AMERICAN UNIVERSITY OF BEIRUT

DOES METHIONINE ENHANCE IMMUNITY IN *MYCOPLASMA GALLISPETICUM* F STRAIN VACCINATED BROILER BREEDER PULLETS?

by
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AN ABSTRACT OF THE THESIS OF

Walid Ghazi Al Hakeem for Master of Science
Major: Poultry Science

Title: Does Methionine Enhance Immunity in *Mycoplasma gallispeticum* F strain Vaccinated Broiler Breeder Pullets?

This experiment was conducted to assess a protocol combining 20% excess dietary methionine above the modern breeder pullet's requirement and AviPro® MGF vaccine in controlling *Mycoplasma gallispeticum* infection. A total of 276 six-week old breeder pullets, of the Ross 308 strain were randomly allocated into four treatments, each with three pen replicates of 23 birds/pen. Pullets were given water *ad libitum* and feed as per the Breeder Manual recommendation provided by the breeding company. The trial was designed in a completely randomized design. The treatments were: 1) Vaccinated with excess methionine (VEM), 2) Vaccinated with adequate methionine (VAM), 3) non-vaccinated with excess methionine (NVEM), 4) non-vaccinated with adequate methionine (NVAM). Birds were assigned to different treatments, including vaccination via drinking water with AviPro® MGF at 6 weeks of age, and provision of dietary methionine as 100% and 120% of Met requirements in the adequate and excess groups, respectively. Results observed in this experiment indicate a slow rate of growth and colonization of MGF vaccine strain at the level of trachea. A delayed increase in colony forming unit that extended up to 6 weeks post-vaccination offers limited competitive exclusion of the F-strain against other *Mycoplasma gallisepticum* infections for a considerable period. Furthermore, the addition of 20% excess methionine above the recommended levels hindered the tracheal colonization rate and log10 values of AviPro® MGF vaccine colony forming units from 5.9 in VAM group to 0.82 / ml of tracheal swab suspension in VEM group. These results highlight the ability of excess methionine in enhancing innate structural immune response. Measured sera titer using an Elisa kit showed that eight weeks post-vaccination were required by the vaccine to stimulate the humoral immunity response against MG. The 20% excess methionine numerically increased the IgG titer against MG in comparison to other groups, yet this increase was not enough to generate protective titer count. The 20% excess methionine above the modern breeder requirements proved to have a key role in enhancing adaptive and acquired immunity.

Keywords: AviPro Vaccine, Immunity, Methionine, *Mycoplasma gallispeticum*, Pullets.

CONTENTS

FIGURES

Figure Page

TABLES

ABBREVIATIONS

AA: Amino Acid

AAFACO: Association of American Feed Controls Official

CRD: Chronic respiratory disease

CP: Crude Protein

CFR: Code of Federal Regulations

DL: racemate

DNA: Deoxyribonucleic acid

E.coli: Escherichia coli

EDTA: Ethylenediamine tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EM: Electron microscopy

FAO: Food and Agriculture Organization

F-strain: Mycoplasma gallispeticum F-strain

FC: Feed Conversion

FCR: Feed Conversion Ratio

FDA: Food and Drug Administration

FI: Feed Intake

F-strain: Mycoplasma gallispeticum F-strain

g: Grams

GALT: Gut-associated lymphoid tissue

GapA: Cytadhesion gene

GRA: Granulocytes

HCT: Hematocrit

HI: Hemagglutinin inhibition test

HGB: Hemoglobin

IB: Infectious bronchitis

IBDV: Infectious bursal disease virus

IFNy: Interferon gamma

IgA: Immunoglobulin A

IgD: Immunoglobulin D

IgG: Immunoglobulin G

IL: Interleukin

IP: Ideal Protein

Kg: Kilogram

LPS: Lipopolysaccharide

LAMP: lipid-associated membrane protection

Log: Logarithm

LYM: Lymphocytes

m: Meters

ME: Metabolic Energy

Met: Methionine

MCH: Mean capsular hemoglobin

MCHC: Mean capsular hemoglobin concentration

MCV: Mean corpuscular volume

M.gallisepticum : Mycoplasma gallispeticum

MG: Mycoplasma gallisepticum

µl: Microliter

MON: Monocytes

NRC: National Research Council

NPIP: National poultry improvement plan

P: Probability value

PCR: Polymerase chain reaction

pH: potential hydrogen

pVPA: Phase variable putative adhesion protein A

q-PCR: Real-time polymerase chain reaction

RAPD: Random amplification of polymorphic DNA

RBC: Red blood cells

R-strain: Mycoplasma gallisepticum R-strain

RDW: Red blood cell distribution width WBC: White blood cells

RFLP: Restriction fragment length polymorphism analysis

ROS: Reactive oxygen species

rRNA: ribosomal ribonucleic acids

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: Standard error of mean

SOD: Super dioxide dismutase

SPA: Serum Plate Agglutination

T-cell: T-lymphocytes

Th: T-helper cells

Ts-11: Mycoplasma gallisepticum Ts-11 strain

%: percentage

6/85 strain: Mycoplasma gallisepticum 6/85 strain

CHAPTER 1

INTRODUCTION

Mycoplasma is the smallest self-replicating prokaryote with a relatively small genome and a complete absence of cell wall (Razin S. et al., 1998). *Mycoplasma gallisepticum* (MG) is the main causative agent for Chronic Respiratory Disease (CRD) in chicken and infectious sinusitis in turkey (S. H. Kleven, 1998). It can infect wide variety of avian species such as pheasant, finches and pigeons. The transmission of the disease occurs in ovo when MG spreads from air sacs into the oviduct and therefore infecting the progeny (Roberts & H.McDaniel, 1967); Moreover, the disease is spread horizontally between susceptible birds. Respiratory rales, nasal discharge, coughing , conjunctivitis, and commonly infraorbital sinusitis in turkeys are signs of mycoplasma disease (Majumder, 2014).MG ability to express diverse surface proteins allows it to escape the immune system and initiate a long chronic disease (Razin S. et al., 1998).Typically, *Mycoplasma gallisepticum* is coupled with other infections to form a multi-factorial disease complex. Several reports described the interaction of MG with respiratory viruses such as ND and IB and E.coli. The clinical manifestation of MG is exacerbated in the presence of several co-infections which leads to higher mortality and morbidity (S. H. Kleven, 1998).

In laying hens, the commonly highest loss production occurs when the flock is infected at the peak lay (Glisson et al., 1984).*Mycoplasma gallisepticum* has been associated with the inflammation of the fallopian tubes and reproductive system (T. Nunoya et al., 1997). As a result

of infection embryo mortality occurs after the embryo is usually weakened leading to a struggle in hatching (pipped embryo) or low-quality chick (Levisohn et al., 1985).

Poultry sector worldwide sustain annually significant losses due to the presence of *M. gallisepticum* infection in meat-type chicken and in commercial layers, these losses occur even in the absence of clinical signs. Downgrade of carcass (S. H. Kleven, 1998), reduced feed consumption efficiency and death in meat-type chicken, in addition to reduced egg production (Glisson et al., 1984), embryo mortality and low-quality chicks in commercial broiler are factors that shape MG as the costliest disease facing poultry producers worldwide (Ley et al., 2008). Maintaining MG-free commercial breeding stock, in addition to eradication, wide biosecurity and extensive surveillance are part of National Poultry Improvement Plan adopted by the poultry sector in the United States. However, with the dramatic increase in production in many parts of the world an MG outbreak persists and remains an economic threat to the poultry sector.

Alternatives to chemical drugs for control of MG include, but not limited to, vaccination and nutrition. In nutrition, amino acids provide a promising approach to control *Mycoplasma gallispeticum* in chicken. Methionine is an essential amino acid that has recognized immune regulatory function (Shini et al., 2011). These functions that are associated directly and indirectly to the immune response include: Vital component of protein synthesis glutathione precursor that lower reactive oxygen species therefore defending the cells from oxidative stress, and it is essential for the synthesis of spermidine and spermine, that play a key role in nucleus and cell division (Mehrdad Bouyeh, 2012a). Furthermore, methionine is a key metal donor for methylation reactions of the DNA and several different molecules. Methionine is believed to mediate the proliferation of the cellular and humoral immune response (Mehrdad Bouyeh,

2012a). It is also responsible for the increased phagocytotic ability of leukocytes (Elmada et al., 2016), detoxification (Kim et al., 2006), resistance to the coccoidal infections (Rao et al., 2003) and stimulates serum lysozyme activity (Chen et al., 2011).

Typically, the NRC requirements (1994) of methionine for broilers are 0.5% for starters (0-3 weeks), 0.38% for grower (3-6 weeks) and 0.32% for the finisher phase (6-8weeks) in order to obtain the expected optimal growth. However, several reports suggest the need for higher levels of methionine to improve overall performance and stimulate an immune response in different animals. Methionine involvement in elevating the immune response is not well documented and described in literature. The 20% excess supplementation of dietary methionine has proven a successful enhancer, and it has portrayed a vaccine performance in MG-infected broilers (Ramadan et al., 2019).

This study combines the use of excess methionine in the diet, and a live MG vaccine of the F strain in order to establish a protocol for the control of MG in breeder pullets. Accordingly, the aim of this study is to assess and investigate the immunopotentiating role of excess dietary methionine by 20% above the recommended level on *Mycoplasma gallispeticum* challenged birds. This assessment will be based on the measurement of bird's performance, vaccine colonization pattern, hematological parameters and immunological response criteria.

CHAPTER 2

LITERATURE REVIEW

2.1 Mycoplasma Gallisepticum Infection:

Mycoplasma gallisepticum is the causative agent of chronic respiratory disease in chicken and infectious sinusitis of turkeys (Ley et al., 2008). Respiratory rales, nasal discharge, coughing, conjunctivitis, and commonly infraorbital sinusitis in turkeys are signs of *Mycoplasma gallisepticum* manifestation (Majumder, 2014).Symptoms and signs of are slow to develop and usually the *M.gallisepticum* infections develop into a long-lasting disease. Furthermore, *M. gallispeticum* or *M. syoviae* infection when combined with a respiratory virus infection (IB or Newcastle) or Escherichia coli results in serve airsacculitis commonly known as Air sac disease.

2.1.1 Economic significance:

Mycoplasma gallisepticum high pathogenicity causes significant economic losses in the poultry sector in the absence of clinical signs (S. Levisohn & Kleven, 2000). Airsacculitis resulting from MG infections and other complications of avian pathogens, results in carcass quality deterioration, reduced feed and egg production and increased medication, vaccination and control programs. As a result, MG infections are one of the costliest infectious diseases threating the poultry sector worldwide (Ley et al., 2008).

2.1.2 Etiology:

2.1.2.1 Classification:

Mycoplasma gallisepticum is host-specific highly pathogenic specie that belongs to class mollicutes within the genus *Mycoplasma* of family Mycoplasmataceae. It is the smallest self-replicating prokaryotes that can be cultivated on a cell-free media (Razin, 1992). *Mycoplasma gallisepticum* are eubacteria that lacks cell wall, have relatively small genome and minimal genetic information that accounts for its complex nutritional requirements (Semashko et al., 2017). Using serotyping, Mycoplasma gallisepticum was first distinguished from other avian mycoplasmas (Yamamoto et al., 1958). At first it was referred to as serotype A (Yoder et al., 1964) which later was changed in 1960 by Edward and Kanarek (Edward & Kanarek, 1960) into Mycoplasma Gallisepticum. In 1993, Molecular techniques helped in differentiating mycoplasma that share phenotypic and antigenic similarities with MG and found that *M.imitans* is quietly related (Harasawa et al., 2004).

The application of molecular tools such as DNA hybridization (Brown et al., 2007), DNA sequence analysis of the 16S rRNA gene (Muto et al., 1992), 16S rRNA PCR and denaturing gradient gel electrophoresis (McAuliffe et al., 2005), and tRNA gene PCR (Stakenborg et al., 2005) aided in the re-examination of mycoplasma phylogeny and taxonomy. Furthermore, a comparative genomic approach was available after determining the whole genome sequence for MG strains: R-low (Papazisi et al., 2003) R-high, and F (Szczepanek et al., 2010).

2.1.2.2 Susceptibility to chemical and physical agents:

Phenol, formalin, β-propiolactone, and thimerosal are chemical disinfectants that are believed to be effective in deactivating *M. gallisepticum.* On the other hand, *M. gallisepticum* is resistant to penicillin and low concentration (1:4000) of thallous acetate which are added into the MG media to eliminate any bacterial or fungal contamination. These additives provide a valuable selectivity for Frey's broth.

Typically, MG cultures stay viable for 2-4 years in broth media when kept at -30C, also MG remained viable in a lyophilized broth culture when kept at 4 C for 7 years. Moreover, MG was recovered from lyophilized infective chicken turbinate that was kept for 14 years at 4C (Yoder et al., 1964). In 1965, MG isolates were cultured in a broth media and stored at -60C, 20 years later they were found viable upon sub culturing. After 10-15 years in storage, lyophilized broth cultures of MG, *M. synoviae* (MS), and *M. meleagridis* (MM) were commonly found viable when sub-cultured. Yet, the viability of MG isolates in liquids can be assumed by relying on the strain, medium or diluent and temperature. A solution made up of skim milk, phosphate-buffered saline (PBS), tryptose phosphate broth, and distilled water can be used to store M. gallisepticum F strain for 24 hours at 4-22 ºC, however, it cannot be used at 37ºC (S. Kleven, 1985a). An after extreme freezing effect was detected in an MG inoculum stock in which a decrease of titers of 10^3 in 24 hours at 4ºC and 10^5 at room temperature. Moreover, in a 12-14-hour heating experiment that reached 45.6ºC, the inactivation of MG in an infected chicken hatching eggs was illustrated (Yoder Jr & W, 1970). In the last study done in 1983 by Rotten, two different layers were found to exist in the MG membrane. One of those layers constitutes the intra-membrane found in a liquid state and composed of cholesterol-rich lipid, whereas the other

layer is made of cholesterol-poor phospholipid which is responsible for the order-disorder transition.

2.1.2.3 Antigenic Structure and Toxins:

Antigenic variations presented on the mycoplasma surface is encoded by the minimal genetic information located in the Mycoplasma's genome (Ose, 1979; Stanley, 2000). The ability to possess large antigenic variation is a key survival factor that allows *M. gallisepticum* to escape the immune system, invade and persist in the host cells (Noormohammadi, 2007; Purswell et al., 2011).The identification of the organisms is based on determining the antigenic attribute (through growth inhibition and immunofluorescent test) and species-specific polyclonal antibody response to organisms (through ELISA, HI, SPA) (Ley et al., 2008). Two-third of Mycoplasma's membrane mass constitute of protein and the other third is made up of lipids (Razin S. et al., 1998). The plasma membrane of MG comprises about 200 polypeptides (Jan et al., 1996) that is linked with surface antigenic variation, nutrient transport and mycoplasma motility and cell adhesion (Miyata, 2005).

Significant time and resources were spent to determine the MG antigens specifically those associated with adhesion or hemagglutinin properties that may be directly responsible for MG pathogenicity and immunogenicity. Adhesions are essential membrane protein with areas exposed on the cell surface. These visible areas adhere to receptor sites on epithelial cells to initiate invasion and colonization, and such regions are observed as a key virulence factors and antigens (Ley et al., 2008).

According to different studies, Immunodominant adhesion or hemagglutinins of the *M. gallisepticum* are proteins or lipoproteins with molecular weights ranging from 60-75KDA (Barbour et al., 1989; Markham et al., 1992). Originally, MG main surface proteins that shape its pathogenicity and immune evasion abilities are coded by 2 main MG genes: pMGA: hemagglutinin protein A and pvpA: phase variable putative adhesion protein A (Markham et al., 1992). Typically, an inimitable homologous pMGA gene is expressed by each MG strain (Glew et al., 2000) which is expressed as various surface lipoproteins (Markham et al., 1992). However, and according to several reports that highlighted the fact that several main cell surface lipoprotein hemagglutinins are the result of pMGA (p67) multigene expression (Jan et al., 1996; Markham et al., 1993). Surface antigens p67 (pMGA) and p52 were explicitly linked to MG and nearly related to *M. imitans* using immunoblotting techniques. The use of anti-p52 serum didn't reveal any antigenic variations, however, this antigenic difference was established using anit-p67 serum (Jan et al., 2001). Approximately, the pMGA gen family constitutes 16% of the R strain genome and 7.7% of the F strain genome (Baseggio et al., 1996). These significant genomic percentages accounts for antigenic differences and predicted function of immune evasion (Markham et al., 1993). The pMGA gene undergoes antigenic switching: an ability mediated by trinucleotide repeat length variations, that allows MG to express surface proteins based on the environmental surroundings and threat of antibodies (Glew et al., 2000). A promoter: (GAA) 12motif'5 to pMGA1.1 mediates the changes in the length of a unique trinucleotide GAA repeat, which is responsible for the on-off switching of the pMGA gene (Glew et al., 2000). The instability of the GAA repeat in MG results different pMGA gene expressions (Markham et al., 1998).

The MG pvpA gene, found as a one chromosomal copy, is translated into an expected cytahesin-linked protein found on the cell surface (Boguslavsky et al., 2000). Different size deletion in its C-terminus, several proline codons, 2 nonstop repeated sequences accounts for the high-frequency phase and further antigenic variation in MG (Boguslavsky et al., 2000; Jiang et al., 2009).The correlation between antibody response and the antigenic variations of PvpA and p67a (VlhA), indicate that immune modulation might have a major role in producing surface diversity (Levisohn et al., 1995). Additionally, several adhesins were found in MG such as GapA (Mgc1) and Mgc2 (Goh et al., 1998). These proteins are like PvpA, in which they are found linked to organelle structure at the surface level. Coordination between GapA: primary cytahesin and CrmA: cytadherence-linked protein mediates the associated phase variation in expression (Goh et al., 1998; Papazisi et al., 2003). Furthermore, it has been established that expression of these 2 cytadhesins is linked with attachment to erythrocytes (F. Winner et al., 2003). Therefore, these studies verified that both GapA and CrmA are vital for MG cytadherence and pathogenesis (Papazisi et al., 2002).

A few number of MG cytadhesin genes and proteins include a homologue in other *Mycoplasma spp.* some of those spp are human pathogens. This indicates that between pathogenic mycoplasmas infecting widely different hosts some conservation of cytadhesion genes and proteins may occur (Goh et al., 1998; May et al., 2006).

Finally, potent toxins have not been linked to MG. (further information will be discussed in virulence factor part).

2.1.3 Strain classification:

Some of *M. gallisepticum* isolates are known either by their isolate designations or occasionally are termed strains. Low pathogenicity and transmissibility of certain field isolates and the low rate of isolation and recovery success are factors that resulted in naming some Mg isolates as “variant” or “ atypical” (Yoder Jr & W, 1986). Also, MG well-known reference strains are established based on antigenic phenotype variability which may differ evidently based on their virulence-linked surface properties and antigen profile (Rosengarten & Yogev, 1996). Hence, sensitive methods to describe and recognize MG strains variability became highly important. Reproducible and discriminatory molecular (genotypic) methods in addition to serologic techniques (phenotypic) and electrophoretic analysis of the cell’s protein helped to recognize the intraspecific genotypic and phenotypic heterogeneity (Grodio et al., 2008).

2.1.3.1 Antigenicity:

Experimentally, antigenic variations of MG strains and isolates has been identified as atypical or variant strains (Kempf et al., 1997)and confirmed by serologic assays (Markham et al., 1992), immunoblots and monoclonal antibodies (Rosengarten et al., 1995).

Specificity and sensitivity of serological tests can be considerably influenced by the antigenic variability among MG strains. Kleven (S. H. Kleven, 1998) examined MG strains with homologous and heterologous hemagglutination-inhibition (HI) assays and reported that homologous HI titers were typically higher than heterologous titers. Likewise, experiments performed to detect the antibody response to vaccination using MG strain ts-11 stated that the main membrane antigen of MG had slightly different antigenic profiles in different strains. As a

result, it is required to use homologous (autologous) antigens in serodiagnositc assays in order to improve the sensitivity of mycoplasma's antibodies tests (Noormohammadi et al., 2002). Development and optimization of antigen-antibody based tests is necessity to face the challenges presented by antigenic variability. .

In conclusion, the organism's genomic mechanisms of immune evasion and adaptation to host environment fluctuation through switching, immune modulation and expression of different antigenic surface protein accounts partially for MG strain antigenicity and its variability (Glew et al., 2000; F. Winner et al., 2003).

2.1.3.2 Immunogenicity and protective characteristics:

With their protective characteristics, and low to mild immunogenicity, three known MG strains (F, ts-11, 6/85) have been used for live vaccines development (ref vaccines). House finch and house finch-like strains immunogenic protective characteristics have been reported suggesting these strains as vaccine candidates (Ferguson et al., 2003). Also, Gt5 and M7 strains which includes major cytadhesion GapA (Papazisi et al., 2002) and dihydrolipoamide dehydrogenase gene respectively have been also reported as vaccine candidates (Gates et al., 2008).

2.1.3.3 Molecular Genetic:

Direct comparison of the protein binding patterns generated by the application of SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and RFLP (restriction fragment length polymorphism) aided in the differentiation of MG strains from on another (S. H. Kleven, 2008). MG-intraspecific differences are illustrated by DNA and ribosomal RNA gene

probes (Khan et al., 1987)and physical chromosomal mapping (Tigges & Minion, 1994). However, these methods are known to be expensive, complicated, and tedious.

Several DNA fingerprinting test such as arbitrary primed PCR (AP-PCR) or random amplification of polymorphic DNA (RAPD) proved their efficiency in identification of different MG strains, epidemiological studies, and in distinguishing of vaccine strains and collected field isolates (Barbour et al., 2005; Cherry et al., 2006). Yet, the interpretation of RAPD banding patterns has been difficult and subjective in which these patterns are liable to variability and are hard to reproduce and standardize. Genomic variability of different Mycoplasma species including MG have been discovered using amplified fragment length polymorphism (AFLP) (Cherry et al., 2006).Furthermore, MG strain molecular differentiation was sought using pulse-field gel electrophoresis (PFGE) however, it was reviewed as expensive, arduous, and dull (Marois et al., 2002). The application of DNA fingerprinting methods requires the availability of pure cultures, a prerequisite which may be hard to fulfill in field situations. To alleviate this situation, a method was proposed to isolate the fast growing nonpathogenic avian Mycoplasmosis from the slower-growing *Mycoplasma gallisepticum* field strains (Boettger et al., 2006).

Recently, MG strain identification was done using pvpA, mgc2, gapA, crmA, crmB, and crmC gene PCRs, succeeded by RFLP(PCR-RFLP) of the amplicon (Lysnyansky et al., 2005). Nevertheless, in research and in case of outbreak investigation targeted sequencing of single or multiple genomic loci mgc2, pvpA, gapA, MGA_0319—and 16/S-23S rDNA ISR became the method of choice in MG strain differentiation (Jiang et al., 2009).

2.1.3.4 Pathogenicity:

Method of propagation, dosage, challenge, number of passages for their preservation, and their genotypic and phenotypic features are factors that determine the relative virulence of different MG strains and isolates. Commonly, yolk-passaged organisms obtained from MG-inoculated embryonating chicken eggs were thought to be more infective than the broth-passaged organisms. Highly pathogenic neurotropic S6 strain of Zander that can cause encephalitis was isolated from the brain of a turkey diagnosed with infectious sinusitis (Zander, 1961). A pathogenic culture was isolated by Van Roekel which was later named by Jungerr as A5969 strain (Jungherr et al., 1955). In poultry diagnostic research center located in the University of Georgia Dale Richey in 1963 was able to isolate the R strain from a chicken suffering from airsacculitis. Later, in MG challenge experiments the R strain was utilized as a bacterin or a virulent strain (Keeler et al., 1996; S. H. Kleven, 1998). Right after the complete genome sequence of the R (low) strain, the pathogenicity (genotypically and phenotypically) of the low R strain and the high R strain have been rigorously studied (Papazisi, 2003). In a comparison approach, between Rlow and Rhigh the latter showed reduced abilities in cytadherence, cell penetration and pathogenicity (May et al., 2006; Papazisi et al., 2002). R low was successfully re-isolated from a bird inoculated with MG while the attempt to isolate Rhigh failed (Much et al., 2002). Therefore, Much stated in his report that MG R strains differ in their ability and capacity to cross the mucosal layer, and that systemic spread of the MG is dependent upon the MG strain capability in cell invasion (Much et al., 2002).

Several reports (Lin et al., 1982; Rodriguez & Kleven, 1980)declared that F strain was more virulent in turkeys than commonly viewed in chicken. Furthermore, other reports (S. H.

Kleven, 1998; Whithear et al., 1990; Whithear et al., 1990)indicated that 6/85 and Ts-11 live vaccines are less virulent than F strain to turkey and chicken.

House finch and house-like finch strains of MG with their variable ability to induce a disease, to initiate antibody response and to be able to survive and to persist in their novel host (Grodio et al., 2012) are factors that led to consider these strains as low (Ferguson et al., 2004; Ferguson et al., 2003).

2.1.3.5 Virulence factors:

The Mycoplasmal pathogenicity at a molecular level remained indefinable (Razin S. et al., 1998). In addition, clinical image of Mycoplasmal manifestation suggests that the damage occurs as a result of the host immune and inflammatory response rather than a toxic effect related to the pathogen cell components (Razin S. et al., 1998). The ability to alter the immunogenicity of the surface protein is a key factor associated with MG pathogenicity and its ability to pass undetected by the immune system and to adapt to host environment (Dusan Bencina, 2002; Markham et al., 1998). Furthermore, mycoplasma's gliding motility and cytadhesin protein are vital virulence factors in cell host's infection (Chen et al., 2011; May et al., 2006), as well as its ability to penetrate and invade the cells (Much et al., 2002; Vogl et al., 2008).

Moreover, in an experiment, the expression of lipoprotein A (MsIA) was lower in the attenuated F vaccine and in R low vaccine, signifying that lipoprotein A is factor in MG virulence (Szczepanek et al., 2010).

Hydro-peroxide resistance for MG in host environment is a vital virulence factor granted by OsmC-like adhesion protein (Jenkins et al., 2008). Furthermore, CysP (cysteine protein) ability to digest IgG chicken antibodies, aids in MG protection and prolong its presence in host's cell despite the presence of antibody response (Cizelj et al., 2011).

With the complete genome sequencing of MG strain R low by Papazisi (source), a potential virulence factors were identified related to binding , cytadherence and heat shock proteins (Papazisi et al., 2003). Dihydrolipoamide dehydrogenase related gene was recognized using signature sequence mutagenesis (Hudson et al., 2006). Factors related to pathogenesis and ability to survive in the host harsh environment were identified using comparative genomic evaluation between Rlow, attenuated Rhigh, and F MG strains (Szczepanek et al., 2010). This method discovered that mutations occur in several genes, and that a variety of proteins are included in virulence. Aside from cytadherence related roles, which are key factor in MG virulence, different original virulence factors that includes glycerol metabolism associated with H2O2 production, sutilases and haloacide dehalogenase were defined. Few mutual genes between MG strain (F,ts-11 and 6/85) were found missing using comparative genomic hybridization, indicates that no sole gene is accountable for their attenuation (Szczepanek et al., 2010).

2.1.4 Pathobiology and Epidemiology:

2.1.4.1 Incidence and Distribution:

Worldwide M. gallisepticum infection is considered a significant flock health concern in chicken and turkeys' commercial production (S. Levisohn & Kleven, 2000). Control programs such as National Poultry Improvement plan (NPIP) aided in reducing the MG outbreak incidence in the United States in the last 50 years. Although, the extensive NPIP has proven to be effective in controlling MG infections, outbreaks of MG in meat type chicken and commercial egg layers continue to occur. Different indication suggests that small backyard and free-range poultry may be sub-clinically infected and act as a foundation of commercial flock infection (Thekisoe et al., 2003)

MG was recognized as the main cause of conjunctivitis affecting mainly chicken and turkey. Later the infection list expanded to amazon parrots , songs birds, ducks , Japanese quail and geese (Duckworth et al., 2003).

2.1.4.2 Natural and Experimental Hosts:

M. gallisepticum infection happens mainly in gallinaceous birds specifically chicken and turkey. Nevertheless, several reports described the isolation of naturally occurring MG from bobwhite, pheasants, grey partridge, Japanese quail, peafowl and quail (Benčina et al., 2003; Vitula et al., 2011). Further, M. gallisepticum has been found in ducks and geese (Jordan & Amin, 1980), flamingos (El-Shater, 1996) and from the amazon parrot (Bozeman et al., 1984). The common occurrence of M. gallopavnios (Cobb et al., 1992) in wild turkeys is a key factor that is responsible for the rare isolation of MG from wild turkeys. However, Davidson et al

(Davidson et al., 1982) reported the isolation of MG in wilds turkeys that were found in confinements and not in their natural habitat. Eight years later, another report claimed the absence of MG in the same population tested by Davidson, suggesting that MG didn't survive and spread in the wild turkey flock (Luttrell et al., 1991). Other reports and surveys done claimed the presence of seropositive (Cobb et al., 1992) and sero-negative (Luttrell et al., 1991) wild turkeys. Using serum plate agglutination test low spread of MG was recorded antibodies in lesser prairie chicken found in southwestern Kansas.

Before the year 1994, several reports failed to establish a significant occurrence of MG infection in free ranging birds. Likewise, efforts to understand the pathogenesis of MG failed and the image was not very conclusive. However, in the year 1994, a successful isolation of MG from a free-ranging house finch suffering from conjunctivitis and ocular swelling, and MG was revealed as the etiology (Sydenstricker et al., 2006). Rapidly, the disease became prevalent between finches located on the eastern range and began to lower their population (Nolan et al., 2004). Almost 10 years later, the disease was spread into the house finches located in the western side (Duckworth et al., 2003). M. gallisepticum conjunctivitis has been isolated at a lower rate from American goldfinches, grosbeaks and pine grosbeaks, and a sole confirmed isolate was described from a purple finch and blue jay (Mikaelian et al., 2001).

Later studies concerning the disease susceptibility and contagiousness of wild birds' species found that after the inoculation of MG, that the pigeon and house sparrow were barely susceptible to clinical infection and slightly contagious to unaffected house finishes. On the other hand, the American goldfinches established the clinal disease and were infectious to house finishes even after 49 days of inoculation (Gharaibeh et al., 2011).

House-finch were infected experimentally by MG isolate and it was reported that the isolate can cause disease in chicken and turkey, however with the intensive biosecurity measures taken in the experiment the rate of disease transmission (O'connor et al., 1999) was decreased. Despite these measures , a similar MG isolate was found in a commercial turkey flock later, indicating that a natural infection by a songbird like MG strain as a possibility (Ferguson et al., 2003).

Experimentally, SPF or mycoplasma free chicken, turkey or their embryonated eggs are used as MG hosts (Bradbury et al., 1996). Furthermore, partridges, canaries and house finches are used readily as experimental hosts for MG (Dhondt et al., 2008; Hawley et al., 2011; Sydenstricker et al., 2006).

M. gallisepticum possesses the ability to infect a wide range of birds at different ages, yet rarely a natural infection occurs in young birds. Despite that, these young birds showed significant susceptibility to experimental infections (Bradbury et al., 1996; Gaunson et al., 2006).

2.1.5 Clinical signs:

Typically coughing, tracheal rales, nasal discharges, conjunctivitis, and ocular mucus discharge are indications for naturally occurring mycoplasma disease (S. H. Kleven, 1998) in addition to feed consumption decrease leading to weight loss. In small pullets, the start of egg production is delayed (Mohammed et al., 1987). In layers egg production and egg size declines and commonly lower egg quality and embryo mortality are reported. Usually, MG infections are more severe at winter durations and males show more distinct signs. Subclinical infection in

layers also causes a decrease in egg production, and in response to surrounding stressors it might develop into clinical disease. In broilers, the outbreaks occur at 4 weeks of age and severe outbreaks results from the presence of viral and bacterial co-infection in addition to environmental stressors (S. H. Kleven, 1998).

2.1.6 Morbidity and Mortality:

Commonly, MG infection disturbs the whole flock yet, the disease period and severity are variable. In cold environments, the disease tends to be more serious and of extended duration. Furthermore, younger birds are more vulnerable than mature birds, although significant losses in egg production are present in laying flocks.

MG infection commonly causes Chronic Respiratory Disease (CRD); however, it is commonly coupled with bacterial and viral infections. These co-infections including Newcastle virus, Infectious bronchitis virus, and E. coli cause complicated and severe air sac disease (complicated CRD) and highly contribute to MG outbreaks (Gross, 1990).The situation is worsened in the presence of E. coli as a secondary agent when IB , ND and MG were present as primary pathogens. Researchers noted the increase in duration and severity of the disease when MG was coupled with the presence of IB (Soeripto et al., 1989).

2.1.7 Mortality:

In adults, mortality is negligible, yet significant losses in egg production are noted (Mohammed et al., 1987). In broilers, the mortality ranges from low to 30% depending on the presences of co-infections, and cold environment. The mortality in broilers is accompanied by

retarded growth; drop in carcasses quality, while the customers' disapproval of the broiler quality increases the losses.

2.1.8 Pathology:

2.1.8.1 Gross:

Mainly, catarrhal exudate and mucosal congestion found in nasal and paranasal passages, trachea, bronchi, and air sacs are the main feature of MG infected bird's gross lesions. Typically, accumulation of mucoid to caseous exudates in sinusitis is prominent in turkeys, yet it can be noticed in chicken and other infected avian species. Air sacs usually contain caseous exudates either limited to one area or disseminated along the lymphofolicullar presentation, further pneumonia presences may be noticed in some birds. The presence of viral and bacterial co-infections resulting in complicated air sac disease in chicken and turkey, in addition to caseous airsacculitis, fibrinous perihepatitis results in high death rates, downgrade of carcass quality and vast criticisms at processing.

M. gallisepticum related Keratoconjunctivitis in commercial chicken layers are defined with facial subcutis, eyelids and infrequent corneal opacity edema (Nunoya et al., 1995). Different avian species such as house finches, songbirds (Hawley et al., 2011; Mikaelian et al., 2001) and chukar partridges (McMartin et al., 1996) show peri-ocular swelling and irritation when infected with MG. Moreover, salpingitis caused by MG infections marked with exudate congestion in oviducts that leads to decrease in egg production (T Nunoya et al., 1997).

2.1.8.2 Microscopic:

Thickening of the mucous membrane due to penetration of mononuclear cells and hyperplasia of the mucous gland are features that highlight MG microscopic pathology in chicken and turkeys (Hitchner, 1949). MG adherence to inflamed epithelial cells leads to cilia's complete destruction (Charlier et al., 1981). Usually, the accumulation of the white blood cells (lymphocytes), macrophages and plasma cells are noticed in the lamina propria. Mainly the increased mucosal membrane thickness in the trachea is used as an indication of MG infection and disease (K. Whithear et al., 1996). According to 156 reports, there was a significant increase in trachea mucosal thickness from week 1 to 2, however, a decreased in thickness was described from week 2 till 3. At the level of the lungs, lymph follicular changes are observed in addition to appearance of pneumonic areas and granulomatous lesions.

Typically in layers, chicken MG infection is marked by epithelial hyperplasia, subepithelial edema and acute cellular infiltration and stroma of the central fibrovascular connective tissue which leads thickening of the eyelids (Nunoya et al., 1995). While in salpingitis case, thickening of the oviductal mucosa leads decreased egg production (T Nunoya et al., 1997). Asymmetrical elevations of the hyperplastic epithelial layer specifically in the subepithelial lamina propria was caused by the spread of lymphocytes and plasma cells (T Nunoya et al., 1997).

Different cases of MG related encephalitis in turkeys have been examined histologically which described a moderate to severe lymphocytic cutting of vessels, focal parenchymal necrosis , fibrinoid vasculitis and meningitis (Chin et al., 1991).

2.1.8.3 Ultra-structural:

Ultra structural details concerning in vivo and vitro MG interaction with the tracheal epithelium has been described by several experiments (Dykstra et al., 1985; Lam & DaMassa, 2003). These experiments highlight the MG presence with degeneration of epithelial cells in addition to inflammatory cellular infiltration of the mucosa in the tracheal tissues (Tajima et al., 1979). Typically, mycoplasma is present extracellularly around epithelial cells where the attachment occurs using their bleb structures and organelles. This attachment is followed by mucous granules release which accelerates the removal of ciliated and nonciliated epithelial cells and therefore the loss of cilia in the trachea (Dykstra et al., 1985). Edema and cellular infiltration accumulate to increase the epithelial thickness (Dykstra et al., 1985).

Winner reported that MG has the ability to penetrate and survive intracellularly (Winner et al., 2000). This claim was supported when MG was incubated with red blood cells which induced modifications of the cell surface appearance and perforations (Lam, 2004; Lam & DaMassa, 2003).

2.1.9 Immunity:

A certain degree of protection is attained in recovered chicken or turkey from clinical signs of MG infection. Nevertheless, these birds may still carry MG (Benčina & Dorrer, 1984) which can be transferred to vulnerable birds through direct contact or egg transmission.

Luginbuhl et al (Luginbuhl et al., 1967) in his literature described the immunological response produced due to MG infection. Several reports emphasized on the importance of antibodies production and the role of bursa of fabricious in the development of an immune response against MG (Javed et al., 2005). Yet, on the other hand, different reports highlighted the low correlation between protection and the number of circulating antibodies (Noormohammadi et al., 2002; Purswell et al., 2011)

Antibodies against MG were found in recovered birds, and following the re-exposure to MG, higher MG elimination rate and fewer tracheal lesions were noticed in comparison with those of the first exposure. Moreover, higher antibody titers against MG were found in the tracheal samples of infected birds with an associated decrease in MG and tracheal lesions score (Chhabra & Goel, 1981). Therefore, the results obtained, combined with other reports (Hopkins et al., 1990; Javed et al., 2005; Yagihashi & Tajima, 1986) indicated the important role of secreted antibodies in *MG* resistance. At the level of trachea, secreted antibodies in response to MG presence eliminated the *MG* cytadhesion to tracheal epithelial cells (Avakian & Ley, 1993), which is considered the main mechanism of immune mediated response.

In ovo, maternal antibodies against *MG* decreased the virulence of infection and enhance the survival rate of infected embryos (Benčina et al., 2005; Levisohn et al., 1985). Significant efforts and research have been made to indicate, assess, and identify MG antigens, specifically antigens related to cell attachment, which play a vital role in pathogenesis and immune mediated response to MG infection.

Reports highlighted the effect of *MG* on cell-mediated immune system. These reports suggested that MG may induce or suppress B and T lymphocytes proliferation and cytokinin production (Chhabra & Goel, 1981; Lam & DaMassa, 2003; J. Mohammed et al., 2007).

Blood samples taken from MG infected chickens indicated the presences of nitric oxide, lymphoproliferation interferon (Reddy et al., 1998). Moreover, Gaunson (Gaunson et al., 2000) monitored the number of lymphocytes and their distribution in the exposed trachea to mild and virulent strains of *MG* and noticed the presence of suppressor T cells specifically at the acute phase of the infection. Furthermore, Gaunson emphasized the vital role of local antibody response to resist MG infection, yet he indicated the significant role and presence of cytotoxic t cell and natural killer cells in response to this infection (Gaunson et al., 2006; Gaunson et al., 2000).

High frequency variation and switching (on-off) of the dominant surface protein is considered a major adaptive mechanisms that enable MG from escaping the immune system, adjust to different changes in host environment and to finally persist and cause a chronic disease despite the presence of healthy immune response (Benčina & Dorrer, 1984; Glew et al., 2000; Winner et al., 2000). Furthermore, several studies suggested the ability of MG to survive intracellularly (cell invasion), therefore escape the immune system and persist in the host body (Much et al., 2002; Vogl et al., 2008).

2.1.10 Diagnosis:

2.1.10.1 Isolation and identification of the causative agent:

Typically, for MG diagnosis the isolation and identification of the disease is required. However, the fastidious nature of MG and its slow growing rate might require more than 3 weeks for significant growth to appear. Contamination with bacterial species and the overgrowth of saprophytic mycoplasmas present at the upper part of the avian respiratory system may inhibit and impair the isolation of MG. Fluid sinus or tracheal samples and air sac exudates can be cultured directly into MG agar media or broth (S. H. Kleven, 2008). Furthermore, tracheal swabs and choanal cleft samples can be used to culture MG (Zain & Bradbury, 1996) and MG presence in the oviduct (T Nunoya et al., 1997) can be isolated from cloaca of infected birds (MacOwan et al., 1983).

Typically, in the 4-8-week post-infection ,which corresponds to the acute stages of the disease, MG levels peak in the upper respiratory tract and the occurrence and spread rate of the disease are then high (Yagihashi & Tajima, 1986). Consequently, the organism can be recovered by tracheal and choanal clefts swabs from 10-30 live birds, yet a higher number of cultures (30-100) are required to recover MG at later stages of the disease (Kleven et al., 1996). Common sampling and culture methods (S. Levisohn & Kleven, 2000) may fail to isolate the organism from chronically infected egg layers and backyard poultry due to the low numbers of MG in the trachea and cloaca. Samples should be collected prior to the application of antibiotic therapy in order to optimize the isolation possibility (Migaki et al., 1993). Furthermore, water drinking treatments including ammonium chloride could interfere with the isolation of MG from infected birds (Branton et al., 1997).

Ideally, swabs, exudates, tracheal fluids and small tissue samples are cultured in mycoplasma broth media and immediately incubated at 37C. Furthermore, inoculated broth requires short term-storage at 4C, or usually held in cold packs for a period less than 24 hours until they are transferred to the lab for further analysis.

Later and for the MG isolation process, the samples are transferred into mycoplasma agar medium where they proliferate, and colony formation occurs. Direct and indirect immunofluorescence are used to detect mycoplasma isolates present in mixed cultures that contain several mycoplasma species (S. H. Kleven, 2008; Talkington & F.Kleven, 1983). Growth inhibition can be used to identify mycoplasma species (Clyde, 1983; S. H. Kleven, 1998)

Another method used in MG isolation is through the injection of a 7-day old embryonating egg through the yolk sac with samples obtained from suspected lesions. These samples obtained should be bacteria and fungus free to avoid undesired contaminations that might hinder MG isolation (Bradbury et al., 1996). After 5-8 days post inoculation death of the embryos occurs, yet several passages of the collected yolk material is needed before the occurrence of typical lesions and death.

2.1.10.2 Detection of the Causative Agent Genetic Material:

The ease of PCR-based procedures and their high sensitivity , rapidness , and specificity (Boettger et al., 2006; R. Harasawa et al., 2004) are factors that aided this method to supersede DNA and ribosomal RNA gene probes that were used and reported by different researches (Arzey & Arzey, 1992; Garcia et al., 1996; M. Khan & Kleven, 1993). Immediate detection of several organisms can be done using Multiplex PCR protocols (Mardassi et al., 2005). McAuliffe

reported a method that is based on the amplification of the 16S rRNA gene with Mycoplasma-specific primers, later using the gel electrophoresis the PCR products were separated (McAuliffe et al., 2005).

PCR kits and several established protocols are now the lead method used at diagnostic laboratories to detect MG (García et al., 2005). Further reports (Grodio et al., 2008; Raviv & Kleven, 2009; Sprygin et al., 2010) described a quantitative PCR approach that is considered more rapid and specific in detection of MG. Their ability to provide positive and negative results within hours instead of days, and to avoid competition with saprophytic mycoplasma and other contaminates are factors that helped PCR to supersede culture isolation methods in MG detection. Nevertheless, culture and isolation methods remain vital and indispensable in experimental studies, pathogenicity assessment and strain identification

Culture and PCR methods could be both applied to samples obtained from inoculated mycoplasma broth. Furthermore, inactivated MG suspensions are filtered in Flinders Technology Assessment filter paper prior to PCR and DNA-dependent assays application (Moscoso et al., 2004).

2.1.11 Serology:

MG control programs and its diagnostic approach relies heavily on serological procedures and methods. These methods, along with the knowledge of the flock health history and clinical manifestation of MG infections, allows a presumptive diagnosis, which should confirm later by the isolation and identification of the organism.

In the 1960s and 1970s the tube agglutination test was a general method used in MG control programs for turkeys, but nowadays is rarely used. Commonly, Serum plate agglutination antigen (SPA) test is used for initial screening in control programs and serodiagnosis. The test sensitivity and rapidity in addition to its relative low price are factors that aided in the widespread of this method worldwide (Kleven et al., 1996; S. H. Kleven, 1998). However, false results might occur in chicken infected with M.synoviae because of cross-reactive agents (Ben Abdelmoumen & Roy, 1995) or in recently vaccinated chicken either by oil-emulsion vaccines or by using vaccines originating from tissue-culture (Ahmad et al., 1988; Glisson et al., 1984). These nonspecific reactors could be decreased by diluting the test serum (Ross et al., 1990). Two-fold (1:2) dilution of the sera in saline is common methods used by laboratories to establish agglutination end point. Furthermore, the specific and nonspecific reactions are distinguished by the sera reacting at 1:8 dilution or greater are considered positive. IgM antibody is a the first immunoglobin generated in response to infection, this antibody is highly detected by the SPA (S. Kleven, 1975).

Commonly, the hemagglutination inhibition test (HI) is performed to validate the results (reactors) spotted by SPA or by enzyme-linked immunosorbent assays (ELISA). Nevertheless, HI test is relatively work and time-intensive, and some cases may lack sensitivity (Kleven et al., 1996; S. H. Kleven, 1998)

Enzyme linked immunosorbent assays (ELISA) test were designed to enhance testing efficiency and increase specificity, peculiarities and sensitivity of results related to the SPA and HI tests (Czifra et al., 1995; Higgins & Whithear, 1986; Stipkovits et al., 1993). Nowadays, in flock monitoring and serodiagnosis, ELISA test kits are the tools of choice. Generally, ELISA is

less sensitive than SPA but more sensitive than HI. In terms of specificity, ELISA performs better than SPA and worse than HI. (Kaszanyitzky et al., 1994; I. Kempf & Gesbert, 1998).

Commonly in several diagnostic laboratories, ELISA became the method used for MG serology. Extensive efforts continue to enhance MG ELISA sensitivity and specificity, these efforts include identifying, purifying and then using specific immunodominant MG proteins (Czifra et al., 1995; Noormohammadi et al., 2002; Spencer et al., 2002)

Simultaneous serodiagnosis is done using Multiplex ELISA for MG, M.M, and MS (Mardassi et al., 2008). Furthermore, ELISA is used to identify MG local antibodies found in the respiratory tract samples (J. L. Grodio et al., 2009) and in the egg-yolk samples (I. Kempf & Gesbert, 1998). Different studies compared the use of egg yolk and serum for the identification of MG antibodies by HI or ELISA and they reported that for flock screening egg yolk sample could be used instead of serum samples (I. Kempf & Gesbert, 1998). Moreover, several reports described the use of VlhA-based colloidal gold immunochromatography assay (GICA), PvpA-based enzymatic rapid immune-filtration assay (ERIFA), and dot immunobinding assays for the detection of MG antibodies (Avakian & Kleven, 1990; Büyüktanır et al., 2008)

In MG-free monitored flocks, serologic test showed a positive MG result. Moreover, in another healthy flock a few percentages of MG SPA test reactors. These results suggest that low virulence MG strains are held responsible for these positive results (Truscott et al., 1974; H. W. Yoder Jr, 1986)

MG strains that characterized as mild, low transmissible and with poor immunogenicity (Dingfelder et al., 1991) have been isolated from turkeys. Nonspecific reactors that are

encountered when monitoring MG in flocks is partially due to antigenic variation of MG isolates, which was proved using immunoblots (Avakian et al., 1991), HI assays (Dingfelder et al., 1991) and agglutination test (Panangala et al., 1992).

Antibody response is affected by the presence of antimicrobials (antibiotics) in the early phase of the infection (Levisohn, 2000; Stanley, 2001). Several studies demonstrated the effect of antibiotics on the immune response; the results indicated the presence of higher serological response in non-treated chicken or turkey groups in comparison with treated ones (Kleven et al., 1996; Migaki et al., 1993).

2.1.12 Differential diagnosis:

Commonly, the differentiation of MG infection from other respiratory diseases is hard, and it only becomes clinically noticeable when it's complicated with other bacterial infections: E.coli and viral infections: Newcastle disease and Infectious bronchitis (S. H. Kleven, 2008).

In chicken flocks it's important to differentiate MG infection from other viral or bacterial infections. Typically, in chicken Newcastle or infectious bronchitis and their related immune response may occur as a distinctive entity or as a part of Chronic Respiratory Disease. Further, MG may be coupled with M. synoviae infection, thus, complicating the diagnosis procedure and necessitating the selection of powerful tools to identify the causative agent.

2.1.13 Prevention and control:

2.1.13.1 Biosecurity:

Biosecurity and bio-surveillance using serological monitoring, Mycoplasma gallispeticum isolation methods, and DNA-based detection methods (T. Liu et al., 2001). Strict biosecurity coupled with adequate bio-surveillance contributed effectively in reducing MG outbreaks occurring in turkeys' and chicken's breeding stocks (S. Kleven et al., 2004). Broilers flocks "all in all out" followed protocol allows for a complete eradication of Mycoplasma gallisepticum infected flocks. However, the case is different in US laying flocks where M. gallisepticum infects more than 50% of the present flocks (J. Evans et al., 2005). Due to the complexities and size of this sector, eradication of infected flocks is not feasible. For that, different alternative approach had been established to manage Mycoplasma gallisepticum infection using live attenuated MG vaccines (K. Whithear, 1996), antibiotics treatment and dietary manipulation such as the addition of excess methionine in the diet (Ramadan et al., 2019).

2.1.13.2 Vaccines:

Vaccine is a biological preparation used to elevate the immune response of the host against a specific disease. Typically, this biological mix is formed from an agent that mimics the pathogenic microorganism. This agent is commonly used in its attenuated or killed form or sometimes replaced by the toxins and surface proteins produced (K. Whithear, 1996).

Nowadays, *Mycoplasma gallispeticum* vaccines used are either killed or attenuated vaccines. Several strains of *Mycoplasma gallispeticum* have been isolated from chicken, from

which several were used as live vaccine candidates (K. Whithear, 1996). Besides providing protection from *Mycoplasma gallispeticum* respiratory infection; three possible objectives from using these vaccines are highlighted such as: Decreaseing the drop-in egg production, preventing the vertical transmission of MG though the eggs, and enhancing the eradication of MG. This eradication is possible through decreasing the revisor build-up of the pathogenic strain or through replacing these pathogenic strains with milder less virulent ones (N. M. Ferguson et al., 2005; Haesendonck et al., 2014).

The suggested outcomes require an ideal *Mycoplasma gallispeticum* vaccine with specific characteristics. Such features include the safe use of the vaccine without the possibility of causing the disease in the vaccinated host or unintentionally spread of the disease to vulnerable neighboring flocks. Moreover, the vaccine should induce a long solid immunity without the need for intensive boosting. This vaccine should be obtained from marked seed stock of known consistent potency and purity and should be easily produced and used at the field level. The vaccines should have a long shelf-life with no need for specialized storage facilities. Furthermore, vaccines should be easily applicable to large numbers of birds. Unfortunately, all the current vaccines for *Mycoplasma gallispeticum* don't attain this ideal status (K. Whithear, 1996).

This following section will address the current situation of Mycoplasma gallisepticum vaccines and possible future candidates.

2.1.13.2.1 Inactivated vaccines (killed vaccines):

Research for MG vaccines originated in 1970s where *Mycoplasma gallispeticum* infections were endemic in broilers, layer and breeders' flocks. Hildebrand in 1983, reported the use of highly antigenic MG isolate that was utilized to create an inactivated oil-emulsified MG vaccine. This vaccine was able to protect the vaccinated hosts from clinical signs of MG. Furthermore, trials at a commercial egg operation indicated the role of the inactivated vaccine in increasing egg production in comparison with non-vaccinated hosts. Large eggs were also produced by the vaccinated layers in comparison with the naïve ones. These results suggested that the used inactivated vaccine is safe and effective against MG infections (Hildebrand et al., 1983). Several other reports suggested the ability of inactivated bacterin to protect broilers against airsacculitis (Karaca & Lam, 1987; H. Yoder Jr et al., 1984), and layers from decrease in egg production (H. W. Yoder Jr & Hopkins, 1985). On the contrary, Khan et al didn't detect an improvement in previously vaccinated commercial egg layers by MG bacterin affected by MG infection (M. Khan et al., 1986).

Kleven in 1985 reported that the bacterin inoculated chicken have relatively lower population of Mycoplasma gallispeticum in their trachea (S. Kleven, 1985b); similar results were obtained in other experiments (F. D. Talkington & Kleven, 1985; Yagihashi et al., 1992; H. W. Yoder Jr & Hopkins, 1985). These results suggested that bacterin can reduce MG colonization inside the trachea, yet it was not able to eliminate it. Furthermore, bacterin failed to provide a long-term immunity and control against *Mycoplasma gallispeticum* infections in multi-age production site (S. Levisohn & Kleven, 2000). Another study by Feberwee in 2006 to assess the effect of inactivated vaccines on the horizontal transmission of Mycoplasma gallispeticum.

Feberwee reported that although the vaccine decreased MG shedding, it failed to reduce the horizonal transmission of MG between the vaccinated layers (Feberwee, von Banniseht-Wysmuller, et al., 2006).

Mycoplasma gallispeticum inactivated vaccines have been manufactured commercially. Such nonliving vaccines are safe to apply with no risk of cross infection or reversion to virulence strain (K. Whithear, 1996). These vaccines are laborious to administer and several applications (boosters) are needed to achieve optimal protection. Several agents and adjuvants such as formaldehyde, liposomes, saponin, iota-carrageenan and binary ethylenimine (E. K. Barbour & Newman, 1989, 1990; E. K. Barbour et al., 1987; Elfaki et al., 1992; Hussein et al., 2007) are used to enhance the performance of bacterin vaccines. However, adverse vaccine reactions occur when layer chickens are vaccinated intramuscularly in the leg with oil-adjuvanted *Mycoplasma gallisepticum* bacterin. Swelling is detected in the vaccinated leg as result of granulomatous cellulitis in the connective tissues and histopathology revealed that the inflammation is localized in the subcutis and muscle fascia, yet it might spread to tendons and tendon sheaths (E. K. Barbour & Newman, 1990; Whithear et al., 1990). Furthermore, oil-adjuvants bacterin vaccines inoculated at the base of the skull caused a transient edema around the eyes (D Bencina et al., 1988).

2.1.13.2.2 Live attenuated vaccines:

Currently the three commercially approved and used live *Mycoplasma gallispeticum* vaccines are the F strain, ts-11 and 6/85.

- F strain:

Originally the *Mycoplasma gallispeticum* F strain was isolated by Adler and Yamamoto in 1953 and described as a typical virulent strain (Yamamoto et al., 1958). Several pullet immunization programs were based on a relatively mild F strain that originated from Connecticut F strain (Glisson et al., 1984; Rodriguez & Kleven, 1980; Van der Heide, 1977). These immunization programs tend to decrease the possible egg transmission of *Mycoplasma gallisepticum* in the following breeder flocks (Luginbuhl et al., 1967). The use of Connecticut F strain was also reported in young replacement pullets before their introduction into a multi-age flock (Carpenter et al., 1981; Van der Heide, 1977). Several reports described the use of Live F strain *Mycoplasma gallispeticum* vaccine to reduce MG egg transmission and egg production losses (Abd-el-Motelib & Kleven, 1993; Cummings & Kleven, 1986).

F strain vaccine provided partial protection in vaccinated broilers against airsacculitis when challenged with virulent R strain (Levisohn & Dykstra, 1987; Rodriguez & Kleven, 1980). The protection induced by F strain didn't include competition for adherence site or stoppage by prior colonization. Furthermore, F strain couldn't stop tracheal tissue colonization by the challenge strain of MG (Levisohn & Dykstra, 1987) . Several reports (Evans & Hafez, 1992; Kleven, 1981; Lin & Kleven, 1982) reported the transmission of F strain through egg and between pen mates. Nevertheless, the transmission of F strain in pullet vaccinated by eye drop didn't occur in broiler found within the same pen or separated by an empty pen (S. Kleven, 1981).

In the presence of endemic MG, the F strain-vaccinated flock was able to produce more eggs in comparison with unvaccinated one, yet not as many eggs produced from MG-clean flocks (Carpenter et al., 1981). Delay onset of lay and decrease in egg production were observed in a flock following MF vaccination at week 12 (Burnham et al., 2002). In addition to that an increase in eggshell pimpling was recorded following the administration of F strain vaccine to layers during egg production phase (Leigh et al., 2010).

In an approach to increase resistance against MG challenge a priming with several attenuated *Mycoplasma gallispeticum* live vaccines such as 6/85 and ts-11 were used, followed by re-administration of F strain live vaccine (Leigh et al., 2010). F strain remained viable in the upper respiratory tract for the whole life of the vaccinated flock (S. Kleven, 1981).

During laboratory experimental trials, vaccination using F strain led to decrease in population of the challenge strain located in the upper respiratory tract (Cummings & Kleven, 1986). Similarly, in pen trials vaccination using F strain successfully replaced the infective strain (Kleven et al., 1998). Furthermore, the 2 years continuous administration of F strain vaccine in successful displacement of the MG field strain in replacement pullets located in a multiple-age layer complex (Kleven et al., 1990). Through experimental studies, F strain was described as pathogenic in turkeys (Lin et al., 1982) and, it has been isolated when MG outbreaks occur in meat and breeder turkeys found under field conditions (Ley et al., 1993). The F strain vaccine can be given in various routes such as intranasal, coarse spray and by eye drop (Levisohn & Kleven, 2000). The F strain vaccine is usually less stable in comparison with other live MG vaccines in water solution found at room temperature, however, F strain is more stable when found in phosphate buffer saline (PBS) (Leigh et al., 2010). *Mycoplasma gallispeticum*

vaccination is usually applied at 8-14 weeks of age. However, it can be applied as early as two weeks or less in case the chicken are at risk of exposure before 8 weeks (S. Levisohn & Kleven, 2000).

- 6/85 strain vaccine:

The *Mycoplasma gallispeticum* 6/85 strain was originally isolated in the United States, in which its development and vaccine features were reported (Evans & Hafez, 1992). Through several experiments, the MG 6/85 strain vaccine appeared to be of low virulence in chicken and turkey. Minimal transmissibility of 6/85 vaccine was detected , with little or no protection against challenge using a virulent MG (Abd-el-Motelib & Kleven, 1993). Minimal or no recognized serologic response can be noted in the upper respiratory tract at least for four to eight weeks post vaccination (Ley et al., 1997). Originally, in USA, MG 6/85 vaccine is used mainly to prevent the loss in egg production in table-eggs layers. In order to achieve full effectiveness, the vaccine is administered by the aerosol route. This vaccine in its freezed-dried pellet form is applied to 6 weeks or older pullets. The vaccine is usually more stable in PBS (phosphate buffer saline) then when found in water (Leigh et al., 2008).

- Ts-11 vaccine:

Several reports described the features and development of ts-11 *Mycoplasma gallisepticum* vaccine (Whithear et al., 1990; Whithear et al., 1990). An Australian *Mycoplasma gallispeticum* strain known as strain 80083 went through different chemical mutagenesis and selection for temperature sensitivity to produce ts-11 MG strain. The effects of chemical mutagenesis included the disappearance of GapA cytadhesion expression gene (Mudahi-

Orenstein et al., 2003). Ts-11 vaccine is not virulent in chicken or turkey and it can spread from a host to another. When inoculated into a host, ts-11 vaccine stimulates a slow response coupled with a low recognized level of antibodies. Ts-11 vaccine was able to stimulate a protection against several MG challenge strains (Abd-el-Motelib & Kleven, 1993; K. Whithear, 1996)

Long lasting immunity is generated from the presence of ts-11 vaccine in the upper respiratory tract of the vaccinated host (Silveira et al., 1996). Vaccination using Ts-11 vaccine didn't alter egg production or eggshell size and features (Branton et al., 2008). Vaccinated broilers breeder by ts-11 vaccine showed resistance to MG challenge. This solid immunity was vertically transmitted to their progeny, in which broilers showed a better production performance (Barbour et al., 2000). The presence of GapA gene in ts-11 strain stimulated a higher protection in SPF chicken (Shil et al., 2011).

Similarly, to 6/85 strain, ts-11 strain vaccine was originally used to decrease the losses in egg production in egg table layers. Ts-11 vaccine is applied through eye drop route to 9-week pullets or older (Gaunson et al., 2006). Furthermore, Gaunson in 2006 described the possibility of administrating MG ts-11 strain vaccine at early age (between 1-4 weeks) and reported its efficacy in protecting the vaccinated host from severe form of the disease (Gaunson et al., 2006).

In order to ensure the required colonization and immunization a precise concentration of live *Mycoplasma gallispeticum* vaccines is required (Evans et al., 2009; Purswell et al., 2011). With their low virulence and low transmissibility from vaccinated birds to unvaccinated one, ts-11 and 6/85 are considered safer to use in comparison with F strain (Levisohn & Kleven, 2000).

Therefore, 6/85 and ts-11 are the preferred vaccination choice when dealing with susceptible flocks (Levisohn & Kleven, 2000).

Through different experiments, several MG live vaccines were able to decrease the challenge strain colonization but failed in preventing it (Feberwee Landman, et al., 2006; Mohammed et al., 2007). On the other hand , MG live vaccines successfully displaced the wild-type challenge strain in a multiple age complex resulting in resistance and protection against the infection in field trials (Levisohn & Kleven, 2000). F strain is more powerful in displacing virulent field strains in comparison with 6/85 and ts-11 (S. Kleven et al., 1990). However, when F strain vaccination was discontinued in the flock the *Mycoplasma gallisepticum* reemerged (S. Levisohn & Kleven, 2000).

Vaccinating replacement pullets with ts-11 vaccine after being previously populated with F strain, resulted in ts-11 replacement of F strain in the flock. *Mycoplasma gallisepticum* F strain was no longer detected within the flock when ts-11 vaccination was discontinued (Turner & Kleven, 1998). Considering 6/85 strain vaccine no data is found concerning its ability to eradicate MG field strains. Based on the literature mentioned before, vaccination with F strain for one or more reproduction cycles is required to eradicate/displace highly virulent *Mycoplasma gallisepticum* strains. Following the eradication of these virulent strains, 6/85 and ts-11 could be used instead of F strain (Levisohn & Kleven, 2000).

Major safety concerns arise with the use of F, 6/85 and ts-11 MG live vaccines which includes their stability, virulence, and vertical and horizontal transmissibility. Concerning F strain, several reports highlighted the horizontal transmission of *Mycoplasma gallispeticum* F strain to other flocks (Gharaibeh et al., 2011; Ley et al., 1993; Lin et al., 1982). Despite its

known safety in chicken, 6/85 is associated with clinical disease in turkeys transmitted from nearby vaccinated chicken flocks (Kleven et al., 2004). The presence of 6/85 *Mycoplasma gallispeticum* strain was detected using RAPD genotyping from different unvaccinated hosts. One of the detected isolated was genotypically like 6/85 strain, however it was phenotypically different. These contradictory results couldn't suggest the role of isolated 6/85 in causing severe sinusitis (Throne Steinlage et al., 2003). Therefore, it is recommended to vaccinate the whole flock to ensure a uniform solid immunity. In case unvaccinated hosts are present, they should be isolated from vaccinated flocks to avoid horizontal transmission of MG (Throne Steinlage et al., 2003).

Similar to 6/85 strain, ts-11 strain was isolated from different unvaccinated flocks. in these cases, vaccinated farms were located nearby thus suggesting a possible spread to tested unvaccinated flocks . Furthermore, vertical transmission of ts-11 strain was detected in broiler progeny produced from vaccinated broiler breeder. These results were confirmed by genotyping and in vivo experiments were the isolated ts-11 strain shared the same virulence as R strains (El Gazzar et al., 2011). The produced results suggest the possibility of vertical transmission following vaccination using ts-11 strain and ability to revert to virulence and infect non-vaccinated hosts through horizontal transmission (El Gazzar et al., 2011).

Therefore, veterinarians and farm managers should adhere strictly to the vaccine's manufacturer's instructions and use live *Mycoplasma gallisepticum* vaccines compatible with the strain dominating that area. Furthermore, special consideration and restrictions should be considered to ensure the safety of nontarget flocks found within nearby proximity of vaccinated flocks.

F strain is highly pathogenic in turkeys (D. H. Ley et al., 1993), whereas ts-11 lacked the ability to colonize the upper respiratory tract (K. Whithear, 1996). Also, 6/85 failed to induce protection in vaccinated turkey against airsacculitis following aerosol challenge, yet there is some sort of protection against lesions in the upper respiratory tract. Therefore, the three available Mycoplasma gallispeticum vaccines aren't safe/efficient to be used in turkeys (Levisohn & Kleven, 2000).

2.1.13.3 Other vaccines:

Modification of an avirulent high passage R strain containing a gene responsible for GapA (cytadhesion protein) produced a promising live MG vaccine :GT5 strain (Javed et al., 2005). Furthermore, another possible vaccine derived from mildly virulent K strain was investigated for potential use for turkeys (Ferguson et al., 2004; Ferguson et al., 2003). Also, a recombinant vaccine consisting of fowl pox and *Mycoplasma gallispeticum* has been introduced as a vaccine in chicken (Sundquist et al., 1996); nevertheless, further assessment is required concerning its safety and efficacy in vaccinated host (Zhang et al., 2010).

2.2 Antibiotics: an overview:

2.2.1 Treatment:

In the absence of the cell wall, *Mycoplasma gallispeticum* is naturally resistant to different types of beta-lactams antibiotics such as: penicillin and cephalosporin (S. Kleven, 2008). In vitro and vivo experiments, *Mycoplasma gallispeticum* showed sensitivity to different antibiotics such as macrolides, tetracycline, fluoroquinolones and pleuromutilin (Bradbury et al.,

1994; Glisson et al., 1989; Tanner et al., 1993). *Mycoplasma gallispeticum* has been treated by different antibiotics that were able to reduce clinical manifestation severity and lower mortality rates (Jordan et al., 1998; I. Kempf et al., 1992). Through antibiotic treatment, lower *Mycoplasma gallispeticum* shedding rates are recorded, which lower the risk of MG horizontal spread to susceptible nearby flocks (Cummings et al., 1986).

2.2.2 Macrolides:

Macrolides are a large group of antibiotics produced primarily by Streptomyces and their related species. These antibiotics are formed from a macro-lactone ring connected to two sugars that holds an amino sugar. In 1965, the original macrolide complex was isolated as a natural product produced by sacchropolyspora known previously as Streptomyces erytherus. Later on, research has been focused on finding analogues that has extended the antibacterial spectrum specifically against gram negative bacteria, in addition to their increased acid stability and reduced gastrointestinal intolerance (Kaneko et al., 2007).

These analogues include sprimycin that was produced in 1960s as the first macrolide for animal use. This was followed by erythromycin and tilmocsin in the following years (Pyörälä et al., 2014). Macrolides showed significant ability to reduce treat respiratory diseases against mycoplasma and gram-positive bacteria. However, gram negative bacteria are inherently resistance to macrolides due to the absence of the cell wall (Pyörälä et al., 2014).

2.2.3 Tilmicosin:

Tilmicosin a semisynthetic bacteriostatic macrolide is synthesized from tylosin with an extensive spectrum of veterinary use. Tilmicosin ability to accumulate in the lungs with a high

volume of distribution makes it the drug of choice in control and treatment of respiratory diseases. Tilmocsin is used primarily to treat and control respiratory diseases. In poultry, Tilmicosin is used to treat *Mycoplasma gallispeticum, Mycoplasma synoviae , Ornithobacterium rhinotracheale and Pasteurella mutlocoda* (Abu-Basha et al., 2007; Kempf et al., 1992).

2.2.4 Mechanism of action:

Tilmicosin binding to the 23S rRNA found with the 50S subunit inhibits the bacterial protein synthesis. Following its binding, Tilmicosin interferes with rRNA and ribosomal protein functions. This interaction results in blocking prolongation and release of developing peptides which results in protein synthesis inhibition (Dinos, 2017). Primarily, tilmocsin has bacteriostatic action , yet when administered in high concentration it has the potential to perform as time-dependent bactericidal .Tilmicosin has a limited spectrum and is used primarily against gram-negative aerobic bacteria such as *Mycoplasma, Pasteurella multocida, Mannheimia haemolytica, and Histophilus somni* (formerly Haemophilus somnus) (Dinos, 2017).

2.2.5 Absorption:

Macrolides including Tilmicosin are easily absorbed in the gastrointestinal tract if they escaped gastric acid inactivation ability. Within 1-2 hours of macrolide administration, plasma levels peak. This absorption rate might be elevated by the presence of food and different salt and ester used. The absorption of Tilmicosin at the level of the ruminoreticulum is commonly hindered and unreliable (Kahn, 2005).

2.2.6 Distribution:

Macrolide is characterized by its high distribution in the tissues, and high concentration in the plasma. Macrolides tend to accumulate within different cells such as macrophage. Macrophages macrolide concentration is found to be equal to that of the plasma concentration or higher by 20 times. The long dosing interval for Tilmicosin accounts for previously mentioned accumulations. Macrolides commonly concentrate at the level of spleen, liver and kidney and specifically the lungs. Macrolide tend to bind to alpha1-acid glycoprotein in plasma, and they are detected in bile and milk of treated animals (Kahn, 2005).

2.2.7 Excretion:

Macrolides and their metabolites are excreted up to 60% by bile and commonly pass through enterohepatic cycling. Less than 10% of macrolides are cleared by urinary system, a process that is often slow and variable. However, following parenteral administration, urinary system is considered a significant route. In the presence of mastitis, macrolides accumulate concentrations in milk higher than that of the plasma (Lucas et al., 2010).

2.2.8 Tilmicosin precautions:

Tilmicosin injections should be avoided because of cardiotoxicity. Tilmicosin injections increase heart rate and decrease contractility resulting in animal death. Tilmicosin administration should be avoided in lactating dairy cattle and equine species (Kaneko et al., 2007).

2.2.9 Pharmacodynamics properties:

Against extracellular pathogens, macrolides efficacy depends on their free extracellular concentrations and the susceptibility of the targeted organism. Against most of the bacteria located within their spectrum, macrolides show a time-dependent bactericidal effect. The optimum effect of macrolides is detected at pH of 8, with a major decreasing in efficacy when pH level is less than 6. When evaluating the efficacy of macrolides, pH, serum effect, variability of activity and the activity of metabolites are highly considered (Modric et al., 1998). Furthermore, macrolides establish an anti-inflammatory and immunomodulatory features in the host. These features contributes significantly to macrolide overall efficacy (Kovaleva et al., 2012).

2.2.10 Tilmicosin withdrawal period:

Tilmicosin withdrawal period varies between different species. It requires 12 days in chicken, 14 days in pigs and 42 days in cattle. It should be notes that Tilmicosin shouldn't be administered to lactating dairy cattle. Moreover, Tilmicosin is not authorized to be used in egg laying flocks for human consumption, and it shouldn't be used in a poultry flock two weeks prior their onset of egg production (Abdelhakim Elkomy et al., 2018; Ji et al., 2019).

2.2.11 Macrolide resistance:

Initially the clinical importance of resistance against macrolides was originally considered to be low. However, with the abuse use of antibiotics in different parts of the world led to major concerns related to not only macrolide resistance but to all the antibiotics available (Palma et al., 2020).

Macrolide common mechanism of resistance occurs following the modification at the level of ribosome. furthermore, other mechanisms include antibiotic efflux systems, reduced uptake or permeability into the cell, several mutations to ribosomal RNA and proteins, and change of the antibiotic structure by inactivating enzymes (Li, 2016; Palma et al., 2020).

2.3 Methionine:

Methionine is an essential amino acid that plays a vital role in poultry growth and production (Lee et al., 2020). Methionine is considered to be the first limiting amino acids in different poultry diets. Corn-soybean base diets usually fail to fulfill methionine levels required by poultry species (Fagundes et al., 2020). This deficiency is usually corrected during feed formulation with crucial addition of sulfur amino acids. The sulfur amino acids: methionine and cysteine provide the organic sulfur needed by the avian species and joined together they form the total sulfur requirement within the avian body (Pacheco et al., 2018).

Methionine plays a vital role as a building block for proteins involved in the immune system, and to ensure the proper development of feathers (Martínez et al., 2017). Methionine is present in high amounts in sesame meals, Brazilian nuts, corn gluten meal, alfalfa and sunflower seed meal (S. Li, 2015). Yet, it is deficient in most protein plant-based sources used in poultry diet. Therefore, methionine supplementation is needed to correct its deficiency (S. Li, 2015).

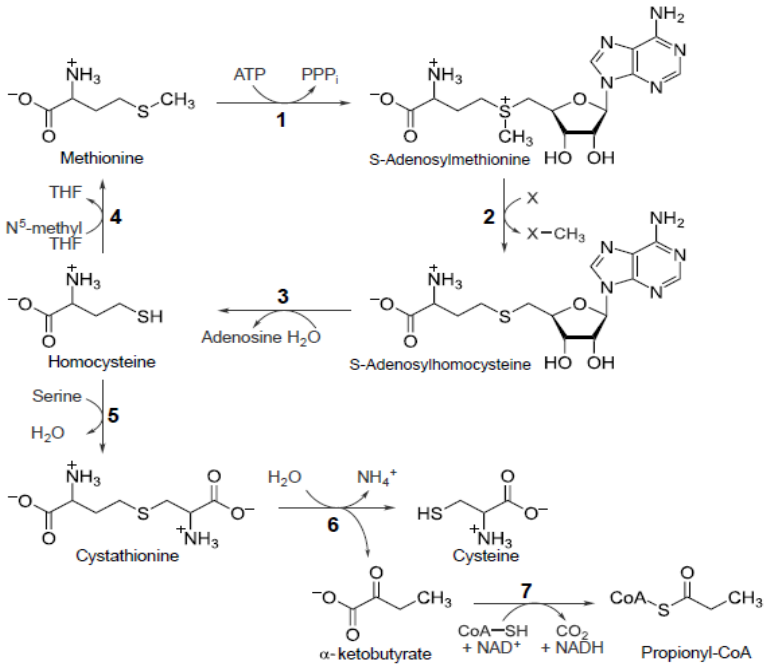
2.3.1 Methionine metabolism:

Methionine IUPAC name 2-amino-methylthiobutanic acid with a chemical formula of C5H11NO2S. Methionine is characterized with its white crystalline powder appearance.

Methionine has a density of 1.340 g/cm3 and a molar mass of 149.21 g/mol, and it is soluble in water (Weast & Astle, 1981).

The liver is considered the main site of methionine metabolism (Finkelstein, 2003). The methionine metabolism (Figure 3) is essential for several physiological processes as it aid of methyl transfer reactions in which methionine is converted into its active S-adenosylmethionine (SAMe) by the catalyzation of methionine adenosyltransferase I (MAT-1) and methionine adenosyltransferase II (MAT 2) (Mato et al., 2008). Moreover, methionine provides a methyl source alongside choline, folic acid and betaine (Bunchasak, 2009). Yet, these compounds differ in their methylation availability and reactions. Methionine is involved in protein synthesis, whereas choline is required primarily for cell membrane and as a neurotransmitter (Metzler-Zebeli et al., 2009).

Figure 2.1: Methionine Metabolism



Following its conversion, SAMe acts as a methyl donor that is involved in methyltransferase reactions , enabling the production of choline, DNA, creatine, epinephrine and several other important compounds (Metzler-Zebeli et al., 2009). Following its methyl group donation, SAMe is transferred into S-adenosylhomcysteine (SAH). Then the adenosyl group is removed from SAH to produce homocsyteine. Finally, homocysteine is either changed into cysteine or methylated back into methionine (Finkelstein, 1990).

First, through the activity of cystathionine B-synthase, the homocysteine molecule is fused into serine to produce cystathionine. Then, using cystathionine-y-lyase enzyme and Vitamin B6 as a cofactor (Samakai, 2016), the produced cystathionine is split into cysteine and alpha-ketobutyrate (Brosnan et al., 2007). Later on, alpha-ketobutyrate is converted by alpha-ketoacid dehydrogenase into propionyl-CoA that undergoes a final conversion into succinoyl-CoA before entering the Krebs cycle and generating ATP/energy. On the other hand, two molecules of cysteine serve as structural blocks for keratin, which is the major protein found in hair, nails, and feathers (Baker, 2009).

Second, through methionine synthase (MS) and the help of vitamin B12 as a cofactor homocysteine is re-methylated from N5-methyltetrahyrofolate to methionine (Brosnan et al., 2007). Furthermore, following choline oxidation into betaine; betaine-homocysteine methyltransferase (BHMT, enables the re-methylation of homocysteine into methionine (Finkelstein, 1990).

The main reason behind considering methionine as an ideal sulfur amino acid rather than cysteine is mainly due to the fact that methionine is an essential amino acid whereas cysteine is not. Moreover, methionine serves as precursor for the production of cysteine; therefore, methionine metabolism alone can provide the total sulfur amino acid requirement. Yet, this doesn't apply to cysteine metabolism in which no enzyme/ metabolic pathway is found to produce methionine from cysteine (Wheeler & Latshaw, 1981).

2.3.2 Methionine requirement:

2.3.2.1 Nutritional requirement:

National Research Council Section Nutrient Requirement of Poultry serves as the major reference for poultry feed formulation. Like any other animal, poultry eat in order to satisfy its metabolic energy requirements. The crude protein incorporated in the diet should contain enough essential amino acids, and enough nitrogen to produce non-essential amino acids. Several factors including: Sex, age, strain, reproductive stage, genetics, immunological system and house temperature etc. affect the nutritional requirement of poultry species (Leeson & Summers, 2001). According to Wu (2009) Dietary amino acid requirement can be separated into quantitative and qualitative need. G.Wu elaborates that qualitative dietary amino acid requirement represents "what" are the amino acids required by poultry species for maintenance, reproduction, optimal performance, and growth. Wu then adds that the quantitative dietary amino acids represent "how" much we actually need from these amino acids are needed (Wu et al., 2014).

Generally, the nutritional requirements viewed in broilers changes with age in which less crude protein is supplied in the diet and more metabolic energy is included. Broilers diets are divided into 3 phases: starter, grower, and finisher. The level of crude protein corresponding to the different phases decreases from 23 in starter to 20 in grower, and to 18% in finisher (Moran Jr et al., 1992).

In poultry, unbalanced diets result in poor growth and performance. Diets with an unbalanced ratio of ME/CP (metabolizable energy/ Crude protein, obliges the birds to over consume to satisfy their amino acids requirements. Such imbalances should be avoided during feed formulation to obtain the optimal growth and performance in poultry flocks (Fanatico, 2010).

Commonly, methionine is the first limiting amino acids in broilers diets, and the second limiting amino acids in corn-soybean diets fed to laying-hens (Liu et al., 2017). According to the National Research Council (1994), methionine requirement in broiler are 0.5% in starter (0-3 weeks), 0.38% in grower (3-6 weeks) and 0.32% in the finisher phase (6-8 weeks).

2.3.2.2 Nutrient requirement in heat stress:

Poultry's nutritional requirements are altered as a response to high ambient temperature. In high temperature and high humidity feed intake is decreased, which results in reducing growth, egg production and poor performance. This feed reduction eventually alters the function of the immune system which makes the birds more vulnerable to disease, leading to higher mortality rates. Therefore, high temperatures significantly reduce the welfare of birds and increase economic losses in the poultry sector (Daghir, 2008).

A combination of methods is implemented to alleviate heat stress waves ranging from housing, feeding practices and management (Daghir, 2008). Moreover, feed formulation manipulation during heat stress is an additional method that could ease the heat stress waves. High ratio of amino acids in diet fed to heat stressed birds results in decreased feed intake (Fi), lower carcass yield and negative body weight gain (BWG) (Attia & Hassan, 2017). Similarly, Leeson and Summer reported that feed intake, body weight gain and carcass yield decreased in heat stressed birds fed high amino acids diets with low apparent metabolizable energy (AME) (Leeson & Summers, 2001).

Significant economic losses in broiler and decreased egg production in laying hens are observed whenever the nutritional requirement of essential amino acids is not met (Jankowski et al., 2014). For that, methionine is supplemented synthetically in the form of D, L-methionine, MHA, and MHAC to meet the dietary requirement. During feed formulation, it's vital to adjust the feed components with respect to the metabolic energy in order to obtain a balanced diet. Birds fed diets that have a high ME to protein ratio tend to over consume from the diet to meet their Essential amino acid requirement, and this resulted in higher fats deposition and lower water content in the carcass (Nahashon et al., 2005). Due to genetic selections, management practices and feed alterations, poultry requirements nowadays differ greatly from those of commercial birds before 1991 (Applegate & Angel, 2014). Others suggested increasing the level of methionine more than the value recommended by NRC in 1994 (Bouyeh, 2012b). Moreover, several studies indicated that the level of methionine required for optimal growth differs from the level required for optimal immunity (Jankowski et al., 2014).

2.3.3 Methionine in oxidative stress:

Physiological and non-physiological processes that take place in the body from Fenton reaction, mitochondrial dysfunction, cellular respiration and stress produce reactive oxygen species (ROS). The produced ROS is removed from the system by endogenous antioxidants such as glutathione peroxidase, glutathione reductase, Superoxide dismutase (SOD) and catalase (CAT). This system allows the removal of low concentrations of ROS, yet when high production occurs, vast cellular disruption, DNA alteration and cell death could occur (Freitas et al., 2016).

The increased cellular level of free radicals and ROS can yield two outcomes: cell death or cell senescence. Cell death is observed as necrosis or apoptosis. Deficiency of ATP molecules lead to cell necrosis whereas apoptosis occurs in the presence of energy conditions. On the other hand ,cell senescence is portrayed as initiation of autophagy and cease of cell cycle (Martínez et al., 2017).

Methionine catabolism in the body yields taurine, glutathione and other metabolites. These metabolites play a vital role in the body's immune system (Ren et al., 2013). In the liver, methionine is catabolized into glutathione which serves as an antioxidant. methionine reduces oxidative stress at the level of the tissue by chelating and removing lead (Martínez et al., 2017).

2.3.4 Effects of methionine on the immune system:

The avian immune system responds to pathogens through two main components: innate and acquired immunity. The latter is composed of :1) humoral immunity that includes the production of specific antibodies by B cells (Sproul et al., 2000) and 2)cell-mediated immune response that involves T cells (Radoja et al., 2006). On the other hand , innate immune system

forms the first line of defense against pathogens and includes physical and chemical barriers, immune cells and gut associated lymphoid tissue (Wershil & Furuta, 2008).

The assessment of immune response magnitude relies on the serval approaches. Classical approach in measuring the immune response includes assessing the level of serum antibodies titers, mainly IgG, which reflects the humoral immunity response. T cell proliferation test, CD4/CD8, blood lymphocytes subgroups as well as serum concentrations of immune mediators like cytokines can also predicts of immune response magnitude. The weight of lymphoid organs, morbidity and rate of recovery from infectious disease provide an insight regarding the functionality of immune system (Bouyeh, 2012a).

Nutrition plays a vital role in supporting immune response against pathogenic microbes (Kogut, 2009). Nutritional factors, including enzymes, antioxidants, amino acids, fatty acids and other components modulate metabolic processes that affect immunoregulatory mediators and, subsequently, the immune response. In 1997, Takahasi described the beneficial effect of both sulfur-containing amino acids,(methionine and cysteine) on immune and inflammatory response. In his experiment, Takashi used two different levels of Cysteine (0.185 or 0.37%) ; the results indicated that higher mononuclear cell proliferation at the level of the spleen was detected in chicken fed higher-cysteine diet in comparison to chicks fed low-cysteine diet (Takahashi et al., 1997). The results align with those obtained Tsiagbe experiment in 1987. Tsiagbe stated that dietary supplementation of methionine enhances the immune system under different catabolic conditions. Enhanced T cell proliferation and IgG titer levels in the presence of Newcastle infection was markedly detected as dietary methionine levels increased. Similar results were obtained as dietary cysteine was increased from 0.185 to 0.37%. However, detrimental effects on

growth and immune response of chicken was observed when methionine and Cysteine levels increased to 1.8 and 0.37% respectively (Tsiagbe et al., 1987). This toxic effect resulted from the high production of toxic substance such as homocysteine and sulfuric acid (G. Wu & Meininger, 2002). Therefore, higher cysteine levels in the diet is considered toxic (Li et al., 2007).

Methionine is essential for the development of humoral and cellular immune response (Swain & Johri, 2000) The proliferation of immune cells that are sensitive to different levels of glutathione and Cysteine levels provides a mechanism to understand methionine interaction with the immune system (S. Shini et al., 2005). Increasing methionine levels above the recommended ones for optimal growth can aid the immune system through protein synthesis and breakdown (direct effect) and through effects of methionine derivatives (indirect effect) (Bunchasak, 2009). Methionine role in cell-mediated system was highlighted by Wu in 2012. In his experiment, methionine deficiency led to ultrastructural pathological changes in the thymus, decreased T-cell population and reduction in Interleukin-2. Therefore, methionine is essential for establishment of a proper cell-mediated immune response (Wu et al., 2012). Similar results were obtained with Wu et al., in 2013, as methionine-deficient diets impaired the growth of bursa of Fabricius and led to decreased B lymphocyte number in the follicles contributed to mitochondrial swellings (G. Wu, 2013). Therefore, a 0.5% of methionine is required for proper growth in commercial broilers (Deng et al., 2007; Rao et al., 2003; Swain & Johri, 2000); however, higher levels of methionine are needed to induce a better immune response.

Bouyeh in 2012 investigated the effect of increasing methionine content from 0.5 to 0.65 and 0.7% in starter period and from 0.4 to 0.52 and 0.56% in the grower period. Results in Bouyeh experiment indicate that IgG titer against Newcastle infection increased as dietary methionine increased. Furthermore, the increase in methionine increased lymphocytes in the

serum (Bouyeh, 2012b). These findings highlight the need of excess methionine than the NRC recommended levels to obtain optimal immune response. Excess methionine inclusion in the diet influenced the cecal microflora by enhancing Streptococcus and reducing *Clostridium perfringes* (Dahiya et al., 2007). In layer hens, the effect of different dietary methionine levels 0.3, 0.36, 0.42) was investigated. The obtained results highlighted the need for additional dietary methionine (0.36%) than the recommended level (0.3%) in order to achieve optimal immunity (Panda et al., 2007).

CHAPTER 3

Materials and methods

3.1 Housing:

This experiment was approved by the Institutional Animal Care and use Committee (IACUC) of the American University of Beirut. It was conducted at the Advancing Research Enabling Communities Center in the Beqaa region where qualified poultry houses are available. The selected poultry house was divided using a nylon cover sheet to separate between vaccinated and non-vaccinated treatments. The house was cleaned with soap and then disinfected. Sterile wood shavings of 5 cm depth were spread in each pen. Moreover, two adjustable feeders and two bell-matic waterers were added to each pen as well. Heaters were used to maintain the temperature throughout the experiment. On the entrance of the poultry house a platter containing a disinfectant was placed. Shoe cover, gloves and lab coats were used during sample collection. Strict biosecurity measures were applied to avoid cross-contamination between treatments.

3.2 Experimental period:

The experiment was approved by the Institutional Animal Care and Use committee (IACUC) of the American university of Beirut and was completed at AUB-AREC over a period of 8 weeks effective of the birds' arrival date.

3.3 Birds, Treatment and Experimental Design:

A total of 276 six-week old breeder pullet, of the Ross 308 strain, were randomly allocated into four treatments, each with three pen replicates of 23 birds/pen. Pullets were given water *ad libtuim* and feed as per the Breeder Manual recommendation provided the breeding company. Upon arrival, Swab samples were taken from the trachea of 12 birds to confirm that the birds were MG-free using Frey's culturing method and real time-Polymerase Chain Reaction (q-PCR). Furthermore, blood samples were drawn from the wing vein of 12 birds and collected in EDTA-tubes. ELISA test was performed to confirm the absence of antibodies titer to *Mycoplasma gallisepticum*.

The trial constituted of four treatments in completely randomized design. The treatments were: 1) Vaccinated with excess methionine, 2) vaccinated with adequate methionine, 3) non-vaccinated with excess methionine, 4) non-vaccinated with adequate methionine. Birds were assigned to different treatments, including vaccination via drinking water with AviPro MGF, and provision of dietary methionine as indicated in table 3.1.

Table 3.1 Treatment allocation to different groups of broiler breeder pullets

Treatment number	Treatment	Abbreviation	Vaccination with AviPro MGF*	Methionine level
1	Vaccinated-Excess methionine	VEM	+	Excess
2	Vaccinated-Adequate methionine	VAM	+	Adequate
3	Non-Vaccinated Excess methionine	NVEM	-	Excess
4	Non-Vaccinated Adequate methionine	NVAM	-	Adequate

*AviPro MGF vaccine was administered to broiler breeder pullets in drinking water at six weeks of age.

3.4 Diets:

A corn-soybean meal-based diet was formulated to meet company's requirement. Groups 2 and 4 were fed the adequate Methionine diet containing 0.38g Methionine/Kg of diet. Groups 1 and 3 were fed diets that contained twenty percent methionine in excess of the required amount, containing 0.456g methionine/Kg die. The composition of the experimental diets is presented in Table 3.2.

Table 3.2 Experimental diets:

	Control diet	Excess methionine diet
Energy per kg	2800	2800
Amino acids	Total	Total
Lysine	0.68%	0.68%
Methionine + Cysteine	0.63%	0.72%
Methionine	0.38%	0.47%
Threonine	0.54%	0.54%
Valine	0.64%	0.64%
Isoleucine	0.56%	0.56%
Arginine	0.84%	0.84%
Tryptophan	0.16%	0.16%
Leucine	0.84%	0.84%
Crude protein	14-15%	14-15%
Minerals		
Calcium	0.90 %	0.90 %
Available phosphorous	0.42%	0.42%
Sodium	0.18-0.23%	0.18-0.23%
Chloride	0.18-0.23%	0.18-0.23%
Potassium	0.4-0.9%	0.4-0.9%

3.4 Evaluation of MG colonization in the trachea:

3.4.1 Sample collection:

A total of 10 individual tracheal swabs per pen (30/trt) were taken at 4 different dates post-vaccination, namely 9, 11, 12, and 14 weeks of age. Swab rubbings were collected in 2 ml of Frey's broth and then were equally divided into two separates sterile microtubes (1ml/tube) to be tested for the presence of *Mycoplasma gallispeticum* using Frey's culturing method (Frey et al., 1968) and Polymerase Chain Reaction (Grodio et al., 2008)

3.4.2 Quantification of MG colony forming unit using culture and real time PCR (qPCR):

3.4.2.1 Culture:

3.4.2.1.1 Yeast Extract:

1-An amount of 250g of yeast is soaked in 1 liter of distilled water for 1 hour and then heated until boiling

2-After cooling, the sample is distributed in 50 ml tubes and centrifuged for 20 mins at 3000 xg

3- Then the collected supernatant is filtered through Whatman No.1 filter paper and its pH is adjusted to 8.

4-The extracts are then filtered through 0.8 Mm filter paper and filter sterilized through a 0.22µm filter paper.

5-Aliquots of 15 ml of the sterile extract are distributed in sterile cups and stored at -20C.

3.4.2.1.2 Inactivation of the swine serum:

1- Sterile swine serum (Gibco, Grand Island, N.Y.14072,USA) is heat inactivated in a water bath at-55C for 30 minutes.

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

3.4.2.1.3 Frey's broth preparation:

The broth was prepared as described by (Frey et al., 1968) as follows:

1- In order to prepare 150ml of Frey's broth: 3.37g of Mycoplasma Broth base (OXid LTD. Bansingstoke, Hampshire,England), 375 microliter of 10% w/v phenol red and 425 microliter of 10% thallium acetate are added to 113.425 ml of distilled water.

2- The mixture is boiled, and the pH is adjusted to 7.8 using 0.1 N NaOH

3- After sterilization at 121C and 20 psi for 15 minutes, the mixture is cooled and 15 ml of yeast extract, 18 ml of heat inactivated swine serum, 750 Microliter of Penicillin (150,000 I.U) and 2 ml of filter sterilized dextrose (0.225g/ml) are added to the mixture.

4- The pH of the mixture is then readjusted to 7.8 with sterile 0.1 NaOH

5-The broth is distributed into sterile screw-capped tubes (2ml/tube)

3.4.3 Culturing protocol:

The first aliquot of the tracheal rubbings in Frey's broth (1 ml/tube; 10 samples per replicate) was incubated at 37C for one week. Positive samples were recorded when the broth color turned from red to orange indicating bacterial growth at the log phase. This color change is a result of sugar fermentation and drop in pH, within a range of four to seven days.

3.4.4 Real time PCR assays:

Mycoplasma gallisepticum DNA was extracted by immersing the second aliquot of tracheal rubbings in Frey's broth (1 mL/tube; 10 samples per pen) in water at 100°C for 10min and then placing it in ice for 10 mins. The q-PCR protocol used to detect MG-Colonization was divided into three phases, 1) Denaturation: 95°C for 3 mins, 2) Annealing at 95°C for 12 sec, and

3) extension at 60°C for 1 min, for 40 cycles (Grodio et al., 2008). The dilution of the added forward/reverse primers and probes were done according to the manufacturer recommendation.

The 20 µl PCR mixture analysis was performed in CFX96 Touch™ Real Time PCR detection system (BioRad laboratories, 2000 Alfred Nobel Drive, CA, USA). The q-PCR reaction targeted the *mgc*2 segment of MG and used 10 µl TaqMan iTaq PCR Mix (BioRad laboratories, 2000 Alfred Nobel Drive, CA, USA), 1 µl of each of 5 pmol forward and reverse primers and the probe , 4 µL DNase-free water and 3 µl DNA of samples or standards. Table 3.3 indicates the volume of each reagent used in the prepared q-PCR mixture per sample

Table 3.3 Reagents used in the q-PCR mixture to amplify mgc2 gene of MG

Reagent	Concentration	Volume (µL)
iTaq	2X	10
Probe	5 pmol	1
Forward primer	5 pmol	1
Reserve primer	5 pmol	1
PCR-water	-	4
Sample DNA	-	3
Total		20

3.5 Blood collection for Complete Blood Count (CBC) and seroconversion studies

Blood samples were collected from 5 birds per pen (15 birds per treatment) before vaccination, and at weeks 9, 11 and 14 of age for *Mycoplasma gallisepticum* seroconversion and Blood CBC. A volume of 3 ml of blood samples was drawn from the wing vein, in EDTA tubes (Ethylenediaminetetraacetic acid). Hematology analysis, namely Complete Blood Count (CBC)

was done in the same day using Mythic^{TM} veterinary hematology analyzer (Orphée, CH 1228 Plan les Ouates, Switzerland). The analyzed hematological parameters were: (WBC, LYM, MON, GRA, RBC, HCT, HGB, MCH, MCHC and RDW). Then, the blood was allowed to settle for one hour at room temperature and then centrifuged for 10 minutes at 2000 rpm to collect the sera that were later preserved at -20°C until further analysis with ELISA. ELISA kits (IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine 04092, United States) were used to assess the levels of anti-MG sera titers at 6, 9, 11 and 14 weeks of age. The ELISA protocol was as follows:

3.5.1 Reagents and Samples preparation:

1- All samples and Elisa kit reagents were brought to room temperature for 2 hours before starting the ELISA procedures
2- All samples were vortexed before the dilution

3.5.2 Sera dilution:

1- Chart for each plate was designed to locate the corresponding sample in each well.
2- First serum dilution (1/50): a volume of 200 microliters of the diluent was added into each well of the dilution plate, then 5 microliter of the serum sample was adde3d and mixed 6 times with the diluent.
3- Second serum dilution 1/(500): a volume of 225 microliters of the diluent was added to wells of a new dilution plate, then 25 microliters of each diluted serum samples (from step 2) were added and mixed with the diluent 6 times.

3.5.3 ELISA procedure:

1- A 100 microliter volume of the negative control was dispensed into duplicate plates
2- A 100 microliter volume of the positive control was dispensed into duplicate plates
3- A 100 microliter volume of diluted samples (1/500) were dispensed into appropriate plates.
4- Plate was incubated for 30 min at 18-26C
5- Each well is washed with 350 microliters of distilled or deionized water 3-5 times. Aspirate completely
6- A 100 microliter volume of conjugate was dispensed in each well
7- Plate was incubated for 30 minutes (+/-) 2 mins at 18-26C.
8- Step 6 was repeated
9- A 100 microliter volume of TMB substrate solution was dispensed in each well
10- Plate was incubated for 15 mins (+ -1 minute) at 18-26C
11- A 100 microliter volume of stop solution was dispensed into each well to stop the reaction
12- The ELISA test values were measured at 650 nm absorbance using ELISA reader Thermo Scientific™ Multiskan™ go microplate spectrophotometer (Thermo fisher Scientific, 2 Friras Drive, Hudson, New Hampshire 03051, US)

For the ELISA test to be valid, the difference between positive control absorbance mean and negative control mean (PCx-NCx) should be greater than 0.075. The negative control reading

should be less than or equal to 0.150. Endpoint titers are automatically calculated using the xCheck software equation described in the calculation below (IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, Maine 04092, United States):

Positive-mean-control= $\mathrm{PC}\bar{x} = \frac{\mathrm{PC1\ A(650)+\ PC2\ A\ (650)}}{2}$

Negative mean control= $\mathrm{NC}\bar{x} = \frac{\mathrm{NC1\ A(650)+\ NC2\ A(650)}}{2}$

S/P ratio: $\frac{Sample\ Mean - NC\bar{x}}{PC\bar{x} - NC\bar{x}}$

Log_{10} Titer= 1.09(log_{10} S/P)+ 3.36

3.6 Determination of Spleen and Thymus indices:

At the end of the experiment (14 weeks of age) 3 birds/pen (9 birds/treatment) were sacrificed by CO_2 suffocation and the weight of each bird was measured. The spleen and thymus were then removed from each bird and weighed. The weight index of each organ was calculated as per the below formula:

Organ index = organ weight (g) x 100/ Bird live body weight (g)

3.7 Determination of trachea and air sac macroscopic lesion scores:

Tracheas and air-sac macroscopic lesions were determined for the same birds that were sacrificed as per the above paragraph. Tracheas were examined for lesions which were given a scale of 0 or 1 with 0= negative tracheitis, 1 = mild tracheitis. Airsacs were also examined for the

presence of lesions which were given a score of 0 or 1 with 0 = negative airsacculitis and 1 = mild airsacculitis.

3.8 Statistical analysis:

This study was designed in a complete randomized design with 4 treatments of 3 replicates of 23 birds/pen. Statistical analysis was done using One-way ANOVA on SPSS. Tukey's honest significance test was used to detect statistical significance ($P<0.05$) between the log MG CFU means, sera titers means and blood parameters. Frequencies of positive swab culture MG and lesion scores were analyzed using Chi-square.

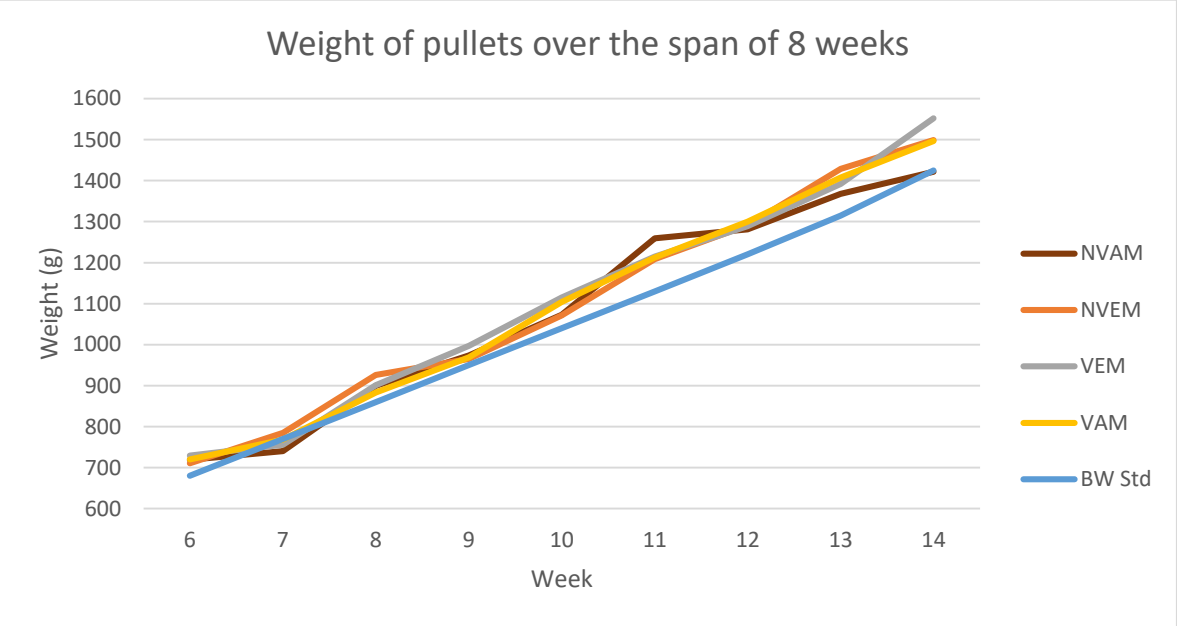
CHAPTER 4

Results and Discussion

4.1 Weight:

The effect of methionine supplementation and AviPro® vaccination on the live body weight of pullets over the span of eight weeks was recorded in Figure 4.1. No significant difference ($P<0.05$) was observed across the four different treatments. These results indicate that the vaccine administration did not affect the body weight. These results are in agreement with results recorded in an experiment conducted previously by (Farran et al., 2019). Highlighting the safety of AviPro® vaccine as the as the birds were not affected by an immune over-reaction or frustration. Furthermore, methionine level in the diet whether in adequate or excess quantities did not impact the body weight of the pullets. Yet, several works reported that increasing methionine level in the diet can significantly decrease the abdominal fat content in white pekin ducks, Yangzhou geese and broilers (Andi, 2012; Wang et al., 2010; Xie et al., 2006). Moreover, recorded body weights were similar to those expected in the Aviagen manual (Figure 4.1), indicating good feed and management practices.

Figure 4.1 Live body weight of Ross 308 breeder pullets from 6 till 14 weeks of age. NVAM = Non-vaccinated, adequate methionine, NVEM = Non-vaccinated, excess methionine, VEM = Vaccinated with AviPro® MGF, excess methionine, VAM = vaccinated with AviPro® MGF, adequate methionine. BW Std= Body weight standard (Ross 308 AP: Parent Stock: Performance Objective).



4.2. Evaluation of MG colonization in the trachea:

4.2.1. Real-time PCR and MG DNA load in tracheal tissues:

In the present experiment, the MG load was assessed in tracheal tissues, one of *Mycoplasma gallispeticum* major site of colonization. q-PCR was used to deduce the bacterial load as it proved to be a highly sensitive, reproducible and cost-effective method for detection and quantification of avian Mycoplasmosis (Grodio et al., 2008). Negative samples reflect that *Mycoplasma gallispeticum* DNA load are less than the sensitivity threshold of the used RT-PCR which is 1.4 ng. q-PCR implied Colony Forming Units (CFU) for each pen namely at 9, 11, 12

and 14 weeks of age were subjected to log transformation before analysis. Results are shown in Table 4.1.

Table 4.1 Log 10 *Mycoplasma gallisepticum* Colony Forming Units (CFU) count per mL of Frey's broth tracheal swab suspensions as implied from q-PCR assays.

Treatment*	qPCR implied-Log10 MG CFU count/ ml Frey's broth at***				
	Week 9	Week 11	Week 12	Week 14	SEM
NVAM	0.00	0.00[a]	0.00[a]	0.00[a]	0.00
NVEM	0.00	0.00[a]	0.00[a]	0.00[a]	0.00
VEM	0.00	0.92[b]	0.82[b]	0.75[b]	0.169
VAM	0.00[1]	1.85[b,2]	5.90[c,3]	5.31[c,3]	0.263
SEM**	0.00	0.168	0.252	0.225	

*NVAM = Non-vaccinated, adequate methionine, NVEM = Non-vaccinated, excess methionine, VEM = Vaccinated with AviPro® MGF, excess methionine, VAM = vaccinated with AviPro® MGF, adequate methionine

**Standard Error of Mean

***Average of 10 birds/pen (30 birds/treatment)

[a-c]Means with different alphabetical superscripts in the same column are significantly different ($P<0.05$).

[1-3]Means with different numerical superscripts in a row are significantly different ($P<0.05$)

In non-challenged groups (NVAM: non-vaccinated, adequate methionine & NVEM: non-vaccinated, excess methionine) negative results were recorded throughout the experiment as

indicated in Table 4.1. It is known that *Mycoplasma gallispeticum* can be highly transmitted horizontally resulting in high rate of insect and disease manifestation in the flock (Ley et al., 2008). These results indicate the success in implementing strict biosecurity measures from the beginning until the end of the experiment.

As indicated earlier in the Materials and Methods section (3.3), the vaccine was administered at week 6 of age via drinking water. Negative results were recorded at week 9 (three weeks post-vaccination) across both vaccinated groups (VEM: Vaccinated, excess methionine and VAM: Vaccinated, adequate methionine). At week 11 (five weeks post-vaccination), significant q-PCR implied CFU results were observed in groups VEM and VAM (0.92 and 1.85 log10 CFU/ml Frey's broth) in comparison with unchallenged groups NVEM and NVAM (0.0 log10 CFU/ml Frey's broth). A week later, both groups VEM and VAM were still showing significant MG count results in the trachea (0.82, 5.9) in comparison to the non-vaccinated groups NVAM and NVEM. However, CFU count detected in groups VAM was significantly higher than that detected in group VEM. Similar trend was observed at week 14 for VEM and VAM recording values of 0.75 and 5.31, respectively ($P<0.05$).

While PCR lacks the ability to distinguish between live and dead cells, positive swab cultures were used to confirm the presence of live *Mycoplasma gallispeticum* bacteria colonizing the trachea. Percentage of MG positive swab cultures shown in Table 4.2 represent a mirror image and a further confirmation of the results obtained in Table 4.1.

Table 4.2 Percentage of MG positive swab samples (culture) collected from the broiler breeder pullets at different ages

Treatment*	Percentage of positive swab samples at**:			
	Week 9	Week 11	Week 12	Week 14
NVAM	0.0	0.0[a]	0.0[a]	0.0[a]
NVEM	0.0	0.0[a]	0.0[a]	0.0[a]
VEM	0.0[1]	23.3[b,2]	40.0[b,2,3]	50.0[b,3]
VAM	0.0	30.0[b,2]	86.6[c,3]	82.1[c,3]

*NVAM = Non-vaccinated, adequate methionine, NVEM = Non-vaccinated, excess methionine, VEM = Vaccinated with AviPro® MGF, excess methionine, VAM = vaccinated with AviPro® MGF, adequate methionine

**Samples collected from 10 birds/pen (30 birds/treatment)

[a-c]Percentages in a column with different alphabetical superscripts are significantly different ($P<0.05$).

[1-3]Percentages in a row with different numerical superscripts are significantly different ($P<0.05$).

Table 4.2 represent the percentage of MG positive swab samples (culture) collected from the broiler breeder pullets at different ages. Complete absence of positive sample in non-vaccinated groups NVAM and NVEM is another conformation on the success of implanting strict biosecurity measure during the experiment.

Further alignment between CFU count and percentage of MG positive swab samples were observed at weeks 11, 12 and 14. Group VEM showed a significantly higher percentage of MG positive swab samples in comparison with unchallenged groups, yet a significantly lesser percentage of MG positive swabs than that observed in group VAM. These results validate the difference in MG colonization rate of the tracheal tissues between the two vaccinated groups.

The colonization pattern of different avirulent *Mycoplasma gallisepticum* strains at the level of respiratory tissues of poultry is well recorded. *Mycoplasma gallispeticum* requires 1-4 weeks to colonize the mucosal linings depending on the virulence of the strain (Kleven, 1998; Ley et al., 2008). This colonization pattern wasn't similar when AviPro® vaccine was administered for groups VEM and VAM in this experiment. AviPro® required 5 weeks following its administration to significantly colonize tracheal tissues of the pullets. Furthermore, the colonization growth was hindered considerably by 20% excess methionine fed to group VEM.

The colonization pattern of AviPro® vaccine observed in this experiment different from that observed in the previous experiment conducted by Farran et al. (2019), where AviPro® vaccine was detected in the tracheal swabs 3 days following its administration. These results indicate a variable growth pattern of AviPro® vaccine. This variability in growth pattern suggests a batch-effect that could have stemmed from a defect at the level of the chain of production. Moreover, Levisohn and Dykstra (1987) reported that F-strain vaccine did not provide protection by competitive exclusion against Mycoplasma infections in chickens (S Levisohn & Dykstra, 1987). These findings highlight also the low infectious process of F-strain and its low ability to colonize tracheal tissues.

Furthermore, 20% excess dietary methionine altered AviPro® colonization of the tracheal tissues by one week and significantly decreased the amount of log10 CFU present at the tracheal tissues at week 12 and 14 in comparison with the group fed adequate methionine (VAM). These observations are in agreement with abundant literature emphasizing the role of dietary methionine in altering the colonization pattern of several etiologic agents of animal diseases. For instance, Dahiya in 2007 observed a reduction in *Clostridium perfringes* colonization pattern in

response to supplementation of dietary methionine. In juvenile Jian carp (oily freshwater fish) dietary methionine supplementation increased the gastrointestinal tract Lactobacillus count and decreased *Escherichia coli* and *Aeromonas counts* (Tang et al., 2009). In addition, Ramadan et al. (2019) reported the ability of 20% excess dietary methionine in decreasing deciliation, improved coherence of mucosal layers and integrity of goblet cells against *Mycoplasma gallispeticum* infection (Ramadan et al., 2019). These findings highlight the role of dietary methionine in enhancing the integrity of mucosal-associated lymphoid tissues thus enhancing structural innate immunity.

4.3. Sera titers to MG:

Table 4.3 shows the sera titers related to *Mycoplasma gallispeticum* of four different groups recorded at different weeks post-vaccination with AviPro® MGF.

Table 4.3 Sera titers (IgG) to MG of birds vaccinated at 6 weeks of age at different weeks post-vaccination with AviPro® MGF

Treatment*	Sera Titers*** at:		
	Week 9	Week 11	Week 14
NVAM	165	54[a]	198[a]
NVEM	89	113[a]	83[a]
VEM	210	981[b]	1708[b]
VAM	107	560[ab]	2116[b]
SEM**	32.1	96.9	1363.7

*NVAM = Non-vaccinated, adequate methionine, NVEM = Non-vaccinated, excess methionine, VEM = Vaccinated with AviPro® MGF, excess methionine, VAM = vaccinated with AviPro® MGF, adequate methionine

**Standard Error of Mean

[***]Average of 5 birds/pen (15 birds/treatment)

[a-c]Means in a column with different alphabetical superscripts are significantly different (P<0.05).

On the first day of vaccination, the sera titers to MG were below detectable levels of ELISA kit for the all groups, confirming the absence of MG titers in birds at the beginning of the experiment (6 weeks of age).

In non-challenged groups (NVAM, NVEM) low titers against *Mycoplasma gallispeticum* were present in sera samples obtained namely at week 9, 11 and 14 of age. Titers obtained from these groups were lower than 1076 and therefore considered negative as per the indication of the ELISA kit manufacturer (IDEXX MG). These negative results offer an additional proof on the success of strict biosecurity measures implemented to prevent cross-contamination between challenged and non-challenged groups.

At week 9 and 11 (three and five weeks post-vaccination respectively), the AviPro® MGF failed to produce significant levels of IgG titers against *Mycoplasma gallispeticum* in groups both vaccinated groups VEM and VAM. However, at week 14 (8 weeks post-vaccination) positive results (>1076) were recorded in vaccinated groups as AviPro® MGF was able to induce significant (P<0.05) amount of IgG titers to MG (VEM: 1707.5; VAM; 2115.5) in comparison to non-vaccinated groups. The obtained results align with those of Farran et al. (2019) as AviPro® MGF vaccine needed 7 weeks to induce positive IgG titers against MG in breeder pullets. On the contrary, Abdel Motelib and Kleven (1993) were able to detect positive ELISA titers 30 days post-vaccination with an MGF live vaccine (Abd-el-Motelib & Kleven, 1993). Likewise, Rodriguez and Kleven (1980) detected antibodies against *Mycoplasma*

gallispeticum F strain 12 days post-exposure and remained positive until the end of the experiment (4 weeks post vaccination). These results indicate that AviPro® MGF vaccine used in this experiment requires longer duration to induce a humoral immune response.

In this study the vaccine needed 8 weeks to produce seroconversions in pullets. This seroconversions could be linked to the high MG load in the tracheal tissues of birds at week 14 of age in Table 4.1 Similar results were observed using AviPro® MGF in the work of Farran et al. (2019) where the highest IgG titer was detected as CFU in the trachea reached its peak. These results suggest an association between bacterial count at the site of infection and serum antibodies. Additional experiments are needed to investigate such correlation.

4.4 Blood parameters:

Hematological studies and blood parameters are reliable in diagnosing the structural and functional status of the animal's body (Elagib & Ahmed, 2011). Hematological changes are usually monitored to understand different influences of environmental, nutritional and pathological factors (Garacyk et al., 2003). Blood parameters of breeder pullets at various age are presented in table 4.4.

Table 4.4 Blood parameters of Ross 308 breeder pullets at 9, 11 and 14 weeks of age

Age	Treatment*	Blood parameters***										
		WBC (10^3/µl)	Lym (%)	Mono (%)	Gra (%)	RBC (10^6/µl)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (µm)
Week 9	NVAM	143.5^{c}	18.2^{a}	6.9^{a}	75.5^{d}	3.14^{b}	12.1^{b}	27.1^{b}	84.2^{b}	38.5^{a}	45.8^{a}	12.2
	NVEM	143.2^{c}	22.9^{b}	8.0^{b}	69.1^{c}	2.87^{a}	11.5^{ab}	24.3^{a}	84.9^{b}	40.2^{ab}	47.5^{a}	11.9
	VEM	111.6^{b}	39.6^{c}	9.7^{c}	51.6^{b}	2.69^{a}	11.1^{a}	22.3^{a}	82.0^{ab}	41.4^{b}	50.5^{b}	11.8
	VAM	94.7^{a}	47.8^{d}	9.9^{c}	42.2^{a}	2.90^{a}	11.8^{ab}	22.9^{a}	79.0^{a}	41.0^{b}	51.9^{b}	12.0
	SEM**	2.84	1.63	0.20	1.80	0.04	0.11	0.38	0.51	0.27	0.42	0.12
Week 11	NVAM	137.3	24.6	8.1	67.5	2.85	11.2^{a}	22.9^{a}	89.3	39.4^{a}	44.5^{a}	11.6
	NVEM	134.5	26.6	8.1	67.7	2.90	11.6^{ab}	25.5^{b}	87.8	40.8^{ab}	46.7^{b}	11.9
	VEM	129.2	27.1	8.4	64.6	2.88	11.9^{ab}	25.6^{b}	87.9	41.5^{b}	46.6^{b}	11.4
	VAM	133.0	25.2	7.9	66.9	3.05	12.6^{b}	26.4^{b}	88.9	42.2^{b}	47.3^{b}	11.2
	SEM	1.8	0.96	0.11	0.86	0.39	0.15	0.37	0.36	0.3	0.29	0.15
Week 14	NVAM	114.3	30.6	8.7	62.9	3.03^{ab}	13.2^{b}	26.7^{ab}	88.7	43.9^{ab}	49.5^{bc}	11.4
	NVEM	121.0	28.2	8.4	65.3	2.90^{a}	12.4^{a}	25.2^{a}	86.5	44.8^{b}	50.5^{c}	11.4
	VEM	119.6	28.8	8.3	62.0	3.30^{b}	12.8^{ab}	27.6^{b}	86.3	41.5^{a}	46.9^{a}	11.9
	VAM	119.8	28.7	7.9	65.9	3.24^{b}	12.8^{ab}	27.3^{b}	86.4	41.0^{a}	47.2^{ab}	11.9
	SEM	1.362	0.96	0.13	1.06	0.04	0.1	0.29	0.65	0.46	0.38	0.19

*NVAM = Non-vaccinated, adequate methionine, NVEM = Non-vaccinated, excess methionine, VEM = Vaccinated with AviPro® MGF, excess methionine, VAM = vaccinated with AviPro® MGF, adequate methionine

**Standard Error of Mean

***Average of 5 birds/pen (15 birds/treatment). WBC = White blood cells, Lym = Lymphocytes, Mon = Monocytes, Gra = granulocytes, RBC= Red Blood Cells, HGB: Hemoglobin, HCT: Hematocrit, MCH = Mean Corpuscular Hemoglobin, MCHC = Mean Corpuscular Hemoglobin Concentration, RDW = Red Blood Cells Distribution Width

$^{a-d}$Means in a column with different alphabetical superscripts are significantly different ($P<0.05$).

At week 9 (three weeks post-vaccination) significant differences ($P<0.05$) were observed among the four treatments. Highest white blood cells count was observed in NVAM group whereas the lowest was recorded in the vaccinated adequate methionine group (143.5 vs 94.7, respectively). On the other hand, lymphocyte and monocytes percentage were significantly higher ($P<0.05$) in vaccinated groups regardless of methionine supplementation in comparison with non-vaccinated groups (NVAM, NVEM). In response to an infection or injury monocytes are released into blood to reach the site of interest. These monocytes reach the site of infection and phagocytose pathogens and secrete different set of chemokine to recruit other immune cells (Chiu & Bharat, 2016). Therefore, the increase in lymphocyte and monocyte production reflects the immune response stimulated against AviPro® MGF vaccine in groups VEM and VAM. No significant results difference was observed in White blood cells count, lymphocytes and monocytes percentages across the four different treatments at 11 and 14 weeks of age.

In avian species, red blood cell count ranges between 2.2-3.2 x 10^6/µl (Mitchell & Johns, 2008). RBC count across the four treatments lies within the normal range, yet significant difference ($p<0.05$) was recorded as group NVAM showed highest RBC count (3.14 x10^6µl) in comparison with the three other groups at 9 weeks of age .Hematocrit represents the volume percentage of RBC in blood, and hemoglobin is a protein found in the blood cells that carries oxygen from the lungs to the rest of the body. Therefore, an increase in red blood cells is expected to be cause in an increase in hematocrit and hemoglobin levels (Mitchell & Johns, 2008).

In our study, group NVAM demonstrated the highest amount of hematocrit (12.1g/dl) and hemoglobin whereas, group 3 exhibited the lowest amount of hematocrit (11.1g/dl) across the treatments at 9 weeks of age. On the contrary, group NVAM exhibited lowest concentration of hematocrit and hemoglobin at week 11 (11.2 g/dl, 22.9%). The absence of a pattern in the changes between red blood cells, hemoglobin and hematocrit decrease the possibility of finding a correlation between these blood parameters, methionine and AviPro® MGF.

Vogel, in 2008, noted that *Mycoplasma gallisepticum* R_{low} invades chicken erythrocytes during infection (Vogl et al., 2008). As a result, MG decreases RBC count. This wasn't the case with AviPro® MGF vaccine as no significant decrease was observed in RBC in vaccinated groups. Furthermore, Lam (2004) reported morphologic changes in chicken cells in vitro after exposure to *Mycoplasma gallispeticum* (Lam, 2004). Such effect was not recorded in this study, as RDW (Red blood cell distribution width) did not exhibit any change in vaccinated groups. AviPro® MGF vaccine's slow colonization rate and delayed humoral immunity explain the mild pathogenicity of the used strain. Therefore, its inability to invade or alter RBC is another conformation of its low virulence.

In regard to the impact of excess dietary amino acid supplementation on specific blood parameters, Abdul Wahed (2016) reported a significant increase in hemoglobin in chicken fed methionine and lysine in comparison with control group. Abdul Wahed suggested that this increase may be related to increased availability of amino acids utilized in construction of globulins (blood proteins) (Wahed, 2016). These findings are in agreement with the results found by Al-Daraji (2012) as L-arginine dietary supplementation

(3%) improved productive and physiological traits of Japanese quail (Al-Daraji et al., 2012). Such interaction between supplementation of excess amino acids and improved blood parameters was not recorded in this study.

4.5 Lesions score in air sacs and tracheas at the end of the experiment:

Tracheas and air sac macroscopic lesions were determined in 15 birds per treatment. Tracheas were examined for lesions which were given a scale from 0 to 2 with 0 = negative tracheitis, 1 = positive tracheitis. Air sacs were also examined for the presence of lesions which were given a score of 0 or 1 with 0 = negative airsacculitis and 1 = mild airsacculitis. Results are shown in Table 4.5.

Table 4.5 Percentage of Ross 308 broiler breeder pullets showing positive lesions score in Airsacs and tracheas at the end of the experiment (14 weeks of age)

Treatment*	Percentage of pullets showing positive lesions** score in:	
	Air sacs	Trachea
NVAM	77.8	44.4
NVEM	55.5	44.4
VEM	44.4	55.5
VAM	75.0	33.3

*NVAM = Non-vaccinated, adequate methionine, NVEM = Non-vaccinated, excess methionine, VEM = Vaccinated with AviPro® MGF, excess methionine, VAM = vaccinated with AviPro® MGF, adequate methionine

** Lesions observed in 5 birds/pen (15 birds/treatment)

According to Table 4.5, no significant difference is observed in the frequency of positive lesions scores across different treatment groups in airsac and trachea, ranging between 44.4% and 77.8% for airsacs, and 33.3% and 55.5% for tracheas. It's well documented that Mycoplasma strain is crucial in dictating the incident and severity of airsacculitis and tracheitis (Rodriguez & Kleven, 1980). Furthermore, there may be an association between the tissue damage and stimulation of humoral immunity. In their study, Rodrigues and Kleven (1980) observed a correlation between pathogenicity expressed as airsac lesion score and hemagglutination inhibition antibodies, which aid the proposed association. However, it is unclear whether the absence of humoral immune response is related to marginal degree of tissue damage or to a difference in antigenic structure. Therefore, the low airsacculitis and tracheitis observed in vaccinated groups align with low colonization pattern viewed over the span of 8 weeks.

4.6 Thymus and Spleen weight indices of Ross 308 broiler breeder pullets:

The thymus and spleen weight indices of the pullets (14 weeks of age) used in this experiment are presented in Table 4.6.

Table 4.6. Thymus and Spleen weight indices of Ross 308 broiler breeder pullets at the end of the experiment (14 weeks of age)

Treatment*	Thymus index***	Spleen index***
NVAM	0.052	0.080
NVEM	0.043	0.085
VEM	0.037	0.092
VAM	0.041	0.085
SEM**	0.003	0.004

*NVAM = Non-vaccinated, adequate methionine, NVEM = Non-vaccinated, excess methionine, VEM = Vaccinated with AviPro® MGF, excess methionine, VAM = vaccinated with AviPro® MGF, adequate methionine

**Standard Error of Mean

*** Means of 5 birds/pen (15 birds/treatment)

No significant differences in thymus and spleen indices were observed between the four different treatments, recording values ranging between 0.037 and 0.052% for the thymus index, and 0.08 and 0.092% for the spleen index. AviPro® MGF vaccine used in this experiment didn't influence the growth or atrophy of the thymus or spleen. On the contrary, Adler in 1973 reported an increase in spleen indices 10 days after the exposure to *Mycoplasma gallisepticum* infection. In his experiment, Adler et al (1973) viewed increased proliferation at the level of the spleen in challenged groups in comparison with other groups. This was not the case as the spleen indices didn't vary between the treatments. The low pathogenicity of used the strain expressed in its low colonization rate

and delayed stimulation of humoral immunity and mild airsacculitis/tracheitis did not offer enough stimuli to generate additional germinal centers.

The thymus is a primary immune organ responsible mainly for the production of cell-mediated immune response that doesn't seem to play a vital role during *Mycoplasma gallispeticum* infection. Yet, through Th (T helper) cells, the thymus is responsible for inducing/stimulating a humoral response against an infection. Therefore, based on its secondary role in fighting *Mycoplasma gallispeticum*, a larger stimulus is required to modify the thymus indices. Based on the results observed during this experiment, AviPro® MGF vaccine proved to be of a mild strain. Therefore, no subsequent effects were observed in the thymus indices of the experimental birds.

On the other hand, Methionine plays a vital role in thymus development. In case of methionine deficiency, the cellular immune function is altered by ultrastructural pathological changes in the thymus. These changes are coupled with decreased T cell production and proliferation, accompanied by a decrease in serum concentration of Interlukin-2. Several experiments investigated the effect of excess methionine on the relative weight of immune organs. Bouyeh and Gevorgyan (2011) found that additional lysine and methionine in the diet didn't affect the relative weight of thymus in broilers regardless of the presence of a challenge (Bouyeh & Gevorgian, 2011). Bouyeh (2012) reported similar results, as excess and lysine didn't affect the relative weight of the thymus. These results are in agreement with our experimental observation as 20% excess methionine didn't have an effect on the relative weight of the thymus and spleen.

CHAPTER 5

Conclusion and recommendations:

It is emphasized that eradication is the preferred method in controlling *Mycoplasma gallispeticum* infection in poultry. However, in situations where eradication is not feasible, strict biosecurity, vaccination and nutritional manipulation could offer an alternative for MG control. Nutritional manipulation such as amino acid addition to the diet forms a promising alternative/ candidate in fighting bacterial infections.

The aim of our study was to assess a protocol that combine the use of AviPro® MGF vaccine and 20% excess methionine to control *Mycoplasma gallisepticum* infection. Typically, the mechanism by which a vaccine offers protection against MG infection is represented by competitive exclusion and production of neutralizing humoral immunity. In our study both mechanisms were assessed in the presence of adequate or 20% excess methionine above the recommended levels.

Results observed in this experiment indicate a slow rate of growth and colonization of MGF vaccine strain at the level of trachea. A delayed increase in colony forming unit that extended up to 6 weeks post-vaccination offer limited competitive exclusion of the F-strain against other *Mycoplasma gallisepticum* infections for a considerable period. Furthermore, the addition of 20% excess methionine above the recommended levels hindered the colonization rate and colony forming units of AviPro® MGF vaccine. These results highlight the ability of excess methionine in enhancing innate structural immune response.

Humoral immune response stimulated by AviPro® MGF vaccine was not of a better shape than its competitive exclusion ability. Eight weeks post-vaccination were required by the vaccine to stimulate the humoral immunity response against MG. These results disagree with literature reporting a significant titer 2-3 weeks to MG following the latter exposure. Methionine effect on humoral immunity was detected at week 11. The 20% excess methionine numerically increased the IgG titer against MG in comparison with other groups, yet this increase wasn't enough to generate protective titer count.

Based on the above results, 20% excess methionine above the NRC recommended levels proved to have a key role in enhancing structural innate and acquired immunity in MG vaccinated broiler pullets. AviPro® vaccine delayed immune response requires additional research and investigation.

Future research should aim to understand methionine metabolism and its interaction with the immune system of breeder pullets. Moreover, recording body composition and blood chemistry could provide a better understanding regarding excess methionine physiological effects. In addition, future research should also focus on measuring innate immune system mediators such as cytokines, chemokines, and the complement system to form a better understanding regarding methionine influence on non-specific immunity.

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