fabricius as well as decrease in proliferative of thymocytes and bursal cells with lower levels methionine in diet than recommended by NRC (1994).

Thus, methionine deficiency can lead to lymphoid organs dysplasia (Zhang and Guo, 2008, Konashi et al., 2000, Zheng et al., 2008), and decrease the relative weight of thymus, spleen and bursa of Fabricius (Wu et al. 2012b, Wu et al., 2013).

With regards to immune cell response, low level of methionine cause receding tubercularization reaction which proposes decline in Th1 lymphocytes proliferation within inflammation sites (Rubin et al., 2007b). Such results were compatible with Rama Rao et al. (2003) and Swain & Johri (2000) displaying levels of methionine below 0.50% spawn feeble immune response as distinguished to higher concentrations (Rubin et al., 2007b; Rama Rao et al., 2003, Swain & Johri, 2000).

Methionine deficiency can significantly inhibit the activities of superoxide dismutase (**SOD**) and glutathione peroxidase (**GSH-Px**) and the prevention of hydroxyl radicals while augmenting malondialdehyde (**MDA**) levels (Wu et al., 2012a). Further, methionine deficiency causes oxidative stress and lipid peroxidation, forcing the aggregation of free radicals, and hence destroying biofilm structure of lymphocytes. It also greatly hinders the reactivity of phytohaemagglutinin (**PHA**), the stimulus reaction of spleen lymphocytes to concanamycin A (**ConA**) as well as the mitogenesis of thymic cells (Ruan et al., 2017; Wu et al., 2012a).

Histological studies acknowledge congestion in cortex and medulla of thymic lobule with loosely arranged and significantly reduced quantity of lymphocytes in the medulla in methionine deficient formulated diet (Wu et al., 2012). Decline in lymphocytes were also observed in lymphoid follicles with thinner cortices and wider medullae in the bursa of Fabricius (Wu et al., 2012b). The histological structure of spleen was disarranged and its

lymphocytes were significantly decreased in the white and red pulp (Wu et al., 2012b). Vacuolated mitochondria of lymphocytes and greater apoptotic lymphocytes were detected in the spleens of broiler on methionine deficient ration (Wu et al., 2012, Wu et al., 2012b, Wu et al., 2013; Ruan et al., 2017).

Commercial broiler chickens do not require above 0.50% and 0.38% Methionine in starter and grower diets, respectively for optimum growth and feed efficiency, while it has been observed that higher incorporation rates of Methionine are necessary to prompt immune responses (Bunchasak 2009; Jankowski et al., 2014; Swain & Johri, 2000; Shini et al., 2005).

Elevated Methionine content, above the required dose for optimal growth, augments the immune response through both direct effects (protein synthesis and breakdown) and indirect effects involving Methionine derivatives. Few studies show the effects of methionine on nonspecific immune function as stimulating phagocytic activity of the leukocyte (Elmada et al., 2016, Shuai and Zhou, 2006), peripheral blood lymphocyte activity, as well as serum lysozyme activity (Zhang and Wo, 2008, Chen et al., 2010) As with methionine derivatives, methionine is a substrate for the synthesis of choline and therefore resulted compounds phosphatidylcholine and acetylcholine play essential role in leucocyte metabolism and nerve function (Li et al., 2007; Kim et al. 2007). Furthermore, Methionine has demonstrated important physiological function in regards to detoxification (Kim et al., 2006) and resistance for coccidium infection (Rama Rao et al., 2003). Mirzaaghatabar et al., 2011 noted antibody (**Ab**) titers and IgG in broilers by supplemented with 1.2 and 0.9% methionine diet were significantly higher than those of low levels of methionine. Such experiment illustrated significant increase of total leukocyte, percentage

of lymphocytes and heterophils as well as significant weight changes of bursa and spleen. Further, increase body weights and higher feed intake were obtained at high methionine and low energy diet (Mirzaaghatabar et al., 2011).

Turkeys that received methionine supplemented diet with at 5.98 g/kg; only slightly (by 8.7%) above the NCR (1994) recommendations have shown increase percentage in IgM+ B-cell subpopulation in the spleen (Kubinska et al., 2014).

Also, optimizing leukocyte migration inhibition assay required greater content of methionine than the growth promoting level in broiler chicks (Kidd, 2004). Similarly, higher methionine was required for antibody response and thymus derived T-lymphocyte helper cells (**Th**) function in full-feathered broiler, in addition to a higher leucocyte migration inhibition value and enhanced antibody titer of New Castle Disease virus in full-feathered broilers (Bhanja and Mandal, 2007).

The results of recent experiments on poultry are insufficient to define the optimal dietary levels of Methionine for sparking immunoexertory activity. However, a study by Rama Rao et al. (2003) have illustrate an elevated anti-sheep red blood cell (**SRBC**) antibody titers and greater cutaneous basophilic hypersensitivity (**CBH**) response with higher supplemented methionine diet and that the optimal methionine concentration for antibody production was the highest at 0.55% (Rama Rao et al., 2003). The SRBC administration pathway, genetic traits and gender may have influenced antibody production. However, in this study, the female birds of four different genetic strains were used, and SRBC was intravenously injected (Rama Rao et al., 2003). Similar results were observed in different study where male Ross broilers were used and SRBC was injected in the muscle (Jankowski et al., 2014; Rubin et al., 2007).

Findings illustrated that methionine can increase the relative weight of bursa of Fabricius and spleen in chicken (Ruan et al., 2017, Mirzaaghatabar et al., 2011, Al Maya, 2006) and while it can also increase the weight of thymus and bursa of Fabricius of layers in the brood rearing stage, it shows no significant effect on the spleen during this stage (Deng et al., 2007). So, it is concluded that the development of the primary lymphoid organs: thymus and bursa of Fabricius possibly be easily influenced than the secondary lymphoid organs such as the spleen by methionine (Sahu et al., 2014, Deng et al., 2006, Ruan et al., 2017, Konashi et al., 2000).

Appropriate methionine concentration can significantly increase the degree of antibody and the sheep red blood cells antibody titer as previously explained and cited by the following authors (Bhanja and Mandal, 2007, Takahashi et al., 1993, Takahashi et al., 1994, Mirzaaghatabar et al., 2011). Methionine is capable of encouraging T-lymphocyte proliferation of the peripheral blood, thymus and spleen, while methionine deficiency diminishes the transformation of T-lymphocyte proliferation (Kinscherf et al., 1994). It has also been evident that with higher levels of methionine in ration, serum antibody rises in the broilers with coccidium infection (Jin et al., 2005, Ruan et al., 2017), and as several researches have pointed out the leukocyte migration and antibody titer also have increased in chickens infected by Newcastle disease virus (**NDV**) (Bouyeh, 2012, Swain and Johri, 2000).

On the other hand, Wu et al. (2012) reported that methionine deficiency can significant decrease serum IgG, IgA and IgM content which in turn suggests that the humoral immunity is jeopardized (Wu et al., 2012). Zhang and Li (2008) illustrated methionine's ability to significantly affect the content of serum IgM and IgA in a meat rabbit.

Deficiency in methionine influences the relative percentage of T-lymphocyte subsets which includes $CD3^+$, $CD3^+$ $CD8^+$ and $CD3^+$ $CD4^+$ of broilers (Wu et al., 2012).

Added, the level of the methionine is found to be higher in the immune response than in normal circumstance (Rubin et al., 2007c, Ruan et al., 2017) deducing that there exists a close relationship between the methionine and immune response or disease resistance (Rubin et al., 2007c, Ruan et al., 2017).

As with such substantiated extensive research review on methionine immunopotentiating effect, it is hypothesized similar findings of Methionine on the immune system is expected to be observed studying *Mycoplasma gallisepticum* infected chickens.

2.3 MYCOPLASMA GALLISEPTICUM OVERVIEW

Mycoplasma gallisepticum (**MG**) is the causative agent of chronic respiratory disease (**CRD**) in chickens and infectious sinusitis in turkeys, pigeons, passerine birds, finches, game birds of all age groups (Hennigan et al. 2012; Ley, 2008). In chickens, *M. gallispeticum* is outlined by severe inflammation of the trachea, air sacs and lungs; conjunctivitis; rales; nasal and mucosal discharge. *M. gallisepticum* cytadheres to the tracheal epithelial cells mediating infiltration of macrophages, heterophils and lymphocytes to the tracheal submucosa. Yet, the molecular infectious mechanisms associated with the severe inflammatory response of MG is elusive (Levisohn and Kleven, 2000; Razin et al., 1998; Majumder, 2014). Of an infinitesimal size and minimal genetic information, MG lack bacterial cell wall; and hence it is unaffected by ß-lactam antimicrobials which target cell wall synthesis. *Mycoplasma gallisepticum* have the ability to penetrate cells; possess a trilaminar membrane and they are highly polimorphic. MG are facultative anaerobes with optimal temperature of 35-37 °C; and require an enriched media of 10-20% animal serum and yeast extract (Kleven, 1997; Frey et al., 1968).

MG causes significant economic losses despite the absence of clinical signs (Levisohn and Kleven, 2000). Condemnations of carcasses, reduced feed efficiency and egg production, reduced hatchability and growth, aggravating and co-existing with other disease agents and increase in medication or vaccinations, control programs prepare MG as the costliest disease facing poultry production globally (Ley, 2008; Levisohn and Kleven, 2000; Bradbury et al., 1993; Wieslander et al. 1992).

2.4 MYCOPLASMOSIS

2.4.1 Classification of Mycoplasma

Mycoplasmas are classified in the kingdom Bacteria, members of phylum Tenericutes, of the class Mollicutes, Order I, Mycoplasmatales. The Mollicutes are phenotypically distinguished from other types of bacteria by their small size and the absence of a cell wall (Levisohn and Kleven, 2000, Goh et al., 1998; Goren, 1978). *Mycoplasma,* a Genus I classification consists of over 100 species, with a DNA G C content of 23—40%, 600–1350 kb genome capacity, a generally optimum growth temperature of 37°C; demands cholesterol for growth, and exists in both humans and animals. Mycoplasmas are descendants of low G+C ratio containing Gram-positive bacteria; within family *Bacillaceae*; via degenerative evolution (Rogers et al., 1985; Woese et al., 1980). Genus II, *Ureaplasma,* is distinguished by its ability to hydrolyze urea. Acholeplasmas are classified in Order III, Acholeplasmatales, family I, Acholeplasmataceae, genus I, *Acholeplasma.* They do not require cholesterol for thriving (Razin et al., 1998). Taxonomic classification of the Mollicutes and the analyzation of genetic relationships among mycoplasmas is achieved through phylogenetic analysis of the 16s ribosomal RNA gene sequences (Brown et al., 2007; Weisburg et al. 1989). Unidentified isolates also exist from domestic poultry (Kleven, 1997). Recent listing of *Mycoplasma* species can be found on the National Center for Biotechnology Information website: (https://www.ncbi.nlm.nih.gov/taxonomy).

2.4.2 Characterization of Mycoplasma

Mycoplasmas are known to be the smallest self-replicating prokaryotes enclosed solely by plasma membrane and thus are cell wall vacant (Kleven, 1997). They consist of highly variable surface proteins and have the capability of causing disease in humans, various animals, plants as well as insects (USGS, 1999). Mycoplasmas usually apt for host specific profile; some strains infect only a single species while others are capable of infecting several distinct animal species USGS, 1999). For so, mycoplasmas have gained the status of next generation bacterial pathogens because of their intricate properties and unique process in causing pathogenicity in hosts. Characterized by a unique sterol- containing plasma membrane and small genome comprised of reduced chromosomes, limited biosynthetic capability with inadequacy of synthesizing essential molecules including purines and pyrimidines; mycoplamsas rely on external supplies of biosynthetic precursors and nutrients such as AA, nucleotides, and fatty acids (**FA)**, including sterols to maintain cell membrane integrity lacking a rigid cell wall support. Thereby mycoplasmas are commonly classified as parasitic or saprotrophic (Razin and Herrmann, 2002). Mycoplasmas apply stop codon, **UGA** to encode for AA tryptophan (Inamine et al., 1990) and they possess high mutation rates, possibly due to insufficient proof-reading by DNA polymerase enzymes (Woese et al., 1980). Under microscopy, mycoplasma is morphologically characterized by a “fried egg” type of colony. Mycoplasmas fundamentally colonize mucosal surfaces; where they can attach to host epithelial cells, such as in the respiratory tract, causing cell damage and inflammatory response and most species are noninvasive yet hold resistance to antimicrobials that affects cell wall synthesis as well as call for intricate nutritional requirements as they notably lack cell walls (Kleven,

1997).

Culturing mycoplasma species from avian sources generally require a protein-rich medium; known as the Frey's broth (Frey et al., 1968) containing 10—15% added animal (bovine or swine) serum supplemented with yeast extract or component; thallium acetate and penicillin (penicillin G for *Mycoplasama gallisepticum*) and dextrose (Frey et al., 1968). Reasons for such supplementations incorporated to develop mycoplasma medium (discussed further in Frey's broth preparation section; in Materials and methods chapter): The animal serum offers nutritional elements but most importantly it provides fat for which Mycoplasma favors. On the other hand, mycoplasma is resistant to thallium acetate and penicillin, and the reason for their supplementation into the medium is for hindering and/or averting any contaminations growth of other bacteria and fungi, thus rendering the medium selective solely for mycoplasma. Dextrose is also added for nutritive aspect. While, the addition of nicotinamide adenine dinucleotide (**NAD**) to the media is necessary for the growth of *M. synoviae* species.

Pathogenic mycoplasma species slowly cultivate with an incubation period ranging from 3-10 days (Shaib, 2004; Kleven, 1997). Yet, colonies of nonpathogenic mycoplasma organisms such as *M. gallinarum* and *M. gallinaceum* may form on Frey's agar within 1 day (Kleven, 1997). Mycoplasma colonies are characterized as circular, smooth, with an overall flat yet a much denser elevation in the center of the colony, possessing typical size of 0.1—1.0 mm. Morphological variations in colony do exist, however species distinction cannot be drawn out from such observations. Single cells are described as coccoid to coccobacilliform, ranging from 0.2–0.5 m, though slender rods, filaments, and ring forms

also exist. Mycoplasma species may be branched into those that perform carbohydrate fermentation (onward reduction in pH for acid production) and those that do not. Thus, this accounts for the sugar (dextrose) incorporation into medium preparation for mycoplasma culturing in order to augment the growth of carbohydrate-fermenting organisms along added phenol red as an indicator to provide indication of such growth once acid is produced in the medium (Shaib, 2004; Kleven, 2008). Species that do not ferment carbohydrates usually consume arginine as their main source of energy using the arginine decarboxylase enzyme and phosphatase. Few mycoplasma species however, such as *M. iowae* are capable of hydrolyzing arginine as well as fermenting glucose. Hemagglutination of erythrocytes is peculiar characteristic of some mycoplasma species such as *M. gallisepticum, M. meleagridis,* and *M. synoviae* that is present in both chickens and turkeys. In this case, hemagglutination inhibition (**HI**) tests using hemagglutinating antigens for these pathogenic organisms are serologically performed (Kleven, 1997; Ley, 1997; Kleven, 2008).

2.3.2 MYCOPLASMA *GALLISEPTICUM*

With a world-wide distribution, *Mycoplasma gallisepticum* (MG) is the greatest economically significant mycoplasma pathogen of avian species. As a highly transmissible avian pathogen, it is the causative agent of chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys, chickens, pigeons, passerine birds, game birds of all age groups (Hennigan et al. 2012). Primary description of the disease by Dodd (1905) in England 1905 named “epizootic pneumoenteritis” was observed in turkeys. *M.*

gallisepticum diseases are characterized by respiratory rales, nasal and ocular discharge, coughing, and conjunctivitis. In turkeys, often infraorbital sinusitis is present. Clinical manifestations generally establish gradually and the infection may abide for lengthy duration; as such once infected, birds become carriers and remain infectious throughout their lifetimes posing threats to other bird populations (Levisohn and Kleven, 2000; Chen et al., 2012). Decrease in growth and production are prominent in MG infection. (Bradbury, 2005). Initial lesions are typically composed of heterophils and macrophages, and subsequent lesions demonstrate large quantity of lymphocytes along high proportions of T-lymphocytes (Szczepanek and Silbart, 2014).

MG convoluted with a respiratory virus infection such as infectious bronchitis (**IB**), Newcastle disease virus (**NDV**), Avian Influenza virus (**AIV**) and very often along with a secondary infection of *Escherichia coli* (E. *coli*) (Kleven, 1998; Kleven, 2008); *Haemophilius,* or avian rhinotracheitis virus causes severe air-sacculitis, also known as "Air sac disease", leading to aggravated clinical CRD, high morbidity, mortality and/or increased condemnations at processing (Ley, 1997; Ley, 2003; Sullivan et al., 1956; Sid et al., 2016). Economic impact of MG is highly significant despite the absence of clinical signs (Levisohn and Kleven, 2000).

Mycoplasma gallisepticum features a small genomic size comprised of only 996,442 bp nucleotides for R_{low} strain (Tan et al., 2015) with limited biosynthetic capabilities. With the lack of cell wall, MG membrane proteins are crucial in establishing MG morphology, nutrient transport and colonization of the host (Cleavinger et al. 1994; Panicker et al. 2012). As an opportunistic pathogen, MG depends on its parasitic life-style and despite several

limitations, their degenerative evolution allow them to travel over inert surfaces, such as glass, plastic and eukaryotic cells, absentee of locomotory appendages such as flagella or pili (Jarrell and McBride, 2008). Antigenic variation, phase variation, superantigens are a few of the mechanisms adopted by MG to evade the host's lymphatic system.

2.3.2.1 Classification

Mycoplasma gallisepticum is a pathogenic species belonging to the Class Mollicutes within the genus *Mycoplasma* of the family Mycoplasmataceae (Razin, 1992). *M. gallisepticum* are the smallest self-replicating eubacteria that lack cell walls. *M. gallisepticum* are prokaryotes with reduced genome (Semashko et al. 2016); minimal genetic information (Levisohn and Kleven, 2000), many metabolic pathways and the capability of growing on artificial cell-free media (Razin, 1992; Semashko et al. 2016). These attributes are mirrored with a high degree of interdependence between *M. gallisepticum* and the host species as well as in the fastidious nature of the organism *in vitro* including its difficulty to culture, selective antibiotic sensitivity, inhibition of phagocytosis, and its intimate association with host cells. MG constitutes two copies of a 23S-5S rRNA gene cluster, both not found near the single 16S rRNA gene (Chen and Finch, 1989). Differentiating from other avian mycoplasmas, *M. gallisepticum* was first classified by serotyping (Adler et al., 1957; Yamamoto and Adler, 1958) and was entitled as serotype A (Yoder, 1964). Molecular techniques aided in distinguishing mycoplasmas with phenotypic and antigenic similarities to *M. gallisepticum* by 1993 and *M. imitans* was found to be closely similar (Bradbury et al., 1993)

2.3.2.2 Morphology and Staining

M. gallisepticum is described as a weakly (Ley, 2008) gram-negative bacteria that stains well with Giemsa staining. Species typically appear coccoid under light microscopy with approximately 0.25—0.5 um in size. *M. gallisepticum* demonstrate filamentous or flask-shaped polarity of the cell body through electron microscopy and such polarity is present preceding cell division (Morowitz and Maniloff, 1966) and can be explained for the well-organized terminal organelles such as the blebs or tip structures that exist (Carson et al., 1992). This tip structure is a polar, tapered cell extension with an electron-dense core cytoplasm and is key in pathogenesis process as it is responsible for motility (Indikova, 2014; Josenhans and Suerbaum, 2002; Ley, 2008). For decades, it has been theorized that these MG structures dictate the host-pathogen interactions (for instance cytadhesins) or the motility and therefore depicting pathogenicity (Levisohn and Dykstra, 1987; Bredt, 1973).

2.3.2.3 Growth Requirements

There exist several kinds of broth or agar media supporting the thrive of avian mycoplasmas (Kleven, 1998) as indicated in Swayne et al. (1998) Laboratory Manual for Isolation and Identification of Avian Pathogens. MG replication necessitates a preparation of a complex medium enriched with 10–15% heat-inactivated animal (swine or horse) serum. Frey et al. (1968) developed an MG culture medium that embodies all fundamental elements including yeast autolysate and glucose. Preparation of MG media with 10–15% swine serum is highly effective and conducive for culturing majority of mycoplasmas. Commonly, bacterial and fungal contamination is governed by the supplementation of

thallium acetate (1:4000) and penicillin (penicillin G) (**Pen G**) (up to 2000 IU/mL); granting Frey's media to be MG-selective (Frey et al., 1968). MG is a carbohydrate-fermenting avian *Mycoplasma* spp., specifically fermenting glucose; resulting in a decrease in pH where the phenol red indicator changes in colour from red to orange at log phase and rendering observation of MG growth in broth media (Frey et al., 1968; Ley, 2008).
The optimal pH for growth in broth medium is at 7.8 with an incubation period ranging from 3-5 days at 37°C. However, some field isolates may demand for lengthier incubation period as well as multiple passages. This is because in the MG cultured broth more than one isolate may exist and one isolate may overgrow another (Shaib, 2004). In such case, the MG broth (where growth of multiple isolates may not be evident) can be cultured into Frey's agar to observe growth colonies of differing isolates. This may require 2 or 3 serial passages at 5–7-day intervals to increase the quantity of isolations. Primary culturing of samples into MG broth is an isolation technique that is highly sensitive, yet direct culturing onto MG agar plates may also result in colonies post 4 to 5 days of incubation. Mycoplasma-cultured agar plates ought to be placed at 37°C incubation in a highly moist atmosphere (a wet tissue paper may be placed near the agar plates) and they need a minimum of 3—7 days of incubation prior for large colonies of mycoplasma can be observed under a dissecting microscope (Shaib, 2004). Moreover, MG can be also isolated or propagated in embryonated chicken eggs (Ley, 2008).

2.3.2.4 Colony Morphology

Development of MG colonies can either be on a serum-enriched agar medium which must be inoculated directly or post the passage from broth or agar cultures (Kleven, 1998;

Swayne et al., 1998). It is rather usually very challenging to acquire colony growth directly from clinical samples. Utilizing dissecting microscope under indirect lighting is best for analyzing any indication of MG colony growth. *M. gallispeticum* colonies are characterized as infinitesimal; ranging from 0.2—0.3 mm in diameter; circularly smooth, translucent with a much denser central area, also known as the "fried egg" appearance. They also often appear in ridges along the streak line, this is due to the fact that closely adjacent colonies quickly coalesce. Distinct species of avian mycoplasmas have manifested differences in colony morphology which have been reported in early stages of research by many studies such as Shifrine et al., 1962; Yoder, 1963; Nelson, 1936; Hitchner, 1949; Groupe and Winn, 1949; McKay and Taylor, 1954 and according to Gentry, 1960; the pathogenic mycoplasma strains are designated as true Pleuropneumonia-Like Organisms (**PPLO**) or Mycoplasma, while the nonpathogenic strains revert to bacteria and are designated as L forms. Yet, mycoplasma species classification cannot be determined by its colony morphology.

2.3.2.5 Biochemical Properties

MG is carbohydrate-fermenter as it is known to ferment glucose as well as maltose and dextrose; with production of acid and no production of gas. It does not however ferment lactose, dulcitol, or salicin. Sucrose is found to be rarely fermented and fickle results are observed with galactose, fructose, trehalose, and mannitol. MG is phosphatase-negative and does not hydrolyze arginine. It, however, reduces 2,3,5-triphenyl tetrazolium and neotetrazolium (Ley, 1997). Further, MG induces complete hemolysis of horse erythrocytes

supplemented into agar medium and can agglutinate chicken and turkey erythrocytes (Ley, 1997; Ley, 2008).

2.3.2.6 Susceptibility to Chemical and Physical Agents

Inactivation of MG has been effective by phenol, formalin, propiolactone, and merthiolate. As mentioned previously, MG is resistant to low concentrations of thalium acetate (1:4000) and to penicillin in which their incorporation into a mycoplasma cultivation media thwarting bacterial and fungal contamination and thus imposing the media as selective to MG.

Several studies have demonstrated the viability of MG even post years of its culturing. According to (Yoder, 1964), MG broth cultures remained viable for 2 to 4 years stored at a temperature of 30°C. Viable MG were also recovered from lyophilized broth culture stored for minimum of 7 years at 4°C; while from lyophilized infective chicken turbinates stored for 13 to 14 years at 4°C (Yoder, 1964). Yoder (1988) discovered various MG broth cultures which had been stored for over 20 years at 60°C temperature and were found to be viable upon subculturing. Further, a research by Kleven (1985) on the stability of *M. gallispeticum* F-strain in powdered skim milk, phosphate-buffered saline (PBS), tryptose phosphate broth, and distilled water stored at 4°, 22°, and 37°C. had demonstrated that MG **F-strain** was stable in all abovementioned solutions for 24 hours stored at 4° or 22°C. However, MG F-strain illustrated to be viable in PBS only once stored at 37°C for 24 hours. Another study has illustrated MG's inactivation in infected chicken hatching eggs as temperature reached 45.6°C (Ley, 2008; Yoder, 1970). According to Rotten et al. 1983,

there exist segregated lipid domains in the MG membrane, one of a cholesterol-poor phospholipid that is able to undergo order-disorder transition, and a the other is a cholesterol-rich lipid that is fixed in a fluid state and consisted of the intramembranous particles.

2.3.2.7 Diagnostic Techniques

2.3.2.7.1 Isolation and Identification of Agent

Isolation and/or the identification of the microorganism is a cornerstone for *Mycoplasma gallisepticum* diagnosis. Isolation techniques of MG constitutes culturing, suspension and resuspension of tissues such as trachea or airsac, lungs, turbinates or fluid sinus exudate on mycoplasma broth or agar medium; commonly on Frey's broth or Frey's agar (Frey et al., 1968). Kindly refer to sections 2.4.2 'Characterization of Mycoplasma' and 2.3.2.3 'Growth Requirements' for culturing and isolation of MG). Using a particular fluorescent antibody, MG can be directly stained on Frey's agar and immunoperoxidase tests (OIE, 2008). Molecular techniques include sequencing of the rRNA gene (15), DNA probes (16), polymerase chain reaction (**PCR**) and a combination of PCR followed by restriction fragment length polymorphism analysis (**RFLP**). molecular methods increase sensitivity and specificity of detection. Bradbury and Kleven, 2008; Nascimento et al., 1993; Slavik et al., 1993; OIE, 2008).

2.3.2.7.2 Serology

Serologic procedures include Enzyme Linked-immunosorbent Assays (**ELISA**s) that detect MG antibodies in blood, respiratory tract or egg yolk. ELISA demonstrate increase in efficacy and specificity in detection; and it is widely used today for flock serodiagnosis and monitoring. Serum Plate Agglutination (**SPA**) antigen is high specificity test used to detect MG antibodies. SPA is quick, inexpensive, and have been widely accepted for flock screening. However, SPA shows cross-reactivity with M. synoviae or flocks administered vaccines of oil-emulsion or of tissue culture origin. The hemagglutination inhibition (HI) test aids in confirming reactors shown via SPA or ELISA but it may lack sensitivity, it is time-consuming and its reagents are not readily commercially available.

Other serology tests include Dot immunobinding assays and tube agglutination test. Serologic methods useful in MG control programs and in the diagnosis of suspected infection (Ley, 2008; Avakian and Ley, 1993; Yagihashi and Tajima, 1986; Mohammed et al., 1986; Qasem et al., 2015).

2.3.2.7.3 Differential Diagnosis

MG infections ought to be differentiated from other respiratory diseases. Clinically, MG is apparent with the presence of conjunction and concurrent respiratory disease such as ND, IB or *E. coli.* Identification of MG followed by seroconversion is necessary to differentiate MG from other respiratory diseases (OIE, 2008; Ley, 1997).

2.3.3. Genomics and Antigenic Structures

A significant portion of mycoplasma's minute genome encodes for antigens that are associated with provoking the production of surface variability by phase, antigenic or size variation (Ose et al. 1979; Stanley et al. 2001). This spawning of phenotypic diversity is an important mycoplasma survival strategy permitting them to elude immune detection, and thereby colonize and persist in the host species (Noormohammadi, 2007). In fact, MG are known to possess the capability to penetrate cells (Bencina et al. 1994). The MG antigenic features and approximately species-specific polyclonal antibody response to the animal are considered in determining 1) the organism; via growth inhibition and immunofluorescence tests and 2) the immunologic response to infection; through Serum Plate Agglutination (**SPA**), Hemagglutination Inhibition (**HI**), and Enzyme-Linked Immunosorbent Assay (**ELISA**) tests (Kleven, 1997; Ley, 2008). Over two-thirds of mycoplasma plasma membrane comprises of proteins while the remaining being membrane lipids (Razin et al., 1998). The plasma membrane of MG consists of roughly 200 polypeptides (Jan et al. 1996.) which function with nutrient transport, antigenic variation and adhesion to host cells (Wieslander et al. 1992).

Substantial effort has been made to identify MG antigens, especially those with adhesin or hemagglutinin (cytadhesin) properties, which may be of vital pathogenesis process of, and immune response to, MG infection. As integral membrane proteins, adhesins possess regions exposed on the surface of the cell that adhere to receptor sites on host epithelial cells, granting colonization and infection; thereby they are perceived as crucial virulence factors and antigens.

According to several research studies, proteins or lipoproteins of *Mycoplasma*

gallispeticum with molecular weights of 60—75 kDa have been deduced as immunodominant adhesins or hemagglutinins (Markham et al. 1992; Barbour et al. 1989; Avakian and Ley. 1993, Kleven, 2008; Ley, 2008; Ley, 1997; Kheyar et al. 1995; Forsyth et al. 1992; Boguslavsky et al., 2000; Markham, 2012).

MG gene families of hemagglutinin protein A **(pMGA)** and phase variable putative adhesion protein A **(pVPA)** are acknowledged for encoding major surface proteins accompanying attributes of pathogenicity, antigenic, and immune escape. MG cells express pMGA as an abundant surface lipoprotein (Markham et al., 1992). Generally, each MG strain expresses only one homogeneous, unique pMGA molecule (Glew et al., 2000). Yet, all MG strains examined to today consist large pMGA multigene families ranging from 32 to 70 genes in size (Baseggio et al., 1996; Glew et al., 2000; Liu et al. 2002). The pMGA gene family of MG strain S6 encodes variant replicates of key cell surface lipoprotein hemagglutinin of 67 kDa (**p67**) (Glew et al. 1995., Baseggio et al. 1996; Glew et al.1998. Moreover, the pMGA gene family holds genome of **strain F** of at least 7.7% and 16% of the genome of **strain R** (Baseggio et al. 1996.), which are powerful genomic integrals contributing to antigenic variability and theorized for immune dodging activity (Markham et al. 1994; Markham et al., 1993.). The pMGA gene family also offers antigenic switching; a mechanism for quick and reversible switches in its proteins' expression in response to antibodies or different environmental factors and it has been shown to be mediated by trinucleotide repeat length variation (Glew et al., 2000). The transcriptional switching between pMGA genes is decidedly connected with changes in the length of a unique trinucleotide **GAA** repeat; particularly, a $(GAA)_{12}$ motif 5′ to a pMGA1.1 promoter is required for the expression of that gene (Glew et al., 1998). Alterations in pMGA gene

expression appear as a result of the inherent instability of GAA repeats in MG (Glew et al., 1998; Glew et al., 2000; Markham et al., 1998). On the other hand, PvpA is an MG size-variable integral membrane protein found on the cell surface, notably at the terminal tip structure (Boguslavsky et al., 2000) which demonstrates high- frequency phase variation in its expression, which furthers the complexity of antigenic variation in MG (Yogev et al. 1994). It PvpA size variation ranges from 48—55kDa in MG strains and can be detected by immunoelectron microscopy. In *vivo* studies, have shown that antigenic variation and expression of two major immunogenic surface proteins; PvpA and p67a; to be associated with antibody response conveying evolution of surface diversity as a function of immunomodulation (Levisohn et al. 1995). Numerous research studies and reports (Athamna et al. 1997; Bencina et al. 1994; Brown et al. 1997; Garciaand Kleven. 1994; Ley, 2008; Garcia et al. 1994; Tan et al., 2015; Xu et al., 2015; Szczepanek et al., 2010; Cizelj et al., 2011) argue for *Mycoplasma gallisepticum*'s role in antigenic variation and variable expression of surface proteins. Additionally, there exist few MG cytadhesin genes and proteins bearing homologues in other *Mycoplasma* spp.; some of which are human pathogens. This rather suggests that conservation of cytadhesin genes and proteins may occur within pathogenic mycoplasmas infecting extensively various hosts (Mohammed et al. 1987; Hnatow et al. 1998; Goh et al., 1998; Rocha et al., 2015). Attenuated virulence in chickens via mutants development indicate virulence role of certain MG-associated proteins (Szczepanek et al., 2010; Tseng et al. 2013; Masukagami et al. 2013; Hudson et al., 2006; Gates et al. 2008). For so, transposon mutagenesis of pathogenic MG strains has been applied to inactivate genes encoding for dihydrolipoamide dehydrogenase (**Lpd**) (Hudson et al., 2006; Gates et al. 2008), **MslA**; a polynucleotide binding protein (Masukagami et al.

2013; Szczepanek et al., 2010) and the ABC sugar transport permease MalF (Tseng et al. 2013). On the potent toxins side, none have been shown to correlate with mycoplasma species (Razin et al., 1998).

2.3.4 Strain Classification

Mycoplasma gallisepticum species are distinguished by their isolates. Mainly, those isolates which had been characterized are called strains. However, there exist various strains of MG and these ought to not be associated with the several determined serotype designations of avian mycoplasmas before speciation within the genus *Mycoplasma* (Ley, 1997). A great variability exists between MG strains in respect of tissue tropism (proclivity for brain, eye, join), culturing ability, virulence and transmissibility and antigen structure (Stipkovits et al., 2017). Few MG isolates from chickens and turkeys have been classified as variant or atypical as they are difficult to isolate and are less pathogenic, transmissible, and immunogenic than wonted of field isolates (Ley, 2008; Avakian et al. 1992; Dingfelder et al. 1991; Kleven, 1997; Fiorentin et al. 1997; Levisohn and Kleven, 2000; Yoder, 1986.). Also, due to their variability in antigenic phenotype, MG strains, along with well-confirmed reference strains, can be notably distinguished from their antigen profiles and virulence-related surface properties (Rosengarten and Yogev, 1996). Recently, the analysis of the nucleotide sequences of distinct MG strain amplicons have demonstrated various melting curve profiles related to a unique DNA sequence of each strain (Ghorashi et al., 2010). For these reasons, it has become crucial to develop methods to identify and classify *M. gallispeticum* strains and strain variability. Sensitive and discriminatory power of molecular (genotypic) typing methods added to serologic tests or electrophoretic analysis of cell

proteins will aid in the identification of intraspecies (i.e. strain) genotypic as well as phenotypic heterogeneity of MG (Kleven, 1997; Ley, 2008; Geary et al., 1994; Grodio et al., 2008; Lysnyansky et al., 2008).

2.3.4.1 Antigenicity of Mycoplasma gallispeticum Strains

Antigenic variation of *M. gallisepticum* strains and isolates has been well- established as atypical or variant strains (Dingfelder et al. 1991; Yoder, 1986; Ley, 1997) illustrated through several studies by serologic assays (Lin and Kleven, 1982; Panangala et al.,1992; Bencina and Dorrer, 1984; Kleven et al., 1988; Ley, 2008; Noormohammadi et al. 2007; Rodriguez and Kleven, 1980), immunoblots, and monoclonal antibodies (Czifra et al. 1995. Brown et al. 1997; Silveira et al. 1993; Kheyar et al. 1995; Rosengarten and Yogev. 1996; Garcia and Kleven, 1994; May et al. 1994).

It is confirmed that antigenic variability among MG strains can significantly influence the sensitivity of serological methods, this is contingent upon the strain infecting the flock as well as the strain adopted for antigen preparation (Ley, 1997). Kleven et al. (1988) studied homologous and heterologous hemagglutination inhibition (HI) assays of MG strains and discovered that homologous HI typically demonstrated higher titers than heterologous HI. According to Kleven et al. (1988), MG strain A5969, commonly used in laboratories as an HI antigen strain, was insensitive for recognizing antibodies against all of the strains tested while none of the antigens studied was efficient in recognizing HI antibodies against all other strains tested (Kleven et al. 1988). Relatively speaking antigenic variability whether among and/or within MG strains, offers great impediment to the formation and optimization of antigen/antibody-based assays such as serologic assays,

immunofluorescence, as well as growth inhibition organism identification tests. MG strain antigenicity multitude; auxiliary to its variability; can be the least denoted for the species' genomic quirk to immune evasion and its adaptation to modifications in the host environment, which ensue in MG antigenic surface proteins expression holding high degree of variation, switching, and immunomodulation (Levisohn et al. 1995; Levisohn and Kleven, 2000; Bencina et al. 1994; Garcia et al., 1994; Glew et al., 2000).

2.3.4.2 Pathogenicity, Immunogenicity and Vaccine Strains of *Mycoplasma gallispeticum*

MG infection is commonly entangled with intricate environment and disease factors (Ley, 1997). The various MG isolates and strains greatly differ in their pathogenic or immunogenic characteristics; these are summarized in (Table 2.2), depending on their genotypic and phenotypic attributes, including propagation mechanism, number of passages and challenge route and dosage (Ley, 1997, Whithear, 1996). The immunogenicity including low to mild virulence and the protective characteristics of three known MG strains which are the MG F-strain, MG ts-11 strain, and MG 6/85 strain have been utilized for inducing artificial immunity with their application in commercial live vaccines (Ley, 1997, Whithear, 1996). As opposed to live vaccines, killed vaccines or bacterins do not jeopardize vaccine's safety yet their impoverishment is for requiring above one administration for an optimal protection. Liposomes, iota carrageenan; polymeric microspheres, adjuvants are examples of antigen delivery systems examined to augment the efficiency of killed MG vaccines. (Evans and Hafez, 1992; Abd El Motelib and Kleven.

1993; Chen et al., 2012; Ley, 2003; Abdelmoumen and Roy, 1995; Garcia and Kleven, 1994; Garcia et al., 1994; Bencina et al., 1988).

Table 2.2. Pathogenic and protective (Vaccination) properties of *Mycoplasma gallisepticum* strains*.

Isolate/Strain	Pathogenic and Protective Properties
Yolk-passaged organisms	Embryonated chicken eggs of which have been MG-inoculated were considered to be much infective than those of broth-passaged.
S6 strain	S6 is pathogenic strain isolated by Zander from the brain of a turkey with infectious sinusitis.
A5969 strain	A5969 is a pathogenic strain isolated by Jungherr et al. 1955
R strain	Dale Richey isolated R strain from a chicken with air-sacculitis in 1963 at the University of Georgia. It is largely used for bacterin production (Killed vaccine) and in MG challenge research studies as a pathogenic strain.
F strain	F strain is a live vaccine administered in drinking water, aerosol, eye-drop, or intranasal; mainly used in pullet immunization programs. It helps in reducing EP decrease and has been administered to displace endemic strains in multiple-age poultry. Yet, the F-strain has been shown to be more pathogenic for turkeys rather than chickens and its main disadvantage its inherent virulence.

6/85 strain	The 6/85 strain is a live vaccine strain stimulating a weaker protective immune response and thus with less pathogenicity, virulence and infectivity than the F strain indicated for both chickens and turkeys. It has demonstrated little or no transmissibility and is resistance against virulent MG challenge. It is administered by aerosol, and is unlikely to persist in vaccinated birds and may fail to stimulate a detectable systemic antibody response. Mainly used to prevent EP losses.
ts-ll	The ts-ll is an Australian MG field isolate (strain 80083); a dose –dependent temperature sensitive (at 33°C) vaccine strain administered by eye-drop. It is less pathogenic and virulent for both chickens and turkeys than the F strain; providing a weaker yet effective long-term immunity with a detectable though variable systemic Ab response. The ts-11 is safe to be used in combination with respiratory virus vaccines.

(*Information in table credited to references: Whithear et al. 1990a; Whithear et al. 1990b; Whithear, 1996; Ley, 1997; Evans and Hafez, 1992; Rodriguez and Kleven, 1980; Ley, 2003; Abd El Motelib and Kleven. 1993; Chen et al., 2012; Fabricant, 1958; Shil et al., 2011; Jungherr et al. 1955; Ley et al. 1993.; Glisson et al. 1989; Kleven et al. 1998b; Markham et al., 1998).

2.3.4.3 Genetic and/or Molecular Classification of *Mycoplasma gallisepticum* Strains

Strains of MG can be distinguished from one another via protein banding patterns emanated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**) or through restriction fragment length polymorphism (**RFLP**) of DNA, with the latter providing higher sensitivity (Carrión et al. 2012; Santha et al. 1988; Liu et al., 2001; Tyler et al. 1997; Khan

et al., 1987a; Majumder, 2014). Depicting MG strains (intraspecies differences) can also be achieved via DNA and ribosomal RNA gene probes (Yogev et a. 1988; Hong et al., 2005; Nagai et al.,1995; Khan et al. 1987b.) and physical chromosomal mapping (Tigges and Minion, 1994). Such abovementioned methods are known to be costly, tedious and intricate (Ley, 1997).

Valuable molecular PCR methods for the identification of vaccine and field strains as well as for suitable for epidemiological research include the arbitrary primed PCR (**AP- PCR**) and the random amplification of polymorphic DNA (**RAPD**) (Turner and Kleven, 1998; Charlton et al. 1999; Shaib, 2004; Hartup et al. 2000; Fan et al. 1995.). RAPD however have not been used as often recently as it is time-consuming and holds lack of specificity as it may amplify the DNA of almost the entire species being tested (Shaib, 2004).
Yet, PvpA gene PCR followed by RFLP of the amplicon has been used for MG strain identification and proven to be the optimal/favorable combined method today, as in addition it is achievable without pure-culture MG isolation preceding the analysis (Liu et al., 2001).

2.3.5 Virulence Factors, Receptor Specificity and Replication

The capability of varying in surface components; a process referred to as cytadhesion; has been suggested and argued for holding fundamental function of the hypothesized immune evasion and the adaptation of MG to host environments and likely the reasoning behind its quirk movement in invading host cells. In such a manner, cytadhesion is a virulence factor

of MG. This is where the pathogenic microorganism establish infection in hosts through the usage of a surface adhesin protein to attach to the receptors on host cells (Bíró et al. 2006; Tzeng et al. 2011).

On another hand, motility is observed as a virulence factor in several pathogenic bacteria. Movement qualification aid microorganisms to either get to a particular niche and/or to exist unfavorable environments. Diverse motility mechanisms have evolved among motile bacterial species. For instance; in *Bordetella bronchiseptica* (**B. *bronchiseptica***), E. *coli*, and *Salmonella enterica* (**S. *enetrica***) serovar Typhimurium flagellar motility has been observed to be vital in infection particularly during the premiere stages. In other bacterial species such as *Legionella pneumophila*, in order to maintain infection motility is required (Josenhans and Suerbaum, 2001). However, in some bacterial species, such as *Helicobacter*, *Campylobacter*, and *Pseudomonas aeruginosa* (***P. aeruginosa***) organisms; motility may be downregulated to support a certain living conduct. In such case and for successful infection, these species rely on their flagella as locomotory appendage (Josenhans and Suerbaum, 2002; Indikova et al., 2014). Several studies have demonstrated that motile bacteria, solely, have the capability of reisolating post infection within a combined population of motile and non-motile species indicating the power of motility in infection process and association as virulence factor (Eatonet al., 1989). MG is known to possess gliding motility; first observed in 1968, that enables it to glide over solid surfaces (Indikova et al., 2014). Until very recently, MG strains and clonal variants have been analyzed for locomotion and results have demonstrated the mandatory presence of cytadherence proteins **GapA** and **CrmA** for motility. While, the absence of GapA or CrmA

cytadherence proteins generated a loss in gliding as well as in hemadsorption which subjected the features of cells' flask-shape to modifications (Indikova et al., 2014). Xu et al. (2014) reported a putative lipoprotein, MGA_0676, comparable to bacterial thermostable nucleases is important virulence factor as it a Ca^{2+}-dependent cytotoxic nuclease of MG with a staphylococcal nuclease (**SNc**) region. Upon deletion of SNc section, MGA_0676 lost its functions of nuclear localization, nuclease activity, and cytotoxicity. Further, sialidase, a neuraminidase was identified as a potential virulence factor due MG adherenace to host cells via sialic acid receptors taking into account the virulence role sialidases imply in other bacterial pathogens (May et al. 2012; Bercic et al. 2008.). The preferred sialic acid for attachment of MG is N-acetylneuraminic acid (**Neu5Ac**) with no strict specificity to one of the following linkages (α-2,3 or α-2,6 linked sialic acids) was illustrated (Glassglow and Hill, 1980). While attenuating the virulence of sialidase knock-out mutants in chickens; sialidase complementation on the other hand did not fully restore virulence in MG (May et al. 2012). Cellular division of MG commences with elongation of the cell supervened by the formation of bleb structures. The occurrence of constriction between the two compartments of MG induces the generation of two daughter cells post 140 minutes of incubation (Morowitz and Maniloff 1966).

2.3.6 Pathogenesis

Mycoplasmas powerful capability to dramatically alter the repertoire and immunogenicity of surface antigens is a vital constituent of its pathogenesis infectious process that grant them to hover undetected by the host's lymphatic system. Apart from vertical transmission,

the upper respiratory tract and/either conjunctiva are commonly acknowledged as portals of entry for naturally acquired MG infections. MG attachment to host cells is a vital virulence factor as it is imperative for successful infection, colonization and pathogenesis (Razin and Jacobs.1992; Dallo and Baseman,1990. Matyushkina, 2016; Razin et al., 1998). Gliding motility is a unique phenomenon observed in MG involving the polarized tip structure that mediates movement on solid surfaces and tight attachment to host cell membrane (Majumder, 2014; Indikova et al., 2014). MG attach to epithelial cells via the aid of their terminal organelle known as the bleb or tip structure binding closely to the host cell membrane (Carson et al., 1992; Collier and Clyde, 1971; Uppal and Chu, 1977; Tajima et al., 1979). A ruthenium red-staining capsule may mediate in cytadsorption to avian tracheal epithelium, but no fusion (of MG to host cell) and no injury (to host cell membrane) has been evident (Tajima et al. 1979; Tajima et al. 1982). Electron microscopy (**EM**) illustrated capsular material associated with MG cells binding with chicken TEC (Gaunson et al., 2000). Howbeit, this baffling interaction amid MG and host epithelial cells is most likely crucial in MG's pathogenic mechanism (Tajima et al. 1979; Ley, 2008). Post attachment, MG infection discharges mucous granules, followed by the exfoliation and annihilation of both ciliated and non-ciliated epithelial cells. This occurs in *vivo* and *in vitro* and causes alternations to tracheal epithelial surfaces (Dykstra et al. 1985. Charlier et al. 1981) which; along with ciliostasis, may have primary and secondary functions in infections process (Ley, 1997). Diversified surface-exposed proteins adhere constituents of the extracellular matrix (**ECM**) is suggested to contribute to attachment and colonization of MG (May et al., 2006; Jenkins et al., 2007; Chen et al., 2011). Further, the ability of MG to invade host cells

relies on the molecules comprising the host ECM (e.g. fibronectin, collagen type IV, plasminogen) (Furnkranz et al., 2013; Indikova, 2014; Yavlovich, 2004).

Normal function of TEC in mucosal membrane is disturbed upon MG infection due to generation of diverse metabolites, toxic substances and depletion of AA, FA and DNA precursors (Stipkovits, 2015). As a single lipid bi-layer, the MG membrane constitutes numerous anchored integral, peripheral proteins and membrane embedded lipoproteins (Chambaud et al., 1999). Key changes are localized in variable lipoprotein hemagglutinin A (**VlhA**) genes upon the adaptation of MG to eukaryotic host cells (Matyushkina et al., 2016), which occupies 10.4% of genome or size of 103kb representing the importance of lipoprotein for mycoplasma's bacterial fitness (Papazisi et al., 2003; Majumder, 2014). Lipoproteins are solely the pathogen-associated molecular patterns (**PAMP**) on MG surface that are known for inflammatory properties and mounting innate immune response (Mogensen, 2009). Lipoprotein haemagglutinin VlhA also plays a role in the attachment and the generation antigenic diversity (Markham et al., 1992; Markham et al., 1994). MG has the potential of invading non-phagocytic host cells such as erythrocytes, fibroblast an HeLa cells, RBCs, with low passage virulent strains more efficient in invasion than high passage virulent strains. Such attribute grants MG the opportunity for resisting host defenses in addition to selective antibiotic treatment (Ley, 1997; Majumder, 2014). In turn, aiding in the development of chronic infections as well as systemic infections (Winner et al. 2000). It has been reported that a major adhesion protein on the surface of MG-HS strain, known as pMGA1.2 is required for MG infection in chicken, as it able to specifically bind to a ~30 kDa host protein (Hu et al., 2015). Moreover, Hu et al., 2015 identified

apolipoprotein A-I **(ApoA-I)** may be of an essential host factor in MG as it cooperates with pMGA1.2 and blocking ApoA-I prevented cilia loss and damage in chicken trachea cells (Hu et al., 2015). MGA_0676 localized on MG membrane surface can induce apoptosis and pathological damages in chicken cells (Xu et al., 2014). Cytokine induction and the excitation or suppression of B and T lymphocytes can occur during the infectious process (Ley, 1997). In R_{low} virulent strain, *pneumoniae*-like protein A (**PlpA**) and HMW3-like protein (**Hlp3**) were classified as fibronectin-binding proteins; yet they do not exist in attenuated derivative $R_{high\ strain}$ (May et al. 2006). While, the **OsmC**-like protein, known as MGA_1142, was demonstrated to bind heparin (Jenkins et al., 2007). Plasminogen binding of MG demonstrated to be mediated by α-enolase; MG adhesion to chicken fibroblast **DF-1** shown to be significantly inhibited by more than 77% in the presence of α-enolase-specific antibodies (Chen et al., 2011). Tracheal MG infection may be considered self-confined in the absence of concurrent infection or environmental stressors (Ley, 2008; Levihson and Kleven, 2000; Yagihashi and Tajima, 1986), but the bird may remain a carrier even in the presence of humoral or local antibody (Yagihashi and Tajima, 1986; Kleven, S. H. 1985); i.e. sustained continuously through the life of the bird as a subclinical form (Bencina *et al*., 1987). MG is characterized by high frequency antigenic or phenotypic variation of major surface antigens allowing the microorganism to modulate its surface proteins in order to evade the host lymphatic response (Chambaud et al., 1999; Ley, 1997; Rosengarten and Yogev. 1996; Bencina, 2002; Noormohammadi, 2007). In addition, MG has high frequency phase variability; a mechanism for coping with rapid varying environments without the need of random mutation. (Yogev et al., 1994; Garcia et al.,

1994; Glew et al., 2000; Levisohn et al., 1995). This is a reversible on/off switch of an "all-or-none" expressing phase, inducing variation in the degree of expression of either one or more proteins between cells of a clonal population. The peculiarity of this variation apart from gene regulation and genetic noise is the fact that it occupies for genetic or epigenetic means granting the variability to be heritable (Van der Woude and Bäumler, 2004). Such elusive properties imply for chronic infection subsistence, in comptemt of a robust immune response (Levisohn and Kleven, 2000). Majumder (2014) reported that MG lipid-associated membrane proteins (**LAMP**) from virulent and non-virulent strains were capable of up-regulating several inflammatory genes from tracheal epithelial cells (**TEC**) in both vitro as well as ex-vivo including, and not limited to IL-1β, IL-6, IL-8, IL-12p40, CCL-20 and NOS-2. Moreover, the interaction of a virulent strain with TECs induces significantly greater macrophage chemotaxis than a non-virulent strain, and the interaction of R_{low} with TECs motivates greater gene up-regulation efficiency from macrophages as compared to R_{high} (Majumder, 2014). MG is a primarily etiological agent of the respiratory tract and conjunctiva, causing destruction and motility of cilia and the excretion of mucus is altered. In effect, MG travels down damaging the lung and air sacs. although can spread to other organs and colonize various pulmonary tissues including blood, heart, spleen, liver and brain; leading to mycoplasmaemia and spreading as well to joints, ovary and oviduct illustrating the development of systemic infections resulting in acute and chronic diseases. Any presence of secondary bacteria in the upper respiratory tract such as *H. paragallinarum* or *E. coli* follows MG; aggravating the pathological processes and its severity. Colibacillosisas, concurrent infections (e.g. *E. coli*), infections from live vaccines,

including factors of poor environmental conditions and immunosuppression result in severe cases of MG (Ley, 1997; Grodio et al., 2008; Indikova, 2014; Winner et al., 2000; Majumder, 2014;Furnkranz et al., 2013; Much et al., 2002; Jerkins et al., 2007; Stipkovits, 2015; chin et al., 1991; Naylor et al. 1992; Nakamura et al. 1994; Gross, 1990; Jordan, 1972). As cryptic the molecular mechanisms of MG infectious process remain to be, it is clinically suggested that MG pathogenesis is to a great extent involves destruction due to host immune and inflammatory responses as a substitute to direct toxic effects via MG cell components (Razin et al., 1998; Ley, 1997). The interaction of mycoplasmas with the lymphatic system was observed to call for macrophage activation, cytokine induction, constituents of mycoplasmas cell behaving as superantigens, in addition to autoimmune expressions (Razin et al., 1998).

2.3.7 Epidemiology

MG-infections have negatively struck the health and rammed in the production of poultry in commercial, free-range and organic flocks globally. It mainly infects gallinaceous birds specifically chickens and turkeys. However, it has been isolated from different avian species not limited to pheasants, finches, partridge, Japanese quails, bobwhite quails, peafowl, Amazon parrot, flamingos; song birds, ducks, geese, free range and backyard poultry. Lower incidence of MG infection has been reported in goldfinches, pine grosbeaks, evening grosbeaks, purple finch and blue jay (Murakami et al. 2002; Bencina et al. 2002; Vitula et al. 2011; Hartup et al., 2000; Mikaelian et al., 2001; Bencina et al., 1988; Ley et al., 1997b; Buntz et al., 1986; Mikaelian et al., 2001; Gharaibeh et al., 2011; McMartin et

al., 1996; Roussan et al., 2015; Reece et al., 986; Ibrahim et al., 2000; Lo et al, 1994; Saadh and Hasani, 2016). MG accompanies emerging problem for commercial flock and endemic outbreaks persist in multiple-age layers (Ewing et al., 1996; Lehvisohn and Kleven, 2000; AAAP/AVMA, 2001; Kelly et al., 1994; OIE, 20009). World-wide distribution of *M. gallisepticum* is highlighted in Figure 2-7 map below according to sources and references provided by CABI (2018), OIE (2009) and OIE Handistatus (2005).

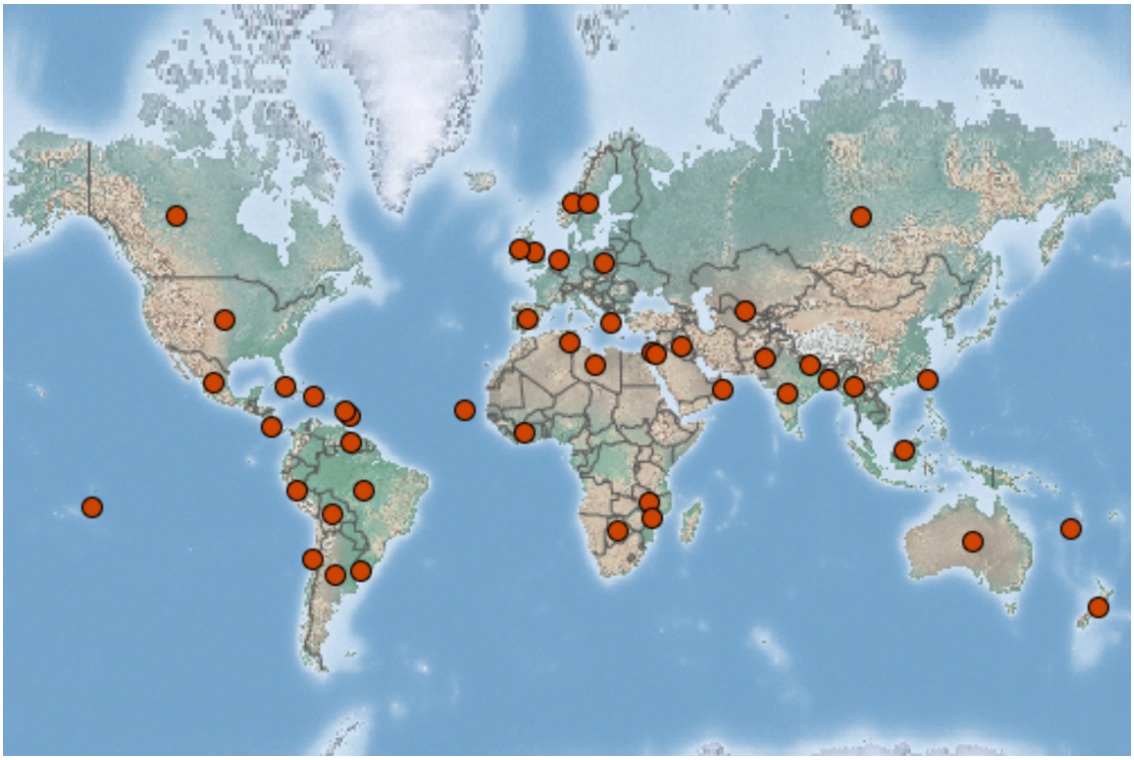

Figure 2-7. World-wide distribution of *Mycoplasma gallisepticum*. Red dots on map display the presence of *M. gallisepticum* infection that is apparent in various countries around the globe. References and sources obtained of reported present infection, localized infection and evidence of pathogen provided by CABI (2018); OIE (2009) and (OIE Handistatus, 2005); map and density of infection is generated via CABI (2018) website.

2.3.8 Transmission, Carriers, and Vectors

Vertical or transovarian (*ovo*) transmission is one route for MG infection (Lin and Kleven, 1982; OIE, 2018; Ortiz et al., 1995; Glisson and Kleven, 1985). The second route is via horizontal transmission throughout the flock mainly through aerosols or inhalation of respiratory droplets from infected birds, infected hatching progeny, or by conjunctival exposure through the upper respiratory tract. In addition, direct or indirect contact of susceptible birds with infected clinical or sub- clinical birds and via fomites including contaminated airborne dust, droplets, or feathers, along with poor biosecurity, personnel practices and management which can lead to rapid outbreak and widespread infection (McMullin, 2014; Ley, 2003**).** An increase in flock density increases the rate of horizontal transmission (Ley, 2003). MG horizontal transmission within a chicken flock has been described by McMartin et al. (1987) and is listed in Table 2.3 below.

MG transmission-rate greatly depends on its survival outside the host; a vital epidemiological factor. MG is capable of remaining viable in egg yolk for 18 or 6 weeks at temperatures of 37°C and 20°C, respectively; in feces or muslin cloth for up to 3 days at temperature of 20°C (Chandiramani et al.,1966), on straw, cotton, and rubber for 2 days; on human hair for 3 days; on feathers up to 4 days, in tap water 2-6 days and in human nasal passage for 24 hours (Christensen et al. 1994; Jordan, 1990).

The highest rates of MG transmission were observed during the infection's acute phase. This is because MG demonstrates a peak in the colonization of the respiratory tract during this phase (Gaunson et al., 2000; Lin and Kleven, 1986; Glisson and Kleven 1984; Ley 2003). While, the source of MG-infection is unknown; cold months, poor air quality,

crowding of birds, secondary infections, and few live virus vaccines may assist and exacerbate MG disease and its transmission (OIE, 2008; Chandiramani et al.,1966; Ley, 1997; Levihson and Kleven, 2008; Christensen et al., 1994).

Table 2.3. Phases of horizontal transmission of *Mycoplasma gallisepticum*[1]

Phases	**Description**
Phase 1	A latent phase Period of 12— 21 days Prior to first detection of Ab in inoculated birds
Phase 2	Infection gradually occurs Period of 1—21 days 5—10% of flock infected
Phase 3	Development of Ab Period of 7—32 days 90—95% of the remaining flock
Phase 4	A terminal phase Period of 3—19 days Positive for MG in remaining flock

[1] McMartin et al. (1987).

2.3.9 Incubation Period

Meaningful incubation periods under natural (not experimental) conditions cannot be stated as it is very difficult to estimate the possible date of exposure based on the appearance of clinical signs; as numerous and highly variable factors (depending on MG strain virulence, complicating infections, and environmental and other stressors) may influence the onset and extent of clinical disease (Ley, 1997). On the other hand, research experimental MG challenges of high dosage and uniform administration, the incubation period of MG ranges between 6 to 21 days.

2.3.10 Clinical Signs

Dominant signs of MG disease in chicken flocks are nasal discharge, tracheal rales and coughing. Conjunctivitis, oral and ocular mucus discharge are also very likely to occur. Decrease in FI and BW are apparent. Majority of outbreaks in broilers occur between 4–8 weeks of age. Clinical signs are more evident in males. In pullets, the onset of lay is delayed (Burnham et al., 2002) and in laying hens reduced egg production and egg size along poor egg quality and increased embryo mortality are reported (Burnham et al., 2002, Yoder and Hofstad, 1964; Patterson, 1994; Mohammed et al., 1987). MG disease is greater in severity during the winter season. Asymptomatic signs of MG can occur and certainly at a younger age yet serologic evidence can confirm presence of MG infection. Low subclinical infection may generate close to the onset of egg production, most likely due to vertical transmission; and can develop into a clinical infection due to stressors. This is frequent in progeny of infected chickens hatched from eggs immersed in antibiotic as a reason to control MG (Ley, 1997). Serious outbreaks of MG with high morbidity and mortality in broilers are commonly due to concurrent infections and environmental aspects (Kleven, 1998; Furnkranz et al., 2013; Much et al., 2002). Yet, source of infection is not always clear and may linger unnoticed due to asymptomatic clinical signs.

2.3.11 Morbidity and Mortality

2.3.11.1 Embryos

The highest concentration of MG can be found in the yolk, yolk sac as well as in the chorioallantoic membrane (**CAM**). Embryo death is very evident within period of 5-7 days post MG yolk sac inoculation at 7 day-old embryonated chicken egg. Most prominent clinical signs include dwarfing, edema, enlarged spleen and liver necrosis. Moreover, MG strains vary in their *ovo* pathogenicity. It has been shown that there is no correlation existing between *in ovo* and other *in vivo* or *in vitro* techniques in term of MG pathogenesis evaluation (Ley, 2008; Levisohn et al., 1985).

2.3.11.2 Chickens

MG commonly infects all chickens in a flock yet it is variable in terms of its span and severity. During cold weather, MG is more severe with a lengthier duration having a greater effect on younger birds in addition to substantial decrease in egg production in layers (Charleston et al., 1998). Aside of being the primary agent of CRD, MG earmarks tracheatis, airsaculitis, inflammation of the lungs, conjunctivitis, rales and many other respiratory distresses. Decrease in growth and condemnations at processing establish further losses. NDV, AIV, IB, or E. *coli* infection can hasten outbreaks of MG and exacerbate the condition. It has been shown that *E. coli* could not readily colonize the air sac without previous infection by MG (Soeripto et al., 1989). Concurrent infections with

MG increase the severity and span in chicken flocks and cause complicated outbreaks (Ley, 2008; Levihson and Kleven, 2000; OIE, 2008; Qasem et al., 2015).

2.3.12 Economic Significance

MG is ascribed as of a chiefly crucial economic significance among all pathogenic mycoplasma species confronting poultry (Ahmad et al., 2008). Significant losses ensue from poor hatchability, drop in egg production (by layers and broiler breeders up to 20%), decrease in egg quality, increase rate of embryonic mortality and the culling of day old chicks, decrease in feed efficiency and performance, reduced growth, increase in morbidity and mortality (up to 30% in uncomplicated cases; and at much higher rates with concurrent infections and/or environmental stressors), carcass condemnations, medication and/or vaccination costs (Charleston et al. 1998; Ley and Yoder ,1997; Kleven, 2008; Bradbury, 2005; Mohammed et al., 1987; Osman et al., 2009; Okwara, 2016; Carpenter et al. . 1981; Vaillancourt and Ley, 2001; Purswell et al., 2012). Of approximately 6 months economic loss in a North Carolina company in 1999 were estimated between 500,000 and 750,000 dollars (Rhorer, 2002). During 1984 in United States, layers that were infected with MG placed 15.7 less eggs compared to healthy layers. This resulted in a 127 million loss of eggs corresponding to 125 million dollars in loss a year (Mohammed et al., 1987; Yoder, 1991; Osman et al., 2009).

2.3.13 Gross Pathology

Catarrhal exudate in nasal and paranasal passages, trachea, bronchi, and air sacs and oedema of the airsac walls predominately highlight MG infected gross lesions. While sinusitis is prominent in turkeys, it can be observed in chickens and other infected birds. Conjunctivitis is commonly observed in broilers and turkeys infected with MG and it is epidemic in house-finches, songbirds. Pneumonia presence may be observed and air sacs comprise caseous exudate, possibly along either beaded or lymphofollicular presentation. Combination of airsaculititis, including fibrunous or fibrinopurulent perihepatitis along with adhesive pericarditis are apparent in severe cases of air sac disease of MG infection. As a result of such case, death rate and pervasive condemnations increases (Charleston et al., 1998; Ley, 1997; Jordan, 1996; Mikaelian et al., 2001; Ley et al., 1997). MG-associated kerato-conjunctivitis observed in layers is characterized by eyelids, facial subcutis and often coneral capacity edema (Nunoya et al., 1995). Salpingitis caused by MG-infection features exudates packed swollen oviducts induces dropped EP (Nunoya et al., 1997; Domermuth et al., 1967).

2.3.14 Histopathology

Thickening of mucosal membranes is highlighted in MG-infected tissues (trachea, chorioallantoic membrane, sinus membrane, lateral borders of lungs, air sac) due to the penetration of mononuclear cells and the hyperplasia of mucosal glands (Whithear et al., 1996; Wijesurendra et al., 2015; Hitchner, 1949; Islam et al., 2011). In tracheal tissues,

destruction and loss of cilia is indicated in MG-infected birds, along swollen epithelial cells (Ley, 1997). Commonly, lymphoid hyperplasia is observed in submucosa (Ley, 1997). Ciliostasis and the cytadherance of MG to host's villi are apparent. (Ley, 2008; Ulgen et al., 1998; Takagi and Arakawa, 1980). Lung, inflammation caused by MG is characterized with pneumonic regions, alterations in the lymphofollicles and granulomatous lesions (Ley, 2008). Keratoconjunctivitis in MG-infected layers is outlined by epithelial hyperplasia, sub-epithelial edema, drastic cellular infiltration, and stroma of the central fibrovascular connective tissue that lead to thickening of the eyelids (Nunoya et al., 1995). While in salpingitis cases, thickening of the oviductal mucosa and lymphoplasmacytic infilt ration are notable (Nunoya et al., 1997). Proliferation of lymphocytes and plasma cells following germinal centers is also indicated in the lamina propia, specifically in the sub-epithelial region with a consequence of irregular elevations of the hyperplastic epithelial layer (Nunoya et al., 1997). Encephalitis caused by MG has been examined from moderate to severe forms comprising fibrinoid vasculitis, lymphocytic cuffing of vessels, parenchymal necrosis, and meningitis (Chin et al., 1991; Ley, 2008).

2.3.15 Ultrastructural Pathology

Ultrastructural pathology includes degeneration of the epithelial cells and inflammatory cellular infiltration of the mucosa in tracheal tissues (Tajima et al., 1979). The attachment of MG to epithelial cells close to host cell membrane via the aid of their bleb structure and their release of mucous granules were observed. This ensued the exfoliation of ciliated and

non-ciliated epithelial cells (Dykstra et al. 1985; Levisohn and Dykstra, 1986). Moreover, MG has been predominately observed in extracellular regions with rare presence in phagocytic vacuoles (Wen et al., 2000; Ley, 2008; Hod et al., 1982). MG has also been shown to inhibit the antiviral gene expression of Avian Influenza virus, and influencing the virus' attachment to the host cell by desialylation of α-2,3 linked sialic acids (Sid et al., 2016).

2.3.16 Immunity

Recovered gallinaceous birds are suggested to possess particular immunity to MG as antibodies against MG persisted; hence upon re-exposure of infection birds possessed faster rate of MG elimination (Ley 2008). Yet recovered birds remain carriers (Bencina and Dorrer, 1984) thus, potentially transmitting MG-infection to susceptible birds either vertically or horizontally. Antigenic variability of MG immunodominant surface proteins, in addition to spontaneous high frequency phase variability and switching proposes such immune modulation of MG may play a vital role in their adaptive mechanism (Razin and Herrmann, 2002; Glew et al., 2000; Levisohn et al., 1995; Garcia et al., 1994; Bencina et al., 1994). The function of the bursa of Fabricius and antibody generation indicated resistance and serological response to MG (Lam et al., 1984; Avakian and Ley, 1993; Adler et al., 1973; Ley, 2008; Luginbuhl et al., 1967). However, poor correlation has also existed between levels of specific antibody and their protection against MG-infection (Lam and Lin, 1984; Ley, 1997). High antibody titers against MG along decrease in tracheal gross

lesion scoring were demonstrated (Yagihashi and Tajima, 1986; Ley, 1997). According to Avakian and Ley (1993) antibodies in respiratory tract triggered response to MG that repressed the attachment of MG to TECs. Further, maternal antibodies against MG found in embryonated eggs significantly decreased the *in ovo* pathogenicity of MG and augmented the probability of MG-infected embryo's survival (Lin and Kleven, 1984; Levisohn et al., 1985; Ley, 2008).

2.3.17 Management Procedures

A combination of eradication, extensive biosecurity of premises and surveillance with periodic serological monitoring is a well control *M. gallisepticum* program. The United States has adopted the National Poultry Improvement Plan (**NPIP**); particularly at hatcheries and breeding flocks in the past 40 years demonstrating significant decrease in MG incidences. Outbreaks persists in many countries around the globe, and management ought to be the first and key priority for reducing the spread of MG in flocks. Because MG is vertically transmitted, maintaining flock free of MG at hatchery level is essential and is possible by introducing MG-free birds followed by a well biosecurity program. This should be maintained with serologic monitoring at short intervals; for turkeys every 21 days weeks and chickens every 14 days for detection and prevention of MG-infection transmission (Ley, 2008; Lehvison and Kleven, 2000).

Yet as poultry production density have increased, and with few infected birds resulting in horizontal transmission involving the entire, preserving MG-free flock may be difficult and even impossible (Levisohn and Kleven, 2000). In such case, appropriate antimicrobial

therapy and dosage may be administered to reduce morbidity and mortality (Ley, 2008; Lehvison and Kleven, 2000).

2.3.18 Treatment

Antibiotic medications have been administered for treatment of chronic respiratory disease and MG infections and transmission in poultry (Ose and Tonkinson, 1985; Glisson et al. 1989; Boone et al., 1957; Jordan and Horrocks, 1996; Jordan et al., 1999; Kempf et al., 1997). MG has demonstrated sensitivity *in vitro* and *in vivo* to antimicrobials including macrolides, tetracyclines, fluoroquinolones, and others (Bradbury et al. 1994; Jordan et al. 1998b; Behbahan et al., 2008; Grózner et al. 2016; Wang et al. 2001; Levisohn and Kleven, 2000) but is resistant to penicillins and those antibiotics that inhibit cell wall biosynthesis. MG has also shown to be cross-resistant and may develop resistance against commonly administered antimicrobials such as the resistance (>/= 1 mg/ml) to streptomycin post 2-3 passages, resistance to erythromycin and spiramycin post 5-8 passages, resistance to enrofloxacin post 8-10 passages, and resistance to tylosin post 9-11 passages (Zanella et al., 1998; Levisohn and Kleven, 2000).

2.4 MACROLIDES AND TILMICOSIN OVERVIEW

2.4.1 Macrolides

Macrolides are acknowledged bacteriostatic administered at therapeutic concentrations but they can gradually become bactericidal, particularly against streptococcal bacteria; illustrating bactericidal at high concentration. Hence, macrolides bactericidal action is characterized as time-dependent. The majority of macrolides were isolated from soil bacteria of the genus *Streptomyces*. The antimicrobial mechanism for all of the macrolides appears to be similar. Macrolides interfere with protein synthesis by reversibly binding to the 50S subunit of the ribosome; preferentially to the 23S rRNA of the 50S subunit; inhibiting translocation that is required for the elongation of peptide chain via their attachment to the donor site. Such antimicrobial action is augmented by a high pH level; ranging from 7.8-8.0; and thus, suppressed by lower pH levels. This allows them to be effective in abscesses, necrotic tissue, or acidic urine (The United States Pharmacopeial Convention, 2007).

Spiramycin; introduced in 1960s; was the first macrolide intended for animal use; followed by erythromycin and tylosin in 1970s (Pyolara et al., 2014). The use of macrolides as growth promoters began within same period as for its therapeutic use. This is where spiramycin as well as tylosin were used in Europe until 1998 (Council Regulation EC2821/98 of 17 December 1998). Macrolides have shown to be effective in treating and preventing respiratory diseases according to many studies (Jordan et al., 1993; Jordan et al., 1999; Kempf, 1997; Christodoulopoulos et al., 2002; Abu-Basha et al., 2007; Pyolara et

al., 2014; ElSayed, 2014; Lappin, 2017). The first one dose long-acting injectable macrolide for food animal use was tilmicosin (Pyolara et a., 2014).

Macrolides have demonstrated to be effective against *Mycoplasma* species and Gram-positive bacteria including *Streptococcus* spp. and *Staphylococcus* spp. However, they are not highly competent against Gram-negative bacteria (Botsolou and Fletouris, 2001), this is due to the fact that the lack of cell wall permeability of Gram-negative bacteria renders them to hold resistance against macrolides.

2.4.2 Tilmicosin

Tilmicosin (20-deoxo-20-(3,5-dimethylpiperidin-1-yl) desmycosin); synthesized from tylosin; is a broad-spectrum bacteriostatic macrolide antimicrobial administered solely for veterinary use. Tilmicosin has been first developed by Elanco Animal Health (a division of Eli Lilly and Company USA) in 1980s, and is synthesized by sequential hydrolysis of mycarose and by reductive amination of the aldehyde in demycarosyltylosin with 3,5-dimethylpiperidine (Wu et al., 2015). It has a molecular formula of $C_{46}H_{80}N_2O_{13.}$ It is essentially effective against *Mycoplasma* spp., *Pasteurella* spp., and distinct Gram-positive organisms including Gram-positive anaerobic species and Gram-negative respiratory pathogens such as *Mannheimia haemolytica* and *Pasteurella multocida* and there exist variation as to potency and activity (Boothe, 2017; Jordan et al., 1993).

Tilmicosin is administered for the treatment of respiratory tract infections in poultry caused by *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Ornithobacterium rhinotracheale*, and *Pasteurella multocida, Pseudomonas aeruginosa* (Boothe, 2017; Abu-Basha et al. 2003; Jordan et al., 1993; Kempf et al., 1997; ElSayed et al., 2014; Prescott, 2000).

2.4.3 Mechanism of Action

Macrolides reversibly bind to the 23S ribosomal RNA (**rRNA**) in the 50S ribosomal subunit, inhibiting translocation of peptides by the dissociation of peptidyl-tRNA, and thus preventing protein macrolides to bind to mitochondrial ribosomes (Adams, 2001; Prescott and Baggot, 1993; Menninger, 1995; Champney and Tober, 1999). Macrolides have a bacteriostatic action, with a potential for a time-dependent bactericidal performance; when administered in high concentrations (Wu et al., 2005; Adams; 2001; Barragry, 1994). They are incapable of crossing the mitochondrial membrane; hence they do not generate bone marrow suppression in mammals (The United States Pharmacopeial Convention, 2007).

2.4.4 Absorption

If not inactivated by gastric acid, macrolides are absorbed from the gastrointestinal tract. Generally, plasma concentrations are elevated within 1–2 hours. However, macrolide absorption patterns may be erratic because of contact with food and is also dependent upon the salt or ester used in the drug preparation (The United States Pharmacopeial Convention, 2007).

2.4.5 Distribution

Macrolides are distributed in tissues, and its concentration may either be similar as found in plasma or higher. They accumulate within various cells, including macrophages demonstrating ≥20 times the plasma concentration. White blood cells (WBCs) then aid in macrolide distribution to inflammation site. Macrolides commonly concentrate in the spleen, liver, kidneys, and especially in the lungs. Macrolides also concentrate in bile and

milk, with up to 75% of the dose is constrained to plasma proteins. Macrolide accretion is contributed to the length of dosing interval; as shown by tilmicosin. Tissue concentration are particularly high in the case of spiramycin although its plasma concentrations are rather low (The United States Pharmacopeial Convention, 2007). In lung tissue, tylosin and timicosin have demonstrated higher concentration than in sera from 2 to 26 hours post intracmuscular administration and at least 96 hours post single subcutaneous administration, respectively (Thomson and Lawrence, 1994; Van Duyn and Folkberts, 1979).

2.4.6 Tilmicosin Precautions

Tilmicosin should not be administered intravenously in all species in order to avoid cardiotoxicity (The United States Pharmacopeial Convention, 2007).

2.4.7 Excretion

Macrolides are subjected to enterohepatic cycling and they are excreted along with and their metabolites above >60% in bile. Urinary exception may be slow and variable (often <10%) yet it depicts a more significant route of elimination (Baietto et al., 2014; Nightingale et al., 2002).

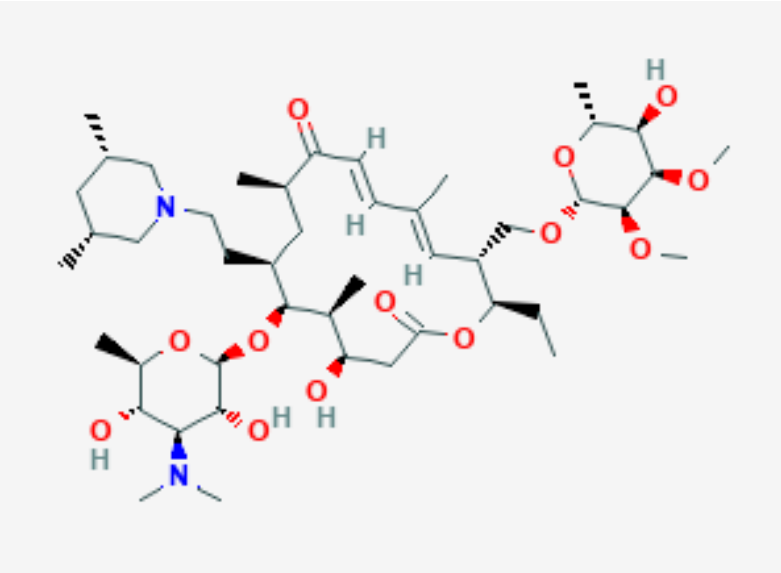

Figure 2-8. Tilmicosin chemical structure. Tilmicosin has a molecular formula of $C_{46}H_{80}N_2O_{13}$.It characterized by a multi-membered lactone ring with one or more amino sugars attached. Tilmicosin is the semi-synthetic derivative of tylosin that consists of 16-membered-ring group; i.e the number of atoms comprising the lactone ring (Shyrock et al. 1998; Bryskier et al., 1993; Kirst, 1992).

2.4.8 Pharmacodynamic Properties

Tilmicosin phosphate has a molecular weight of 967.13 (The United States Pharmacopeial Convention, 2007). Tilmicosin is characterized as a semi-synthetic antibiotic of the macrolide group, behaves a bacteriostatic but at high concentrations it may possess bactericidal activity. This antibacterial activity is predominantly against Gram-positive microorganism. Tilmicosin activity has been effective on Mycoplasma *hyopneumoniae*, Pasteurella *multocida* and Actinobacillus *pleuropneumoniae* in pigs; on *Mycoplasma gallisepticum* and Mycoplasma *synoviae* in chickens and turkeys; and on Mannheimia *haemolytica*, Pasteurella *multocida*, *Mycoplasma bovis* and M. *dispar* in calves (The United States Pharmacopeial Convention; 2007; HPRA, 2016; Shyrock et al. 1998). Tilmicosin and macrolide have been suggested to act synergistically with the host immune system;

augmenting phagocytosis of bacteria. Further, tilmicosin has demonstrated the ability to inhibit the replication of both Porcine Reproductive and Respiratory Syndrome virus in *vitro* of alveolar macrophages as dose-dependent (Kovaleva et al., 2011). It is worth noting that cross-resistance has been illustrated between tilmicosin and other macrolides as well as lincomycin (HPRA, 2016).

2.4.9 Tilmicosin Withdrawal Period

The tilmicosin withdrawal periods according to species are outlined in Table 2.4 below. It is important to note that tilmicosin is not authorized for use in laying birds producing table eggs for human consumption and is not authorized for usage within 14 days of onset of the laying. Tilmicosin should also not be administered in lactating animals.

Table 2.4 Tilmicosin withdrawal period(s) in different species.

Species	***Withdrawal period (days)***
Calves	42
Turkeys	19
Pigs	14
Chicken	12

2.4.10 Tilmicosin and *Mycoplasma gallisepticum*

Studies *in vitro* have demonstrated the effectiveness of tilmicosin adverse to MG infection with a minimum inhibitory concentration of 0.048 ug/ml, particularly considering filed isolates originating from North Africa as well as Europe (Ose, 1987; Reeve-Johnson, 1996). Moreover, tilmicosin-based antimicrobial illustrated to significantly reduce ($P < 0.05$) MG infection symptoms of retarded growth and respiratory clinical signs at dosage of 50-300 mg/liter (Kempf et al., 1997). Shryock *et* al*.,* 1994 suggests treating MG infection in broilers via the administration of 300-500 mg/kg tilmicosin in feed. Both, peritonitis and airsac lesions were significantly decreased ($P < 0.05$) with tilmicosin treatment of 100, 200 or 300 mg/liter for 5 days (Kempf et al., 1997). Charleston et al., 1998 also reported the efficacy of tilmiconsin in lessening severity as well as the incidence of airsacculitis caused by MG.

An observed significant decrease in the proportion of MG-positive birds was obtained at a dose level of 50 mg/liter ($P < 0.05$) according to Kempf et al. (1997). Further increasing tilmicosin dose improved the results causing reduction in the number of MG shedding birds. Dosage of 200 and 300 mg/liter ensued no serologically positive results on day 21 as compared to 46/58 positively MG- infected untreated birds (Kempf et al., 1997).

The minimum effective concentration of tilmicosin in drinking water was illustrated to be equal to or less than 50mg/1 for a period of 3 or 5 days. Although tilmicosin significantly alleviated the severity of airsacculitis caused by; no impact was shown morbidity of birds (Charleston et al., 1998).

El-Samie (2014) has shown that treatment of MG- infection with tilmicosin phosphate in drinking water for 3 days was successful. Tilmicosin phosphate demonstrated to be effective in to conquering weight loss issues and marketability due to MG (El-Samie, 2014).

2.4.11 Macrolide Resistance

Macrolide resistance can be intrinsic or plasmid-mediated and constitutive or inducible (Rosato et al., 1999; Leclercq, 1991; Emond et al., 1989). Resistance can occur by target site modification, drug inactivation, or drug efflux (The United States Pharmacopeial Convention, 2007). Macrolide resistance may be rapidly established as observed with erythromycin or gradually as with tylosin (Leclercq and Courvalin, 2002; Pyorala et al., 2014). Thomas et al. (2003b). Macrolide resistance commonly result in cross-resistance between macrolides. In vitro susceptibility testing of 50 various strains of MG isolated in Israel during the period 1997-2010 have indicated acquired resistance to tylosin and 50% of the MG strains demonstrated resistance to tilmicosin (Gerchman et al. 2011).

In gram-positive bacteria, resistance to macrolide is a cause of alterations in ribosomal structure; either of target site methylation or mutation and loss of macrolide affinity (Tait-Kamradt et al., 2000; Skinner et al., 1983). Another, resistance mechanism is due to efflux from cells demonstrating less frequently drug inactivation (Vester and Douthwaite, 2001; The United States Pharmacopeial Convention, 2007).

CHAPTER 3

MATERIALS AND METHODS

The following procedures were followed to isolate and identify *M. gallisepticum* collected randomly from local breeds.

3.1 Isolation and Identification of *Mycoplasma gallisepticum*

3.1.1 Frey's Broth Preparation for Samples Collection

3.1.1.1 Fresh Yeast Extract Preparation

1. 250g of baker's yeast are soaked in 1 liter of distilled water for 1 hour and then heated until boiling.
2. After cooling, the suspension is distributed in tubes and then centrifuged at 3000 xg for 20 minutes.
3. The supernatants are then collected and filtered through Whatman No. 1 filter paper and their pH is adjusted to 8.
4. The extracts are then filtered through a 0.8µm filter paper then filter sterilized through a 0.22µm filter paper.
5. Aliquots of 15 ml of the sterile extracts are distributed in sterile cups and stored at -20 °C.

3.1.1.2 Inactivation of the Swine Serum

1. Sterile swine serum (Gibco, Grand Island, N. Y. 14072, USA) is heat

inactivated in a water bath at 55°C for 30 minutes.

2. Aliquots of 18 ml are then distributed in sterile conical tubes and stored at -20 °C.

3.1.1.3 Frey's Broth Preparation

1. The selective broth was prepared as described by Frey et al. (1968).
2. For the preparation of 150 ml of broth 3.37g of *Mycoplasma* broth base (Oxoid Ltd. Basingstoke, Hampshire, England), 375µl of 10% w/v phenol red and 425 µl of 10% thallium acetate are mixed in 113.425 ml of distilled water.
3. The mixture is boiled and pH is adjusted to 7.8 with 0.1N NaOH.
4. After sterilization at 121°C and 20 psi for 15 minutes, the mixture is cooled and 15 ml of fresh yeast extract, 18 ml of heat inactivated swine serum, 750µl of penicillin containing 150000 I.U. and 2 ml of filter sterilized dextrose (0.225g/ml) are added to the mixture.
5. The broth in then distributed into sterile screw-capped tubes (5ml/tube).
6. The pH is readjusted to 7.8 using sterile 0.1N NaOH.
7. The tubes are ready for sample collection. It is worth noting that for highly contaminated samples it is recommended to raise the level of the thallium acetate concentration and put 750µl of 10% thallium acetate and 3 ml of 1% thallium acetate. Penicillin could be also raised to 450000 I.U. in 150 ml of Frey's broth.

3.1.1.4 Frey's Agar Preparation

The agar is prepared using the same materials and in their respective amounts. However, 1.5g of Bacto-agar (Difco Lab, Detroit, Michigan, USA) is added to the mixture of MG broth base, phenol red, and thallium acetate before boiling and sterilization. The mixture is allowed to cool till 55°C in a water bath. Fresh yeast extract, swine serum, dextrose and penicillin are then added to the mixture and the pH is adjusted to 7.8 with sterile 0.1N NaOH. Frey's agar is then poured in small sterile plates (10 ml/plate).

3.1.2 Sample Collection from Local Breeds

1. *M. gallisepticum* field isolates were collected from broilers, layers, broiler breeders, egg type breeders and pet birds from commercial pet shop in Ouzaii, Beirut, Lebanon.
2. Tracheal samples are obtained by rubbing the trachea of 13 random chickens with a sterile cotton swab. These swabs were then vigorously shaken in the tubes containing the Frey's broth.

3.1.3 Culture Methods for Detection of Mycoplasmas

1. Tubes containing tracheal suspensions are incubated at 37ºC for 3-4 days or once observation of phenol red colour change (from red to orange) is examined.
2. A volume of 20ul of the MG suspected broth is then subcultured onto Frey's agar and incubated in a bag with a moistened tissue at 37 ºC for 5-7 days or until the formation of Mycoplasma-like colonies that could be identified under 100x

magnification with a raised, dense central area.

3. Under sterile conditions, three separate fried-egg-like single colonies were selected for each isolate from each plate and subcultured into 3 separate tubes containing 5 ml of Frey's broth then incubated at 37 ºC for 3-4 days until a color change occurs.
4. The tubes are then stored at -80 ºC. These 3 tubes/sample will be then used for speciation and typing of the isolated colonies.

3.1.4 Rapid Slide Agglutination (RSA) test for speciation of Mycoplasma

1. The three tubes (above mentioned-suspected MG) were centrifuged at 15557*(gravity) for 15 minutes.
2. MG pellets were obtained and then resuspended in 0.5ml sterile saline to perform Rapid Slide Agglutination test.
3. A drop of re-suspended Mycoplasma pellet in sterile saline is added to a slide, and a drop of MG anti-sera (KPL, 37 Birch street, Milford, MA, USA) is added next to it.
4. The slide is then rotated slowly for approximately 2-4 minutes to allow mixing of the serum with the bacterial suspension.
5. Positive slide agglutination test illustrates formed granules (agglutinates) rapidly due to combination of homologous antigen and antibody.

3.1.5 PCR and Sequencing for Typing of the Isolated MG Colonies

3.1.5.1 DNA extraction

MG suspension, as confirmed by RSA test were subjected to 95°C for 10 minutes. Then, the tubes were subjected to -80°C for 10 minutes to extract the DNA.

3.1.5.2 PCR and Sequencing for M. gallisepticum strain identification

3.1.5.2.1 *pvpA* PCR

1. Amplification reactions for the pvpA gene of the DNA extracted from the pure culture samples were performed in a 50ul reaction volume as follows.
2. Protocol was followed according to REDTaq®ReadyMix™PCR Reaction Mix (SIGMA-ALDRICH®), Catalog Number R2523, (St. Louis, MO 63103 USA).
3. The following reagents were added to a 0.2 ml PCR tube in the following order according to the Table 3.1.
4. All amplifications were performed in a C100 Bio-Rad.
5. The amplification was performed accordingly with 30 cycles: Denaturation at 94°C for 1 min. Annealing primers at 55 °C for 2 min. Extension at 72 °C for 3 min.
6. Agarose gel preparation:

a) Add 8 ml of 50X TAE buffer to 400ml of distilled water into Erlenmeyer flask to make 1% TAE solution
b) A 0.80g of 2% Agarose powder is added to a 40ml of 1% TAE solution and brought to careful boil.
c) Allow agarose solution to cool down for 3-5 minutes to approximately 50°C

Table 3.1 Reagents and Volumes for *pvpA* conventional PCR.

Reagent	***Volume***
REDTaq ReadyMix	25 µL
Forward Primer (5 pM) pvpA3F (5' GGTAGTCCTAAGTTATTAGGTC3')	4 µL
Reverse Primer (5 pM) pvpA2R (5'CTATCCTACTATGAGAAAAATGAG3')	4 µL
Template DNA	3 µL
Water	14 uL
Total Volume	*50 µL*

d) The agarose is then poured into a gel tray slowly to avoid the formation of bubbles with the well comb in place.

e) Once solidified, place the agarose gel into electrophoresis unit.

7. Loading Samples and Running an Agarose Gel:

   a. The gel box was filled with 1% TAE until the gel was covered (or line indicated on the box).

   b. For the ladder: 2ul of EZ vision was mixed with 8ul of 1%TAE buffer and 2ul of ladder then carefully loaded into one well of the 2% agarose gel.

c. For the samples: 2ul of EZ vision was mixed with 10ul of each amplification reaction and were carefully loaded into the wells of the 2% agarose gel.

8. Agarose gel electrophoresis was run for 50 min at 100 V, and the gel was visualized under UV light using gel doc system (BioRad; 2000 Alfred Nobel Drive, CA USA).

3.1.5.2.2 Sequence analysis

Amplified products from *M. gallisepticum* strains were purified with a QIAquick PCR purification kit (Qiagen, Inc.).

3.1.5.2.2.1 Gel Purification

Gel purification was performed according to QIAquick® Gel Extraction Kit (50) (QIAGEN 2006 edition, QIAGEN GmbH, D-40724 Hilden) manual.

1. Successfully amplified amplicons (MG, MG1 and MG2 isolates) were excised from the agarose gel using a gel-cutting ruler.
2. Three volumes of Buffer QG (containing Guanidine Thiocyanate) were added to one volume of gel.
3. The mixture was incubated at 50°C for 10 minutes.
4. One volume of isopropanol was mixed with the sample.
5. The mixture was applied to the QIAquick column and centrifuged (8,000 rpm) for one minute.

6. An amount of 0.5 ml of Buffer QG was added to the QIAquick column and centrifuged (8,000 rpm) for one minute.
7. The mixture was washed with 0.75 ml of Buffer PE to QIAquick column and centrifuged for one minute.
8. The flow-through was discarded and the QIAquick column for an additional one minute at full speed of 15577xg.
9. PvpA gene DNA was eluted by adding 30 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) to the QIAquick column and centrifuged (5,000 rpm) for one minute.

3.1.5.2.2.2 Gene Sequencing

1. Two litters of gel purified prion amplicon DNA of *Mycoplasma gallisepticum* isolates were each mixed with 1uL of a 1.6 pmole/ l reverse primer and 2ul of Big Dye Terminator Version 1.1 (Applied Biosystem).
2. PCR was performed in PE9007 thermocycler (Applied Biosystem) using 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 minutes. Amplicons were finally sequenced in a ABI-Prism 3130 type sequencer (Applied Biosystem).

3.2 Preliminary Study: Evaluation of virulence of field isolates of *Mycoplasma gallisepticum* colonies

3.2.1 Disinfection and Preparation of the Animal Rooms

Walls, floors and windows of an animal room were cleaned, flushed with water and disinfected by soaking the floor overnight with 10% sodium hypochlorite. The room was

separated into 4 equal pens. Wood shavings were spread over the floor of each pen to a depth of around 5 cm. Each animal pen was supplemented with one feeder of 60 cm length, one waterer of 35 cm diameter and one height-adjustable 250 Watts infrared lamp. A prepared 10% sodium hypochlorite was placed in a platter in front of room door entrance to soak and disinfect shoes prior to placing on a shoe cover and entering the room; and a new batch was added when needed.

3.2.2 Bird and Treatments

A total of 40 day male broilers of Ross 308 strain aged one week were raised in floor pens at the American University of Beirut Animal Houses. Before purchasing the experimental birds, day-old chicks originating from the same breeding flock and hatchery were tested to confirm they were M*ycoplasma gallisepticum* free. Tracheas were collected from 12 chicks and put in 5ml of Frey's broth. The media was incubated at 37C for 10 days. No positive samples to MG were revealed.

3.2.3 Challenge

Birds were challenged with distinct field isolates of *Mycoplasma gallisepticum* of $8.3x10^6$ CFU/ml. The challenge strains were administered intrathoracically and intratracheally with 0.2 ml and 50 ul of the *Mycoplasma gallisepticum* suspension, respectively, at 10 days of age. Birds within pen 1 were challenged with MG isolate, birds within pen 2 were challenged with MG 1 isolate, birds in pen 3 were challenged with MG 2 isolate and birds in pen 4 served as control and remained unchallenged. Birds were observed every other day

for a period of 10 days. Birds were observed at different dates post challenge for clinical signs of rales, conjunctivitis, sneezing and coughing and oral and nasal mucosal discharge.

3.2.4 Diets

Starter, corn-soybean meal based diet was formulated to contain the recommended level or slightly exceed nutrient requirement suggested by the Ross 308 company (Table 3.3). Feed and water were offered *adlibitum* and a continuous lighting was provided.

3.2.5 Experimental Design

Birds were allocated to 4 treatments with a complete randomized design consisting of 10 birds per treatment. Disease signs frequency namely conjunctivitis, rales, sneezing and/or coughing and nasal and/or oral mucosal discharge are compared among different groups using chi-square analysis (SPSS v.24, 2018).

3.3 Experimental Research Thesis Study: Evaluation of the immunopotentiating effect of DL-Methionine on *Mycoplasma gallisepticum infected broiler*

The following procedures were followed to evaluate the immunopotentiating effect of 20% excess DL-Methionine on *Mycoplasma gallisepticum* infected broilers.

3.3.1 Disinfection and Preparation of the Poultry Houses at AREC

Walls, floors, windows of two poultry houses at AREC were cleaned, flushed with water and disinfected by soaking the floor overnight with 10% sodium hypochlorite. A prepared 10% sodium hypochlorite was placed in a platter (shoe-dip) at the entrance and at all points

being used to access the poultry houses to immerse and disinfect shoes prior to placing on a shoe cover and entering the room; and a new batch was added when needed. Gloves, lab coats, head covers, masks and biosecurity measures were strictly used.

3.3.2 Birds and Treatments

A total of 1200 day-old male broilers of Ross 308 strain were raised on the floor in two identical environmentally-controlled poultry houses. The experiment was conducted at Agricultural Research Education Center (AREC). On the same day of purchasing the experimental birds, day-old chicks originating from the same breeding flock and hatchery were tested to confirm they were *Mycoplasma gallisepticum* free.

The facility includes two poultry houses of which were converted to a closed system (environmentally-controlled) poultry houses with air fans exhaust ventilators and cooling pads. Each house included 12 floor pens accommodating 50 broilers per pen using wood shavings as litter materials, one large manual circular feeder and an automatic hanging bell shaped drinker. The MG-challenged birds were raised in the first building whereas the second building housed the unchallenged birds. The trial was composed of 8 treatments with 3 replicates per treatment. The following treatments were allocated as per Table 3.2.

3.3.3 Challenge

Birds within the first house were challenged with a virulent strain *Mycoplasma gallisepticum* ("MG2" of preliminary study) of $8.3x10^6$ CFU/ml. The challenge strain was administered

intrathoracically and intratracheally with 0.2 ml and 50 ul of the *Mycoplasma gallisepticum* suspension, respectively at 10 days of age. In the second house, birds remained unchallenged.

3.3.4 Antibiotic

Challenged and unchallenged birds were administered Tilmicosin active-based antibiotic *Pulmotil AC*® in drinking water during the first 3 days of age and from 18-20 days of age as recommended by Elanco Company.

3.3.5 Diets

Starter, grower and finisher corn/soybean meal based diets were formulated to contain the recommended (or slightly above) level of Methionine to meet the modern breed requirement suggested by the modern Ross 308 Broiler company, as illustrated in Table 3.3. Feed and water were offered *adlibitum* and a continuous lighting program was provided. Other diets were formulated to contain 20% additional Methionine above the recommended level for the modern Ross 308 Broiler requirement with feed and water offered *adlibitum* and a continuous lighting was program provided.

3.3.6 Experimental Design

The trial constituted a (2x2x2) factorial arrangement of treatments in a completely randomized design with interactions. The main factors were 1) *Mycoplasma gallispeticum* challenged vs. unchallenged; 2) Methionine levels: a) adequate and b) excessive; and 3) Antibiotic: a) *Pulmotil AC*® vs. b) Non-treated.

Table 3.2. Treatments designed in 2x2x2 factorial arrangement to evaluate the immunopotentiating effect of 20% excess DL-Methionine on *Mycoplasma gallisepticum* infected broilers with a comparison to an antimicrobial administration.

Treatment Number	*Treatment Name*	*ABR*	*MG Challenge (+/-)*	*Methionine Level*	*Antibiotic*
1	Unchallenged No Pulmotil Adequate Methionine	UN NP AM	-	Adequate	None
2	Unchallenged Pulmotil Adequate Methionine	UN P AM	-	Adequate	Pulmotil
3	Unchallenged Pulmotil Excess methionine	UN P EM	-	Excessive	None
4	Unchallenged No Pulmotil Excess Methionine	UN NP EM	-	Excessive	Pulmotil
5	Challenged No Pulmotil Adequate Methionine	CH NP AM	+	Adequate	Pulmotil
6	Challenged Pulmotil Adequate Methionine	CH P AM	+	Adequate	None
7	Challenged Pulmotil Excess Methionine	CH P EM	+	Excessive	Pulmotil
8	Challenged No Pulmotil Excess Methionine	CH NP EM	+	Excessive	None

Table 3. 3. Nutrition Specifications for As-Hatched Broilers - Target Live Weight 1.70 - 2.40 kg (3.75 - 5.30 lb) adopted from Aviagen, 2014.

		Starter		Grower		Finisher	
Age Fed	days	0 - 10		11 - 24		25 - market	
Energy	kcal	3000		3100		3200	
	MJ	12.55		12.97		13.39	
AMINO ACIDS		**Total**	**Digest[1]**	**Total**	**Digest[1]**	**Total**	**Digest[1]**
Lysine	%	1.44	1.28	1.29	1.15	1.16	1.03
Methionine + Cystine	%	1.08	0.95	0.99	0.87	0.91	0.80
Methionine	%	0.56	0.51	0.51	0.47	0.47	0.43
Threonine	%	0.97	0.86	0.88	0.77	0.78	0.69
Valine	%	1.10	0.96	1.00	0.87	0.90	0.78
Isoleucine	%	0.97	0.86	0.89	0.78	0.81	0.71
Arginine	%	1.52	1.37	1.37	1.23	1.22	1.10
Tryptophan	%	0.23	0.20	0.21	0.18	0.19	0.16
Leucine	%	1.58	1.41	1.42	1.27	1.27	1.13
Crude Protein[2]	%	23.0		21.5		19.5	
MINERALS							
Calcium	%	0.96		0.87		0.79	
Available Phosphorus	%	0.480		0.435		0.395	
Magnesium	%	0.05 - 0.50		0.05 - 0.50		0.05 - 0.50	
Sodium	%	0.16 - 0.23		0.16 - 0.23		0.16 - 0.20	
Chloride	%	0.16 - 0.23		0.16 - 0.23		0.16 - 0.23	
Potassium	%	0.40 - 1.00		0.40 - 0.90		0.40 - 0.90	
ADDED TRACE MINERALS PER KG							
Copper	mg	16		16		16	
Iodine	mg	1.25		1.25		1.25	
Iron	mg	20		20		20	
Manganese	mg	120		120		120	
Selenium	mg	0.30		0.30		0.30	
Zinc	mg	110		110		110	
ADDED VITAMINS PER KG		**Wheat based feed**	**Maize based feed**	**Wheat based feed**	**Maize based feed**	**Wheat based feed**	**Maize based feed**
Vitamin A	IU	13,000	12,000	11,000	10,000	10,000	9000
Vitamin D3	IU	5000	5000	4500	4500	4000	4000
Vitamin E	IU	80	80	65	65	55	55
Vitamin K (Menadione)	mg	3.2	3.2	3.0	3.0	2.2	2.2
Thiamin (B1)	mg	3.2	3.2	2.5	2.5	2.2	2.2
Riboflavin (B2)	mg	8.6	8.6	6.5	6.5	5.4	5.4
Niacin	mg	60	65	55	60	40	45
Pantothenic Acid	mg	17	20	15	18	13	15
Pyridoxine (B6)	mg	5.4	4.3	4.3	3.2	3.2	2.2
Biotin	mg	0.30	0.22	0.25	0.18	0.20	0.15
Folic Acid	mg	2.20	2.20	1.90	1.90	1.60	1.60
Vitamin B12	mg	0.017	0.017	0.017	0.017	0.011	0.011
MINIMUM SPECIFICATION							
Choline per kg	mg	1700		1600		1500	
Linoleic Acid	%	1.25		1.20		1.00	

Table 3.3 continued

Digest[1] = Digestible
Crude Proetin[2] = Formulation priority is to meet the recommended minimum essential amino acid levels. These crude protein levels are not requirements *per se*, but instead are levels which will likely occur when achieving the aforementioned essential amino acid minimums.
NOTES: "*These feed specifications should be used as a guide. They require adjustment for local conditions and markets. A withdrawal feed should be fed to meet local requirements for drug withdrawal times. This can be formulated to the same standards as the final feed listed above."* (Aviagen, 2014).

3.3.7 Parameters

3.3.7.1 Performance

3.3.7.1.1 Growth rate, FCR and Mortality

Performance parameters included final body weight, cumulative feed conversion ratio, corrected feed conversion and mortality; which was recorded as it occurred and dead birds weighed. All performance parameters were measured at 10, 17 and 34 days of age.

3.3.7.2 Pathological and Immunological Criteria

3.3.7.2.1 Maternal Immunity to *Mycoplasma gallispeticum*

Trachea of 1 day of age (11 samples) were collected in prepared Frey's Broth and incubated to test for maternal immunity to MG.

3.3.7.2.2 Air sacs and Tracheal Lesion Scores

Five birds were randomly chosen from each pen, with a total of 15 birds per treatment. Birds were sacrificed by CO_2. Air sac lesion scoring was confined to only the left air sac of the bird. Airsac were given scores of 0 for negative, clear airsac; 1 for cloudiness of the membrane; 2 for foamy spots and thickened membrane, and 3 for a meaty appearance with cheesy exudates. Tracheal scores were as follows, (-) for negative tracheatis; (+) for mild tracheatis and (++) for severe tracheatis.

3.3.7.2.3 MG DNA Load in Respiratory Organs

3.3.7.2.3.1 Pooling and weighing of tissues and organs

Pooling of air sac and trachea of 5 birds per pen; a total of 15 birds per treatment. Empty tubes weighed and weight of pooled air sac and pooled trachea were obtained.

3.3.7.2.3.2 Homogenization of Airsacs and Tracheas

Homogenization of air sac and tracheal samples was performed at 10, 17, and 35 days of age. Homogenization was performed with liquid nitrogen at temperature of -196°C with a mortar and pestle. Aluminum foil on mortar was changed and new one was added between unchallenged and challenged groups as well as between different treatments to avoid cross-contamination.

Post homogenization; a 300ul of sterile saline was allocated to each tube of pooled air sacs. A 2 ml of sterile saline was allocated to each tube of 10 and 17 days of age and 3ml to 35 days of age of pooled trachea.

3.3.7.2.3.3 DNA extraction of Airsacs and Tracheas

DNA extraction of air sac and tracheal samples was performed at 10, 17, and 35 days of age. Pooled air sacs and tracheal samples were subjected to a boiling temperature of 90 °C, then to a freezing temperature of -80 °C for DNA extraction.

3.3.7.2.3.4 Real-Time PCR for Airsacs and Tracheas

MG colonization of air sacs and trachea by Real-Time PCR at 10, 17, and 35 days of age was determined. q-PCR Protocol for cycling and temperature was performed as follows: denaturation at 95°C for 3 min; Annealing at 95°C for 12 sec, extension at 60°C for 1min, for 40 cycles. Dilution of probe and primers were according to manufacturer's recommendation.

q-PCR was performed using iTaq^{TM}(BioRad). For the standard curve, 4 different MG DNA standards (0, 1.5, 15 and 150mg) were used. This was performed using Real time machine (CFX96 touch TM Real-time PCR detection system, BioRad; 2000 Alfred Nobel Drive, CA USA).

The prepared working solution was dependent upon the volumes of reagents which were multiplied according to the number of samples following the below Table 3.4. Probe, reverse and forward primers sequences were confirmed with references to Grodio et al. (2008).

Table 3.4. Reagents and Volumes for preparation of Real-Time PCR mixture.

Reagent	*Volume*
iTaq	10µL
Probe (5pmol) (5'-6-FAM-CCACAGGGCTTTGGTGGCCCA-BHQ-1)	1µL
Forward Primer(MgC_2F)(5pmol) (5'-GGTCCTAATCCCCAACAAAGAAT)	1µL
Reverse Primer(MgC_2R) (5pmol) (5'-CTTGGTTGGTTCATATTAGGCATTT)	1µL
Template DNA	3µL
PCR Water	4uL
Total Volume	*20 µL*

3.3.7.2.4 Thymus and Bursa of Fabricius Indices

Thymus and Bursa of Fabricius indices were obtained at 10, 17, 35 days of age. Five birds were randomly chosen from each pen with a total of 15 birds per treatment. Weight of individual bird prior to dissection was recorded. Thymus and bursa of each bird were individually weighed using a sensitive balanced accordingly.

Indices measurement obtained via equation:

$$Thymus\ Index = \frac{Thymus\ weight\ (g)}{Broiler\ weight\ (g)} \times (100)$$

$$Bursal\ Index = \frac{Bursal\ weight\ (g)}{Broiler\ weight\ (g)} \times (100)$$

3.3.7.2.5 Haematology

Blood samples of 3ml each was drawn from the brachial vein of 5 birds per pen with a total of 15 samples per treatment and were collected in ethylenediaminetetraacetic acid (EDTA) heparinized tubes as an anticoagulant. Hematology analysis of blood obtained at 35 days of age was performed to determine quantity of Red Blood Cell (RBC) (10^6/ul), Hemoglobin (HGB) (g/dl), Mean Corpuscular Hemoglobin (MCH) (pg) and Hematocrit (HCT) (%). Samples of 35 days of age were coded and offered for manual microscopic and blood count analyzed using veterinary hematology machine.

3.3.7.2.6 ELISA titers against Mycoplasma gallisepticum

The following procedures were performed to obtain titers against *M. gallisepticum* using Enzyme-Linked Immunosorbent Assays.

3.3.7.2.6.1 Sera Samples

Blood samples of 1, 10, 17 and 35 days of age were centrifuged at ¾ speed for 10 minutes at room temperature to extract sera. Sera were stored at -20 °C for further seroanalysis.

3.3.7.2.6.2 Seroconversion and ELISA

Seroconversion to MG at 1, 10, 17 and 35 days of age was determined using Enzyme-linked immunosorbent assay (ELISA):

3.3.7.2.6.2.1 Preparation of Samples

1. Test samples were diluted five-hundred-fold (1:500) with sample diluent prior to being assayed (e.g., by diluting 1 µL of sample with 500 µL of Sample Diluent). It is important to note that controls should not be diluted.
2. Assured that tips were changed for each sample.
3. Samples were vortexed prior to dilution and thoroughly mixed (6 times) prior to dispensing into the antigen-coated plate.

3.3.7.2.6.2.2 Dilution of Sera

1. Charts for each plate was sketched accordingly for sample well and identification. Sera were allowed to come to 18-26°C, then mixed via vortexing.
2. Diluted 1/50; a volume of 200ul of dilution buffer was loaded into dilution plate, then 4ul of each serum sample was added and mixed with dilution buffer 6 times.
3. Diluted 1/10; a volume of 180ul of dilution buffer was loaded into a new dilution plate and 20ul of each step 1 diluted serum sample was added and mixed with dilution buffer 6 times.

3.3.7.2.6.2.3 ELISA

1. The reagents were allowed to come to 18–26°C, then mixed gently by inverting and swirling.
2. Antigen-coated plate(s) were obtained and the samples recorded.
3. A 100 µL of UNDILUTED Negative Control was dispensed into duplicate wells.
4. A100 µL of UNDILUTED Positive Control was dispensed into duplicate wells.
5. A100 µL of diluted sample was dispensed into appropriate wells. Samples may be tested in duplicate but a single well was acceptable.
6. Incubated for 30 minutes (± 2 minutes) at 18–26°C.
7. Each well was washed with approximately 350 µL of distilled or deionized water 3–5 times. Aspirate completely.
8. A100 µL of Conjugate was dispensed into each well.
9. Incubated for 30 (± 2 minutes) minutes at 18–26°C.
10. Repeated step 6.
11. A 100 µL of TMB Substrate Solution was dispensed into each well.
12. Incubated for 15 minutes (± 1 minute) at 18–26°C (in the dark).
13. A100 µL of Stop Solution was dispensed into each well to stop the reaction.
14. Measured and recorded absorbance values at 650nm, A(650) using ELISA reader ThermoScientific™ Multiskan™ GO Microplate Spectophotometer (ThermoFisher Scientific, 2 Friars Drive, Hudson, New Hampshire 03051 US).
15. **NOTE:** For the assay to be valid, the difference between the Positive Control mean and the Negative Control mean (PCx–NCx) should be greater than 0.075. The Negative

Control mean absorbance should be less than or equal to 0.150.

16. The presence or absence of antibody to MG is determined by relating the A(650) value of the unknown to the Positive Control mean. The Positive Control is standardized and represents significant antibody levels to Mg in serum.
17. The relative level of antibody in the sample is determined by calculating the sample to positive (S/P) ratio. Endpoint titers are calculated using the equation described in the calculations section below.

3.3.7.2.6.2.4 ELISA titer calculations

1. Absorbance values were entered into Excel sheet and optical density values were used to achieve titer values.
2. Equations illustrated in Table 3.5; ELISA calculations (IDEXX) were entered into the same Excel sheet to obtain ELISA titers.

Table 3.5 ELISA calculations (IDEXX).

1.	Negative Control mean ($NC\bar{x}$)	$\frac{NC1\ A(650) + NC2\ A(650)}{2} = NC\bar{x}$
2.	Positive Control mean ($PC\bar{x}$)	$\frac{PC1\ A(650) + PC2\ A(650)}{2} = PC\bar{x}$
3.	S/P Ratio	$\frac{\text{Sample Mean} - NC\bar{x}}{PC\bar{x} - NC\bar{x}} = S/P$
4	Titer - Relates S/P at a 1:500 dilution to an endpoint titer:	$\text{Log}_{10}\text{Titer} = 1.09\ (\text{log}_{10}\ S/P) + 3.36$

3.3.8 Statistical Analyses

As indicated above, the design of the trial was a factorial arrangement of treatments in a complete randomized design with 8 treatments replicated 3 times with 50 birds per replicate. Data was pooled and analyzed as three-way ANOVA (2x2x2) and two-way ANOVA (2X2) with interactions using the GLM procedure and means were compared using Student Newman-Keuls test for significance of $P<0.05$ (SAS, 2017). Significant interactions were followed by one-way ANOVA and Tukey's test for mean separation (SPSS v.24, 2018). Frequencies of airsac and tracheal lesion scores were analyzed using Chi-Square (SPSS v.24, 2018).

3.3.9 Experimental period

The experimental procedure was approved by Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut and was completed at (AREC) over a period of 35 days effective the date chick arrival. At the end of the experiment, treated birds were sent for rendering in a processing plant in the Beqaa area whereas unchallenged birds were sold.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation and Culture of field *Mycoplasma gallisepticum*

In order to isolate and culture field isolates of *M. gallisepticum*, Frey's media was prepared. A total of 13 tracheal swab samples were then randomly obtained from various chicken breeds at commercial pet shop in Ouzaii area, Beirut. A frequency of 4/10 suspected *M. gallisepticum* were observed to be positive. Figure 4.1 demonstrates a positive suspected MG of a tracheal swab sample 10 (middle tube), compared to a negative control (tube on the right) and to a negative presence of MG from tracheal swab sample 2 (tube on the left). Positive suspected MG sample 10 were then subcultured into Frey's agar to examine for MG colonies. Positive MG colonies were observed post 5 days of incubation at 37°C (Figure 4-2). The local field isolate obtained of *Mycoplasma* gallisepticum grew well in Frey's broth within 5 days of incubation at 37°C where later observed under microscopy 100X objective illustrating mycoplasma's typical colony morphology of single celled "fried egg" type of smooth coccoid translucent with a much denser central area (Figure 4-3).

4.2 Slide agglutination test for *Mycoplasma gallisepticum*

Rapid slide agglutination test for *Mycoplasma gallisepticum* was determined positive for sample 10. Formed granules or agglutinates were observed on slide shortly after the slide was rotated for approximately 3 minutes post combination of homologous antigen and antibody.

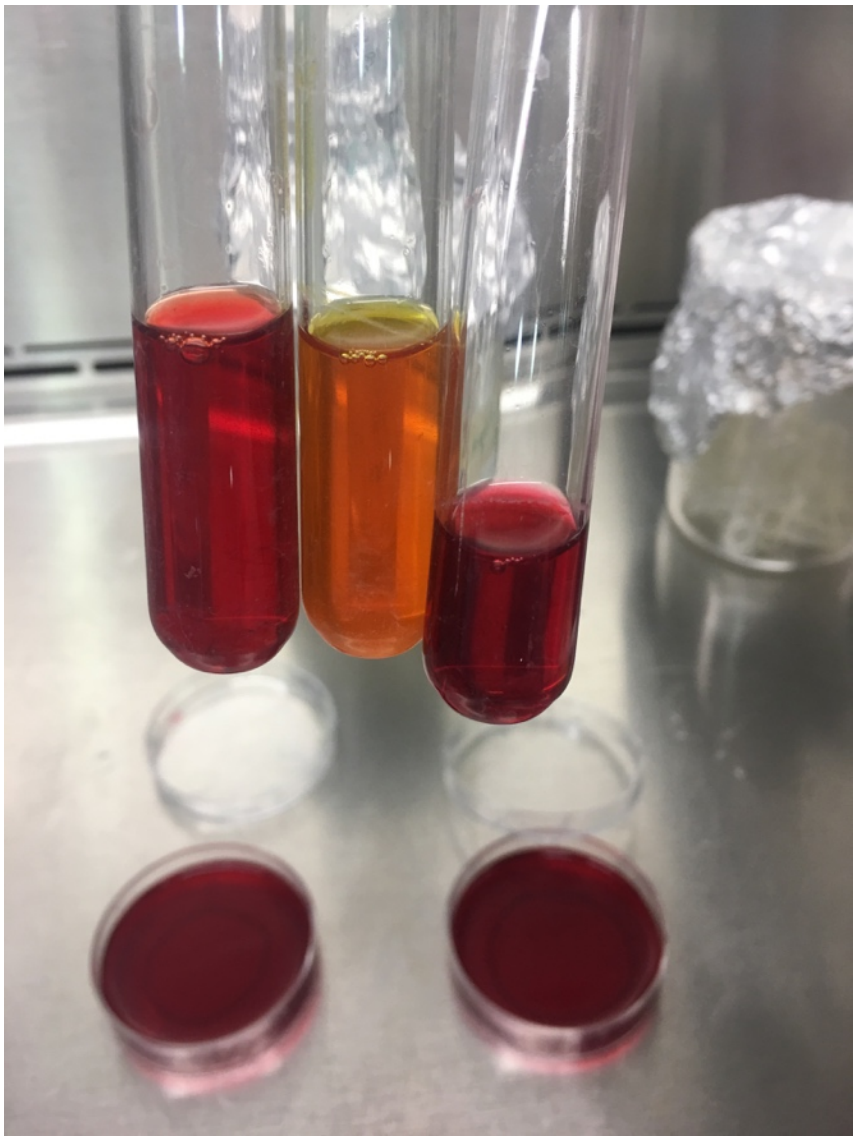

Figure 4-1. Suspected *Mycoplasma gallisepticum.* Sample 10 (middle tube) illustrating suspected *Mycoplasma gallisepticum* (MG) compared to negative control Frey's broth (right), and to another tracheal swab sample 2 (left), showing negative MG. The suspected MG is then subcultured (on October 12th, 2017) on Frey's agar to examine MG colonies presence.

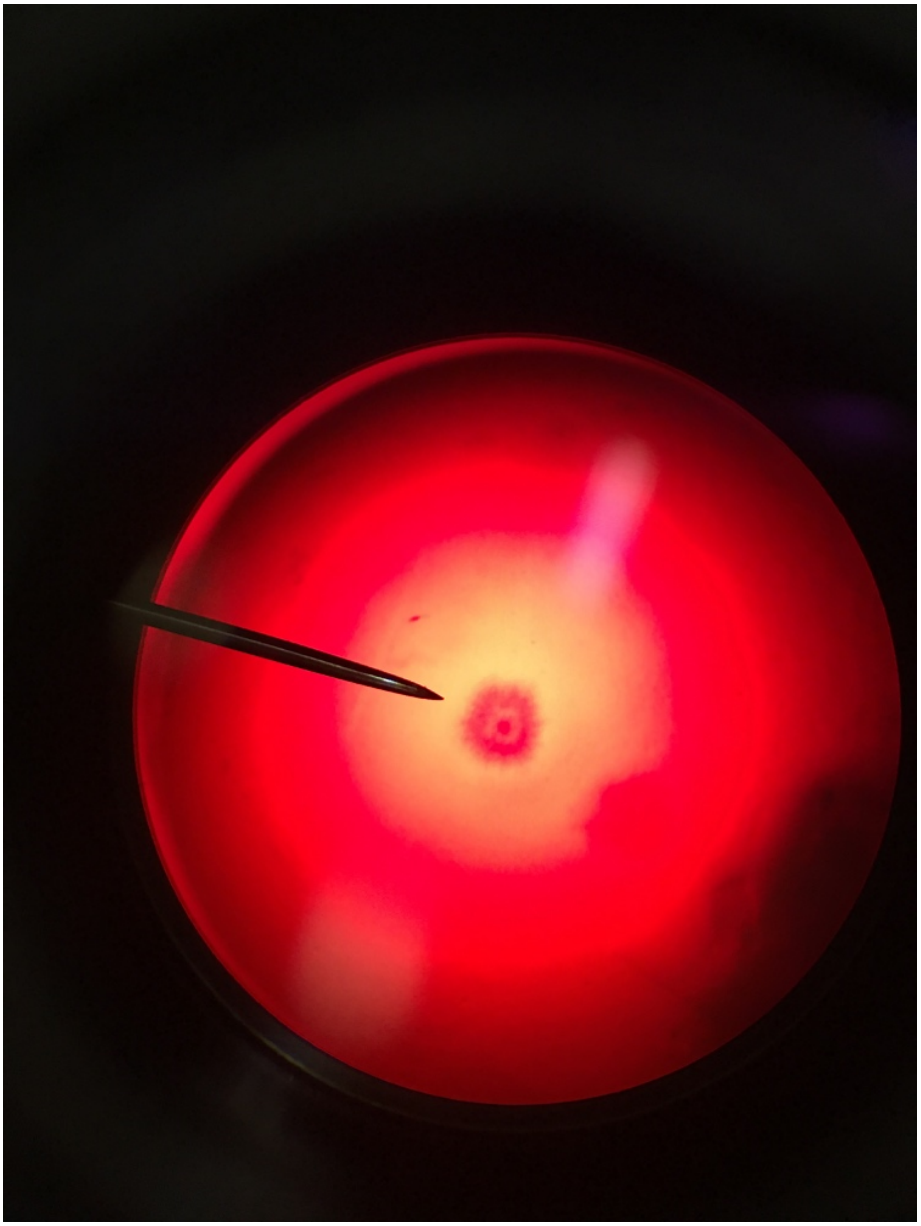

Figure 4-2. *Mycoplasma gallisepticum* colony observation on Frey's agar plate, on October 16th, 2017 post 5 days of incubation at 37° C.

Figure 4-3. Colonies of *Mycoplasma gallisepticum* isolated from broiler appear on Frey's agar post 5 days of subculturing from Frey's Broth (Sample 10, 100x). Note the "fried egg" morphology of MG colonies.

4.3 Results for Study A: Preliminary Study - Evaluation of virulence of field isolates of *Mycoplasma gallisepticum* colonies.

Clinical signs were observed and recorded for each bird in all pens at different dates post challenge included rales, conjunctivitis, sneezing and coughing, and oral and nasal mucosal discharge. Table 4.1 displays results of preliminary study (Study A).

Table 4.1. Signs at different dates post challenge (DPC) in experimental groups versus the control (C) of preliminary study.

Days	*Groups*[a]	*Signs at different DPC*[b] *(out of 10 birds)*			
		Rales	**Conjunctivitis**	**Sneezing/Coughing**	**Oral/Nasal Mucus**
Day 1	MG1	0	0	0	0
	MG2	1	0	0	0
	MG	2	0	0	0
	C	0	0	0	0
Day 3	MG1	0	1	0	0
	MG2	1	0	1	0
	MG	0	1	0	0
	C	0	1	0	0
Day 6	MG1	0	1	1	0
	MG2	1	0	1	0
	MG	0	1	0	0
	C	0	1	0	0
Day 7	MG1	0	1	1	0
	MG2	0	1	1	0
	MG	1	1	0	0
	C	0	2	0	0
Day 8	MG1	0	3	1	0
	MG2	1	0	0	0
	MG	0	0	0	1
	C	0	2	0	0
Day 9	MG1	0	2	1	0
	MG2	2	2	2	0
	MG	1	2	0	0
	C	0	3	0	0
Day 10	MG1	0	2	1	0
	MG2	1	0	2	0
	MG	1	1	0	0
	C	0	2	0	0

[a]MG1 group was challenged with MG field isolate number 1.
MG2 group was challenged with MG field isolate number 2.
MG group was challenged with MG field isolate number 0.
C group remained unchallenged and served as the control.
[b] Challenged field isolates were administered of 8.3×10^6 CFU/ml intrathoracically and intratracheally with 0.2 ml and 50 ul of the *Mycoplasma gallisepticum* suspension, respectively at 10 days of age.

Based on the following recorded observatory signs of the three MG challenged isolates (MG, MG1 and MG2) versus the control (C); MG2 has illustrated to be the most virulent isolate. This is apparent from signs at dates post challenge. Birds in pen MG2 have shown greater clinical signs of rales and sneezing/coughing compared to birds in MG, MG1, and C pens. Statistical analysis via chi-square illustrated no significant difference between the groups. Decision for selecting MG2 strain as the most virulent strain compared to other strain in this preliminary study was pegged on the observation trend illustrated from signs at DPC up to day 10.

4.4 Cultured day-old pooled trachea of Ross 308 chicks

Day-old chicks originating from the same hatchery were further tested to confirm they are MG-free by submerging pooled trachea samples (a total of 11 samples) obtained from sacrificed birds via CO_2 into Frey's broth incubated at 37C for 10 days. Negative presence of MG was observed in all pooled samples as illustrated in Figure 4-4.

4.5 Post-dissection Gross Lesion Scoring of Trachea and Air sac

Gross lesion scores for respiratory organs (trachea and air sac) were obtained from sacrificed birds via CO_2. Tracheal lesion scoring was measured accordingly as described in 'Materials and Methods" and shown in Figure 4-5. Air sac lesion scoring were confined to left air sac of the bird and scoring were measured accordingly as described in 'Materials and Methods' Chapter and shown in Figure 4-6, Figure 4-7, Figure 4-8 and Figure 4-9. Missing air sac scoring were given to birds with *E. coli* infection and Ascites.

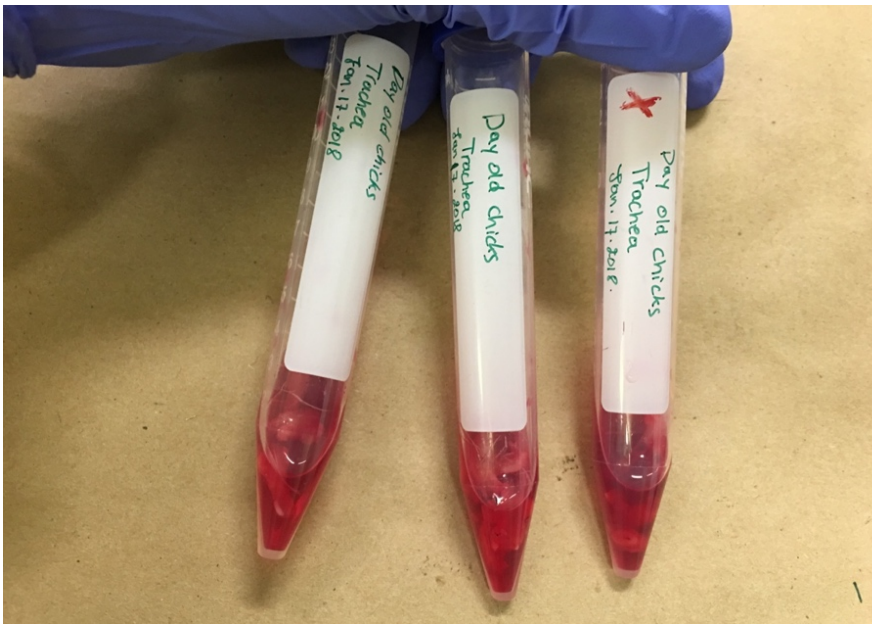

Figure 4-4. Cultured 1-day old male Ross 308 broilers pooled trachea. A total of 11 tracheal samples collected in conical tubes of prepared Frey's Broth and incubated at 37°C to test for *Mycoplasma gallisepticum* colonization. Day old tracheal samples have demonstrated negative presence of *Mycoplasma gallisepticum*; no change in Frey's Broth of phenol red indicator from red to orange was observed.

Negative Tracheatis (-) **Mild Tracheatis (+)** **Severe Tracheatis** (++)

Figure 4-5 Tracheal Gross Lesion Scoring. Negative tracheatis (-) on the left, mild Tracheatis (+) in the center, and severe tracheatis (++) on the right.

Figure 4-6. Airsac Gross Lesion Scoring of 0.

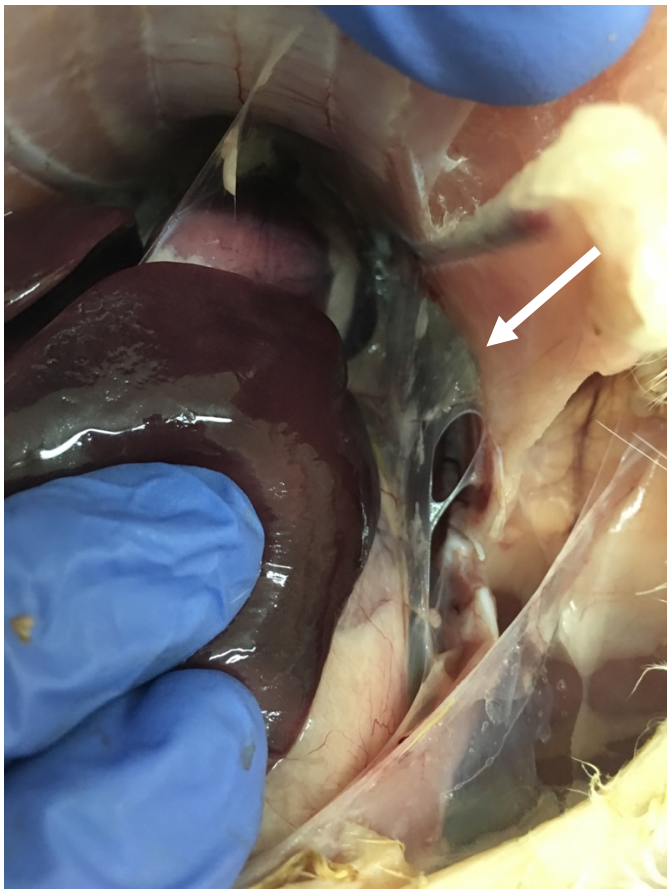

Figure 4-7. Airsac Gross Lesion Scoring of 1.

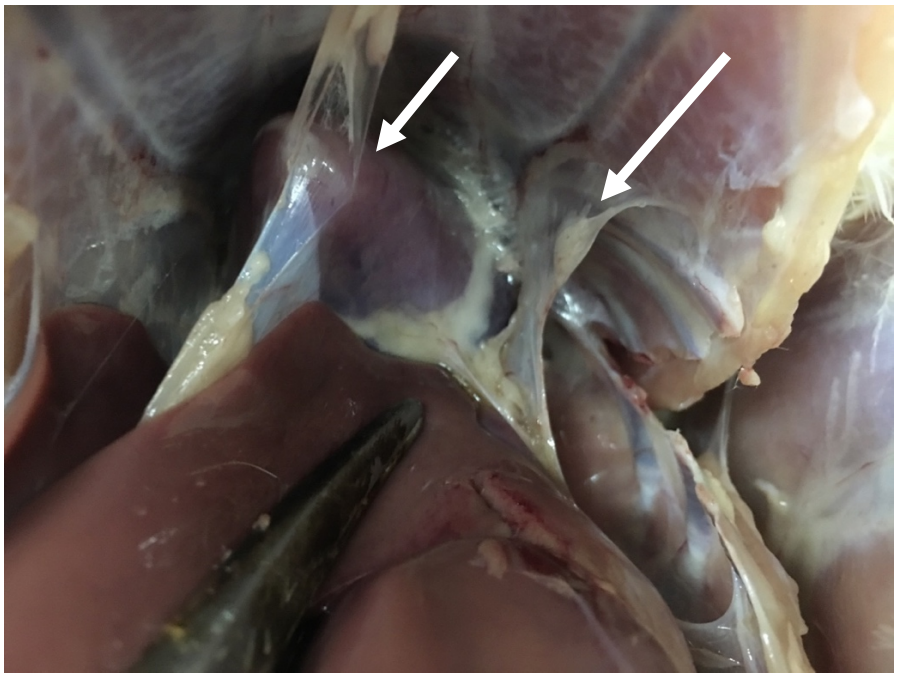

Figure 4-8. Airsac Gross Lesion Scoring of 2.

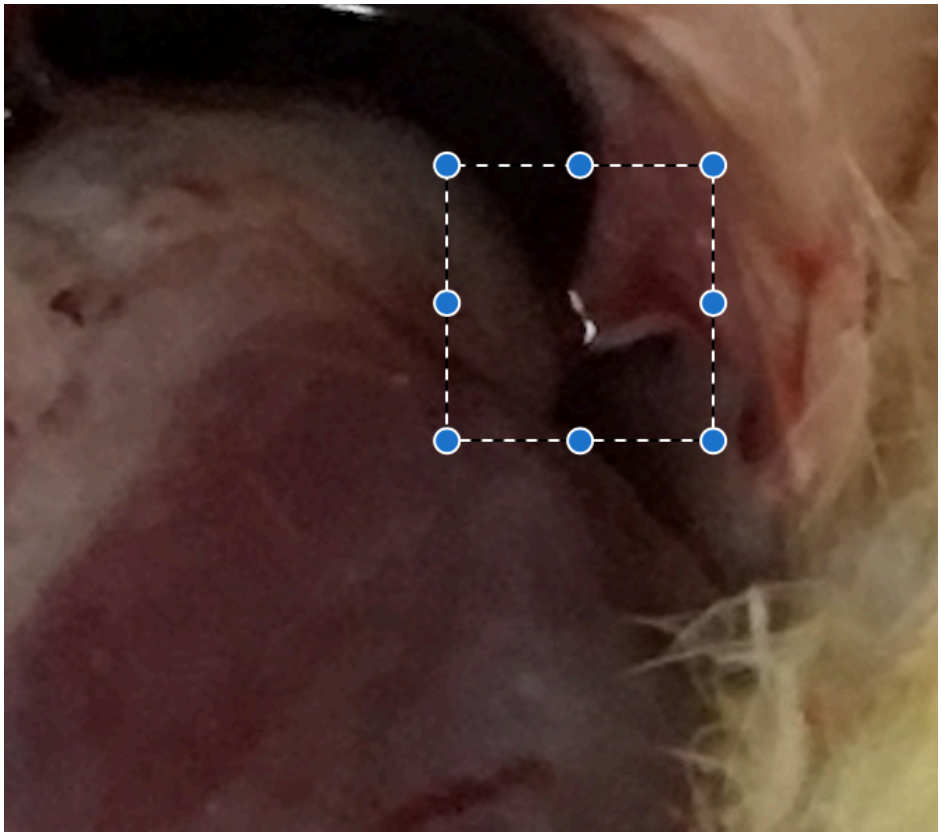

Figure 4-9. Airsac Gross Lesion Scoring of 3.

The thesis experiment was performed to determine the effects of DL-Methionine in excess and above the recommended requirement of NRC (1994) and modern breeder company requirement on the immune response and growth performance of male Ross 308 broiler chickens challenged with *Mycoplasma gallisepticum*. The experiment is divided into 3 part studies. Study A is a preliminary trial for evaluating three separate *Mycoplasma gallisepticum* field isolates on clinical signs of challenged Ross 308 broilers. Study A consisted of 40; seven day-old broilers, arranged in 4 treatments of completely randomized design serving 10 birds per treatment as Control, "MG", "MG1" and "MG2". Study B aimed to identify the strain of each evaluated field isolates adopted in Study A via MG pvpA gene based conventional PCR, purification of DNA and molecular sequencing. Study C comprised a completely randomized design with 8 differing treatments; where levels of dietary DL-Methionine to meet 100 and 120% of methionine requirements were supplemented in the adequate and excess methionine groups, respectively. A total of 1200-day-old male Ross 308 broiler chickens were randomly divided into 8 treatments with 3 replicates of 50 birds per pen. The trial constituted a factorial arrangement of treatments in a completely randomized design. The main factors were 1) *Mycoplasma gallisepticum* challenged vs. unchallenged; 2) Methionine levels: a) adequate and b) excessive; and 3) Antibiotic: a) *Pulmotil AC*® vs. b) Non-treated. Performance and pathological parameters were recorded at 10, 17, and 35 days of age. Growth performance, cellular immunity, and humoral immunity were determined in correlation between bursa of Fabricius and thymus indices. The coefficient of variation (CV) was calculated to show data dispersion by dividing the standard deviation (SD) by the mean. q-PCR of trachea and air sac sample to analyze the MG DNA load per tissue weight as Log_{10} of MG colony forming unit were

assessed. The percentages of airsaculitis and tracheatis were evaluated. In addition, hematological parameters of RBC, HGB, HCT and MCH were determined.

Mycoplasma gallisepticum (MG) is a pathogenic microorganism and the primary etiologic agent of chronic respiratory disease in chickens and infectious sinusitis in turkeys (Bradbury, 1984; Evans et al., 2005). MG is the smallest self-replicating prokaryotes voided of cell wall; possess a reduced genome (Semashko et al. 2016); minimal genetic information (Levisohn and Kleven, 2000), various metabolic pathways and the capability of growing on artificial cell-free media (Razin, 1992; Semashko et al. 2016). These attributes are mirrored with a high degree of interdependence between *M. gallisepticum* and the host species as well as in the fastidious nature of the organism *in vitro* including its difficulty to culture, selective antibiotic sensitivity (as they demonstrate resistance to β-lactam antimicrobials and membrane synthesis inhibitors); inhibition of phagocytosis, and its intimate association with host cells. MG-infection aggravates diseases caused by other agents and it is known to co-exist with various bacterial and viral diseases (*E. coli,* AI, IB, etc.). MG-infection is responsible for tracheatis, conjunctivitis, airsaculitis, rales, inflammation of the lungs, nasal and oral mucosal discharge including other respiratory distresses. MG results in significant losses in poultry production including mortality, reduced growth; decreased egg production and hatchability, and increased carcass condemnation (Ley, 2003; Kleven, 1997; Levisohn and Kleven, 2000; Razin et al., 1998). MG ought to be properly controlled through well surveillance, extensive biosecurity and eradication program.

No previous study has been performed on the evaluation of methionine on *Mycoplasma*

gallisepticum infected birds. As such, comparison of the results of this study are based on the available information considering methionine effect on other disease factors.

4.6 Results of Study B: Strain Identification via Conventional PCR and Sequencing of *Mycoplasma gallisepticum* field isolates

4.6.1 Results for pvpA conventional PCR

Amplification reactions for cultured samples of MG, MG1, and MG2 were performed in a 50 ul reaction volume in addition to a Negative Control. The conventional PCR temperature cycle was performed as follows; the thermocycler was programmed as follows: 94°C for 3 min followed by 40 cycles of 94°C for 30 s; and the annealing temperature was set at 55°C for 30s. This is because it is considered as the optimal annealing temperature for *pvpA* primer extension (Liu et al., 2001) then extension at 72°C for 1 min, and finally at 72°C for 10 min. Figure 4-10 illustrates the position of the implemented pvpA primers; forward primer pvpA3F and reverse primer pvpA2R.

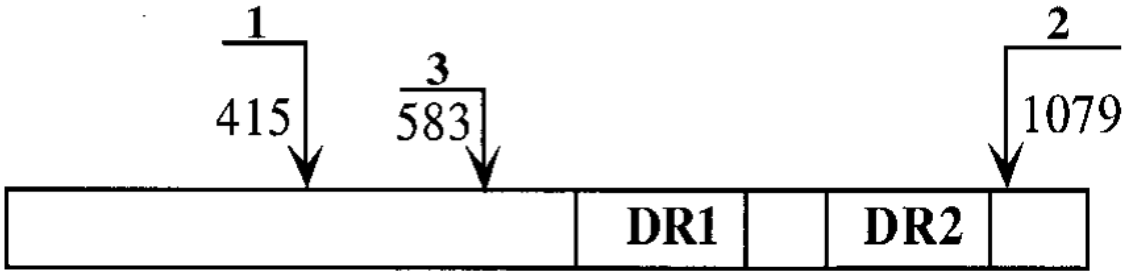

Figure 4-10. The following schematic diagram of *pvpA* adopted from Liu et al., 2001, was followed accordingly for the performance of *pvpA* conventional PCR. The figure illustrates the location of PCR primers. Position of primer is associated to the R strain sequence. The reaction consisted of primer 3; a forward primer pvpA3F, which is placed at nucleotide positions 583 to 604 (5 GGTAGTCCTAAGTTATTAGGTC3), and primer 2; reverse primer pvpA2R that is located at nucleotide positions 1059 to 1081 ('5 GGACGTSGTCCTGGCT GGTTAGC3')(Diagram credit to Lui et al.,2001).

Amplification results in Figure 4-11 observed under UV light using Gel-Doc system demonstrated a band in the third lane belonging to MG1 and a band in the fourth lane for MG2; both possessing a molecular weight of approximately 270 bp.

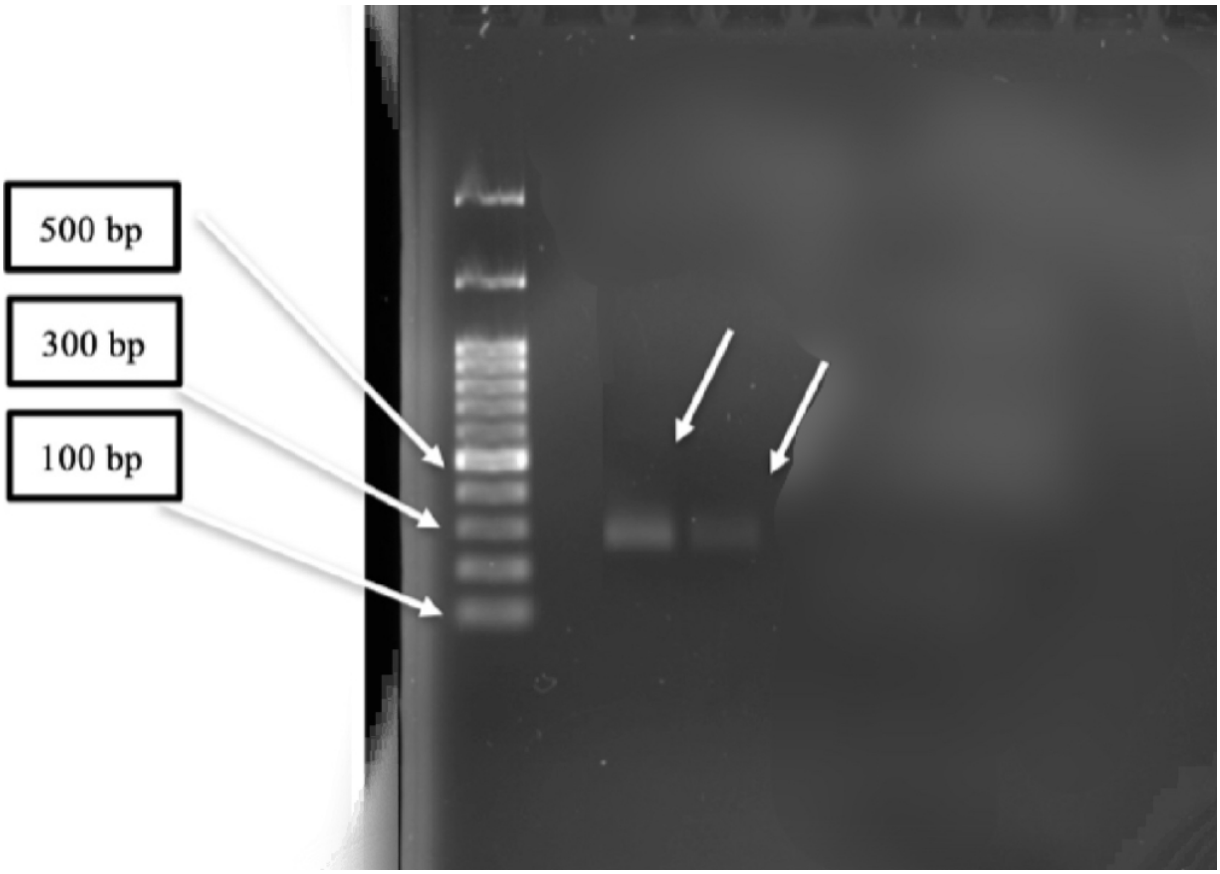

Figure 4-11. Agarose gel electrophoresis of PCR-amplified and purified *M. gallisepticum* field isolates. Using Gel-Doc UV system, bands of MG1 and MG2 filed isolates post purification are demonstrated in third and fourth lanes, respectively. The first lane served for the ladder; a molecular weight marker of 100 base pair DNA and the second lane served for the Negative Control. Band belonging to MG1 and MG2 demonstrating a molecular weight of approximately 270 bp according to the reaction consisting of implemented primer 3; pvpA3F, which is placed at nucleotide positions 583 to 604 (5 GGTAGTCCTAAGTTATTAGGTC3'), and primer 2; pvpA2R that is located at nucleotide positions 1059 to 1081 ('5 GGACGTSGTCCTGGCT GGTTAGC3').

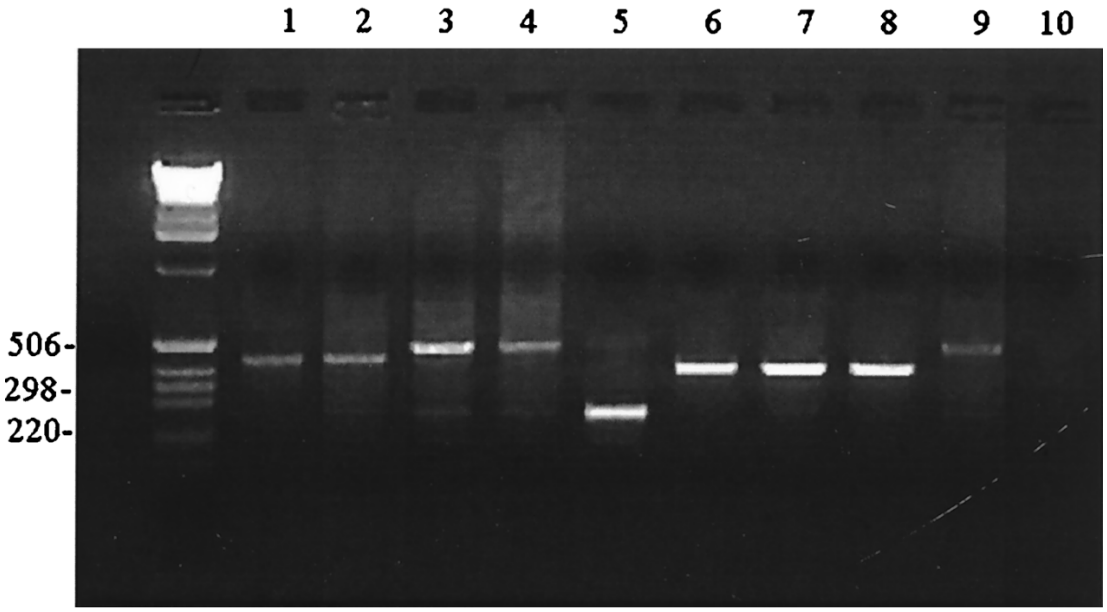

Figure 4-12. PCR of *Mycoplasma gallisepticum* reference performed by Liu et al. (2001) with outer primers pvpA 5 and pvpA 3. The electrophoresis above is used as reference for differentiating *Mycoplasma gallisepticum* strains based on molecular weight markers. The determined strains were as follows: lane 1 with 6/85 strain; lane 2 with field isolate (CK/CA/96/1) strain; lane 3 with R-strain; lane 4 with ts-11 strain; lane 5 with F-strain; lane 6 with K503 strain; lane 7 with K703 strain; lane 8 with K730 strain; lane 9 with A5969 strain; and lane 10 serving for negative control. The observed size variation of amplification products ranged from 497 base pairs (bp) in lanes 3, 4, and 9 to 267 bp in lane 5 (Photo credit to Liu et al., 2001).

4.6.2 Results of Sequencing of 'MG', 'MG1' and 'MG2' Mycoplasma gallisepticum field isolates

Three separate *Mycoplasma gallisepticum* field isolates were used in the preliminary study for evaluating their virulence effect and clinical signs at different dates post challenge.

In this part of the study, these three separate *M. gallisepticum* field isolates were identified at the strain level. The first field isolate termed "MG" was cultured and demonstrated positive *M. gallisepticum* via Rapid Agglutination test at American University of Beirut. Field isolate "MG" underwent strain analysis at Istituto Zooprofilattico Sperimentale delle Venezie (Italy) and tests displayed in Table 4.2 and Table 4.3 below included

microbiological examination, Mollicutes identification, minimum inhibitory concentration and antimicrobial susceptibility were performed with a provided certification of the identified result of the *M. gallisepticum* strain of "MG" as an F-strain.

Table 4.2. Microbiology testing analysis identification of *M. gallisepticum* strain of "MG"[1].

MATERIAL: BACTERIAL STRAIN (POULTRY) ATTIVITA' A PAGAMENTO / CHARGED EXAMINATION		
ANALYSIS (Method)	**Sample**	**Result**
MYCOPLASMA SPP. (MICROBIOLOGICAL EXAMINATION / OIE Manual for Terrestrial Animals 2008 cap 2.3.5)	1 2	**POSITIVE** **POSITIVE**
MYCOPLASMA GALLISEPTICUM MGC2 (OTHER / PDP DIA 129 2015 Rev. 0)	3 - 1 I D A 4 - 2 I D A	**POSITIVE Type Pink** **POSITIVE Type Pink**

MATERIAL: BACTERIAL STRAIN (POULTRY) ANALISI DERIVATA / FREE EXAMINATION		
ANALYSIS (Method)	**Sample**	**Result**
IDENTIFICAZIONE DI MOLLICUTES (DENATURING GRADIENT GEL ELECTROPHORESIS / PDP DIA 101 2015 Rev. 0)	3 - 1 I D A 4 - 2 I D A	**POSITIVE MYCOPLASMA GALLISEPTICUM** **POSITIVE MYCOPLASMA GALLISEPTICUM**

MATERIAL: BACTERIAL STRAIN (POULTRY) RESEARCH, RING TEST, NRL ACTIVITIES / RESEARCH		
ANALYSIS (Method)	**Sample**	**Result**
MISURATION OF MINIMUM INHIBITORY CONCENTRATION OF MYCOPLASMAS OF VETERINARY INTEREST (MICROBIOLOGICAL EXAMINATION / PDP DIA 103 2012 Rev. 0)	5	TILMICOSINA TIAMULINA LINCOMICINA ERITROMICINA TILOSINA OSSITETRACICLINA DOXICICLINA ENROFLOXACINA

[1] Microbiology testing analysis was performed at Istituto Zooprofilattico Sperimentale delle Venezie in Italy.

Table 4.3. Antimicrobial susceptibility analysis for identification of *M. gallisepticum* strain of "MG"[1].

DRUG	RANGE (ug/mL)	BP REFERENCE	BP (ug/mL)			MIC (ug/mL)	RESULTS (R, I, S)	BP(S)/MIC QUOTIENT
			S	I	R			
			≤	=	≥			
Tilmicosin	0,015-32	CLSI 2008	8	16	32	0,03125	S	256
Tiamulin	0,007-16	Hannan 1997	8	-	16	0,015625	S	512
Lincomycin	0,5-32	Behbahan 2008	0,5	2	4	4	R	0,125
Enrofloxacin	0,125-16	Hannan 1997	0,5	1	2	0,125	S	4
Erythromycin	0,5-8	Gautier-Bouchardon 2002	0,5	-	8	<0,5	S	>1
Tylosin	0,007-32	Hannan 1997	1	2	4	0,03125	S	32
Oxytetracycline	0,5-32	Hannan 1997	4	8	16	2	S	2
Doxycycline	0,125-32	Hannan 1997	4	8	16	1	S	4

Legenda: CLSI = Clinical and Laboratory Standards Institute; **BP** = Breakpoint; **R** = Resistant; **I** = Intermediate; **S** = Sensible;

[1] Microbiology testing analysis was performed at Istituto Zooprofilattico Sperimentale delle Venezie in Italy.

Sequence for the two field MG isolates; "MG1" and "MG2" were performed in collaboration with University of St. Joseph (USJ), in Beirut, Lebanon post DNA excision, extraction and purification at America University of Beirut. DNA sequences were viewed through FINCH TV (Geospiza, Inc.); a chromatogram viewer to observe DNA Sequencing; and Standard Nucleotide BLAST achieved via NCBI (National Centre for Biotechnology Information) U.S. National Library of Medicine. Table 4.4 and Table 4.5 demonstrate the nucleotide sequencing properties of "MG1" and "MG2" field isolates, respectively, prior to Standard Nucleotide BLAST.

Table 4.4. Nucleotide sequencing properties of "MG1" field isolates.

Nucleotide sequencing properties of "MG1" field isolates[1]	
Band	Band MG1
Query Length	241
Program	BLAST 2.8.0+
Base pairs	CACGTCCGATGAATGCTCATCCAGGTCAACCACGCCCTCAACAAG CTGGCCCACGTCCAATGGGAGCTGGTGGATCTAACCAACCAAGAC CAATGCCAAATSGTCYACAAAACCCACAAKGTCCACGACCAATGA ACCCTCAAGGCGATCCTCGTCCTCAACCAGCTGGTGTCAGACCTAA CAGCCCACAAGCTAACCAGCCAGTACCACGTCCATWG

[1] Nucleotide sequencing obtained through Standard Nucleotide BLAST via NCBI.

Table 4.5. Nucleotide sequencing properties of "MG2" field isolates.

Nucleotide sequencing properties of "MG2" field isolates[1]	
Band	Band MG2
Query Length	193
Program	BLAST 2.8.0+
Base pairs	CGGGACCMCGKTYCCGATGWAATGCTCATTCCCAGGTTCAAC CACGCCCTCAACAAGCTGGCCCACGTCCAATGKKAGCTGKTGG ATCTAACCAACCAAGACCAATGCCAAATTGTCACAAAACCCAC AAGGTCCATTACCMATGAACCCTTAAGGCTATCCTCGTCCTCAWTC

[1] Nucleotide sequencing obtained through Standard Nucleotide BLAST via NCBI.

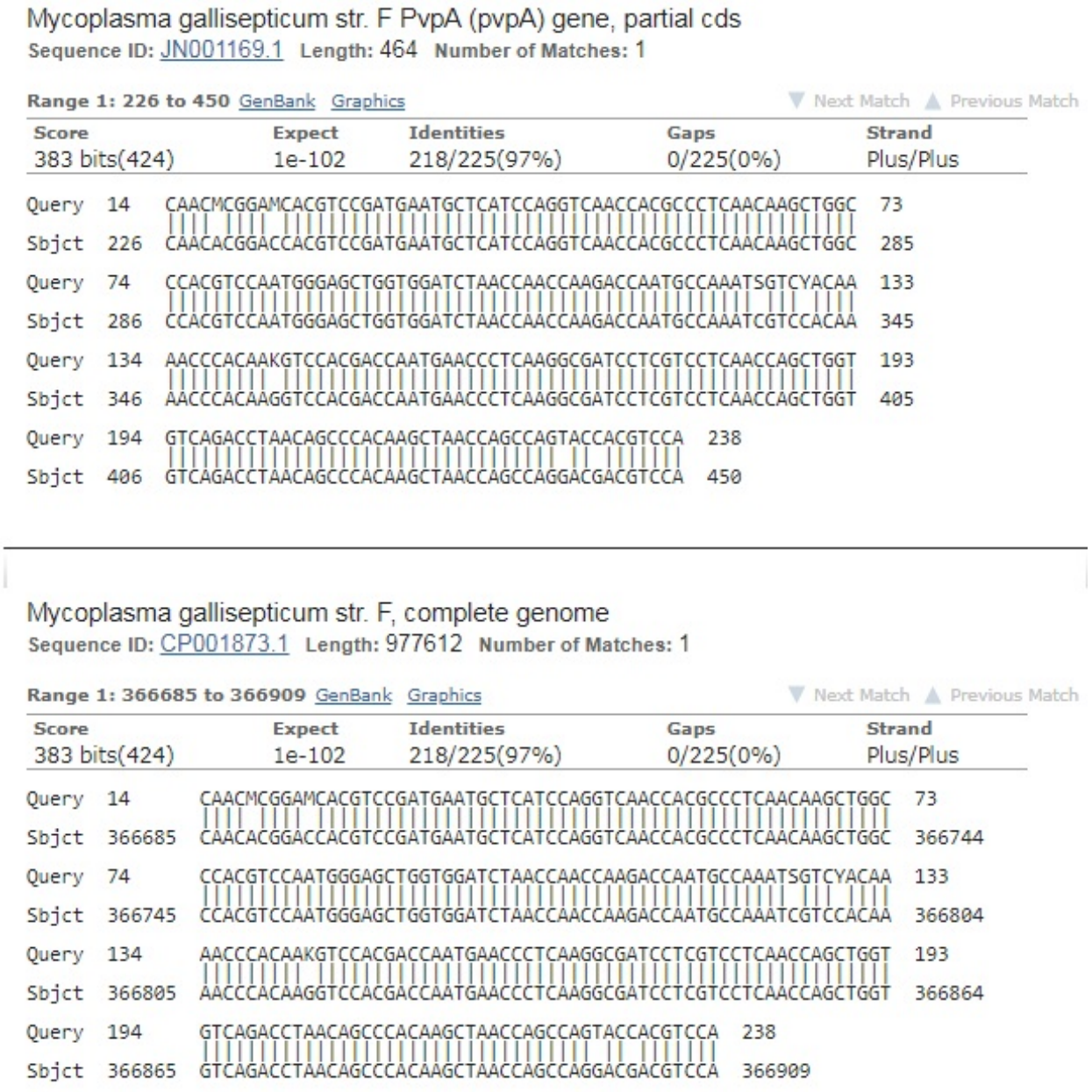

Figure 4-13. Sequences producing significant alignments to band 'MG1' of amplified, purified and sequenced *Mycoplasma gallisepticum* field isolate. Nucleotide BLAST results of lower band MG1 with first and second matches. First match demonstrated identities of 97% to *Mycoplasma gallisepticum* strain, F PvpA (pvpA) gene. This first sequence match comprises a score of 383 out of a total of 424 bits for *Mycoplasma gallisepticum* strain, F PvpA (pvpA) gene. The second sequence match demonstrate identities of 97% to *Mycoplasma gallisepticum* strain, F, complete genome.

Figure 4-14. Sequences producing significant alignments to band 'MG2' of amplified, purified and sequenced *Mycoplasma gallisepticum* field isolate. Nucleotide BLAST results of lower band MG2 with first and second matches. First match demonstrated identities of 89% to *Mycoplasma gallisepticum* strain, F PvpA (pvpA) gene. This first sequence match comprises a score of 224 out of a total of 248 bits for *Mycoplasma gallisepticum* strain, F PvpA (pvpA) gene. The second sequence match demonstrate identities of 89% to *Mycoplasma gallisepticum* strain, F, complete genome; comprising a score of 224 out of a total of 248 bits.

Particular MG strains have the ability to spread hastily and coax a serological response in contact with infected birds in approximately 4 weeks. Therefore, MG disease may range from mild to very severe, can be docile and spread slowly or expand very quickly. In addition, MG strain may either be simply diagnosed via isolation or by induction of strong serological response, or it can be of a difficult diagnosis due to isolation of variant or atypical strains, or the fact that it produces a poor serological response (Yoder 1986).

In this study, MG strain demonstrated a simple diagnostic via isolation. Size variation of pvpA PCR for "MG1" and "MG2" and field isolates wad confirmed by nucleotide sequencing analysis. Amplification product sizes for *M. gallisepticum* reference strains range from 267 to 497 base pairs (bp) (Figure 4-12). A PCR product with the expected size of 267 bp was observed for strains MG1 and MG2 (Figure 4-11); suggesting bands for MG F-strain. The nucleotide sequencing analysis has confirmed MG F-strain for MG1 and MG2 field isolates (Figure 4-13 and Figure 4-14). Percentages with first and second matches of DNA sequences of "MG1" and "MG2" with significant alignments to international strains and isolates have been displayed in Table 4.6. These results confirm that there were no significant differences ($P>0.05$) in pathogenicity between challenged field isolates of MG, MG1 and MG2 in the preliminary study post-chi square analysis (Table 4.1) and indicate that the *Mycoplasma gallisepticum* challenge strain used in this experimental research was in fact an MG pvpA F-strain.

Table 4.6. Percentages of matching identities of sequenced MG isolates to international strains and isolates.

MG isolates	International MG Isolate	Significant alignments matched	Percentage of Identities
MG1	*Mycoplasma gallisepticum* strain F PvpA (pvpA) gene	218/225	97%
	Mycoplasma gallisepticum strain F, complete genome	218/225	97%
MG2	*Mycoplasma gallisepticum* strain F PvpA (pvpA) gene	152/170	89%
	Mycoplasma gallisepticum strain F, complete genome	152/170	89%

4.7 Performance Parameters

Measured performance parameters included average body weight, feed conversion ratio and mortality. The least mean squares of performance parameters are provided in Table 4.7. Significant difference ($P<0.05$) in average body weight (BW) were apparent only at day 10 of age. This was true for *Mycoplasma* challenged birds as compared to unchallenged birds. Unchallenged broilers demonstrated significantly larger (237 g) average body weight at 10 days of age compared to the MG-challenged birds (230 g). Broilers BW was also significantly ($P=0.0118$) larger in pens not administered tilmicosin-active ingredient antimicrobial; Pulmotil *AC*® (237 g) compared to those treated with Pulmotil *AC*® (230 g). While pens treated with 20% excess methionine were observed to have significantly

(P=0.0087) larger BW at 10 days of age (237.61g) compared to pens provided adequate Met levels (229.53g).

Feed conversion (FC) at 10 days of age in the unchallenged MG pens demonstrated significantly (P=0.0420) lower FC than birds with MG-challenge. Morality mean at 10 days of age was significantly (P=0.0163) reduced in control, unchallenged pens contrary to MG-challenged pens. Equivalently, this was also illustrated at 17 days of age with a significant decrease (P=0.0107) in mortality in birds of unchallenged pens compared to those with MG-challenge.

Apart from the above-mentioned results, no further significant influence on FC and BW were observed at 17 or 34 days in MG (challenge versus unchallenged pens) Pulmotil (Treated versus untreated) or Met (adequate versus excess). Thus, Ross 308 broilers in these groups showed no differences among and despite treatments in FI and BW gain.

Several studies have reported that Met addition decreased FI compared to diets deficient in sulfur-containing AA (Esteve-Garcia and Llaurado, 1997). While, improvements in feed utilization as a cause of Met supplementation have been extensively illustrated in chickens, and increases in the level of Met augmented an increase of approximately 12 to 14% in BW gain contrary to birds on a Met-deficient diet (Solberg et al., 1971; Lin et al., 2000; Garlich, 1985; Lin et al., 1996; Zhang et al., 2012; Wu et al., 2012). Lin and Shih (2000), Carew et al. (2003), and Attia et al. (2005) determined that a marginal Met-deficient diet is often satisfied by increased FI. Broilers on a marginally Met-deficient feed are likely to overeat to meet the adequate Met amounts. According to Zhang et al. (2012) Met at 0.4 and 0.32%

was adequate for minimum growth requirement at starter and grower phases, respectively. However, birds receiving a marginally Met-deficient diet were observed to attain similar growth through overeating. Compared to Met-deficient diets, supplemented Met-diets significantly increase BW gain (Fanatico et al., 2007, Fanatico, 2010). Diets supplemented with AA, carbohydrates and vitamins result in intestinal enlargement, and development by increasing the size of the villi and the capacity of digestion. In turn, this augmented feed digestion and absorption (Al-Asadi, 2006; Mirzaaghatabar et al., 2011; Nadeem et al. 1999; Fanatico, 2010).

Besides the effects on BW and FC, HMTBA and DL-Met have shown to have distinct impact on effects on protein and fat deposition when Met is limiting. The yield of breast muscle was greater for DL-Met supplemented broilers than the equivalent amount of HMTBA (Esteve-Garcia *et al*. 1997). HMTBA supplementation demonstrated greater abdominal fat deposition at 41 days for male broilers deficient in TSAA, despite similar live BW.

According to Bouyeh (2012), heart and liver weight in addition to total carcass efficiency and breast muscle yield were all significantly increased ($P< 0.05$), by excess methionine with a rate of 40% supplementation into diet. Feed conversion ratio (FCR) was observed to have been significantly reduced on excess methionine ($P< 0.05$). Bouyeh (2012) found out that the excess methionine had a significantly decreased body weight gain ($P<0.05$) yet there was no significant difference observed on thigh and leg yield.

The finding of this experiment showed that treatments whether via administration of

pulmotil, or 20% excess Met above modern breeder (Ross 308) company requirement, or providing pulmotil plus excess Met to broiler diets were not significantly different from one another; hence demonstrating no significant impact on BW and FCR at 17 and 35 days of age. These results indicate that the adequate Met level provided to MG-challenged and unchallenged male Ross 308 broilers were indeed fully adequate; fixed at a plateau level of a growth curve.

In addition, results of several experiments (Elagin and Elzubeir, 2012; Bouyeh, 2012; Rubin et al., 2007; Deng et al., 2007; Jankowski, 2014) on chickens supplemented different levels of Met; ranging from 0.3 to 1.2% and 0.3 to 0.9% in starter and the grower phases, respectively illustrate that commercial broilers do not require more than 0.50 and 0.38% Met in starter and grower diets, respectively for optimum growth and FE.

Table 4.7. Pooled weights (WT), Feed Conversion (FC) and Mortality (MOR) at 10, 17 and 34 days of age of male Ross 308 broilers in different designated treatments using Least Square Means and Student-Newman-Keuls Test of 3-way (2x2x2) factorial ANOVA with interactions (SAS, 2017).

	Performance parameters[1]								
Treatment[2]	***WT10***	***FC10***	***MOR10***	***WT17***	***FC17***	***MOR17***	***WT34***	***CFC***	***CMORT***
MG									
+	230[b]	1.300[b]	0.010[b]	547	1.292	0.014[b]	2221	1.551	0.033
-	237[a]	1.230[a]	0.000[a]	558	1.281	0.002[a]	2225	1.529	0.033
SEM	1.9108	0.0224	0.0026	6.0839	0.0105	0.0031	14.2817	0.0105	0.0080
Pulmotil AC									
Yes	230[b]	1.287	0.008	544	1.293	0.010	2207	1.547	0.033
No	237[a]	1.243	0.002	560	1.280	0.006	2239	1.543	0.034
SEM	1.9108	0.0224	0.0026	6.0839	0.0105	0.0031	14.2817	0.0105	0.0080
Methionine									
Adequate	229[b]	1.284	0.007	554	1.291	0.010	2242	1.542	0.032
Excess	238[a]	1.247	0.003	550	1.283	0.006	2204	1.538	0.035
SEM	1.9108	0.0224	0.0026	6.0839	0.0105	0.0031	14.2817	0.0105	0.0080

Table 4.7 ***continued*** ***Probabilities***

	WT10	***FC10***	***MOR10***	***WT17***	***FC17***	***MOR17***	***WT34***	***CFC***	***CMORT***
MG	0.0312	0.0420	0.0163	0.2165	0.5048	0.0107	0.8965	0.0912	0.9788
Pul	0.0118	0.1860	0.0926	0.0812	0.4168	0.3502	0.1312	0.8651	0.9788
MET	0.0087	0.2634	0.3844	0.6499	0.6104	0.3502	0.0765	0.7322	0.7744
*MG*Pul*	0.7111	0.2538	0.0926	0.3761	0.4673	0.8498	0.1264	0.9734	0.9788
*MG*MET*	0.2979	0.7078	0.3844	0.7110	0.6556	0.8498	0.1114	0.4604	0.7699
*Pul*MET*	0.8249	0.3257	1.0000	0.6979	0.1245	0.5717	0.5045	0.7316	0.3916
*MG*Pul*MET*	0.1623	0.0884	1.0000	0.9225	0.8965	0.8498	0.8847	0.1035	0.3884

[a,b]Means with differing alphabetical superscript in the same column are significantly different (P<0.05).
Least Square Means of 15 N (birds) per treatment.
[1]Performance parameters: CFC=Cumulative Feed Conversion, CMOR=Cumulative Mortality.

Table 4.7 *continued*

[2]Trt=Treatment; MG=*Mycoplasma gallisepticum*; Challenged field isolate was administered to each bird in poultry house 1 of $8.3x10^6$ CFU/ml intrathoracically and intratracheally with 0.2 ml and 50 ul of the *Mycoplasma gallisepticum* suspension, respectively at 10 days of age. *Pulmotil AC*®: challenged and unchallenged birds were administered Tilmicosin active-based antibiotic *Pulmotil AC*® in drinking water during the first 3 days of age and from 18-20 days of age as recommended by Elanco Company. Methionine: Starter, grower and finisher corn/soybean meal based diets were formulated to contain the recommended level (adequate) of Methionine by modern breeder (Ross 308) company requirement; other diets were formulated to contain 20% additional Methionine above the modern breeder (Ross 308) company requirement.
[3]SEM=standard error of mean.
[4]Interactions between treatments: MG= *Mycoplasma gallisepticum*; Pul= *Pulmotil AC*®; Met=Methionine.

4.8 ELISA and MG antibody response

The presence or absence of anti-MG antibody was determined by comparing the absorbance (650nm) value of the unknown sera to that of the positive control. The standardized positive control represented a significant MG antibody level in chicken plasma. For the analysis of the data, an S/P ratio was required (sample value related to positive control value). The below formula was applied using mean absorbance values for both controls and sera samples and the antibody titer was calculated using the equation provided in the ELISA kit; where:

Negative control (NC) mean:

$$NCX = \frac{(well\ A1(450) + well\ A2(650))}{2}$$

Positive control (PN) mean:

$$PCX = \frac{(well\ A3(450) + well\ A4(650))}{2}$$

S/P ratio:

$$S/P = \frac{SAMPLE\ ABSORBANCE - NC\ ABSORBANCE}{PC\ ABSORBANCE - NC\ ABSORBANCE}$$

Titer relates S/P at a 1:500 dilution to an end point titer:

Log_{10} titer = $1.35(\log_{10}\ \text{S/P}) + 3.36$

titer = antilog of $\log_{10}$ titer

Plasma samples with S/P ratios ≤ 0.2 were considered negative, whereas S/P ratios > 0.2 (titers > 400) were assumed positive.

Sera obtained on 1-day of age showed no titers against MG-infection. This means that no maternal antibody to MG was present in progeny. Male Ross 308 broiler chicks were MG-free as tested prior to the start of experiment via tracheal swabbing examined by MG culture; where no MG isolation was detected in the prepared Frey's broth of all samples tested. IgG titers of all unchallenged treatments demonstrated no or low titers to MG indicating no *Mycoplasma gallisepticum* presence in sera samples obtained at 10, 17 and 35 days of age from poultry house 2. This is due to the fact these sera samples have resulted in ELISA titer of < 400 units and hence were considered negative to MG-infection. Such results of pens in the unchallenged poultry house are indicative of high biosecurity implementation and the prevention of cross-contamination between the two poultry houses; i.e. the unchallenged and the challenged house.

Table 4.8. IgG ELISA titers of *Mycoplasma gallisepticum* of male Ross 308 broilers at 35 days of age of two differing treatment showing positive titers using Least Square Means and Student-Newman-Keuls Test (SAS, 2017).

Treatments	***MG Titer 35***
Challenged No Pulmotil Adequate Met	1842.9[b]
CV[1]	66.38
WA SEM[2]	281.068
Challenged No Pulmotil Excess Met	3170.3[a]
CV	14.84
WA SEM	281.068
P-value	0.0035

[a,b]Means with differing alphabetical superscript in the same column are significantly different (P<0.05).
Least Square Means of 15 N (birds) per treatment.
[1]CV=Coefficient of variation.
[2]WA SEM= Weighted average of standard error of mean.

IgG ELISA titers against MG at 1, 10, 17 days of age were all undetectable and below 400 absorbance. Two positive IgG titers against MG were determined as displayed in Table 4.8. Titer of the non-treated challenged group, i.e. treatment 5; with MG, adequate methionine and no tilmicosin administered demonstrated significantly ($P< 0.05$) higher titer, compared to treatment 1; the unchallenged group showing negative IgG titer to MG. The challenged antibiotic (Pulmotil) treated group (Treatment 6), and the challenged Pulmotil+ 20% methionine treated pens (Treatment 7) demonstrated low MG titers for birds at both 17 and 35 days of age. The greatest titer to MG was illustrated in the challenged excess methionine treated pens (Treatment 8), apparent at 35 days of age with significance of ($P<0.05$). The greater the concentration of the specific antibody in the serum sample, in this case IgG, the higher the titer. Prior to MG-challenge exposure, the antibody titer was very low or undetectable as seen on day 1 and day 10 of age in addition to all of treatments designated in the control house at 17 and 35 days age. Following MG-challenge, the lymphatic system generated antibodies, resulting in an increase in IgG titer. Treatments composed of tilmicosin based antimicrobial administration whether solely or in combination of excess methionine, both demonstrated low IgG immunoglobulin titers of MG-challenged birds.

Two discrete interpretations for the above positive titer results reside. Typically, a rising antibody titer in sera samples collected 2-4 weeks apart serves evidence for exposure to an infection. The tilmicosin based active ingredient antimicrobial; Pulmotil AC, has aided in lowering the IgG titer against MG-infection compared to group of non-treated MG-

challenged birds. Tilmicosin hence aided in tackling down MG-infection in challenged birds thereby decreasing the MG titers in pens with challenged pulmotil administered and challenged pulmotil plus excess methionine; treatment 6 and 7 respectively. However, in the case of birds in group 8, with the challenged excess methionine treated pens; the demonstrated significantly different (P=0.0035) higher MG-titer of a 3170.3 mean compared to challenged untreated group (Treatment 1) with a mean of 1842.9 places an emphasis on the effect of methionine in generating a greater immune response towards MG infection. Ardently, methionine has elevated the antibody response in challenged birds through the production of higher levels of IgG compared to birds in challenged adequate methionine pens. Further, 20% excess methionine treatment holds a coefficient of variation (CV) of 14.84 in MG-challenged birds in comparison to a coefficient of variation of 66.38 in MG-challenged untreated group. A larger coefficient of variation is a characteristic of any disease or infection, and is particularly of interest when evaluating the effectiveness of the treatment (Reed et al., 2002). As a dimensionless number that quantifies the degree of variability relative to the mean, CV is used to compare patterns of homogeneity and or heterogeneity whether in treatment, vaccine, or diseases (Volkow et al. 2002; Quinn et al., 2002). In vaccine studies; the fraction of samples with sera antibody concentrations that increase by more than twofold from baseline post vaccination can be established as an indicator for the immunogenicity and the efficacy of the vaccine (Reed et al., 2002). The uniformity of in broiler blood sera was estimated by serological profiling and expressed as a percentage coefficient of variation (CV%). Good and poor uniformity are coefficients of variation < 20 or > 20%, respectively. Based on that reference value, male Ross 308 broilers had the best uniformity of the MG titer with a CV of 14.84 %,

demonstrating successful homogeneity of excess methionine treatment on MG-infection. In other words, the excess methionine treatment may be depicted of vaccine performance. In certain infectious diseases such as avian influenza virus; vaccine efficacy is determined upon the protection it provides from the infection of which requires a particularly attained antibody titer following vaccination. This grants the vaccine the stimulation needed for antibody response and hence achieve the desired titer. Because antibody titers decay over a period of time, revaccination is thus mandatory to elevate the titer to its protective level. The 20% excess methionine above modern breeder requirement has behaved similarly to a vaccine in terms of boosting IgG titer against MG-challenged birds.

According to Bouyeh (2102), excess methionine resulted in significant increase of lymphocytes while decreasing heterophyls as well as the ratio of heterophyls to lymphocytes as indicator for decreasing the stress index significantly ($P<0.01$). Further, a linear increase was observed in Newcastle titer with elevated levels of methionine apparent at 42 days of age ($P<0.01$) in broilers.

A study by Adler et al. (1973) illustrated that the exposure to MG induced continuous titers of agglutinins within 7 days in chickens compared to bursectomized birds that devoid of hemagglutinin to rabbit RBC prior to the exposure to MG. While, live MG strains significantly generated higher titers of agglutinins.

Rubin et al. (2007) assessed the influence of Met on the immune response of birds submitted to immunological stimuli. Met concentrations of (0.31, 0.51, and 0.66% from 1 to 21 d; 0.29, 0.49, and 0.64% from 22 to 42 d) were tested. Vaccines against IB, SRBC, Marek's disease, fowl pox and avian tuberculin were administered to one group as

immunological stimuli. Results showed that those higher or lower concentrations Met than recommended levels equally did not succeed in impacting humoral immune response of broilers. In contrast, the greatest cell immune response (**CIR**) was illustrated at the intermediate level of Met.

4.9 Real-Time PCR and MG Bacterial DNA load in tissues

In the present study, bacterial load was studied in both tracheal and air sac tissues, the two major sites of MG infection. For quantification of the bacterial load FAM based q- PCR was used and has been found to be rapid, highly sensitive, cost effective, reproducible and reliable for quantification of avian mycoplasmosis (Ley, 2008; Kleven, 1997; Levisohn and Kleven, 2000). Negative DNA-load pertains *Mycoplasma gallisepticum* DNA starting quantity load of zero or less than >516 colony forming unit counts. This is due to the fact that the q-PCR cycle threshold (**Ct**) is 516 counts. As the fluorescence was plotted against the different Nano grams of DNA, the sensitivity of this q-PCR protocol cannot perform below 516 CFU. In this manner, any CFU counts below 516 could not be detected. Positive results were those of >516 CFU detection of MG DNA load in tissue samples.

DNA starting quantity from the q-PCR results of each pen at 10, 17 and 35 days of age for MG-challenged and unchallenged birds were subjected to log transformation prior to analysis. The below equations were determined and used for calculating the Log_{10} MG CFU for the airsac and trachea, respectively. Statistical analysis was determined for all data using one-way ANOVA procedure of (SPSS v.24, 2018). Significance of differences

in the treatment means were separated using Tukey's studentized range test. Differences were considered significant at P ≤0.05.

$$Airsac\ Log_{10}\ MG\ CFU = Log\ \frac{(DNA\ Starting\ Quantity) \times (20) \times (3) \times (8.3x10^6\ CFU/50ul)}{(150\ mg)(Airsac\ weight\ g)}$$

$$Trachea\ Log_{10}\ MG\ CFU = Log\ \frac{(DNA\ Starting\ Quantity) \times (20) \times (3) \times (8.3x10^6\ CFU/50ul)}{(150\ mg)(Trachea\ weight\ g)}$$

q-PCR for the quantification of bacterial load results indicated well standard curve and a linear regression line between the amount of input DNA and the Ct values over log $_{10}$ dilutions with an average R^2 of all q-PCR five runs of 0.985. Efficiency (E) for all PCR runs was determined between 95-97%. Table 4.9 displays results of bacterial DNA load of MG CFU in airsac tissues. It was found that the MG-bacterial concentrations were negative <516 CFU in all airsac samples of all pens, in both the unchallenged and challenged broilers at 10, 17 and 35 days of age. Thus, unchallenged pooled airsac samples demonstrated Log_{10} MG CFU of 0.00 at 10, 17 and 35 days of age, and MG-challenged pooled airsac samples at 17 and 35 days of age demonstrated <3.7 Log_{10} MG CFU. For bacterial DNA load of tracheal tissues, results are displayed in Table 4.10. Unchallenged tracheal pooled samples of all pens at 17 days of age demonstrated Log_{10} MG CFU of 0.00. While, MG-challenged tracheal pooled samples of pens at 17 days of age demonstrated Log_{10} MG CFU of <3.7.

Table 4.9. Bacterial DNA load of *Mycoplasma gallisepticum* colony-forming units via q-PCR at 10, 17 and 35 days of age of male Ross 308 broilers in Airsac tissues in different designated treatments using one-way ANOVA (SPSS, 2018).

			AIR SAC q-PCR[1]		
			10 days	*17 days*	*35 days*
	Treatment				
MG[3]	**Antibiotic[4]**	**Methionine**	**Log_{10} MG CFU[2]**	**Log_{10} MG CFU**	**Log_{10} MG CFU**
Unchallenged	-	Adequate	0.00	0.00	0.00
Unchallenged	+	Adequate	0.00	0.00	0.00
Unchallenged	-	Excess	0.00	0.00	0.00
Unchallenged	+	Excess	0.00	0.00	0.00
Challenged	-	Adequate	NA[5]	<3.7	<3.7
Challenged	+	Adequate	NA	<3.7	<3.7
Challenged	-	Excess	NA	<3.7	<3.7
Challenged	+	Excess	NA	<3.7	<3.7
R^2*					98.5%
E**					95-97%

[1]Log of least mean squares of 15 N samples of air sacs, in each pen 5 air sacs samples from 5 random birds were pooled.
[2]Unit is Log_{10} *Mycoplasma gallisepticum* colony-forming unit (CFU) per gram of tissue.
[3]MG=*Mycoplasma gallisepticum*; Challenged field isolate was administered to each bird in poultry house 1 of $8.3x10^6$ CFU/ml intrathoracically and intratracheally with 0.2 ml and 50 ul of the *Mycoplasma gallisepticum* suspension, respectively at 10 days of age.
[4]Antibiotic= Pulmotil AC®, (+) for Pulmotil administration; (-) untreated with Pulmotil.
[5]NA=Not applicable
*R^2= Coefficient of determination measuring the regression line of q-PCR run.
**E=Efficiency of q-PCR runs.

Tracheal tissues of 35 days of age in unchallenged pens illustrated MG-DNA load of 0.00, similarly found at 10 and 17 days of age. MG-DNA load of <3.7 at 35 days of age was illustrated for pens administered pulmotil solely with adequate methionine, and those pens administered pulmotil in addition to excess 20% methionine. Positive q-PCR was shown in challenged adequate methionine pens and challenged excess methionine pen with 7.50 and 7.53 Log_{10} MG CFU, respectively. Hence, pens not treated with antibiotic.

These results explain that the MG-DNA load was not significantly different between challenged adequate methionine pens and challenged excess methionine pens. Yet, these pens were in fact significantly different from the unchallenged groups with a P-value of 0.001. Results also show that the tilmicosin-based antibiotic, pulmotil had reduced the MG-colonization of the trachea.

Reporting these log_{10} MG CFU indicates that the experimental model was performed and executed well. This is because the MG colonization was consistent in the non-treated anti-microbial pens. A decreasing trend was observed in the least square means of data values of the challenged excess methionine pens prior to log transformation in comparison to the challenged adequate methionine pens; yet this reduction was not significantly different. It was also shown to be not significantly different post log transformation. The positive results of the MG DNA load in tracheal tissue prove that the F-strain has successfully colonized the upper respiratory tract of broilers.

Moreover, according to the airsac MG-DNA load which have shown to be consistent throughout the experimenting demonstrating a Log_{10} MG CFU of 0.00 in the unchallenged

birds at 10, 17, 35 days of age and a Log_{10} MG CFU of < 3.7 in the challenged birds at 17 and 35 days age, indicates that MG-infection did not effectively colonize the lower respiratory tract of broilers. Such results are in accordance with the MG isolation, pvpA PCR and the MG field isolates sequencing results. The corollary of F-strain, i.e. the 'MG2' determined field isolates that was used for the MG-challenge in this study resolute a mild pathogenicity strain. F-strain is a live strain, commonly used for immunization of pullets. It is administered in drinking water, aerosol, eye-drop, or intranasal to aid in reducing EP drop and has been administered to displace endemic strains in multiple-age poultry. Yet, the F-strain has been shown to be more pathogenic for turkeys rather than chickens and its primarily disadvantage reside in its inherent virulence.

However, the MG F-strain in this study endured to be non-highly virulent; and this is due to the fact that it did not aggregate and successfully colonize in the air sacs of challenged broilers at various age groups according to the results of q-PCR MG-DNA loads. This also interprets that the challenged MG F-strain demonstrated to be docile and slow in terms of its infectious process. While, positive Log_{10} MG CFU counts were observed, the F-strain pathogenicity lingered until it was apparent at 35 days of age; 25 days post MG-challenge. The field observations thus confirm the pvpA PCR typing and sequence of MG challenge strain. Performance and disease signs were mild and the MG colonization in airsac compromised reported values below 516 CFU or Log_{10} MG CFU >3.7. The F-strain infer to be not virulent where no deleterious effect had taken place.

Table 4.10. Bacterial DNA load of *Mycoplasma gallisepticum* colony-forming units via q-PCR at 10, 17 and 35 days of age of male Ross 308 broilers in Tracheal tissues in different designated treatments using one-way ANOVA (SPSS, 2018).

TRACHEA q-PCR[1]

			10 days	*17 days*	*35 days*
Treatment					
MG[3]	**Antibiotic[4]**	**Methionine**	**Log_{10} MG CFU[2]**	**Log_{10} MG CFU**	**Log_{10} MG CFU**
Unchallenged	-	Adequate	0.00	0.00	0.00^{b}
Unchallenged	+	Adequate	0.00	0.00	0.00^{b}
Unchallenged	-	Excess	0.00	0.00	0.00^{b}
Unchallenged	+	Excess	0.00	0.00	0.00^{b}
Challenged	-	Adequate	NA[5]	<3.7	7.50^{a}
Challenged	+	Adequate	NA	<3.7	$<3.7^{b}$
Challenged	-	Excess	NA	<3.7	7.53^{a}
Challenged	+	Excess	NA	<3.7	$<3.7^{b}$
SEM[6]					1.59
R^2*					98.5%
E**					95-97%
P value					0.001

[a,b]Means with differing alphabetical superscript in the same column are significantly different (P<0.05).
[1]Log of least mean squares of 15 N samples of trachea, in each pen 5 tracheal samples from 5 random birds were pooled.
[2]Unit is Log_{10} *Mycoplasma gallisepticum* colony-forming unit (CFU) per gram of tissue.
[3]MG=*Mycoplasma gallisepticum*; Challenged field isolate was administered to each bird in poultry house 1 of $8.3x10^6$ CFU/ml intrathoracically and intratracheally with 0.2 ml and 50 ul of the MG suspension, respectively at 10 days of age. [4]Antibiotic= Pulmotil AC®, (+) for Pulmotil administration; (-) untreated with Pulmotil. [5]NA=Not applicable. [6]SEM=standard error of mean.
*R^2= Coefficient of determination measuring the regression line of q-PCR run.**E=Efficiency of q-PCR runs.

4.10 Mild and Severe Airsaculitis and Tracheatis

Tracheal and air sac lesion scores and statistical data analysis were performed for all the challenged pens at day 17 and 35 of age. Tracheal scores were measured as follows (-) Negative, for negative tracheatis, (+) for mild tracheatis, and (++) for severe tracheatis. While air sac lesion scores were confined for the left airsac of the bird (i.e. the right when dissected) and evaluated accordingly as a lesion score of (0) for negative airsaculitis, a score of (1) for cloudiness of airsacs, a score of (2) for thickened membrane, and a score of (3) for "meaty" appearance of membranes, with large accumulations of "cheesy" exudate. For statistical data analysis, airsac scores of '0' and '1' were merged and denoted a statistical code of "1" for negative to mild lesion; while those with lesion scores of '2' and '3' were merged and denoted a statistical code of "2" for severe lesions. On the other hand, tracheal lesion scores of (-) and (+) were merged and denoted a statistical code of "1" for negative to mild lesions, and lesion scores of (++) were denoted a statistical code of "2"; for severe lesions.

According to the air sac lesion scores in Table 4.11, no significant differences were observed in between challenged pens at 17 days of age of lesion scores of 0.00 to 1.00, i.e. no significant differences between treatment in mild lesion scores. This is in coherence with lesion scores of 2.00-3.00 at 17 days of age; where no significant difference is observed between the differing challenged pens: those untreated, those treated with pulmotil or excess methionine, or pens treated with pulmotil plus excess methionine. The severe lesion score at 17 days of age were not significantly different between the MG-challenged house with different designated treatments.

Similar results are obtained at 35 days for airsaculitis of lesion scores compromised of 0.00-1.00, mild lesions, in addition to scores of 2.00-3.00 of severe lesions. No significant difference illustrated at 35 days of age between all the challenged groups with different treatments: untreated pens, pens treated with pulmotil or excess methionine, or pens treated with pulmotil plus excess methionine. This is in coherence with the q-PCR results of pooled airsac samples at 17 and 35 days of age demonstrating negative MG DNA load and hence MG colonization in air sac was infective. This also justifies that the MG F-strain applied in this study was not highly virulent.

Regarding the statistical analysis of tracheatis; results displayed in Table 4.12 confer no significant differences ($P>0.05$) between distinct challenged treatments; i.e no significant differences among challenged adequate methionine, challenged pulmotil with adequate methionine, challenged excess methionine; and challenged administered pulmotil plus excess methionine at 17 days of age. Analogous statistical results were observed at 35 days of age. Differences exhibited no significance ($P>0.05$) amidst treatments: challenged adequate methionine, challenged pulmotil with adequate methionine, challenged excess methionine; and challenged administered pulmotil plus excess methionine. Nonetheless, if statistical analysis of treatments is compared between various age groups rather than between treatment at particular age group; i.e. between results in rows rather than those found in columns in the same table; a significant difference ($P<0.05$) is established in the challenged excess methionine group concomitant to tracheatis. This is a significant decrease in the percentage of birds with severe lesion scores of tracheatis from 40% at 17 days of age to 10% of severe lesion scores at 35 days of age as illustrated in Figure 4-16.

Table 4.11. Airsacutitis percentages of negative to mild lesion scores versus severe lesion scores at 17 and 35 days of age of Ross 308 male broilers at different designated treatments using Chi-square analysis[2] (SPSS, 2018).

AIRSACULITIS

	Treatment		*17 days*		*35 days*	
MG	**Antibiotic**	**Methionine**	**[3]Lesion scores of 0-1 (%)**	**[4]Lesion scores of 2-3 (%)**	**Lesion scores of 0-1 (%)**	**Lesion score of 2-3 (%)**
Challenged	-	Adequate	80	20	73.3	26.7
Challenged	+	Adequate	73.3	26.7	73.3	26.7
Challenged	-	Excess	90	10	90	10
Challenged	+	Excess	80	20	86.6	13.3

Airsac tissues were scored individually; 5 airsacs from each pen; a total of 15 N per treatment.
[2]Chi-sqaure analysis produced frequencies that were converted into percentages.
[3]Lesion scores of 0.00 and 1.00 were merged to produce statistical code score of 1.00 for mild airsacultis.
[4]Lesion scores of 2.00 and 3.00 were merged to produce statistical code score of 2.00 for severe airsaculitis

While, no significant difference is observed in the percentage of birds with severe airsaculitis from 17 to 35 days of age; the challenged no Pulmotil excess Met treatment demonstrated numerically the least percentages of birds with severe tracheatis as shown in Figure 4-15. Data analysis suggests that consequently Pulmotil treatment in challenged group whether solely administered or in combination with excess methionine did not significantly reduce tracheatis or airsaculitis in broilers infected with MG. Although at 35 days of age, there is a small reduction in tracheatis as well as airsaculitis in MG-challenged pens provided pulmotil plus excess methionine as compared to pens treated with pulmotil alone, yet this reduction is not significant.

The challenged excess methionine treatment had consistently shown a trend in alleviation of severe tracheatis and airsaculitis as compared to all other challenged groups; though not significant. Significant difference of the 20% excess methionine was apparent once percentage of tracheatis in birds were compared at distinct age groups, from 17 to 35 days of age.

In MG field cases of in poultry, it is critical to recognize that concurrent infection with additional microorganisms exacerbate the clinical disease. Such microorganisms and viruses include *H. paragallinarum,* (Uchida et al, 1990) *E. coli,* AI, colibacillosisas (Ley et al., 1997; Grodio et al., 2008; Indikova, 2014; Winner et al., 2000). It has been suggested that *E. coli* infection cannot penetrate the air sacs without MG infection (Gross, 1961). Similar synergisity is acknowledged in swine production; where P. multocida does not attain pneumonia without a prior infection of M. *hyopneumoniae.*

Table 4.12. Tracheatis percentages of negative to mild lesion scores versus severe lesion scores at 17 and 35 days of age of Ross 308 male broilers at different designated treatments using Chi-square analysis[2] (SPSS, 2018).

TRACHEATIS

Treatment			*17 days*		*35 days*	
MG	**Antibiotic**	**Methionine**	**[3]Lesion scores of 0-1(%)**	**[4]Lesion scores of 2 (%)**	**Lesion scores of 0-1 (%)**	**Lesion score of 2 (%)**
CH	-	Adequate	66.7	33.3	73.3	26.7
CH	+	Adequate	80	20	73.3	26.7
CH	-	Excess	60	40	90	10
CH	+	Excess	73.3	26.7	86.7	13.3

Tracheal tissues were scored individually; 5 tracheas from each pen; a total of 15 N per treatment.
[1]trt=treatment: Challenge: UN=Unchallenged, CH=Challenged; Antibiotic: NP=No Pulmotil *AC*®, P=Pulmotil *AC*® administered; Methionine: AM=Adequate Methionine; EM= Excess Methionine.
[2]Chi-sqaure analysis produced frequencies that were converted into percentages.
[3]Lesion scores of 0.00 and 1.00 were merged to produce statistical code score of 1.00 for mild tracheatis
[4]Lesion scores of 2.00 remained as statistical code score of 2.00 for severe tracheatis.

While, tracheal MG infection may be considered self-confined in the absence of concurrent infection or environmental stressors (Ley, 2008; Levihson and Kleven, 2000; Yagihashi and Tajima, 1986). The bird may remain a carrier even in the presence of humoral or local antibody (Yagihashi and Tajima, 1986; Kleven, 1985).

Mycoplasmas have the capability of stimulating various aspects of the lymphatic response, this includes complement activation to macrophage ingestion (Simecka *et al*., 1992; Gauson et al., 2000); however, the mechanisms by which they trigger a chronic immune response while simultaneously denoting immune evasion and an adaptation to modifications in the host environment are yet elusive.

Birds with either bursectomy or thymectomy, lacking a functional lymphatic system demonstrated failure to abolishing MG-infection from tracheal tissues (Mukherjee *et al*., 1990; Adler et al., 1973) and thus have significantly increased tracheal lesion scores (Gauson et al., 2000).

Administering bivalent live vaccine of NDV and IBV increases MG-induced lesions scores in the tracheae of broilers (Nakamura et al. 1994). For so, knowledge of the pathogenesis process of various live vaccines in combination with coinciding MG-infection is oughted to prevent severe reactions post vaccination. This is why dates and duration of vaccination programs should always be considered, particularly if high tracheal lesion scores were observed in non-challenged birds.

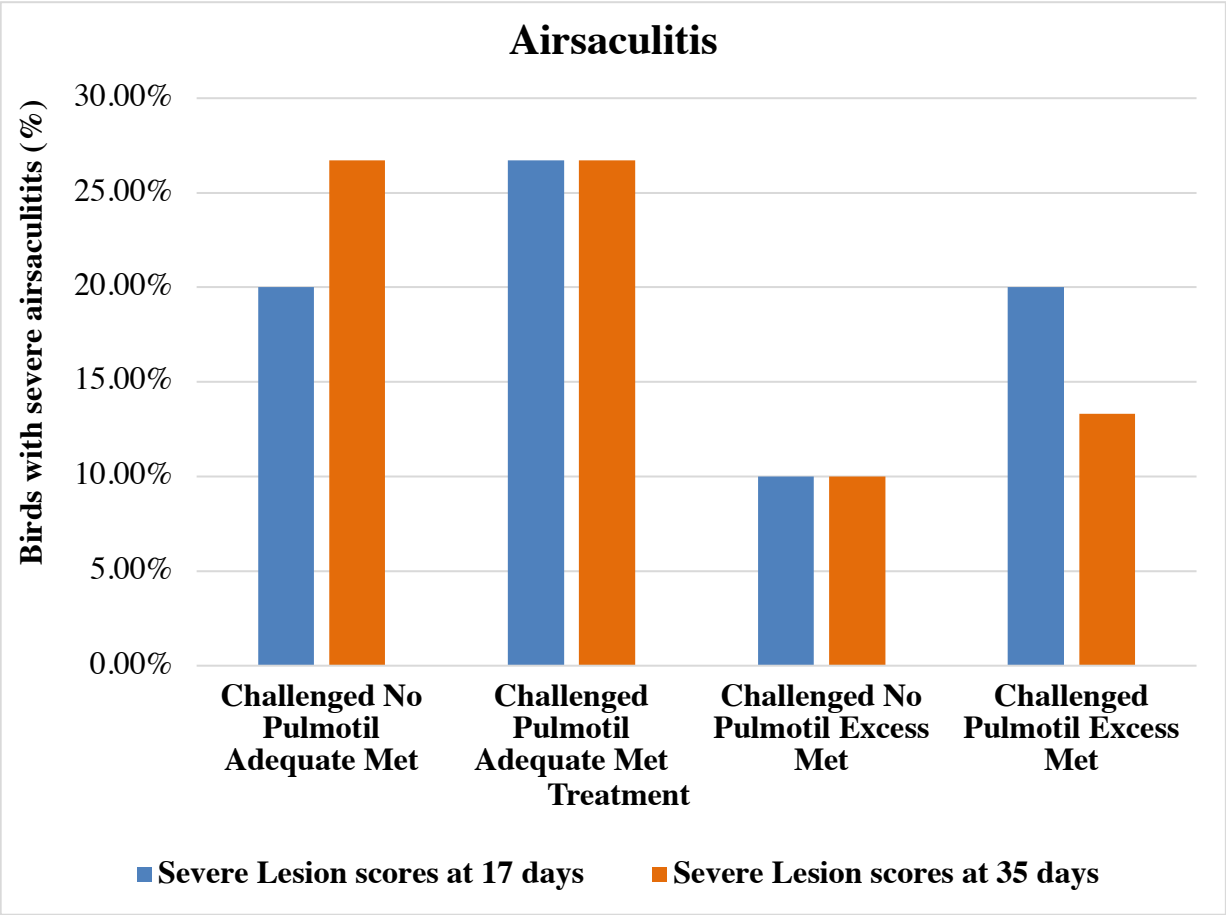

Figure 4-15. Airsaculitis severe lesion score percentages at 17 versus 35 days of age of male Ross 308 broilers. No significant difference observed between severe lesion score of airsaculitis between 17 and 35 days of age. Total samples of 15 broilers (15 N) per treatment; 5 birds selected randomly from each pen.

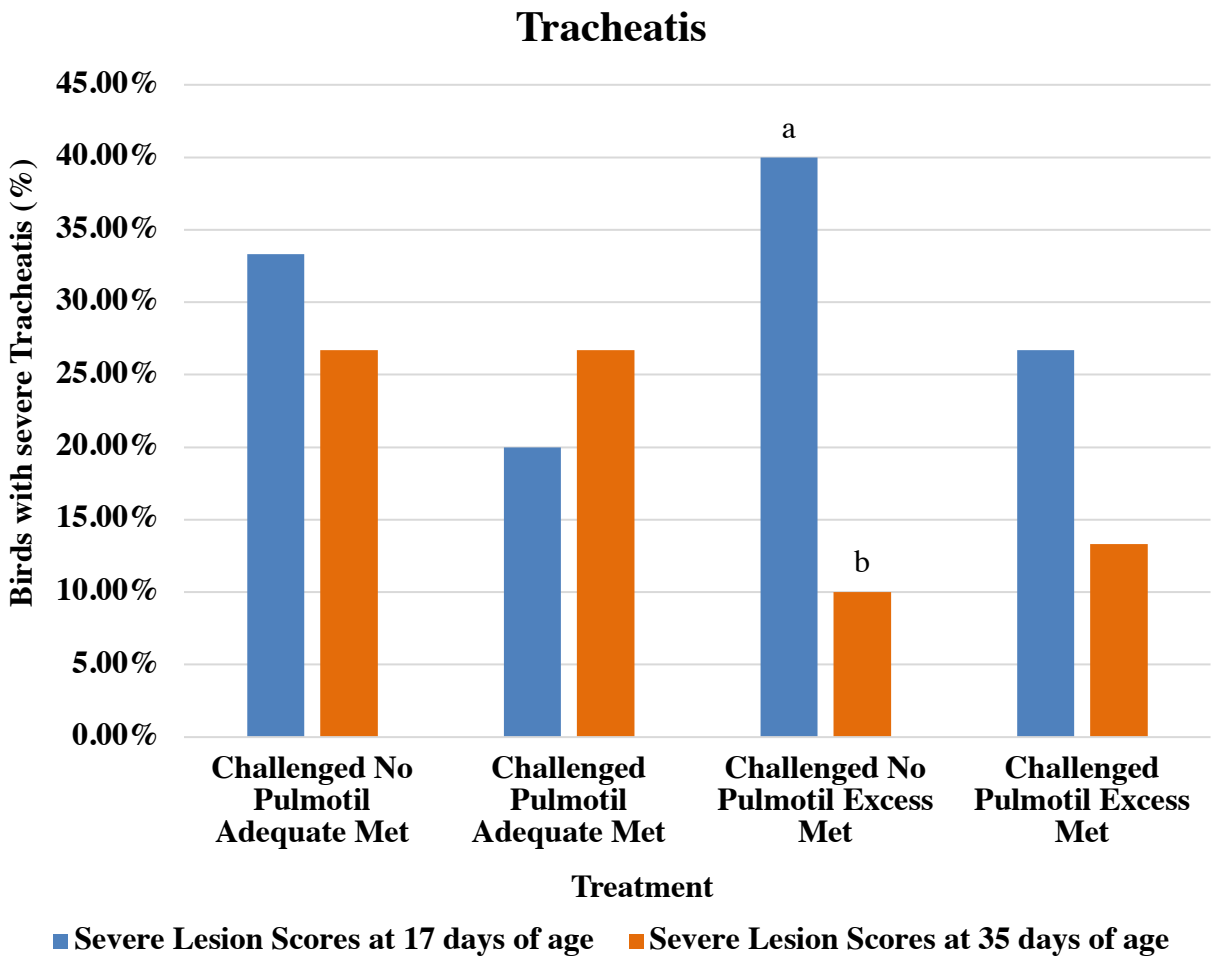

Figure 4-16. Tracheatis severe lesion score percentages at 17 versus 35 days of age of male Ross 308 broilers. Significant difference (P<0.05) observed between severe lesion score of tracheatis between 17 and 35 days of age in the *Mycoplasma gallisepticum* challenged group receiving excess methionine (CH NP EM treatment). Total samples of 15 broilers (15 N) per treatment; 5 birds selected randomly from each pen.

4.11 Hematological Parameters

Hematological and blood parameters are reliable analyses of bird's health condition and productivity and useful diagnostic tools (Woerpel and Rosskopf, 1984; Albokhadaim, 2012; Abdi-Hachesoo et al., 2011). Thereby, it is important to understand and determine the haematological parameters of broilers to better interpret their health status and lymphatic system capability to tolerate pathogens and diseases.

Blood samples of 3ml each were drawn from the brachial vein of 5 birds per pen and they were collected in ethylenediaminetetraacetic acid (**EDTA**) heparinized tubes as an anticoagulant at 1, 10, 17, and 35 days. All samples were used for sera collection post centrifuging. Only samples of 35 days old birds were used for hematological analysis prior to centrifuging to obtain sera and perform serological analysis.
Statistical Analysis for hematology are displayed in Table 4.13. No significant differences were observed in RBC, HGB and MCH in birds that were MG-challenged versus those that remained MG-free. Statistical significance is apparent in the percentage of hematocrit in pens administered tilmicosin based antimicrobial, Pulmotil AC®, and those treated with excess methionine.

Hematocrit value has shown to be the highest in control methionine group, ie in unchallenged birds. Hematocrit is the fraction of the volume of RBCs to the total volume of blood; thus, it reflects closely the RBC and packed cell volume (**PCV**). As such, once RBC and PCV values increase, the HCT % increases. The excess methionine group demonstrated significantly the highest percentage of HCT.

Table 4.13. Hematological statistical analysis of 35-day old male Ross 308 broilers against different designated treatments using Student-Newman-Keuls Test of 3-way factorial ANOVA with interactions (SAS, 2017).

	***Blood Parameters*[1]**			
***Treatment*[2]**	***RBC*** *(10^6/ul)*	***HGB*** *(g/dl)*	***HCT*** *(%)*	***MCH*** *(pg)*
MG				
+	2.75	11.6	26.3	42.4
-	2.77	11.6	26.4	42.3
SEM	0.071	0.25	0.74	0.59
Pulmotil AC®				
Yes	2.70	11.5	24.2[b]	43.1
No	2.81	11.7	28.5[a]	41.6
SEM	0.071	0.25	0.74	0.59
Methionine				
Adequate	2.70	11.3	25.2[b]	42.2
Excess	2.81	11.9	27.5[a]	42.5
SEM	0.071	0.25	0.74	0.59
***Interactions*[3]**		***Probabilities***		
MG	0.8555	0.9290	0.9162	0.9597
Pulmotil AC®	0.2659	0.5581	0.0008	0.0920
Methionine	0.3140	0.1523	0.0452	0.7679
MG x Pul	0.3739	0.6465	0.4739	0.3567
MG x Met	0.6325	0.7268	0.6359	0.8432
Pul x Met	0.8472	0.3563	0.1096	0.2210
MG x Pul x Met	0.4950	0.3441	0.3724	0.7180

Least Square Means of 15 N (birds) per treatment.
[a,b] Least Square Means with different superscripts in the same column differ significantly ($P < 0.05$).

Table 4.13 continued

[1] RBC=Red Blood cell (10^6/ul), HGB=Hemoglobin (g/dl), HCT=Hematocrit (%), MCH=Mean Corpuscular Hemoglobin (pg).

[2] MG=*Mycoplasma gallisepticum*; Challenged field isolate was administered to each bird in poultry house 1 of $8.3x10^6$ CFU/ml intrathoracically and intratracheally with 0.2 ml and 50 ul of the *Mycoplasma gallisepticum* suspension, respectively at 10 days of age. *Pulmotil AC®*: challenged and unchallenged birds were administered Tilmicosin active-based antibiotic *Pulmotil AC®* in drinking water during the first 3 days of age and from 18-20 days of age as recommended by Elanco Company. Methionine: Starter, grower and finisher corn/soybean meal based diets were formulated to contain the recommended level (adequate) of Methionine by modern breeder (Ross 308) requirement; other diets were formulated to contain 20% additional Methionine above the modern breeder (Ross 308) requirement.
[3] Interactions between treatments: MG= *Mycoplasma gallisepticum*; Pul= *Pulmotil AC®*; Met=Methionine.

In HGB, the excess methionine is among the highest values, of 11.9 g/dl (Figure 4-19); this was reflected with the highest HCT 27.5% (Figure 4-20). How does in such case the unchallenged excess methionine group differ from the challenged excess methionine group in regard to HCT %? It is the fact that pens within the control house with excess methionine were more comfortable in terms of MG-free infection. Moreover, as methionine percentage in diet increased, RBC values increased to $2.81x10^6$/ul; as compared to 2.70 $x10^6$/ul in pens provided adequate levels of methionine. The effect of methionine on blood RBC, HGB, and MCH was observed to hold a trend in increasing levels of all three parameters. Pens supplemented with 20% excess methionine, although not significant, had the highest HGB levels, whether it had been in unchallenged or challenged birds. In addition, pens supplemented with 20% excess methionine were amongst the highest in terms of RBC and MCH levels (Figure 4-17 and Figure 4-18). The statistical significant difference had appeared in the percentage of HCT in the blood, where pens with excess methionine

treatment were significantly different with P=0.042 and a least square mean of 27.5% as compared to group administered adequate levels of methionine; with a least square mean of 25.2%. This is true for birds that remained unchallenged as well as for those that were MG-challenged. Could there exist a relationship between Methionine and RBC? As excess methionine was primarily illustrating an increasing trend in all hematological parameters tested provide an indication of the its nutritive elements. Perhaps, as there were more N available, and thus more AAs for the synthesis of RBCs.

Why is such insight of the observed relationship between methionine and blood parameters is important? For one, physiologically, in treatment 4 and 8, tissues were growing better under the excess treatment of methionine. The effect of excess methionine on increasing hematological parameters particularly may also be associated with the role of AAs in the synthesis of globulins. Globulins are classified as hemoglobin-building unit compromised of iron and porphyrin. This translates that the excess methionine had a greater effect in synthesizing globulins and porphyrins (Wahed, 2015).

Vogl et al., 2008 discovered via nested PCR, differential immunofluorescence and interference contrast microscopy that MG R_{low} was detected in the bloodstream of infected chickens demonstrating the capability of MG not only to attach to the surface but also penetrate chicken erythrocytes. It is worth noting that in the blood results of this study, 20% excess Met had great impact comprised of an increase trend in hematological parameters particularly in the presence of MG-infection as demonstrated in Figure 4-17, Figure 4-18, Figure 4-19 and Figure 4-20.

The feeding with excess methionine, though not significantly, had an increased pattern of MCH value compared to the adequate methionine group as shown in Figure 4-18. MCH is coherent with PCV, such that an increase in MCH, strongly correlates an increase in PCV and this is because there is rise in total RBC count (Al-Daraji et al., 2012). This is in agreement with results by Wahed (2015) who found the PCV, which in this study reflect MCH has increased in birds fed methionine. Findings reported by shown increased in TRBC of chicks feed methionine and lysine as compared to control group. Particular higher increase was illustrated in Met-supplemented group than other treated groups (Wahed, 2015). Such increase in TRBC as a result of Met has been suggested to be associated with the ability of Met in synthesizing more globulins.

Secondly, the significant increase is HCT % typically means that blood platelets have also increased. Generally, there exist a strong correlation between RBC count, PCV and HGB contents and HCT% and this also will associate with increase in platelets count. An increase in platelets counts would translate in blood clotting and thus bruising prevention. Nevertheless, economic losses due to bruising and failure of marketability of fowl have been reported (Ekstrand, 1998; Warriss et al., 1992; Mayes, 1980). Easy-bruising and injury also imply that the welfare of bird is threatened. The mean percentage of birds at processing plant arrival with bruising ranges from 0.022 to 25% (Farsaie et al., 1983; Ekstrand, 1998; Nijdam et al., 2004).

In order to prevent blood coagulation clotting, vitamin K is commonly added to poultry feed (Watanabe et al., 2010; Gentry et al., 2008). Poultry require vitamin K in a considerable quantity; yet it is difficult to determine the precise nutritional requirement for

vitamin K due to its short half-life (Powers, 2000; Gentry et al., 2008; Watanabe et al., 2010). In addition, Vitamin K deficiency may be present by a cause of antimicrobial or coccidiostat treatment (Gentry, 2004).

Birds are afflicted by various pathogens that induce hemostatic disorders. For instance, circovirus and polyomavirus may cause thrombocytopenia and vasculitis. Gram-negative bacterial infections generally are diagnosed with systemic fibrinous thrombosis and blood vessel necrosis. Infectious bursal disease virus (**IBDV**) causes significant thrombocytopenia and whole blood recalcification time (**WBRT**). While, *E. coli* generates fibrin in the liver and spleen of flocks. (Zeryehun et al., 2012; Gentry et al., 2008; Buzala et al., 2017; Thomson, et al. 2003.) Moreover, chicken thrombocytes are readily available source of messenger RNA (mRNA) encoding diverse proteins analogous to those already characterized in mammal platelets. Avian thrombocytes are capable of integrating [35S] methionine into various proteins, in addition to glycoprotein IIb and IIIa analogs (integrin complex found on thrombocytes or platelets) during 4 hours of incubation in vitro. Such insight suggests that chicken's peripheral blood nucleated thrombocytes, unlike mammals, have the ability to retain the capacity to synthesize protein. In contrast, antibiotic Pulmotil AC has significantly decreased (P=0.0008) the hematocrit percentage in blood of broilers as compared to groups not administered Pulmotil treatment. This reduction in hematocrit was from 28.5% in non-Pulmotil treated groups to 24.2% in Pulmotil treated groups. RBC and HGB although not significant, were numerically reduced in the Pulmotil administered pens.

Disturbance in normal HCT % can be a result of antimicrobial treatments. Low HCT% and HGB have been observed due to antibiotic administration such as penicillin. low levels of

HCT% or HGB commonly are a sign of anemia. Results of significant decrease in HCT% due to antibiotic administration is in agreement with Al-Mayah and Al-Ahmed (2005). Where, Ampicillin, Enrofloxacine ad Amoxicillin have all demonstrated reduction in hematological parameters. Antimicrobial agenst such B-lactams, Vancomysine and trimethoprim-Suldamethaxazole (TMP-SMX) have been shown to cause leucopenia (Cuhna, 2001). Chloamphenicol have shown to reduce RBC counts (Calwell and Cluff, 1974). According to El-Sayed (2014); tilmicosin caused reduction in the RBCs and WBCs counts but has shown no impact on HBG and PCV. Similar findings by Altunok et al. (2002) and Scorneaux et al. (1999) were determined post tilmicosin administration resulting in significant ($P<0.05$) reduction in RBCs and WBCs of rabbits and demonstrated high levels in phagocytes of avian, swine and bovine (El-Sayed, 2014; Scorneaux et al. 1999). Further, Azithromycin and Clarithromycin (of macrolide class) administered to human patients demonstrated a decline of RBCs and WBCs (Ohtsuka et al., 1996).

As previously explained in the literature review chapter, those cells that are associated with the non-specific, innate immune response include phagocytic cells, NK cells, basophils, and mast cells. Studies have also demonstrated that platelets or thrombocytes as known in Aves have incorporated functions in inflammation and innate immune response (Glick et al., 1964; Clawson and White, 1971; Klinger and Jelkmann, 2002; Elzey et al., 2003; Scott and Owens, 2008; Ferdous, 2014).

According to (Lam, 2003), the pathogenicity of MG was suggested to be linked with in the shape alteration RBCs. MG-infection of RBCs induced perforations in RBCs in addition to causing changes in size, shape, and surface of erythrocytes.

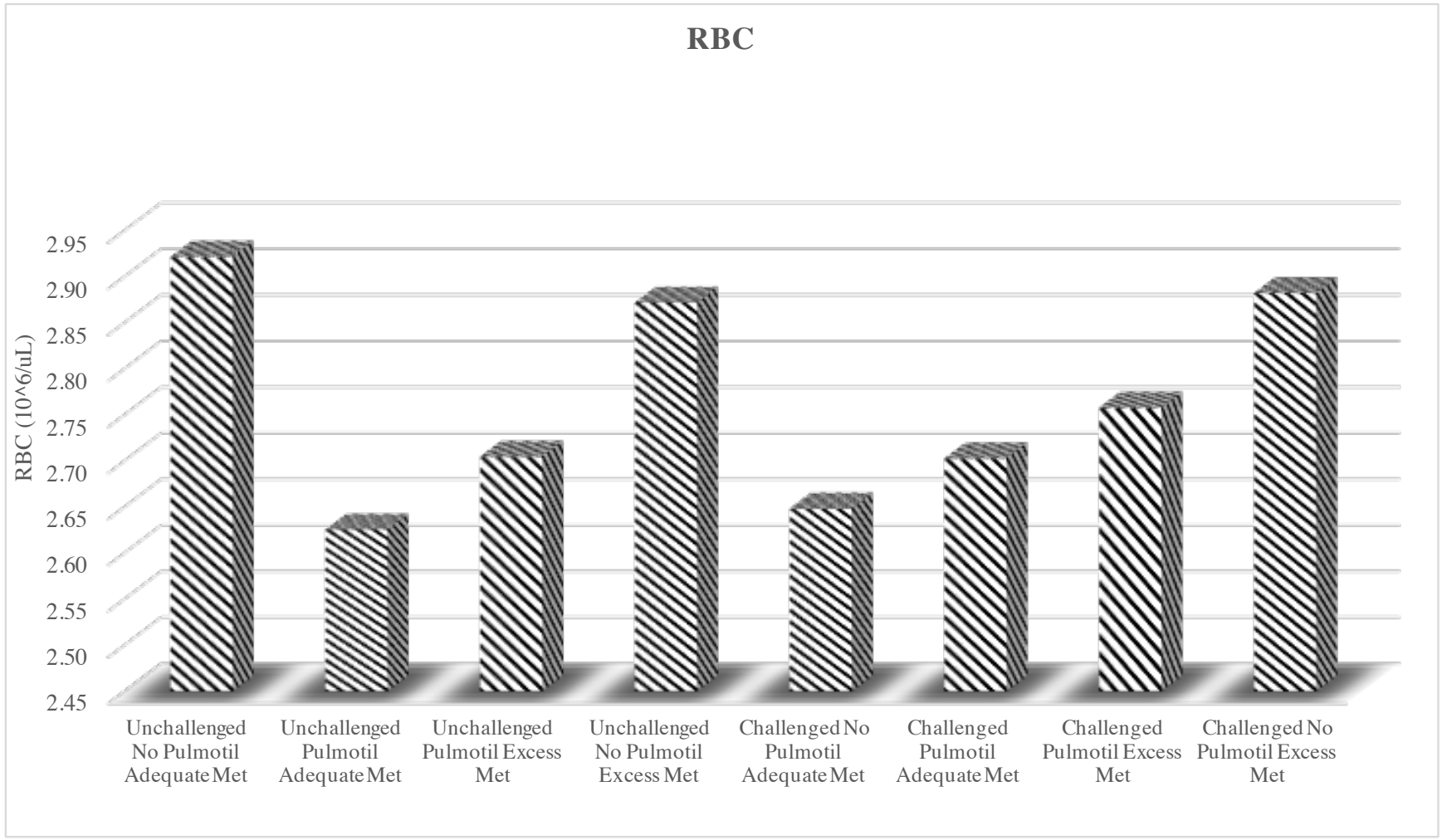

Figure 4-17. Red Blood Cell (RBC) (10^6/ul) trend in male Ross 308 broilers at 35 days of age in different designated treatments. The 20% excess methionine treatment is among the highest in RBC counts whether in MG-challenged or unchallenged broilers.

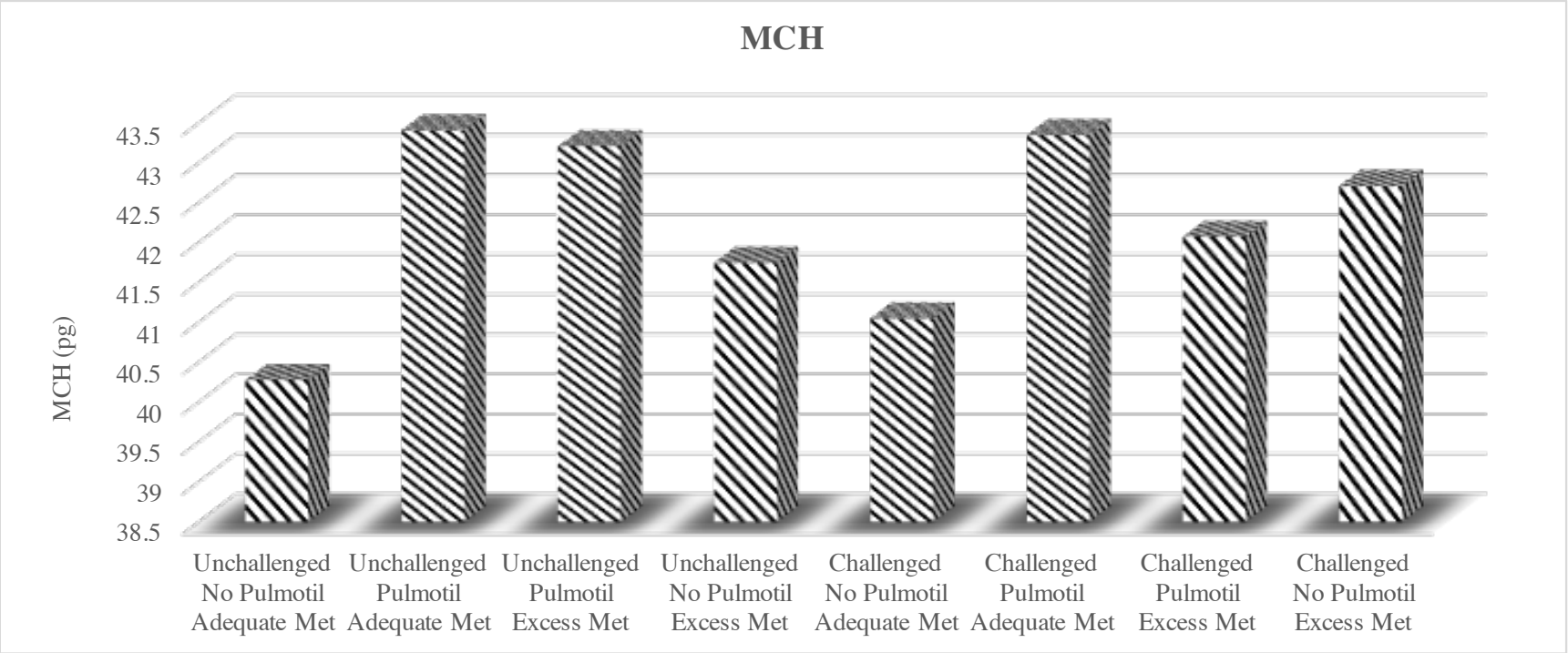

Figure 4-18. Mean Corpuscular Hemoglobin (MCH) (pg) trend in male Ross 308 broilers at 35 days of age in different designated treatments. The Challenged No Pulmotil Excess methionine group is among the highest levels of MCH.

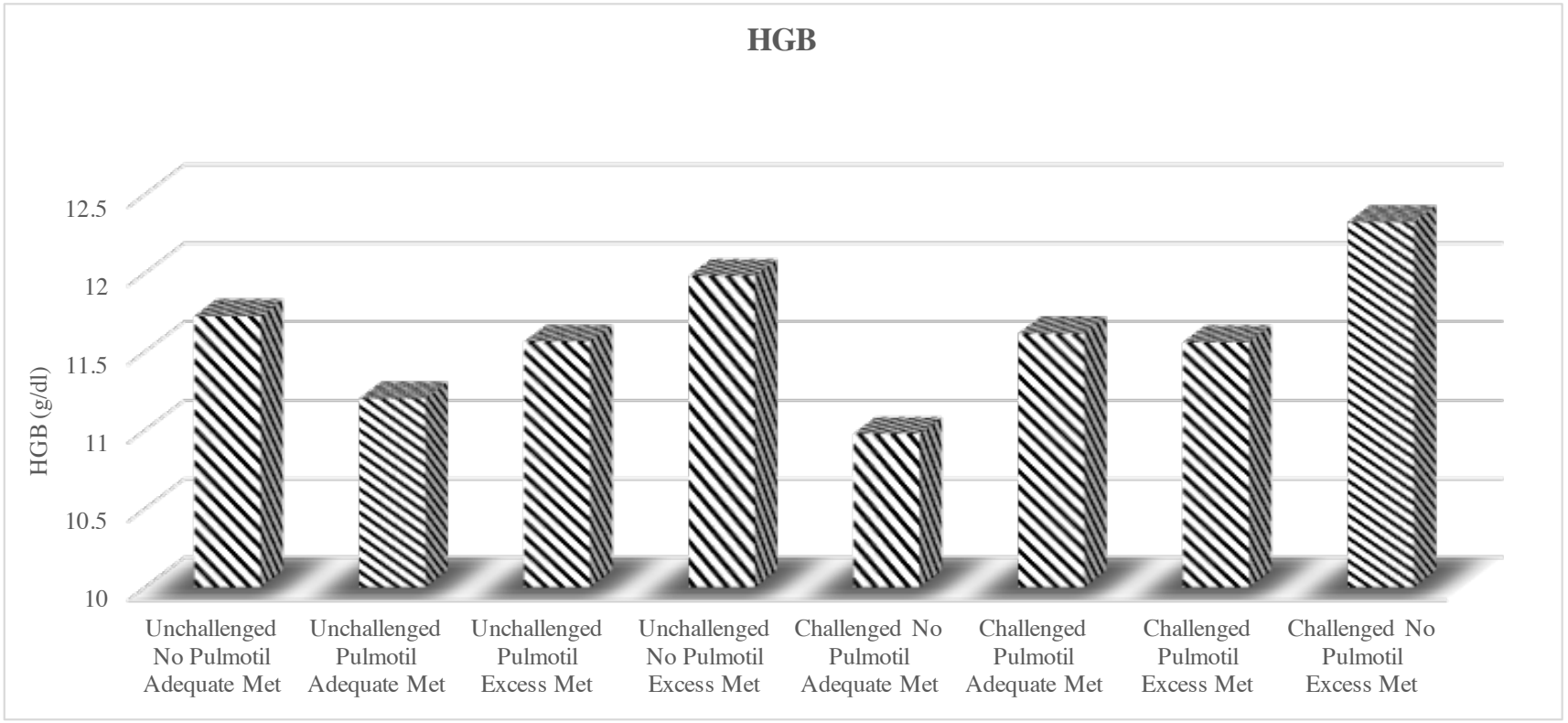

Figure 4-19. Hemoglobin (HGB) (g/dl) trend in male Ross 308 broilers at 35 days of age in different designated treatments. The Challenged No Pulmotil Excess Methionine group demonstrating the highest level of hemoglobin (g/dl). The unchallenged No Pulmotil Excess Methionine treatment demonstrating the second highest levels of HBG.

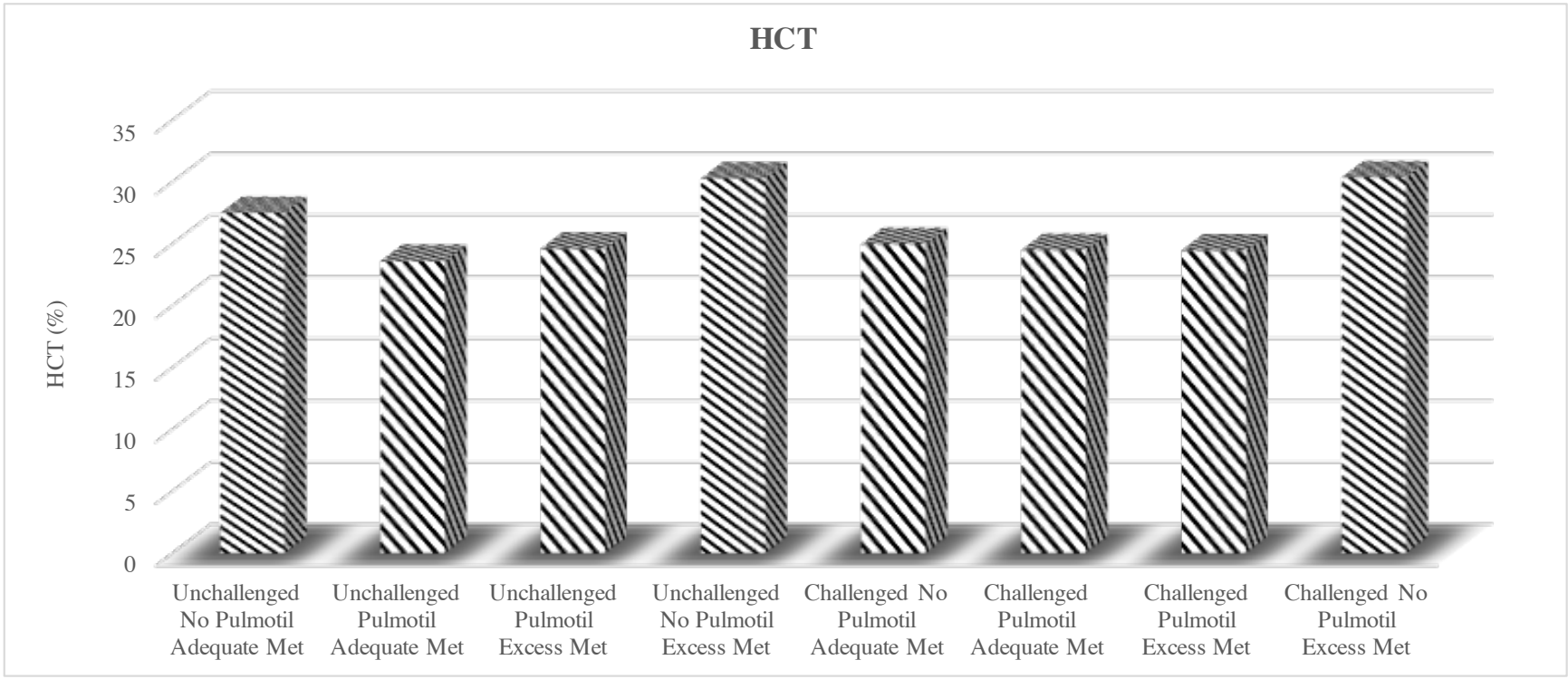

Figure 4-20. Hematocrit (HCT) (%) trend in male Ross 308 broilers at 35 days of age in different designated treatments. Significant differences were observed as illustrated in Table 4.13 between pens receiving 20% excess methionine and pens receiving adequate methionine levels with P= 0.0452; and between pens administered Pulmotil AC and pens untreated with Pulmotil AC with P= 0.000.

4.12 Bursa of Fabricius and Thymus indices

Thymus and bursal indices of broilers at 10 days of age are illustrated in Table 4.14 show no significance difference between treatments ($P>0.05$). According to such results, it implies that both; the tilmicosin antimicrobial Pulmotil AC and the excess methionine treatment did not have an effect on the bursa of Fabricius. Such observations are indicative of normal results as birds in this stage were at younger age and hence it was yet early to observe any potential influence of either treatment.

For bursal indices at 17 days of age; as presented in Table 4.15; all the challenged groups have demonstrated significantly ($P<.0001$) larger bursal indices than the unchallenged groups. This is in fact typical for any infection. The immune system in this case have likely been triggered and was activated to produce more immune cells and antibodies; which was reflected by higher bursal indices.

Several bacterial and viral infections have great impact on the bursa of Fabricius, leading to B-lymphocyte depletion or yet excess proliferation. This in turn jeopardizes the humoral immunity that is contingent upon the normal functioning of the bursa. Examples of viral and bacterial infections that impair bursal cells include Marek's disease, chicken infectious anemia virus (CAV), IB, Adenovirus, Retrovirus, NDV, AI (Levisohn and Kleven, 2008; Ley, 2008).

As discussed in 'Lymphatic System Overview' section of 'Literature Review' chapter, at onset of hatching, the bursal duct opens to transport antigens from cloacal region to the bursal lumen and straight down into the lymphoid follicles (Bar-Shira et al., 2003). These bursal cells migrate to secondary lymphoid organs such as spleen and the gut-associated

lymphoid tissues (Hoskins, 1977). B- lymphocytes originate from the bursa of Fabricius with the capacity to migrate to the circulation to transform into antibody-producing plasma cells when exposed to antigens. Bursa's follicles consist of more than ninety percent of B-cells (Kreier, 2001). However, these rapidly proliferating B-cells, up to nighty nine percent of them undergo apoptosis (Pastoret et al., 1998; Kreier, 2001). Such process illustrates a negative selective pressure on the self-reactivating B- cells (Kreier, 2001). Introduction of antigens into the bursa results in an increase in B-cell responses; however, it does not encourage antibody production directly (McNab, et al., 2002, Spring et al., 2008). On the surface of bursa's lymphocytes, a slow consecutive occurrence of immunoglobulin M-, immunoglobulin G- and immunoglobulin A- determinants illustrates that immature precursor cells convert into immunoglobulin by a genetically antigen-independent transformation (Cooper et al., 1972; Hoskins, 1977). It has been shown that bursectomized chickens are refracted to antigenic stimulation that reflect their inadequacy of the diversity and quantity of antibody-forming precursor cells; that particularly lead to the damage of bursal or non-bursal mediation (Adler et al., 1973; Lerner et al., 1971). Adler et al. 1973 study revealed that 10 days post MG exposure generated greater quantity of lymphoid follicles in the spleen, i.e. more germinal centers compared to the control uninocolted birds yet this was not the case for cecal tonsils.

Zhang et al. 2012 trial consisted of an arrangement of 4 graded levels of LMA meeting 80, 100, 120, and 140% of Met requirements of broilers recommended by Chinese feeding standards for chickens. The trial has shown that the greatest relative weights of bursa and the spleen were observed on day 10 and day 21, respectively were for the group fed 120% of methionine requirement.

Table 4.14. Bursal and Thymus indices least square means at 10 days of age of unchallenged male Ross 308 broilers using 2-way factorial (2x2) ANOVA Student-Newman-Keuls Test (SAS, 2017).

Bursa of Fabricius and Thymus Indices[1]

Trt[2]	***Bursa10***	***Thymus10***
Pulmotil AC		
yes	0.14500	0.13500
No	0.16925	0.16100
SEM	0.01366933	0.01103781
Methionine		
Adequate	0.15458	0.15000
Excess	0.15967	0.14600
SEM	0.01366933	0.01103781

Probabilities

Interactions[3]	***Bursa10***	***Thymus10***
Pulmotil AC	0.2451	0.1344
Methionine	0.7992	0.8042
Pul x Met	0.4204	0.1344

Least Square Means of 15 N (birds) per treatment.
[1] Bursa10= Means of Bursal indices at 10 days of age of unchallenged birds, Thymus10= Means of Thymus indices at 10 days of age of unchallenged birds.
[2]*Pulmotil AC*® : unchallenged birds were administered Tilcomisin active-based antibiotic *Pulmotil AC*® in drinking water during the first 3 days of age and from 18-20 days of age as recommended by Elanco Company. Methionine: Starter, grower and finisher corn/soybean meal based diets were formulated to contain the recommended level (adequate) of Methionine by modern breeder (Ross 308) company; other diets were formulated to contain 20% additional Methionine above the modern breeder (Ross 308) company.
[3] Interactions between treatments: Pul= *Pulmotil AC*®; Met=Methionine.

Table 4.15. Bursal and Thymic indices statistical analysis of male Ross 308 broilers at 17 and 35 days of age against different designated treatments using Student-Newman-Keuls Test of 3-way (2x2x2) factorial ANOVA with interactions (SAS, 2017).

	Bursa of Fabricius and Thymus Indices[1]			
Treatment[2]	***Bursa17***	***Thymus17***	***Bursa35***	***Thymus35***
MG				
+	1.0092[b]	0.1877[b]	0.1227[b]	0.1805
-	0.1895[a]	0.1386[a]	0.1673[a]	0.1742
SEM	0.04051	0.01069	0.00586	0.01995
Pulmotil AC®				
Yes	0.6128	0.1826[b]	0.1593[a]	0.1687
No	0.5858	0.1436[a]	0.1307[b]	0.1860
SEM	0.04051	0.01069	0.00586	0.01995
Methionine				
Adequate	0.5475	0.1714	0.1363[b]	0.1785
Excess	0.6512	0.1549	0.1538[a]	0.1762
SEM	0.04051	0.01069	0.00586	0.01995

Table 4.15 ***continued***

	Probabilities			
Interactions[3]	***Bursa17***	***Thymus17***	***Bursa35***	***Thymus35***
MG	<.0001	0.0051	<.0001	0.8252
Pul	0.6436	0.0200	0.0033	0.5476
MET	0.0891	0.2911	0.0520	0.9351
MG x *Pul*	0.5387	0.4749	0.1253	0.6847
MG x *MET*	0.2033	0.2646	0.0005	0.0516
Pul x *MET*	0.1994	0.6803	0.8202	0.5400
MG x *Pul* x *MET*	0.2249	0.8108	0.6432	0.9074

Least Square Means of 15 N (birds) per treatment.
[a,b] Least Square Means with different superscripts in the same column differ significantly ($P < 0.05$).
[1] Busra17= Means of Bursal indices at 17 days of age, Thymus17= Means of Thymus indices at 17 days of age, Bursa35= Means of Bursal indices at 35 days of age, Thymus35= Means of Thymus indices at 35 days of age.
[2] MG=*Mycoplasma gallisepticum*; Challenged field isolate was administered to each bird in poultry house 1 of $8.3x10^6$ CFU/ml intrathoracically and intratracheally with 0.2 ml and 50 ul of the *Mycoplasma gallisepticum* suspension, respectively at 10 days of age. *Pulmotil AC®* : challenged and unchallenged birds were administered Tilmicosin active-based antibiotic *Pulmotil AC®* in drinking water during the first 3 days of age and from 18-20 days of age as recommended by Elanco Company. Methionine: Starter, grower and finisher corn/soybean meal based diets were formulated to contain the recommended level (adequate) of Methionine by modern breeder (Ross 308) company; other diets were formulated to contain 20% additional Methionine above modern Ross 308 requirement.
[3] Interactions between treatments: MG= *Mycoplasma gallisepticum*; Pul= *Pulmotil AC®*; Met=Methionine.

In addition, regarding the thymic indices at 17 days of age, similar results to bursal indices were observed when comparing the challenged and the control groups. All MG-challenged groups have significantly (P=0.0051) demonstrated higher indices compared to the control groups. This increase in thymic indices is of a comparative enlargement result values; suggesting that the bursal and thymic indices of challenged groups at day 17 were more active compared to the unchallenged groups; as a decrease in indices would translate to lower immune cell and antibody production and an abnormal increase would suggest a first step before atrophy of both lymphoid organs. Moreover, the 20% excess methionine treatment has numerically demonstrated greatest indices for the bursa of Fabricius at 17 days of age indicating an augmented trend where lymphatic cells were more prepared to fight an infection; in this case MG; as more B-lymphocytes, as effector cells being recruited in Bursa and more T-lymphocytes being produced in the thymus.

At 35 days of age, results have shown significant increase (P=0.0033) of bursal indices in pens administered Pulmotil AC compared to pens untreated with Pulmotil. The same is true for excess methionine, where pens treated with 20% excess methionine have significantly increased (P=0.0520) bursal indices at 35 days of age compared to pens with adequate methionine levels.

Moreover, a significant relationship is apparent between MG infection and excess methionine treatment (P= 0.0005) in terms of bursal indices and significant relationship (P=0.0516) in terms of thymic indices at 35 days of age.

Bursal indices at 35 days show opposite results of those appearing at 17 days of age in terms of MG-infection. Where, the unchallenged pens have increased their bursal indices compared to the MG- challenged pens. In this context, it is assumed that there may exist a

certain relationship, where MG infection insinuating to be peaking between 17 and 35 days of age. It could also be due to the antibiotic administration period, as broilers during this stage from 18-20 days of age were administered in Pulmotil in water and observed to be performing well yet later bursal indices retrisized and hence the unchallenged groups bursal

In an overall dynamic, 7 days post challenge, the bursal and thymic indices have been significantly triggered by the immune system. By 35 days of age, the bursal indices of MG-challenged groups have decreased, and as above-mentioned commonly the bursa increases then decreases in size. As shown this reduction is consistent and it is not of very high reduction. While within the unchallenged groups there exist no burden of MG infection, on the contrary in the MG challenged groups the significantly different increase of bursal indices at day 17 is thus expected as the lymphatic system is activated and this is followed by a significant decrease of bursal indices at day 35. This illustrates that the MG challenge had induces the begging signs of bursal atrophy, which is emblematic of an infection. Thymus impact on the MG-challenged pens was insignificant at day 35. This is due to the fact that cell-mediated immunity does not accord with the generation of immunoglobulins, and so the thymus is not heavily involved in acting against MG-infection particularly at this late stage. MG-infection proliferating in mucosal surfaces and epithelia requires humoral immunity along the generation and migration of IgA; specifically, secretory IgA. Secretory IgA has crucial effector function on mucous membrane surfaces, guarding the main entry sites for many pathogens. Secretory IgA is polymeric and due to this reason, it has the capability of cross-linking large antigens with multiple epitopes (Goldsby et al., 2002). When secretory IgA binds to either or bacterial and viral surface, the antigens

prohibit the attachment of the pathogen to the mucosal cells, therefore inhibiting infections (Goldsby et al., 2002).

Dimeric IgA attaches via the Fc receptors to the blood section of the epithelial cells. Once, IgA is bound it becomes internalized and passes through the epithelial cells' cytoplasm. MG is a chronic respiratory disease. Hence, treatments that include vaccination are likely not successful in abolishing the infection, because these treatments are inoculated into the blood rather than directly into the respiratory system.

The only immunoglobulin that can cross into the mucosal epithelium is IgA. The passage through mucosal epithelium allows IgA to function in host defense. IgA may combat antigens in three associated mucosal epithelium compartments: the lumen, the epithelium, or in the lamina propria (Lamm, 1997). Moreover, IgA has an excretory capability of antigens from mucosal lamina propria into the lumen in a non-inflammatory approach. This is due to the fact that IgA possess limited burden in exciting inflammatory aspect as distinguished from IgG or IgE. This excretory immune function of IgA functions under normal circumstances and during local infection due to increased synthesis of local IgA during antigenic challenge. If IgA encounters antigen in the lumen, it prohibits it from penetrating into mucosa. While, if IgA recognizes a virus in lining epithelium (during IgA transit from lamina propria to luminal secretions), it can promptly counter the infection. Moreover, IgA can attach to an antigen, within the lamina propria and excret it via the epithelium and into the lumen (Kaetzel, 2007).

It is important to note that while humoral immunity is considered to solely function extracellularly, a crucial exception to this rule lies within IgA transcytosis reaction of intracellular virus neutralization (Kaetzel, 2007)

Why this is valuable? Analytically, designing a treatment for protection against pathogens that infect mucosal membranes whether locally and/or systemic as in MG, it is cogent to consider IgA diverse roles and identify main susceptible antigens and epitopes of MG. It has been shown that IgA can counter infections *in vivo* against internal viral proteins (Burns et al., 1996) Or, in the case of AI, whose internal proteins apt for conserved antigens; IgA may hold heterosubtypic immunity, contributing to augmented protection against newly emerging variants (Takada et al., 2003; Kaetzel, 2007).

The pattern in thymic index endures to be true as insignificant difference reside in thymal indices at day 35 of pulmotil and excess methionine treatments, indicating no impact of further aggregation of T-lymphocytes against MG.

This however does not declare that the thymus is not associated with antibody production. It would be incorrect to assert such statement. Thymus T-lymphocytes play a crucial role in immunoglobulin production (Refer to T-Dependent Antigens versus T-Independent Antigens in 'Lymphatic System Overview' chapter). For the majority of antigens, the production of immunoglobulins by B-lymphocytes is dependent on the incentive of helper T-cells (Th) (Murphy, 2012). Th are responsible for non-specifically or polyclonally activating majority of B-lymphocytes via Toll-like receptors (Mohey et al., 2009; Lesinski et al., 2001; Obukhanych et al., 2006). These are expressed on the surface of B-lymphocytes post BCR stimulation (Murphy, 2012). T-cell dependent antigens (TD antigens) are able to induce memory B-cells. These are produced in the TD germinal centers and can be recognized by somatic mutations in their immunoglobulin loci or either

via surface expression of secondary immunoglobulin isotypes (Mohey et al., 2009; Obukhanych et al., 2006).

It is not simple to observe variation in the size of the thymus; it is rare and particularly during an MG-challenge. This is because the underlying lymphatic response is dealing with a bacterium that does not primarily depend on the cell-mediated immunity but rather induces humoral response.

Results for pens administered pulmotil at 35 days of age had significantly larger bursa indices as compared to pens that were untreated with pulmotil. For a deeper interpretation of the above results, an understanding of the bursa of Fabricius and the thymus development and morphology is important (Refer to Chapter I).

During (**e)**, the antigen-independent phase of bursal ontogeny commences and then terminates shortly post-hatching. In this period, lymphoid cells colonize the epithelial buds, proliferating and influencing an aggregation of bursal follicles (Taylor et al. 2009; Houssaint et al., 1976; Hodges,1974). The rearrangement of immunoglobulin genes is achieved via V(D)J somatic recombination between day 9e to 10e and 16e (Chrząstek et al., 2011; Ratcliffe, 2006; Reynaud et al., 1992). During the second phase (from 2 to 4 weeks of age), B-lymphocyte development is dependent upon antigens that are readily transported from the bursal lumen into the lymphoid region of the bursa (Ratcliffe, 2006). Lymphoid cells from the bursal medulla begin to propagate in the bursal cortex entering the blood to colonize secondary lymphoid organs (Hodges,1974; Reynaud et al., 1992)

Humoral immunity during the first week of life requires the full function of the bursa. Simultaneously, broad-spectrum antibiotics are usually administered against numerous

diseases (Booker et al., 1997; Burton et al., 1996; El-Banna, 1998; Vancutsem et al., 1990; Chrząstek et al., 2011; Ueda and Suenaga, 1995) and often these are provided in coincident with vaccination. Administration of antimicrobials particularly at an early stage of bird's life can cause morphological alterations in the bursa and on its proliferation of B-lymphocytes. In this study pulmotil has demonstrated to have an upregulating effect on B-cell proliferation, in terms of significantly increasing bursal indices at 35 days of age. Antimicrobials that do not produce a downregulating effect on B-lymphocytes are consequently preferable.

It has been suggested that the quantity of B-lymphocyte in cortex of the bursa of Fabricius was significantly higher than when treated with an antimicrobial as compared to a control group. According to Chrząstek et al., 2011 this was an example of enrofloxacin; yet the quantity of B-lymphocytes in the medulla post enrofloxacin administration (23.81 ± 4.11) was significantly lower as compared to the control group (28.40 ± 5.06; $P < 0.01$). On the contrary, Khalifeh et al. (2009) study demonstrated that tilmicosin, florfenicol, or enrofloxacin reduced the humoral immune response while had beneficial influence on the cell-mediated immune response; producing the highest significant levels of chicken interferon γ (**ChIFN-γ**) upon ConA stimulation ($P < 0.05$).

Chin et al. (2000) discovered that tilmicosin was concentrated in phagocytes; including monocyte-macrophages and heterophils; due to its lipophilic properties and that the uptake of tilmicosin caused an elevated lysosomal enzyme activity, suggesting a complex interaction between phagocytic cells and tilmicosin.

Equivalently in regard to the bursal indices, the excess methionine treatment had significantly increased the bursal indices as compared to pens with adequate levels of methionine. Both treatments (pulmotil and excess methionine) have succeeded in maintaining significantly larger bursal indices which is indicative of the preservation of the humoral immunity via conserving the bursa and its morphology. Peculiarly, both treatments demonstrated effects at later stage; a critical period where bursal atrophy commonly commences. Striking a treatment that has the ability to decrease the shrinking and the restitution of a lymphoid organ at such phase in addition to undergoing contemporary infection is of a favourable outcome. Besides, the outlined significant interaction between MG and methionine (MG x MET) that was time-lapsed to 25 days post challenge such interaction was seen for both the bursal and thymic indices; proving for a successful intervention. Post the repeating stimulation by antigen, B cells can generate immunoglobulin that attach their antigen with much greater affinity. Here, the induction of antigen greatly increases the antibody defense (Goldsby et al, 2002; Albert et al, 2002; Tusculum College; 2016; Ramadan et al., 2017). This was the case of the excess methionine effect on bursal indices at 35 days of age on *Mycoplasma galliseptium* challenged birds; indicating an apparent affinity maturation of B-cells with MG antigens.

4.13 Cost-Analysis of Excess Methionine versus Adequate Methionine

Cost analysis was performed to differentiate between the cost of excess methionine from the adequate methionine provided in diets of Ross 308 male broilers. Table 4.16 highlights the outcomes of the cost analysis of this study and projects the cost of adequate and excess

methionine for commercial settings. Cost analysis in US dollars was calculated based on cumulative average body weight of broilers at 35 days of age and the incorporated quantity of methionine per 100 kg in diet. The highest level of incorporated Methionine into the feed, which was in starter diet; was used to calculate the cost.

Table 4.16. Cost analysis of Excess Methionine versus Adequate Methionine for Ross 308 male broilers.

Methionine Level	***Bird***	***1000 birds***	***10,000 birds***
	$	$	$
Excess	1.709	1709	17090
Adequate	1.729	1729	17290

According to the cost analysis demonstrated in Table 4.16; 20% excess methionine would save $200 US dollars at a commercial setting of 10, 000 birds. This is because broilers provided excess methionine (although was not significantly different from broilers provided adequate methionine levels as illustrated in Table 4.7 of statistics regarding performance parameters) had lower CFC and average BW at 34 days of age.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

The experimental work comprised 1200-day-old male Ross 308 broilers randomly divided into 8 treatments with 3 replicates (pen) of 50 birds each. A 2x2x2 factorial arrangement of treatments with interaction in a complete randomized design was used. The main factors were *Mycoplasma gallisepticum* a) challenged vs. b) unchallenged; Methionine: a) adequate vs. b) excess, and Antibiotic a) Pulmotil *AC®* treated vs. b) Non-treated. Levels of DL-Methionine to meet 100 and 120% of methionine requirements were supplemented in the adequate and excess methionine groups, respectively. Results from increasing DL-Methionine levels demonstrated significant increase in average body weight (BW) gain in broilers at 10 days of age compared to those provided adequate methionine levels, whereas Pulmotil AC significantly reduced BW at the same age. No significant differences in BW and feed conversion ratio (FCR) at 17 and 35 days of age were observed for either the excess methionine or Pulmotil AC treatment.

ELISA titer was significantly the greatest (P=0.0035) in the MG-challenged group fed 120% of methionine requirement. Significant increase in IgG titers of 3170 against MG-infection in the excess Met treatment were detected along a coefficient of variation (CV) value of 14.84% compared to MG-challenged broilers provided adequate Met level with IgG titers of 1843 and CV of 66.38%.

Excess Met significantly increased bursal indices (0.154) in MG-challenged broilers at 35 days of age compared to adequate levels of Met (0.136) along with a significant apparent interaction between Met and MG (P =0.0005). No significant differences were obtained in

MG DNA load in tracheal and airsac tissues between the excess and adequate levels of Met; both groups performed similarly. Treatment of 20% excess Met augmented hematological parameters with a significant increase (P <0.05) of hematocrit (HCT) % compared to adequate Met level; while, Pulmotil AC (tilmicosin antimicrobial treatment) significantly decreased (P<0.01) HCT % compared to broilers untreated with antibiotics at 35 days of age. Significant reduction in the percentage of birds with severe tracheatis due to MG-infection from 40 to 10% at 17 to 35 days of age, respectively in the group fed 120% of methionine requirement.

Results demonstrated increased humoral immunity, alleviation of severe tracheatis by day 35 of age; generation of homogenized response, and significantly higher antibody titers.

In conclusion, the 20% excess supplementation of methionine has proven a successful immune enhancer and it has portrayed a vaccine performance in MG-infected broilers.

It is recommended to incorporate 20% excess DL-Methionine into broiler diets for its immunopotentiating effect and to provide immunological intervention for *Mycoplasma gallisepticum* infection in broilers.

Understanding the underlying mechanisms and metabolism of methionine and the process by which it alleges an impact of the lymphatic system is essential in the complex interactions between nutrition and disease. Specifically, the relationship between methionine and *Mycoplasma gallisepticum*. If fortunate to take this relationship into deeper experimental trial, it is believed that a definite profound comprehension of the intricate connection between these two elements exist.

The treatment or resistance against an infection necessitates an augmented response congregated by the lymphatic system. From nutritional point of view, amino acids are

required to stimulate an immune response including, but not limited to, lymphocytes proliferation, germinal centers enactment in the bursa of Fabricius, immunoglobulins affinity, synthesis of effector B-lymphocytes, nitric oxide, lysozyme, complement in addition to cytokines and eicosanoids (Rubin et al., 2007; Montassier, 2000; Jeevanandam *et al*. 1990; Kidd, 2004; Shini *et al*., 2005; Rubin et al., 2007b). Several reasons were emphasized in this thesis for choosing methionine as the amino acid of choice in evaluating its effect on *Mycoplasma gallisepticum*. However, additional research is also recommended to elucidate the precise gene and factor involvement in the relationship between methionine and *Mycoplasma gallisepticum*. Phenotypic variation of MG surface antigens and its spontaneous phase frequency is a plausible reasoning for the chronic respiratory infection caused by MG despite a strong lymphatic response. It is recommended to study the mechanisms of the key virulence factors of MG and/or the specific MG cytadherence genes with the impact of methionine metabolism; more specifically the methylation function; and evaluating if any possible interactions exist.

Moreover, as MG predisposes birds to secondary infections such as *E. coli* resulting in high mortality rates under field conditions, it would be appropriate to design a specific study in order to test the efficacy of 20% Met on concurrent infections.

In MG vaccines, triggering cellular immune responses in tracheal mucosa is dependent upon natural killer cells (NK) and T-cytolytic-cell responses (Gaunson et al., 2006). In immunocompromised birds, interferon gamma (**IFNγ**) is ineffectively generated in response to an MG vaccine. Thereby inactivated macrophages fail in MG phagocytosis (Muneta et al., 2008). CD8+ that are activated in response to IFNγ may inactivate the intracellular MG in macrophages or other cells (Gobel, 1996) and MG cells may be

abolished via induced NK cells through ADCC (Gondal et al., 2013). IgG can then attach NK cells though its Fc fragment and MG cell via Fab fragment. In free form, NK cells may eradicate MG cells through perforins release (Boyaka and McGhee, 2001; Hilton et al., 2002). It is important to note that albeit the indispensable function of cell-mediated immunity, the intervention on MG ought to heavily rely on humoral immunity and more specifically on IgA production. As previously emphasized, secretory dimeric IgA is equipped with a J-chain and secretory protein with the capability of crossing into the mucosal epithelium demonstrating crucial effector function on mucosal membrane surfaces guarding the main entry sites for many pathogens. MG is a chronic respiratory disease and vaccinations may not perform successfully as they are inoculated into the blood rather than having a direct effect onto the respiratory system. It is recommended to study immunoglobulin A role on *Mycoplasma gallisepticum* infection in poultry, and one methodology is to simply test for IgA preferably in mucosal surfaces or to test for serum IgA through ELISA. Finally, it is recommended to evaluate or design a treatment that has the ability to pass through mucosal epithelium encountering directly with MG.

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