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**Efficacy of *in ovo* delivered prebiotic on health,
performance and meat quality of Ross 308
broiler and Kuroiler chicken reared under
temperate and tropical climatic conditions**

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*To all who in one way or the other played a
mentorship role in my life. Your confidence in
my ability got me over the final hurdle!!*

*For the gracious gifts and
calling of God are
irrevocable.*

-Romans 11:29

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ABSTRACT

In ovo technology has been developed to enable delivery of sustainable bioactives, such as pre-/probiotics, directly into the egg air chamber at day 12 of embryonic incubation. Thus, improving the effectiveness of these compounds by administering them to the animals under fully controlled conditions and as early as possible. However, research on the efficacy of prebiotics delivered *in ovo* on gut health, performance and meat quality is still in exhaustive having been concentrated mainly on meat-type chickens. This neglects the richness of chicken biodiversity and climatic variations. The aim of the current thesis (divided in two parts) was to evaluate the efficacy of prebiotics delivered *in ovo* on egg hatchability, gut health, performance, and meat quality of Ross 308 broiler and Kuroiler chickens reared under temperate and tropical climatic conditions.

Study one carried out in Italy used a commercial prebiotic Bi²tos (BI) injected *in ovo* to assess the effects of prebiotics on performance, quality and oxidative stability of meat from broiler chickens reared under temperate climatic conditions. The eggs used in this experiment were from Ross 308 broiler (meat-type) chickens. On day 12 of incubation, 300 eggs that had viable embryos were randomly divided into three experimental groups: prebiotic group (BI), injected with 200 μ L Bi²tos (3.5 mg/embryo); saline group (S), injected with 200 μ L of physiological saline solution; and un-injected control (C). Hatched chicks were scored and sexed for each experimental group and 120 males (40 chicks/group) were grown to 42 days of age in floor pens (4 replicates of 10 birds each), with free access to an outdoor area. Birds were fed *ad libitum* starter and grower-finisher diets with free access to water. Along the rearing period, chickens were weighed and counted within each pen. At 42 d, 20 randomly chosen birds per treatment, of similar estimated body weight, were individually weighed and slaughtered. Hot carcass weight was recorded and carcass yield was calculated. Main commercial cuts were removed from the carcass and weighed and their percentages (yield) calculated. Pectoral muscle pH, colour and water holding capacity (WHC) were measured 24 hours *post-mortem*. In addition, fatty acid composition and meat oxidative stability were also analyzed. Data were analyzed by one-way ANOVA. The multi-comparison Scheffe's test was used to separate the differences among the mean for statistical significance ($P < 0.05$). Results from the study showed a satisfactorily high hatchability (> 90%) though similar among experimental groups. Mortality of the chickens during this study was very low (2%) and not dependent on the substance injected *in ovo*. Initial body weights were the same among all experimental groups. In the period from week one to three, BI group showed a

significantly higher weight gain in comparison to the C (+2.5 %) and S (+1.9 %) groups. While for the entire rearing period (week 1 - 6), both BI and S groups showed higher BWG in comparison to the C group (+7.5% and 6.8%, respectively). Chickens from BI group were significantly heavier at slaughter than those from C group, but similar to those of S group. Similarly, carcass weight was higher in S and BI groups as compared to C. However, no significant differences among experimental groups were found for carcass yield, main commercial cut yields (breast, legs and wings) and back+neck yield. pH₂₄, colour (L*, a*, b*) and WHC were the same among treatments. Intramuscular fat (IMF) content was higher in BI group compared to C group, with intermediate values for S group. The treatment did not affect total amounts of saturated fatty acids (SFA) and individual SFA content of meat. The most concentrated SFA in all experimental groups were palmitic acids (C16:0; 23.45 – 24.95%) followed by stearic acid (C18:0; 9.49 – 10.21%). No effect of treatment was observed in the composition of monounsaturated fatty acids (MUFA) as well as total MUFA contents. Quantitatively, oleic acid (C18:1n 9) was the most abundant MUFA (24.49 – 25.53%). *In ovo* delivered prebiotic lowered (-2.6%) the total poly unsaturated fatty acid (PUFA) content compared to the S group. Total n-6 and n-3 PUFA contents of meat from BI group birds were also significantly lower than those of the S group. For individual fatty acid, only docosahexanoic acid (C22: 6n 3) was affected by the treatment being lower in the BI group in comparison with the S group (-0.31%) and C group. Regardless of the treatment, the most abundant PUFA were linoleic (C18:2n 6; 25.13– 25.73%) and arachidonic acids (C20:4n 6; 5.22 – 6.44%). Regarding selected fatty acid indices, n-6/n-3 ratio was significantly higher in the prebiotic treated group (+16.2%) than in S group. In addition, P/S tended to be lower in BI group compared to S and C. The treatment did not have any effect on atherogenic and thrombogenic indices. The treatment had no negative effect on lipid peroxidation. In fact, TBARs values were slightly lower in BI compared to the control group after 72 hours of storage. All in all, the results obtained from this study clearly proved that the *in ovo* prebiotic administration improved production performance in Ross 308 broiler chickens throughout the rearing period without negatively affecting meat quality.

In the second study, the efficacy of prebiotics, antibiotic-chick formula and a combination of the two on growth performance, carcass traits and gut health in the face of a natural coccidiosis infection was assessed in Kuroiler chickens reared under field condition in Uganda. At the 12th day of incubation, 150 eggs from Kuroiler (dual purpose) chickens with viable embryos were randomly divided into two equal groups. One group was injected with 0.2 mL of Bi²tos (3.5mg/embryo); and the other was left uninjected as control. Hatched

chicks from each of the two experimental groups above were further randomly divided into two groups: one group received antibiotic chick formula (poltricin with oxytetracycline at a dose of 1g/litre of drinking water for 7 days) while the other was left without the antibiotic chick formula. Thus, giving rise to four experimental groups: Control (C), Antibiotic (A), Bi²tos (B) and Bi²tos + Antibiotics (AB). The birds (half males and half females) were reared in a local poultry farm in Gulu District where coccidiosis infection was previously confirmed by field veterinarians. All birds were reared under semi-intensive confined system for a period of 18 weeks. Chickens were fed *ad libitum* starter, grower and finisher diets and had constant access to water. Body weights were taken per pen on a weekly basis and also faecal samples were collected for parasitological analysis to check for possible infection with *Eimeria* parasites. At d 12 and at the end of the experiment, 6 birds/group were randomly chosen to assess the severity of coccidial lesions in the intestines. At slaughter, carcass and meat quality traits were evaluated as described in experiment one above. The treatment with Bi²tos (B) significantly increased body weight and body weight gain especially at 6 weeks of age. On the other hand, AB group was the heaviest of all experimental groups at the end of the experiment. The B group had a slightly higher carcass weight compared to the rest of the treatments. Breast yield was generally higher in all treatment groups compared to the C, although significant differences were found only with AB group (+6.0%). Leg weight and yield were not significantly different among experimental groups but tended to be higher in prebiotics treated groups. Wings yield was higher in AB group compared to the C group, intermediate values were observed in A and B groups. Sexual dimorphism was clearly evident with males being heavier at slaughter and displaying better carcass traits. pH and WHC values were similar among experimental groups and between the two sexes. Significant interaction between treatments x sex was observed for breast yield and wings weight. With reference to the effect of the treatment on fatty acid composition, meat from the B group displayed the highest (+ 3.72 %) amount of total PUFA compared to the control group. Conversely, breast muscles of the B group as well as those of the AB group had, in general, lower amounts of total MUFA compared to C and A groups. Total amount of SFA was not affected by the treatments. Total n-3 fatty acids were higher in B compared to the control groups A and AB had intermediate values. Total n-6 fatty acids were the same among treatment groups. The ratio of PUFA to SFA was not significantly different among experimental groups. On the other hand, the n-6/n-3 ratio was lower in B and AB groups compared to C and A groups. A comparison of the two sexes showed only minimal effects on fatty acid profile. Total MUFA was higher in males compared to the females; while PUFA

were higher in females than males. The fatty acid ratios were not affected by sex. There were significant treatments x sex interaction effects on total n-3 as well as n-6/n-3 ratio though.

Overall, prebiotic (Bi²tos) with or without antibiotics reduced the severity of coccidiosis lesions induced by natural infection with *Eimeria* spp. as well as oocyst excretion compared with the control and also improved meat quality. In conclusion, the study has demonstrated that use of *in ovo* delivered prebiotics in broiler production can lessen the depression in growth due to coccidial challenge with positive effects on meat quality. Regardless of the treatment and experimental conditions, Kuroilers had lower n-6/n-3 ratio compared to Ross 308 broiler chickens.

La tecnologia di iniezione *in ovo* consente di somministrare componenti bioattivi di origine naturale, quali pre- e probiotici, direttamente nella camera d'aria dell'uovo al 12° giorno di incubazione. Questa via di somministrazione permette di veicolare tali composti il prima possibile ed in condizioni altamente controllate, aumentandone così l'efficacia. Tuttavia, gli studi condotti finora per provare e validare l'efficacia dei prebiotici somministrati *in ovo* sulla salute intestinale, sulle performance di crescita e qualità della carne non sono del tutto esaustivi, essendo stati tutti condotti su polli da carne, trascurando sia la ricchezza della biodiversità avicola, nonché le diverse condizioni climatiche di allevamento. L'obiettivo del presente lavoro di tesi, articolato in due studi, è stato quello di valutare l'efficacia dei prebiotici iniettati *in ovo* sulla schiudibilità delle uova, sulla salute dell'intestino, sulle performance produttive e sulla qualità della carne di polli Ross 308 e Kuroiler allevati in zone climatiche temperate e tropicali, rispettivamente.

Il primo studio, condotto in Italia, ha inteso valutare gli effetti della somministrazione *in ovo* di un prebiotico commerciale Bi²tos (Clasado Ltd., Malta) sulle performance produttive, e sulle caratteristiche qualitative e stabilità ossidativa della carne di polli allevati in condizioni climatiche temperate. Per lo studio sono state utilizzate uova di polli da carne di razza ibrida (Ross 308). Al 12° giorno di incubazione, 300 uova fertili sono state divise a random in 3 gruppi: BI, gruppo iniettato con 0,2 ml di Bi²tos (3,5 mg/uovo di transgalactooligosaccaridi); S, gruppo di controllo positivo iniettato con 0,2 ml di soluzione fisiologica; C, gruppo di controllo negativo non trattato. Dopo il sessaggio, 120 pulcini maschi (40 pulcini/gruppo) sono stati pesati e trasferiti presso un'azienda agro-zootecnica ed allevati in box a terra (n = 10 polli per box, 4 repliche), con libero accesso ad un'area esterna. Gli animali sono stati alimentati *ad libitum* con diete commerciali formulate in funzione della loro età ed hanno usufruito di acqua fresca, sempre disponibile. Per tutto il periodo di allevamento i polli sono stati monitorati e rilevato il peso vivo, per ogni singolo box, e calcolato il relativo accrescimento medio di riferimento. A 42 giorni d'età, sono stati scelti 20 polli per gruppo, presi a random tra quelli di peso simile, sono stati pesati e macellati. È stato registrato il peso della carcassa e calcolata la relativa resa. La carcassa è stata quindi sezionata nei principali tagli commerciali (petto, cosce e ali), pesati e calcolate le rispettive rese. A 24 ore post-mortem, sul muscolo pettorale, sono stati misurati il pH, colore e la capacità di ritenzione idrica (WHC); inoltre, sul muscolo pettorale sono state condotte analisi

chimiche per la determinazione del profilo lipidico e della stabilità ossidativa (TBARS). I dati sono stati analizzati mediante ANOVA ad una via. Le differenze tra le medie sono state valutate mediante il test di Scheffé. L'iniezione *in ovo* del prebiotico e della soluzione salina non hanno avuto alcun effetto negativo sulla percentuale di schiusa, che è risultata elevata (> 90%) e simile tra i tre gruppi sperimentali. Il tasso di mortalità è stato molto basso (2%) ed indipendente dalla sostanza iniettata *in ovo*. Il peso iniziale dei pulcini è risultato simile tra i 3 gruppi sperimentali. Gli animali del gruppo BI, iniettati *in ovo* con il Bi²tos, hanno mostrato nei primi 21 giorni di vita un miglior ($P < 0,01$) incremento ponderale rispetto a quelli dei gruppi C (+2,5%) e S (+1,9%). I valori dell'incremento totale di peso registrati nell'arco dell'intero periodo di sperimentazione (6 settimane) sono risultati significativamente superiori ($P < 0,05$) nei gruppi BI e S rispetto al controllo (+7,5% and 6,8%, rispettivamente). Per quanto riguarda il peso vivo degli animali macellati, i polli dei gruppi iniettati *in ovo* (S e BI) sono risultati più pesanti ($P < 0,05$) rispetto a quelli di controllo. Anche il peso della carcassa è risultato maggiore nel gruppo BI ed S rispetto a C ($P < 0,05$); mentre la resa in carcassa e la resa dei principali tagli commerciali (petto, cosce ed ali) sono risultate simili tra i gruppi. Il pH₂₄, il colore (L*, a*, b*) e WHC sono risultati simili tra i gruppi sperimentali. La carne del gruppo trattato con prebiotico (BI) ha mostrato un contenuto di lipidi totali significativamente più elevato rispetto al gruppo C, con valori intermedi per il gruppo S. Il trattamento con prebiotico non ha avuto alcun effetto sia sul contenuto totale di acidi grassi saturi (SFA) che su quello dei singoli acidi grassi saturi. Gli acidi saturi più abbondanti sono risultati l'acido palmitico (C16:0, da 23,45 % a 24,95 %) e l'acido stearico (C18:0, da 9,49 % a 10,21 %). Il contenuto totale di acidi grassi monoinsaturi (MUFA), come anche quello dei singoli MUFA è risultato simile tra i gruppi sperimentali. L'acido oleico (C18:1) è risultato essere il più abbondante (da 24,49 % a 25,53 %). Il contenuto totale di acidi grassi polinsaturi (PUFA, da 33,80 % a 36,40 %) è risultato significativamente più basso (- 2,6%) nel gruppo BI rispetto al gruppo S, così come il contenuto totale degli acidi grassi PUFA n-3 e n-6. Relativamente al contenuto dei singoli PUFA, ad eccezione dell'acido docosaesaenoico (C22:6 n-3), risultato più basso ($P < 0,05$) nel gruppo BI rispetto ai gruppi S e C, il contenuto dei restanti PUFA è risultato simile tra i gruppi sperimentali. Gli acidi grassi polinsaturi più abbondanti sono risultati l'acido linoleico (C18:2, da 25,13 % a 25,73 %) e l'arachidonico (C20:4, da 5,22 % a 6,44 %). Riguardo gli indici nutrizionali, il rapporto n-6/n-3 è risultato significativamente più elevato (+16,2%) nel gruppo BI rispetto a quello S. Il rapporto acidi grassi polinsaturi/saturi (P/S) è risultato tendenzialmente più basso nel gruppo BI rispetto ai gruppi S e C. Il trattamento non ha avuto alcun effetto sull'indice aterogenico e quello

trombogenico. L'ossidazione lipidica non è stata influenzata negativamente dal trattamento. Infatti, il gruppo BI ha mostrato un più basso contenuto di TBARS dopo 72 ore di refrigerazione aerobica rispetto al controllo. Concludendo, i risultati ottenuti dal presente studio consentono di affermare che la somministrazione *in ovo* di un prebiotico commerciale ha avuto effetti positivi sulle performance di crescita, mentre non ha avuto alcun effetto negativo sulle caratteristiche chimico-fisiche e nutrizionali della carne.

Il secondo studio, condotto in Uganda, ha inteso valutare gli effetti della somministrazione di un prebiotico, di una formulazione antibiotica, e della loro combinazione, sulle performance produttive, sulle caratteristiche qualitative della carne, nonché sulla salute intestinale (in seguito ad un'infezione naturale di coccidiosi) di polli Kuroiler allevati in condizioni climatiche tropicali. Per lo studio sono state utilizzate uova di galline Kuroiler, razza a duplice attitudine. Al 12° giorno di incubazione, 150 uova fertili sono state divise a random in 2 gruppi. Un gruppo è stato iniettato con 0,2 ml di Bi²tos (3,5 mg/uovo), l'altro gruppo non iniettato è stato lasciato come controllo. Alla schiusa, i pulcini di ciascun gruppo sono stati ulteriormente divisi in due gruppi: un gruppo a cui è stata somministrata la formulazione antibiotica (ossitetraciclina, 1g/l in acqua per 7 giorni), mentre l'altro gruppo non ha ricevuto antibiotici. I gruppi, quindi, erano i seguenti: controllo (C), antibiotico (A), Bi²tos (B) e Bi²tos + Antibiotico (AB). Gli animali (metà maschi e metà femmine) sono stati allevati presso una piccola azienda avicola nel distretto di Gulu dove l'infezione da coccidiosi era stata confermata da veterinari. I polli sono stati allevati per 18 settimane in regime semi-intensivo. Gli animali sono stati alimentati *ad libitum* con diete commerciali formulate in funzione della loro età ed hanno usufruito di acqua fresca, sempre disponibile. Per tutto il periodo di allevamento, a cadenza settimanale, è stato rilevato il peso vivo dei polli, per ogni singolo box, e prelevati campioni di feci per le analisi parassitologiche al fine di evidenziare possibili infezioni da *Eimeria*. A 12 giorni e alla fine dell'esperimento, sono stati sacrificati 6 animali per gruppo per valutare il grado di severità delle lesioni da coccidi a livello intestinale. Le caratteristiche della carcassa, così come le caratteristiche chimico-fisiche e nutrizionali della carne sono state determinate come precedentemente descritte nell'esperimento 1. L'iniezione *in ovo* con Bi²tos (B) ha determinato un aumento significativo del peso dei polli a 6 settimane di età; mentre, a 18 settimane, i polli del gruppo AB sono risultati più pesanti rispetto ai polli degli altri gruppi sperimentali. Anche il peso della carcassa è risultato lievemente maggiore nel gruppo B rispetto agli altri gruppi. In generale, la resa del petto è risultata superiore nei gruppi trattati (A, B e AB) rispetto al controllo, anche se le differenze sono risultate statisticamente

significative solo tra i gruppi AB e C. Il peso e la resa delle cosce sono risultati simili tra i gruppi sperimentali anche se il gruppo trattato con prebiotico ha mostrato valori tendenzialmente più elevati. L'incidenza delle ali è risultata superiore nel gruppo AB rispetto al gruppo C, con valori intermedi per gli altri due gruppi. Come atteso, per il dimorfismo sessuale, i maschi sono risultati più pesanti e con caratteristiche della carcassa migliori rispetto alle femmine. Un'interazione significativa tra trattamento e sesso è stata riscontrata per il peso del petto e l'incidenza delle ali. Per quanto riguarda il profilo degli acidi grassi, la carne del gruppo trattato con prebiotico (B) ha mostrato un contenuto maggiore di PUFA rispetto al controllo; mentre, il contenuto totale di MUFA è risultato essere più basso nei gruppi B ed AB rispetto agli altri due gruppi sperimentali. Il contenuto totale di SFA è risultato simile tra i gruppi. Il contenuto di PUFA n-3 è risultato maggiore nel gruppo B rispetto a quello A, con valori intermedi per il gruppo AB. Nessuna differenza statisticamente significativa è stata rilevata sia per il contenuto di PUFA n-6, che per il rapporto PUFA/SFA. Al contrario, il rapporto n-6/n-3 è risultato inferiore nei gruppi B ed AB rispetto agli altri due gruppi. Marginale è stato l'effetto del sesso sulla composizione acidica. La carne dei maschi ha mostrato un contenuto superiore di MUFA ed inferiore di PUFA rispetto alle femmine. Il contenuto di SFA e gli indici nutrizionali sono risultati simili. Sono state evidenziate interazioni significative tra trattamento e sesso per alcuni acidi grassi, per PUFA n-3 e per il rapporto n-6/n-3.

Nel complesso, il prebiotico (Bi²tos) con o senza antibiotici, ha ridotto la gravità delle lesioni indotte da infezione naturale con *Eimeria* spp. e l'escrezione di oocisti rispetto al controllo, nonché ha migliorato alcune caratteristiche qualitative della carne. In conclusione, lo studio ha dimostrato che l'uso di prebiotici iniettati *in ovo* può contrastare gli effetti negativi della coccidiosi sulle performance di crescita degli animali nonché sulla qualità della carne.

ACRONYMS / ABBREVIATIONS

ADG	average daily gain
AGP	antimicrobial growth promoter
AI	atherogenic index
ANOVA	analysis of variance
BW	body weight
BWG	body weight gain
CLA	conjugated linoleic acid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DPM	deep pectoral myopathy
d	day
EPA	eicosapentaenoic acid
FA	fatty acids
FAME	fatty acid methyl esters
FCR	feed conversion ratio
FI	feed intake
FOS	fructooligosaccharide
GI	gastrointestinal
GIT	gastrointestinal tract
GOS	galactooligosaccharide
IMCT	intramuscular connective tissue
IMF	intramuscular fat
MCFA	medium chain fatty acids
MDA	malondialdehydes
ME	metabolizable energy
MOS	mannanoligosaccharides
MUFA	monounsaturated fatty acids
NSP	non-Starch Polysaccharides
PSE	pale, soft and exudative
PUFA	polyunsaturated fatty acids
SCFAs	short chain fatty acids

SEM	standard Error of the Mean
SFA	saturated fatty acids
TBARS	thiobarbituric acid reactive substances
TEP	tetraethoxypropane
TI	thrombogenic index
WB	wooden breast
WHC	water-holding capacity

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PART 1: INTRODUCTION AND LITERATURE REVIEW

Chapter 1

TRENDS IN POULTRY PRODUCTION AND CONSUMPTION

1.1. Introduction

Currently, livestock is one of the fastest growing agricultural subsectors in developing countries. Its share of agricultural Gross Domestic Product (GDP) is already 33% and is quickly increasing (Thornton, 2010) while in the developed countries its growth seems stagnant even though its contribution to the GDP is about 53%. This growth is driven by the rapidly increasing demand for livestock products, as a result of population growth, urbanization and increasing incomes in developing countries (Delgado, 2005). According to Thornton (2010), the global livestock sector is generally characterized by a dichotomy between developing and developed countries. Total meat production in the developing world was reported by the World Bank (2009) to have tripled between 1980 and 2002, from 45 to 134 million tons; with much of the growth concentrating in countries that had experienced rapid economic growth, particularly in East Asia. This growth was mainly observed in the poultry and pig subsectors.

It is estimated that the global poultry flock stands at 23 billion birds which is about three birds per person on the planet (FAOSTAT, 2016). This is almost five times higher than the previous five decades. The birds are kept and raised in a wide range of production systems, with the main aim of providing meat, eggs and manure for crop fertilization. Globally, poultry meat is the most widely consumed animal source food across a diversity of cultures, traditions and religions. In the livestock sector, Mottet and Tempio (2017) noted that poultry emerges as the most efficient subsector in terms of the utilization of natural resources and the provision of protein to meet the global growing demand. Furthermore, poultry is of importance particularly to small holders, poor rural and peri-urban population, and also in large scale intensive production systems. This makes it one of the fastest growing agricultural subsectors.

1.2. Poultry production systems

Poultry production is very diverse mainly because of the diversity in the feed base, breeds, the orientation and type of housing. Globally, poultry are kept under a wide variety of

production systems, that range from those with very rudimentary night shelters to those with fully automated, environmentally controlled systems. In developing countries, the housing, management and feeding of indigenous poultry stock in rural areas is for the most part basic. Poultry are kept in simple night shelters with very limited management and disease prevention inputs (Conan *et al.*, 2012), and minimal supplementary feeding using household scraps and small amounts of grain. Because of natural selection and their capacity for foraging, the birds are able to survive, grow and lay eggs in these environmental conditions, and in so doing, make a significant contribution to food security and protein intake of the human populations. However, the normally low productivity of these genotypes makes it economically unfeasible to rear them under intensive management systems. Irrespective of the size of operation, the large majority of commercial production units utilize commercial rather than indigenous genotypes.

Commercial production systems with highly selected meat or egg types of poultry require a suitable physical environment, optimal nutrition and efficient protection from the effects of diseases. To achieve these, the birds must be at least partially confined, and therefore need to be provided with all or most of their nutritional requirements. Foraging is generally not used except in free-range systems, where only a small proportion of the birds' nutrient requirements are typically met from the range. Commercial egg and meat birds have high requirements for protein and energy and do not tolerate high fibre levels in their diets (Chad, 2012). Constraints for feeding in developing countries are the very wide variation in the quality and composition of poultry feed, which is often of questionable quality. Because of the high cost associated with the provision of an optimal physical environment, particularly in hot tropical regions, sophisticated environmentally controlled housing is generally only used in large-scale operations.

Generally, there are three main types of poultry production systems recognized at global level (Table 1.1; Gerber *et al.*, 2013). These are: broilers, layers and back yard systems.

Table 1.1. Poultry production systems

System	Housing	Characteristics
Broilers	Broilers assumed to be primarily loosely housed on litter, with automatic feed and water provision	Fully market-oriented; high capital input requirements (including Infrastructure, buildings, equipment); high level of overall flock productivity; purchased non-local feed or on farm intensively produced feed
Layers	Layers housed in a variety of cage, barn and free range systems, with automatic feed and water provision	Fully market-oriented; high capital input requirements (including infrastructure, buildings and equipment); high level of overall flock productivity; purchased non-local feed or on farm intensively produced feed
Back yard	Simple housing using local wood, bamboo, clay, leaf material and handmade construction resources for supports (columns, rafters, roof frame) plus scrap wire netting walls and scrap iron for roof. When cages are used, these are made of local material or scrap wire	Animals producing meat and eggs for the owner and local market, living freely. Diet consists of swill and scavenging (20 to 40 percent) and locally- produced feeds (60 to 80 percent)

Source: Gerber *et al.* (2013)

1.2.1. Poultry production and consumption

GLEAM 2 (2016) puts the global production of eggs at 73 million tons and that of poultry meat at about 100 million tons. Of these, backyard systems contribute only 8% of global eggs production and 2% of global poultry meat. The majority (92%) of poultry meat comes from industrialized broiler systems while layers contribute only 6% of the total production. It is important on the other hand, to note that there are significant regional differences in these figures. For instance, backyard systems make significant contribution to eggs and poultry meat production in Eastern Europe, South Asia, Sub-Saharan Africa and to a lesser extent in East Asia and Latin America and the Caribbean (Figures 1.1a, b).

Poultry, one of the fastest growing agricultural subsectors, has shown the fastest growth trend in demand and consumption in the past few decades. The average annual growth rate over the last 5 decades was 5% compared to only 1.5% for beef, 3.1% for pork and 1.7% for

small ruminants' meat (Alexandratos and Bruinsma, 2012). Global per capita consumption of eggs increased from 4.55 kg to 8.92 kg between 1961 and 2010, while global per capita consumption of poultry meat increased from 2.88 kg to 14.13 kg (FAOSTAT, 2016). The composition of meat consumption in the United States and most parts of the developed world has also shifted from predominantly beef to predominantly chicken, a trend driven by lower cost and health concerns (Gerber *et al.*, 2013). In the United States, per capita beef consumption has fallen 40% since its peak in the 1970s, while poultry meat has emerged as the most popular type of meat by far.

Production has been particularly dynamic in developing countries, especially East and South East Asia (Figure 1.2), with an annual growth rate in poultry meat production of 7.4%. The biggest poultry meat producers are the United States, with almost 20 million tons a year, followed by China, with 18 million tons, the EU and Brazil with about 13 million tons. Thornton (2010) observed that production response to increased demand were historically characterized by systems as well as regional differences, with confined livestock production systems in industrialized countries being the source of much of the world's poultry and pig meat production. He further reported that such systems were being established in developing countries, particularly in Asia, to meet increasing demand. Bruinsma (2003) estimates that at least 75% of total production growth by 2030 will be in confined systems, although much less growth of the same systems is expected to occur in Africa.

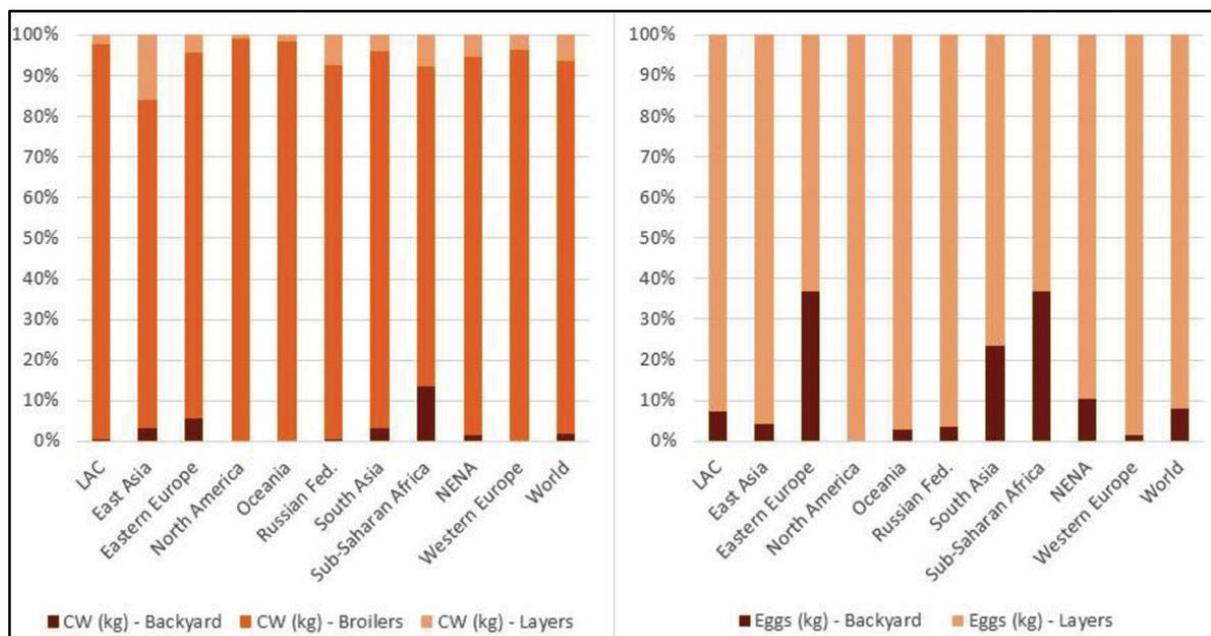


Figure 1.1a. Eggs and poultry meat production by production systems and regions (GLEAM 2, 2016)

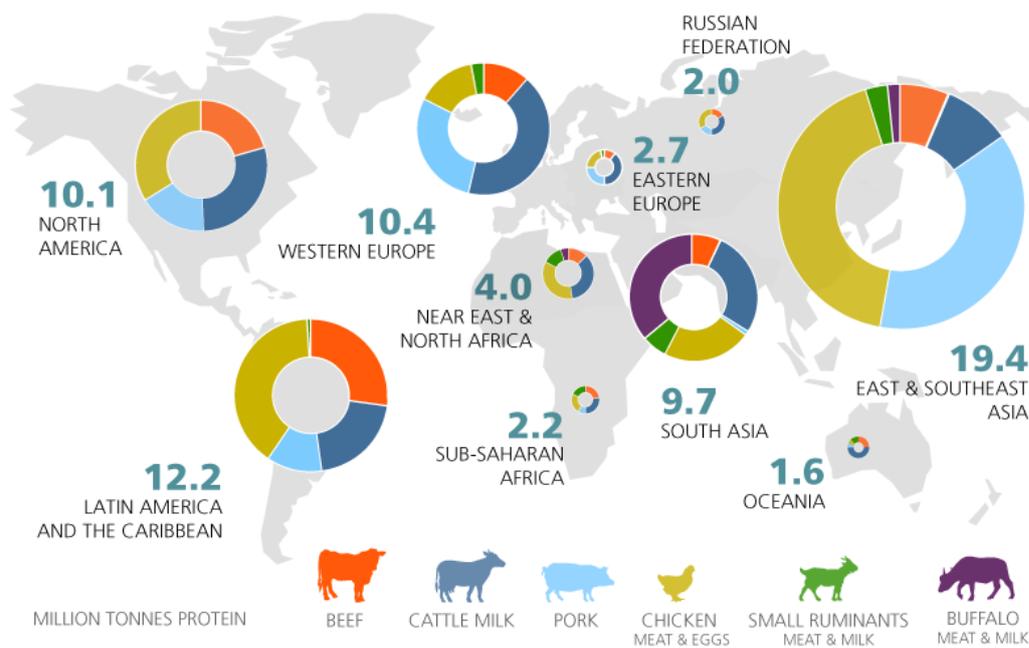


Figure 1.1b. Regional production of meat, eggs and milk (GLEAM 2, 2016).

Mottet and Tempio (2017) reported that technological changes in production practices were one of the main drivers of the poultry subsector’s growth. The move from free-ranging to confined poultry operations dramatically increased the number of birds per farmer, facilitated the substitution of capital for labour, and led to a significant increase in labour productivity. Narrod *et al.* (2012) showed that between 1985 and 1996, the share of poultry farms with more than 10,000 heads grew from 42% to 78% in the regional Center West of Brazil. Advances in breeding to improve animal size, fecundity, growth rate and uniformity, have also been cited as potential contributors to increased outputs (Thornton, 2010).

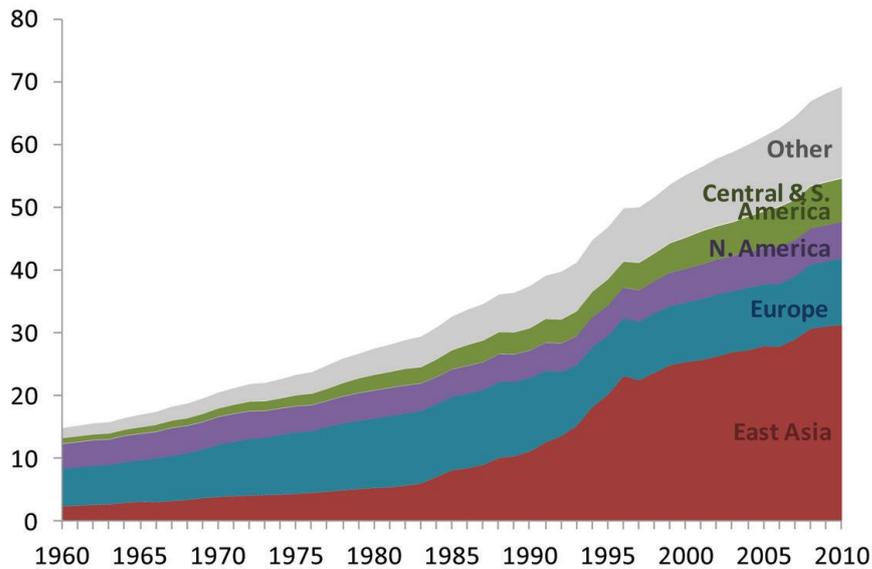


Figure 1.2. Global eggs production by region (Mottet and Tempio, 2017)

1.3. Future trends in poultry production and consumption

The growth of the global livestock sector is expected to continue. Global human population is estimated to reach 9.15 billion in 2050, with a range of 7.96-10.46 billion (UNDP, 2008). Most of the increase is projected to take place in developing countries. For instance the population in sub-Saharan Africa will still be growing at 1.2 per cent per year. Rapid population growth could continue to be an important impediment to achieving improvements in food security in some countries. Additionally, according to the same report, about 70% of this population will be living in urban areas, while incomes could increase by 2% annually.

The next few decades are therefore likely to see unprecedented urban growth, particularly in Africa and Asia. Urbanization has considerable impacts on patterns of food consumption in general and on demand for livestock products in particular: urbanization often stimulates improvements in infrastructure, including cold chains, which allows perishable goods to be traded more widely (Delgado, 2005; Thornton, 2010). In this context, Alexandratos and Bruinsma (2012) projected that the demand for animal source food could grow by 70% between 2005 and 2050. Poultry meat is expected to have the highest growth, with up to 121%. Demand for eggs is projected to go up by 65%. Global demand for eggs is also expected to keep on growing. Still the fastest growing subsector, poultry meat production would however increase at a slower rate than in the past decades. By 2050, its annual growth rate is estimated to reach 1.8% at global level, and 2.4% in developing countries. Most of the growth will be driven by Asia. Average per capita consumption of poultry meat is still

relatively low in Asia, with less than 10 kg per year, twice as less as in Western Europe and 5 times less than in Northern America. For example, OECD/FAO (2016) estimate that, in East and South Asia, meat production will increase by 1.8 Mt annually by 2025, with pork and poultry accounting for the bulk of this expansion.

In Sub-Saharan Africa, poultry consumption has been increasing so fast that domestic supply is no longer adequate in meeting the demand. Therefore, almost 40% of the additional consumption is imported. For the period 2015 - 2025, OECD/FAO (2016) estimate that imports will supply 66% of the growth in poultry meat demand in the region, while this share will only be 16% for beef and veal, 2% for sheep and 45% for pork. Globally, 10% of meat output will be traded in 2025, with most of the increase coming from the poultry sector (Mottet and Tempio, 2017).

Overall, the poultry sector needs to respond to the growing demand for meat and eggs and enhance its contribution to food security and nutrition. However, to be sustainable, it needs to consider its roles beyond just providing food. It needs to produce more with less, while benefiting all. It has a key role in providing secure livelihoods and economic opportunities for hundreds of millions of smallholder and rural farmers especially in sub-Saharan Africa. Enhancing this role requires a specific attention to market access. It needs to use natural resources efficiently, mitigate and adapt to climate change and reduce other environmental impacts. Finally it is necessary that the sector enhances human, animal, and environmental health and welfare. Thus, there is urgent need to evaluate the efficacy of possible alternatives to antibiotics growth promoters (AGP) in improving productivity and mitigating antibiotic resistance under tropical and temperate climatic conditions in the wake of the ban on AGP use, since 2006, in poultry production by the EU.

1.4. Poultry production in Uganda

There has been a general linear increase in production of poultry products over the years (Figure.1.3). According to the Ministry of Agriculture Animal Industry and Fisheries (MAAIF), poultry production in Uganda in 2014 stood at 54,868 metric tonnes and is projected to increase to 63,647 metric tonnes in 2020 (MAAIF, 2016 unpublished). The poultry industry in Uganda is composed mainly of chicken. Over 90% of which are chicken breeds that are indigenous to Uganda and are raised by small scale farmers under the backyard system (Kugonza *et al.*, 2008; Natukunda *et al.*, 2011). These birds produce on average 50 eggs per hen per year. The eggs are either for hatching chicks or used as table eggs. While the exotic commercial layers and broilers kept under the intensive system of

husbandry mainly in urban and semi-urban areas constitute only 10% of the national flock. The intensively managed commercial enterprises fall under three categories: i) small units of between 50 and 500 birds, ii) medium sized units of 500-1,000 birds; iii) very few usually less than 5% of the large scale units of over 1,000 birds. The village flocks on the other hand comprise of unimproved native chickens, typically 5-20 birds per family (Okot, 1990). Apart from chickens, other species of birds kept in rural areas include turkeys, ducks and guinea fowls and pigeons.

Chickens represent an efficient and sustainable resource, helping villagers meet increasing food demands, as well as providing rural women with a source of income, which further improves their social standing and overall livelihood. Indigenous chickens are kept for meat, eggs, income and socio-cultural roles. Indigenous Ugandan hens however, produce just 20 to 40 eggs per year, with a typical male chicken weighing about 1.5 to 2 kg after about nine to 12 months of growth. Even with the introduction of exotic broilers and layers in Uganda, the demand for local chickens still exceeds supply. Indigenous chickens are preferred to their exotic counterparts because of their colour, organoleptic qualities, leanness and suitability for special dishes. Given that Ugandan chicken flocks typically consist of just 5 to 20 chickens, the failure of traditional varieties to meet basic nutritional and economic needs of village families is not uncommon. Many efforts over the years have attempted to address this problem, though until recently, results have not been satisfactory.

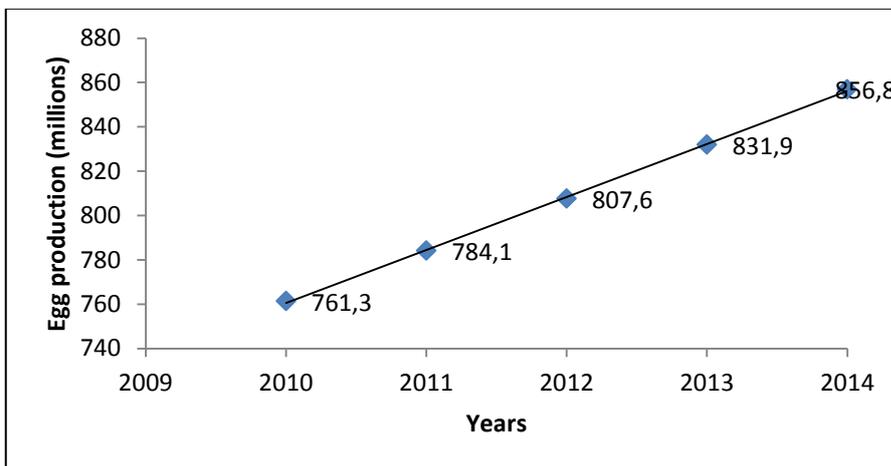


Figure 1.3. Egg production trend in Uganda (UBOS, Statistical abstract, 2015)

1.4.1. Introduction of the Kuroiler chickens in Uganda

In Uganda just like in most African countries as aforementioned, more than 85% of families live in rural village conditions, where small-scale backyard poultry operations are ubiquitous. In addition to providing sustenance, the birds can help residents achieve

economic and social independence in areas often plagued by cycles of destitution and deprivation. This is particularly true for rural women who are the traditional keepers of the flocks. Unfortunately, indigenous chicken breeds of Uganda generally perform poorly under scavenging and semi-scavenging conditions, leaving families struggling to make ends meet financially and failing to adequately supply their basic dietary needs. This is so because, despite low productivity, village households rely on these flocks for family nutrition and income from selling surplus eggs and meat to neighbours or at the village market. It is well accepted that increasing productivity of backyard flocks would improve nutrition and income of rural households resulting in a better livelihood (Sharma *et al.*, 2015). This is particularly the driving force behind the introduction of the Kuroiler chickens in Uganda.

Kuroiler chickens (KC) are high-efficiency dual-purpose scavenger chickens developed in India that closely resemble indigenous chickens (IC) (Ahuja *et al.*, 2008). They produce almost five times the number of eggs per year and attain almost twice the body weight in less than half the time of indigenous backyard chickens. KC were obtained by crossing several pure genetic lines of chickens to select for a high-performance dual-purpose hybrid chicken capable of thriving in village environment under scavenging or semi-scavenging conditions. The selection included phenotypic similarity to the local backyard chickens called “Desi” raised by most rural farmers in India (Sharma *et al.*, 2015). Because of this, the KC can coexist and blend very well with the indigenous chickens (Figure 1.4) that populate the small holder flocks in Uganda. KC became commercially available in the early 1990s in India.

This chicken is preferred by most rural small-holder farmers because of higher egg and meat production in comparison with local chickens. A field study in India revealed that at 5 months of age, the KC had an average body weight of 2.5Kg compared to 800g for the Desi birds (Ahuja *et al.*, 2008). At 9 months, the body weights were 1.23 Kg and 2.70 Kg for Desi chickens and KC respectively (Ahuja *et al.*, 2008). These observations indicated that under village management conditions, KC gained weight more rapidly than Desi chickens. It has been estimated that a Kuroiler hen produces 150-200 eggs in a laying cycle compared to 35-40 eggs produced by a Desi hen (Isenberg, 2007). In addition, because the broodiness gene is poorly expressed, the production by the Kuroiler hen is continuous through the laying cycle; Desi hens lay eggs in clutches of 5-10 eggs per clutch. After each clutch, the hens get broody and stop laying until the progeny chicks hatch and are of several weeks of age (Sarkar and Bell, 2006). Because of superior meat and egg production under rural scavenging or semi-scavenging conditions, KC have been considered a suitable replacement for local (Desi) chickens in India (Ahuja *et al.*, 2007, 2008). Indeed over the last two decades, over one

million rural households in diverse parts of the country have raised KC. A field evaluation involving extensive interviews with KC farmers in four districts of West Bengal, India, revealed that KC rearing improved family nutrition and income (Ahuja *et al.*, 2008).



a) Kuroilers



b) Kuroilers



Indigenous and Kuroiler chicken flock



The East African bwana

Figure 1.4. The indigenous chickens versus the Kuroilers (slides a and b above)

In most countries in Africa, small scale poultry farming by rural households is a cultural norm and over 80% of total poultry production is attributed to family flocks. In Uganda, approximately 46M chickens are produced annually; 86% of this production is in small village backyard or free-range flocks owned by individual rural households (UBOS, 2013). The indigenous chickens (IC) that populate these flocks do not produce adequate meat or eggs to meet the needs of an average household. Because KC have shown great promise in increasing returns from backyard flocks in India, the possibility of introducing this chicken hybrid to Uganda has been evaluated under field conditions.

In a recent study, Sharma *et al.* (2015) compared body weight gain of KC with that of IC raised under similar scavenging conditions in rural households in Uganda. At 25 weeks of age, the average body weight of male KC was 2.6 Kg compared to 1.6 Kg for the IC. And at 43 weeks, the respective body weights were 3.0 Kg and 2.2 Kg. Overall, the study results showed that KC could thrive in Ugandan villages and perform better than the IC under scavenging conditions indicating that returns from backyard poultry production in Uganda and other African countries with similar scavenging conditions could be improved by the introduction of KC. However, information on the nutritional property and carcass traits of the KC is still scanty.

Chapter 2

POULTRY MEAT QUALITY AND NUTRITIONAL COMPOSITION

2.0. Introduction

Meat quality is a general term that describes the sum total of all attributes of meat such as its physico-chemical, biochemical, microbial, technological, sensory and nutritional properties (fat and cholesterol contents) that could influence consumers' satisfaction with the final product. On the basis of this, quality attributes such as appearance, tenderness, juiciness, flavour and fat contents are more important to the consumers while properties such as water holding capacity, shear force, pH, shelf life, protein solubility, and fat binding capacity are considered to be of utmost importance to the processors. Generally, consumer evaluation of eating quality is the major determinant of meat quality (Maltin *et al.*, 2003). Increased concerns related to meat quality are prevalent especially with the rise in life style diseases incidence thought to be linked to the consumption of meat and meat products (Petracci *et al.*, 2015). This is not surprising since the move towards further processing has led to increased handling of the product; thus, creating new appearance issues that were not apparent in the traditional whole or live bird market (Fletcher, 2002; Petracci *et al.*, 2015). In addition, the age at which slaughter of broilers occurs has been continually declining. Thus making meat quality attributes a matter of concern to both the consumers and producers.

The determinants of meat eating quality are multifaceted given that the muscle that makes up meat is highly organised and complex structurally. A myriad of intrinsic and extrinsic factors including genotype, diet, rearing system, pre- and post-slaughter handling among others (Cavani *et al.*, 2009) have been reported to have impacts on the various attributes of meat quality. An understanding of the role of these factors in defining the quality of meat is crucial to improving the quality of the meat products by producers and processors thereby increasing acceptability by the consumers.

2.1. Chemical and nutritional composition of poultry meat

2.1.1. Macro- and micronutrient composition and energetic value of poultry meat

Meat and meat products provide significant quantities of essential nutrients at greater concentrations compared to non-animal source foods. Poultry meat is made up of approximately 60 to 80% water, 15 to 25% protein, and 1.5 to 5.3% lipids (de Oliveira *et al.*, 2016). Usually, there is no significant variation in the nutrient content of the animal's musculature between species. On the contrary, the ratio between fat and muscle mass in the edible part does vary considerably. The quality of animal fat and the amounts of nutrients basically depend on the animal's diet or its genotype, age, rearing environment, and anatomical cut.

Cooking and heating processes usually have only minimal effects on the nutritional profile of meat, mostly corresponding to the concentration of nutrients (including fat) and a decrease in water content. In particular, the energetic value of poultry meats varies between chicken breast and chicken thighs with skin (Gnagnarella *et al.*, 2008): the presence of skin (due to its fat content) increases the caloric value by around 25 - 30%. It must be noted that cooking also affects energetic value, which increases by 30 - 50% for meat with skin (essentially due to a loss of water during the cooking process) (Lofgren, 2005).

2.1.2. Protein

Proteins constitute 16–22 % of skeletal muscle tissue and are generally categorized according to function. Of the total amount of protein in skeletal muscle tissues; myofibrillar (contractile) protein is usually approximately 11.5 %, sarcoplasmic (metabolic) protein is normally about 5.5 % and 2 % stromal (connective or support) proteins (Keeton and Eddy, 2005). Myofibrillar proteins are soluble in high salt solution (about 0.6 M). These proteins build up the myofibril of skeletal muscle that consists chiefly of myosin and actin (Smith, 2010). This group of proteins, situated entirely into the cell, could be divided in three subgroups: muscle contraction protein (22% actin and 43% myosin), enzyme and proteins that aid muscle contraction, and cytoskeletal proteins that make up the cytoskeleton and are responsible for muscle cells integrity and rigidity (8% titin, 5% tropomyosin, 5% troponin, 3% nebulin, 2% C protein, 2% a-actinin, 2% M protein and <1% desmin; Barbut, 2002; Smith, 2010). Sarcoplasmic proteins are found in the sarcoplasm and form about 30% (w/w)

of the total muscle tissue. They include hemoglobin and myoglobin pigments and a wide variety of enzymes. Sarcoplasmic proteins are good emulsifiers, though less effective compared to myofibrillar proteins (Keeton and Eddy, 2005). Myoglobin (MW 16,000) is the main sarcoplasmic protein and is responsible for the color of meat. Myoglobin (Mb) concentration varies depending on the species, breed, sex, age, type of muscle, exercise, and also nutrition. Stromal or connective tissue proteins are not soluble in water or in salt solutions. They are mainly comprised of collagen and elastin and some membrane proteins. In general, collagen represents the most diffuse protein in animal's body, since it is the most important protein that constitutes the connective tissue. Its quantity depends mostly on the muscle's type and physical activity. For instance, the leg meat is generally less tender compared to meat from more inactive muscles due to higher collagen concentrations resulting from increased physical activity and supporting body weight (Barbut, 2002).

Poultry meat, like other meats, milk, and eggs, has a protein component usually defined as 'high quality' (Marangoni *et al.*, 2015). It is well established that animal derived foods have a Protein Digestibility Corrected Amino Acid Score (PDCAAS) value equivalent to or slightly below one. On the other hand, plant-derived foods, which despite containing a relevant quantity of protein have a less favourable protein profile. They are generally lacking in one or more essential amino acids and are more difficult to digest; and have a substantially lower PDCAAS value (e.g. 0.75 for beans and 0.5 for wheat). The protein content of most meat including poultry meat ranges between 15 and 35%, depending on the water and fat content of the product.

Marangoni *et al.* (2015) further noted that cooking also causes an increase in protein concentration, which reaches up to 60% in weight for skinless turkey drumstick and skinless chicken drumstick. Proteins are also the least contributors to the daily caloric intake of all macronutrients. It is also worth noting that protein is the only macronutrient for which a precise recommended intake has been established just like for micronutrients. The low content of collagen which is a structural protein in meat is another favourable characteristic of poultry meat (Maiorano *et al.*, 2012). Collagen reduces the digestibility of meat, and high levels of this protein in muscular meat are associated with a lower percentage of digested products per unit time (Marangoni *et al.*, 2015).

2.1.3. Fat

Meat is generally high in fat particularly saturated fatty acids and its consumption therefore is associated with an increased risk of lifestyle diseases incidence. Nevertheless, the

suggested dietary target for fat in the general healthy population ranges from 25 to 35% of total energy, thus, a typical average intake of 2,000 kcal results in 70 or more grams of these nutrients per day (reviewed in Marangoni *et al.*, 2015).

In addition, when consumed in the right quantities, fat plays a number of important roles such as:

- a) providing essential fatty acids such as linoleic and alpha-linolenic acids, needed for healthy skin, normal brain and nervous system functioning and normal growth in children;
- b) helping the body absorb lipophilic vitamins A, D, E and K, and carotenoids such as beta-carotene;
- c) represents a major source of energy;
- d) promoting a sense of satiety due to slowing effects on gastric emptying;
- e) reducing, for the same reason, the bioavailability of carbohydrates (and, hence, the glycemic response);
- f) enhancing the taste, smell, and texture of foods thereby giving food a palate appeal.

It must also be noted that the muscular part of animals, lacking visible fat, has a fairly limited lipid content, which was further reduced over the past decades, due to the changes and improvement in farming techniques and feed quality and profile. Lipid intake associated with poultry meat is variable and dependent on the cut considered. Fat is nonetheless mainly found in skin and can, therefore, be easily removed (Marangoni *et al.*, 2015). The lipid content of chicken is around 1% in the leanest cuts, such as the breast, and around 17% at the opposite extreme in cooked chicken wings with skin. The inclusion of skin can increase these values. Cooking can also increase the fat content although it is less so in fat compared to protein content, by removing water from meat, or by adding fat present in the condiments used during preparation. Nonetheless, when compared to other types of meat, poultry appears to be relatively low in fat content.

From a nutritional point of view, the composition of poultry fat is generally favourable: it has significant amounts of monounsaturated fatty acids with saturated fatty acids constituting only a third of the total fat. In comparison with bovine, ovine, or pig meat, poultry fat has substantial amounts of polyunsaturated fatty acids, especially the omega-6 linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6), which can be found mostly in the skin (Marangoni *et al.*, 2015), and also some amounts of long-chain omega-3 fatty acid alpha-linolenic acid although the amount varies depending on diet. In most Western countries, where fish

consumption (a major source of omega-3) is relatively low, poultry meat may thus represent an important source of these fatty acids.

Besides fatty acids, cholesterol is another nutritionally important component of meats. Cholesterol exists in meat as free cholesterol and esterified cholesterol. Free cholesterol is associated chiefly with cellular and subcellular membranes of muscle and intramuscular adipocytes. Because intramuscular adipocytes are essentially lipid-filled spheres with very little membrane content, the amount of cholesterol associated with membranes is small (usually about 25 %). Esterified Cholesterol, located within the triacylglycerol-rich central lipid vacuole, comprises about 75 % of the total cholesterol in adipose tissue. According to Smith *et al.* (2004), muscle fibers have nearly 75 % of their total cholesterol associated with membranes and the rest are in the form of neutral lipids.

Poultry meat is characterized by a low cholesterol content (broiler *Pectoralis* muscle, 47.41 mg/100 g muscle; Chizzolini *et al.*, 1999) making it healthier than other meat products i.e. beef 66 mg/100 g, pork 65 mg/100 and lamb 50 mg/100 (Chizzolini *et al.*, 1999). However, other studies on broiler chicken reported higher cholesterol values; for example, Salma *et al.* (2007) reported an average cholesterol content of 93.6 mg/100g of meat in *Pectoralis major* of 56 day old male Chunky broilers; while, Maiorano *et al.* (2012) reported cholesterol values ranging from 70.45 to 78.12 mg/100g in 42 days old broiler chickens and de Oliveira *et al.* (2016) reported values ranging from 47.88 to 68.15 mg/100 g in breast and thigh respectively. The reported discrepancies in cholesterol content could be explained by the use of different analytical methodologies for cholesterol quantification and sampling (Bragagnolo and Rodriguez-Amaya, 2002), diet, breed (de Oliveira *et al.*, 2016), diet, age, and sex (Wang *et al.*, 2005).

2.1.4. Carbohydrates

Animal-derived foods including chicken meat contain very few carbohydrates, which, conversely, are found abundantly in plant-based foods. The only naturally occurring carbohydrate in muscle is glycogen, whose content rapidly decreases following butchering. Carbohydrates are present in a relatively small concentration in living muscle tissues, ranging from 0.5 % to 1.5 %. The main carbohydrate is glycogen, a branched polysaccharide composed of α -D-glucose units linked by α -1,6 glucosidic and α -1,4 glucosidic bonds, which in living animal functions as an energy store supplying energy for muscle contraction through aerobic glycolysis and subsequent conversion of lactic acid back to glycogen. After

death, no oxygen is available from blood, aerobic pathways stop, and this causes, for a short period of time, a conversion to anaerobic glycolysis, in which glucose is converted into lactate. At 24 h postmortem, glycogen concentration drops to less than 1 % while lactate concentration builds-up, consequently, the pH of the meat drops. On the other hand, insufficiency of glycogen prior to slaughter results in muscles with a high pH usually showing dark firm and dry meat defect with a short shelf-life due to an extremely high water holding capacity. Other carbohydrates include glucose, other mono- and disaccharides (0.1 – 0.15 %), and intermediates of glycogen metabolism (Warriss 2000; Keeton and Eddy 2005).

2.1.5. Vitamins and minerals

Meat represents an excellent source of the majority of hydrophilic vitamins, and it is the ideal dietary source of vitamin B12 (reviewed in Marangoni *et al.*, 2015). The amounts of B-group vitamins (e.g. niacin, vitamin B6, and pantothenic acid) in poultry are very similar to those of other meats and do not significantly diminish during cooking. Although red meat is the most abundant in terms of vitamin B12, poultry remains an important source of niacin. Lipophilic vitamins such as vitamins E and K, contained in muscles, are less abundant in meat compared to plant-based foods.

Meat also provides several minerals. Despite a large variability in iron concentration across different types of meat, poultry also provides this mineral (100 g of chicken thighs provide 1.4 mg of iron, compared to 1.3 mg for an equal amount of rump steak from an adult bovine). Sodium is only minimally present in fresh meat and in poultry too, and does not significantly contribute to total dietary intake. Processed meat products, on the other hand, can contain high or very high quantities of sodium, added as a preservative or flavour enhancer. Chicken meat is also an excellent source of selenium.

Additionally, lean meat contains factors that promote the bioavailability of a variety of nutrients, which is often larger compared to that of the same nutrients present in plant-based foods. Besides heme iron, zinc, copper, and B vitamins are also highly bioavailable when consumed with meat. At the same time, meat also promotes the bioavailability of nutrients found in other foods when consumed concurrently. For example, the absorption of non-heme iron contained in other foods is increased when they are consumed with meat.

2.2. Chicken meat sensorial and functional quality traits

The major poultry meat quality attributes are appearance, texture, juiciness, flavour, and functionality. Appearance and texture are the most important of the aforementioned attributes since they influence consumers' choice and ultimate satisfaction with poultry meat products. Although juiciness and flavour are extremely important attributes, Fletcher (2002) observed that except for isolated defects they are most often more a function of preparation than of the product itself. The relative importance of meat functionality and sensory quality attributes have increased in recent years as a result of the increase in trends in further processing as consumers prefer more processed products. Complex products such as sausages, marinated fillets, breaded products, fully cooked heat-and-serve items, frozen entrees, and complete dinners require an understanding of the contribution of poultry meat to these products as well as their influence on sensory properties of the food. Functional properties such as water holding capacity are critical for successful product formulation. A basic understanding of the live production and processing factors that influence these poultry meat quality attributes, especially colour and texture, is necessary to produce consistently high quality poultry products.

2.2.1. Appearance

Appearance is the most critical quality attribute for the selection of many foods, including poultry products. The consumers' decision to purchase or reject a product most often is solely based on its appearance. Appearance is also critical for final product evaluation due to its effects on other sensory properties. One of the major contributing components of appearance is colour. Colour has long been known to be a major selection criterion for fresh poultry and meat products as well as for final product satisfaction.

For poultry meat products, colour is important for skin, meat, and bone. Skin colour is most critical for the marketing of fresh whole birds or parts. Meat colour is most important both for the selection of deboned and skinless raw meat. It is as well a critical factor for the final evaluation of many cooked products. Pink or red appearance of cooked poultry meat is generally associated with undercooking and is highly undesirable. Dark or black bones are also considered to be a defect in fully cooked products. Bone darkening is primarily associated with frozen products prior to cooking. Other visual defects are associated with bruises, haemorrhages, blood pooling, or any number of other possible discolourations.

2.2.1.1. Skin colour

Market studies in the early 1960's clearly showed pronounced regional differences in consumers preferences for fresh whole broilers based solely on skin colour (reviewed in Fletcher, 2002). These studies showed that consumers generally prefer broiler skin colours ranging from white, to pale yellow, to deeply pigmented on the basis of traditional regional supplies. Earlier studies on this subject reported a general preference by consumers of poultry skin colours which were traditionally available and based on feeding practices as well as genetic stock (Fletcher, 2002). Even in the modern markets, the trend is still almost the same with consumers favouring their traditional market forms. For instance, in the Eastern United States, deeply pigmented birds are the most desired; in the South-eastern US, moderately pigmented birds are preferred; in the North-western US, pale skin colour is preferred; in the United Kingdom, consumers prefer a white, non-pigmented skin. Similar differences in colour preferences exist all over the world, and are based primarily on historical and regional supplies, traditional genetic stock (i.e. ability of some breeds to deposit carotenoid pigments in the skin), and availability of carotenoid containing feedstuffs.

Because of its market impact, much is known about the factors affecting skin pigmentation. By as early as 1915, researchers had already recognized the principal pigment involved in the colouration of egg yolks and tissues of poultry (reviewed in Fletcher, 2002). Pigmentation, or the deposition of pigments in the skin of the bird, depends upon the genetic capability of the bird, dietary source of pigments, health of the bird, and processing. Broilers must have the genetic ability to deposit carotenoid pigments in the epidermis. With the exception of the Cornish breed, English Class birds lack this genetic ability to deposit carotenoid pigments in the skin thus these birds have a white appearance regardless of diet or other factors. Those birds that have the genetic disposition to deposit the carotenoid pigments in the skin must also have the pigments supplied in the diet.

For this reason, numerous studies have been conducted to evaluate the skin pigmenting properties of a variety of both natural and synthetic sources. Diseases, particularly coccidiosis, have been shown to have dramatic negative effects on pigmentation. Flock health is therefore critical to uniform pigment absorption and deposition. Since the carotenoid pigments are deposited in the epidermis, care must also be exercised in processing not to remove this layer by over-scalding or damaging the skin during picking (Fletcher, 2002).

However, with the increase in further processing of poultry and changing markets with increased cut up, deboned meat, and fully cooked products, the relative importance of skin colour has decreased in recent years, primarily in developed countries. As the skinless raw

and further processed products increase in the markets, the demand for whole birds and skin-on parts is gradually reducing. The increased trend for further processing, which includes numerous breaded or coated products, has also resulted in a requirement by further processors to remove the epidermal layer of skin during scalding so as to increase coating adherence to the underlying dermal layer of the skin during further processing and cooking (Fletcher, 2002). The epidermal layer, also known as the cuticle, is loosened at scald water temperatures above 54°C and is removed during picking leaving only the underlying dermal layer of skin. Thus even in areas which may prefer a yellow skinned bird, further processing demands for removing the epidermis has resulted in decreased economic incentives to maintain high carotenoid levels in the feed.

2.2.1.2. Meat colour

Colour and variations in colour are important quality attributes that affect selection and acceptability of many foods. Colour of raw poultry meat is critical for consumer selection while colour of the cooked meat is critical for final evaluation. Colour variation and colour defects in poultry have long been recognized as a major problem (Qiao *et al.*, 2002). Consumers will often reject products with colours which differ from the expected pale tan to pink raw meat or from the tan to grey cooked meat. This is especially true with the appearance of pinkness in fully cooked meat which is a major defect in poultry meat products.

A survey by Fletcher (1999a) indicated that approximately 7% of skinless-boneless breast fillets, packaged 4 fillets to a pack, had one or more fillets which were noticeably different from the other 3 fillets in the same package. Fletcher (1999b) surveyed five commercial broiler processing plants and found that breast meat colours had a wide range with lightness values (43.1 to 48.8 using the CIELAB colour description system) which had a strong negative correlation to muscle pH. While in another study, Fletcher *et al.* (2000) showed that variations in raw breast meat colour were sufficient in causing variations in cooked product appearance. All these results demonstrate that significant variations in breast meat colour do exist and that they are present at the retail level.

A plethora of factors affect poultry meat colour. These include: bird's sex, age, strain, processing procedures, chemical exposure, scalding temperature, cooking temperature, irradiation, and freezing conditions, pre-slaughter conditions, reaction of the major meat pigment, myoglobin, as well as effects of nitrates and nitrites, ovens and environmental

gasses (primarily carbon monoxide and nitric oxide), haemochromes, and cytochrome C (Maga, 1994; Froning, 1995).

Stress immediately prior to and during slaughter has been shown to affect meat colour. Studies have shown that antemortem temperature stress and excitement just prior to slaughter affect meat colour in turkey (reviewed in Fletcher, 2002). Stimulating antemortem stress using epinephrine injections just prior to slaughter resulted in darker breast meat due in part to an increased muscle pH and haemoglobin content of meat (Walker and Fletcher, 1993). On the other hand, thermal preconditioning and heat shock in chicken resulted in breast meat that appeared pale, soft, and exudative (Northcutt *et al.*, 1994). While, studies on the effect of stunning on broiler chicken meat found little or no direct effect on broiler breast meat colour (reviewed in Fletcher, 2002). It was however reported that electrical stunning at high currents increased blood spots in broiler breast meat.

Gas stunning or gas killing has been shown to affect breast meat colour. Mohan Raj *et al.* (1990) reported that broiler breast muscles from argon killed birds were less dark than those killed conventionally or killed with carbon dioxide. Fleming *et al.* (1991a) found that stunning with carbon dioxide resulted in significantly less red breast and thigh meat compared to electrically stunned turkeys. On the other hand, Froning and Uijttenboogaart (1988) reported that postmortem electrical stimulation resulted in darker broiler breast meat.

The effect of chilling and leaching of haem pigments on poultry meat colour is unclear. Fleming *et al.* (1991b) reported no effect of immersion *versus* air chilling on broiler breast or thigh muscle colour or haem content. However, Boulianne and King (1995) attributed pale boneless broiler breast fillets to loss of haem pigments during storage in ice slush tanks. Yang and Chen (1993) reported a decrease in lightness and redness values of ground breast and thigh meat with storage time.

The major factors that contribute to poultry meat colour are myoglobin content, chemical state of the haem structure, and meat pH. Furthermore, myoglobin content is known to be mostly related to species, muscle, and age of the animal. Whereas muscle pH has been shown to be primarily related to the biochemical state of the muscle at time of slaughter and following *rigor mortis* development. Both of these factors contribute to meat colour and the occurrence of meat colour defects. The relationship of animal species, muscle type, and animal age on meat myoglobin content and visual colour was reported by Miller (1994).

The colour of meat is very important especially for fresh and processed meat products. Thus the biochemistry of the haem pigments and their reactions that affect meat colour are extremely well documented in the general meat science literature. Bodwell and McClain

(1978) presented a comprehensive coverage of the myoglobin reactions associated with fresh meat colour and cooking. The various ionic and covalent complexes of both the ferrous and ferric state of the haem with oxygen and other compounds to form the basic meat colour variations from the purplish red of deoxygenated myoglobin to the bright red of oxymyoglobin to the brown/gray of metmyoglobin are well established. Bard and Townsend (1978) discussed numerous haem reactions involved in meat curing. The reactions with various nitrogen compounds and heat to form stable nitrosyl haemachrome complexes produce the desirable pink colour of cured red meats or the undesirable pinkness of some poultry products. A series of research reports by Ahn and Maurer (1990a, 1990b, and 1990c) presented a comprehensive coverage of the complex haem reactions that affect turkey breast meat colour. This series of papers clearly illustrates the complexity of the numerous potential compounds and their effects on turkey meat colour. Their work also illustrated the pronounced effect of muscle pH on the formation of these complexes.

2.2.2. Texture (tenderness)

Texture/tenderness, defined as the ease of mastication, is probably the single most critical quality factor associated with the consumers' ultimate satisfaction with a poultry meat product. Many external factors contribute to the wide variation in meat tenderness. These factors can be related to the bird and environment, processing conditions, and cooking methods. Age, strain, and sex as well as environmental and nutritional stresses have all been shown to influence the variation in tenderness among meat samples (Owens *et al.*, 2004). The two major intrinsic factors contributing to poultry meat tenderness are the maturity of the connective tissues and contractile state of the myofibrillar proteins (Fletcher, 2002).

Maturity of the connective tissue involves the chemical cross bonding of the collagen in the muscle. Collagen is an abundant protein in the body and the predominant form of protein that makes up the epimysium, perimysium and endomysium in muscle tissue. Because of the multiple crosslinks in its structure and the ordered array of the collagen fibrils, collagen is fibrous and has high tensile strength. As animals age, heat stable crosslinks form between collagen fibrils thus further increasing tensile strength (Bailey and Light, 1989) which remains during cooking resulting in tough meat (Maiorano *et al.*, 2012).

The second factor, the contractile state of the myofibrillar proteins, is predominantly a function of the rate and severity of *rigor mortis* development. With the new developments in the broiler industry where birds reach market weight much earlier than in the past, the issue of age related toughness resulting from connective tissue cross-linking has virtually vanished.

Except for spent hen and older bird utilization, or for specialty markets such as for capons, age related connective tissue toughness is not a major factor in broiler meat quality since the market age of broilers is less than 7 to 8 weeks of age. The myofibrillar protein impacts on ultimate meat tenderness are primarily a function of the biochemical predisposition of the muscle at the time of slaughter, the rate and severity of *rigor mortis* development, and the physical handling of the carcass and muscle during rigor development.

With traditional broiler industry production practices, processing, and the predominant marketing of whole carcasses the negative impact of the myofibrillar protein reactions were not thought to have a major impact on meat quality. Although the predominant marketing of young broilers minimizes age associated toughness, the economic incentive to cut-up and debone broilers earlier in the processing scheme has resulted in an increased incidence of tough broiler breast meat (Fletcher, 2002). If the carcass is cut-up into parts, or more importantly, if the breast meat is removed from the carcass prior to the completion of *rigor mortis*, the muscles usually contract unimpeded by the normal skeletal restraint, the muscle fibres then contract and shorten the muscle, and the resulting meat is therefore less tender.

The contractile state of the muscle is influenced by the conversion of muscle to meat (*rigor mortis* development), a process which takes approximately 4 h in broilers. During this process, which begins at the time of slaughter, muscle pH declines due to an accumulation of lactic acid in the muscle resulting from the loss of the circulatory system. In addition, ATP content declines as the muscle shifts from aerobic to anaerobic metabolism and as the muscle continues to use energy. Although there is a natural shortening of muscle sarcomeres due to the increased actomyosin bond formation during *rigor*, there are times at which the sarcomeres can shorten to a great extent by external stimuli. For example, if the muscle is stimulated by deboning, prior to *rigor* completion when ATP is present in sufficient amounts, the muscle can shorten thus altering the state of contraction resulting in changes in meat tenderness and acceptance by the consumer (Owens *et al.*, 2004).

However, in recent years with the dramatic increase in cut up, deboned meat, and further processed products, the demands are placed on the slaughter plant to cut up and debone the carcasses as fast as possible. During the past 20 years, intensive research efforts have been focused on determining the live bird and processing factors which affect breast meat tenderness. The ultimate goal has been to develop slaughter methods which would allow for acceleration of post-mortem *rigor mortis* such that carcasses could be cut-up and deboned as soon after slaughter as possible. Innovative techniques such as pulsed electrical stimulation, (Lyon *et al.*, 1989; Sams *et al.*, 1989) wing restraints or tensioning (Papa *et al.*, 1989; Lyon *et*

al., 1992a), post-chill flattening or extended holding of deboned breasts (Lyon *et al.*, 1992b), marination (Alvarado and Mckee, 2007), and various combinations of these techniques (Birkhold *et al.*, 1992; Lyon and Dickens, 1993; Dickens and Lyon, 1995) have been devised to eliminate the need for postmortem aging. However, these techniques have had limited application due to variation in results in commercial settings.

2.2.3. Water holding capacity (WHC) and pH

WHC is defined as the ability of meat to hold its inherent and added moisture during fabrication, processing, and storage. Poor water holding capacity in raw poultry meat results in diminished visual appeal and inferior palatability traits for consumers as well as reduced ingredient retention, protein functionality, and product yields for processors. This is so because the aqueous solution that is lost from postmortem muscle (purge or drip) contains significant amount of protein, on average approximately 112mg of protein per milliliter of fluid. Most of the proteins found in drip are water-soluble, sarcoplasmic proteins (Savage *et al.*, 1990). The light red color of drip is due to the fact that it contains the sarcoplasmic protein myoglobin; the pigment found in meat. Typically very little hemoglobin (pigment in blood) from blood is found in drip (Savage *et al.*, 1990). In addition to myoglobin, glycolytic enzymes and other sarcoplasmic proteins, amino acids and water-soluble vitamins are found in the purge. Many factors: genetics, as well as live animal handling and early postmortem temperature management, have the potential to greatly influence the rate and extent of pH decline, and thus the WHC of the meat.

The ability of meat to bind water is a complex trait that is influenced by structural and biochemical changes that occur during the transformation of muscle to meat by the process of *rigor mortis* (Bowker and Zhuang, 2015). Fresh muscle is approximately 75% water by weight. Water in muscle is classified as bound, immobilized, or free water (Offer and Knight, 1988). Immobilized water makes up to 80% of the water in muscle and is held within the myofibrillar structure, between the myofibrils, and between the myofibrils and the sarcolemma (Offer and Cousins, 1992). Due to changes in muscle structure and pH that occur during the transformation of muscle to meat, immobilized water can escape from the muscle along with the free water as drip loss (Offer and Knight, 1988). The degradation of protein linkages between different structures within the muscle cell are also thought to influence drip loss (Huff-Lonergan and Lonergan, 2005). Broiler breast muscles (*pectoralis major*) are typically comprised of nearly 100% type 2B, fast-twitch, glycolytic muscle fibers (Sams and Janky, 1990). As a result broiler breast meat undergoes a rapid postmortem pH decline

making them susceptible to developing inferior WHC characteristics. Muscle pH and protein denaturation are considered to be the main determinants of WHC in meat (Offer and Knight, 1988). As muscle pH decreases with the progression of postmortem metabolism, the net charges of the muscle proteins are reduced. This decrease in net protein charge results in diminished WHC due to the availability of fewer charged protein sites for binding water and because the lack of repulsive charges allows muscle proteins to become more closely packed, which forces more of the immobilized water into the free water compartment.

Protein denaturation due to a rapid or an extended postmortem pH decline in meat can also negatively influence WHC. In pork and turkey muscle, low WHC and pH are closely associated with high degrees of postmortem myofibrillar and sarcoplasmic protein denaturation and reduced protein solubility (Pietrzak *et al.*, 1997; Warner *et al.*, 1997; Joo *et al.*, 1999; Choi *et al.*, 2010; Chan *et al.*, 2011; Yin *et al.*, 2014). Myosin denaturation in particular is thought to be the key event in the development of poor WHC (Pietrzak *et al.*, 1997). While this phenomenon is routinely cited in literature on WHC in chicken, actual data supporting this theory of WHC in chicken are scarce or contradictory. Some reports have shown that isolated myofibrillar proteins from chicken breast muscle are resistant to denaturation under *in vitro* conditions simulating postmortem muscle (Van Laack and Lane, 2000) and that pale and normal coloured fillets have similar protein solubility levels (Van Laack and Lane, 2000). However, others have observed lower salt-soluble protein extractability in pale breast meat (Barbut *et al.*, 2005) and increased sarcoplasmic protein denaturation in fillets incubated at elevated temperatures (Zhu *et al.*, 2011). In a study on the occurrence of PSE-like breast meat, Zhu *et al.* (2012) observed that meat with low WHC and low pH also exhibited substantially lower sarcoplasmic and total protein solubility than normal meat. As a result of these varied reports, the role that protein denaturation plays in WHC in chicken meat is unclear.

Due to the dynamic nature of postmortem muscle, WHC characteristics in chicken meat have been shown to change during the first 24 h postmortem. Using a salt-induced water uptake method, Zhuang and Savage (2012) observed that WHC was greater at 24 h postmortem than at 2 h post-mortem in broiler breast meat. It is unknown if the relationships between muscle protein denaturation and WHC attributes in broiler breast meat are influenced by postmortem time. In a study to further delineate the relationship between WHC and protein denaturation in chicken breast meat exhibiting divergent WHC attributes, Bowker and Zhuang (2015) showed that other than being weakly related to drip loss accumulation at 2 days postmortem ($r = -0.33$ and -0.31), overall sarcoplasmic protein solubility

measurements, like myofibrillar protein solubility, were not strongly correlated to the WHC attributes measured in the study. These results suggest that the overall degree of sarcoplasmic protein denaturation, as indicated by total sarcoplasmic protein solubility, has limited impact on WHC in commercially processed broiler breast fillets. The results further confirm the findings of Zhuang and Savage (2012).

Chapter 3

QUALITY ISSUES ASSOCIATED WITH POULTRY MEAT

3.0. Introduction

The past few years have witnessed a lot of changes in meat production and marketing, many of which have impaired the image of food products of animal origin to the consumers (Toldrá and Reig, 2011). This is mainly due to their content in fat and saturated fatty acids, cholesterol, sodium and other substances such as nitrosamines that somehow are thought to be involved in diseases like cardiovascular diseases, diabetes mellitus (Micha *et al.*, 2010) and cancer (Cross *et al.*, 2010; Ferguson *et al.*, 2010; Santarelli *et al.*, 2010). Indeed, epidemiological data suggest a relationship between consumption of meat or dietary heme and the risk of colon cancer (Cross *et al.*, 2010; Bastide *et al.*, 2010).

For poultry meat on the other hand, production and consumption have increased rapidly and, in many parts of the world per capita consumption of poultry meat is expected to continue to grow. Relatively low and competitive prices compared to other meats, the absence of cultural or religious obstacles, and dietary and nutritional properties are the main factors behind poultry meat's attractiveness to the consumers. Nutritionally, poultry meat fits well the current consumer demand for a low-fat meat with a high degree of unsaturation in terms of the fatty acids profile, and low sodium and cholesterol levels. Poultry meat also qualifies to be considered as "functional foods", since it provides bioactive substances with favourable effects on human health, like conjugated linoleic acid (CLA), vitamins and antioxidants, and a balanced n-6 to n-3 PUFA ratio.

The growing demand for poultry meat has resulted in pressure on breeders, nutritionists and growers to increase the growth rate of birds, feed efficiency, size of breast muscle and reduction in abdominal fatness. Today, chickens and turkeys are marketed in about half the time and at about twice the body weight compared to 50 years ago. These improvements are mainly due to the high heritability of body weight and body composition during breeding. This kind of selection has obviously put more stress on the growing bird and some believe it has resulted in histological and biochemical modifications of the muscle tissue (Petracci *et al.*, 2015).

Several studies evidenced that fast growing strains exhibit a high incidence of spontaneous or idiopathic myopathies (e.g., deep pectoral muscle disease) and an increased susceptibility

to stress-induced myopathies which may have great implications for meat quality and incidence of abnormal conditions such as pale, soft and exudative (PSE)-like meat (reviewed in Petracci and Cavani, 2012; Petracci *et al.*, 2015). Additionally, selection for muscle growth is also believed to have resulted in an increase in meat quality problems associated with toughness and poor cohesiveness, colour, and water holding properties (Dransfield and Sosnicki, 1999).

These abnormalities are increasing the meat downgrading rates for fresh market retailing and to some extent the nutritional, sensory and technological proprieties of raw meat materials used for further processing. With the evolution of the sale of more processed and/or ready to eat or ready to cook meat, quality of fresh and raw meat is becoming more important economically as traits such as water holding capacity, appearance and texture directly influence purchasing decision. It is therefore imperative to look at the factors that are associated with development of meat quality defects in poultry.

3.1. Muscle Myopathies

With the increase in growth rate and muscle size, there has been an increase in incidence of pectoral myopathies (e.g., deep pectoral myopathy, focal myopathy etc). Among these, deep pectoral myopathy (DPM) has the most significant impact on final product quality. DPM (Figure 3.1) exhibit haemorrhagic appearance with a swollen reddish-brown lesion, typical of an early developing stage, that gradually becomes green as it ages (Petracci *et al.*, 2015). Thus, the presence of DPM may result in significant commercial complaints, if the whole carcass is sold to cut-up as well as processors units or butcher shops. DPM, also known as Oregon disease or green muscle disease, was first described in 1968 as “degenerative myopathy” in turkeys and it was subsequently studied at the Oregon State University. Even though this condition was first recognized in adult meat-type turkey and chicken breeders, it has become more and more common in meat-type growing birds (reviewed in Petracci and Cavoni, 2012). Siller (1985) observed that DPM occurs exclusively in birds that have been selected for breast muscle development. It is largely acknowledged that DPM is an ischemic necrosis that develops in the deep pectoral muscle (*supracoracoideus* or *pectoralis minor* muscle) mainly because this muscle is surrounded by inelastic fascia and the sternum, which do not allow the muscle mass to swell in response to the physiological changes occurring when muscles are exercised, as in wing flapping (Jordan and Pattison, 1998). It has been estimated that, in turkeys and broilers, the supracoracoid

increases in weight by about 20% during activity for the huge blood flow into the muscle. The increased size of the muscle is so marked in the heavy breeds that the muscle becomes strangulated and ischemic, because the increased pressure within the muscle occludes the blood vessels and causes a necrosis of the muscle. The lesion does not impair the general health of birds and is generally found during cut-up and deboning; moreover, it can be both unilateral and bilateral, affecting just one or both *pectoralis minor* muscles, respectively. Apart from being aesthetically undesirable, DPM is of no public health significance. Therefore, the affected fillet should be removed but the rest of the carcass is still fit for human consumption. However, the required trimming operations cause the downgrading of the products leading to economic loss for the industry, especially because it affects the most valuable part of the carcass. The incidence of DPM has been estimated to vary between 0.02% and 1.9% (Kijowski *et al.*, 2014). According to Bilgili *et al.* (2000), the incidence of DPM increases with market weight in broilers, with more cases reported in higher-yielding strains and in males. Recently, Lien *et al.* (2012) noted that DPM appears to begin at approximately 26 and 36 days of age in male and female broilers, respectively. It has also been observed that increased bird activity (flock nervousness, flightiness, struggle, and wing flapping) induced by factors such as feed or water outages, lighting programs and intensity, human activity, and excessive noises in and around chicken houses could trigger the development of DPM in broilers. Bianchi *et al.* (2006) showed a strong relationship between DPM prevalence and breed of birds, suggesting that genetics may play an important role in the determination of this condition. Furthermore, Castellini and Mugnai (2002) studied the behaviour of fast-growing chickens under organic farming conditions and concluded that their lack of movement was more of a genetic predisposition than the effect of rearing condition. As a consequence, genetic selection against DPM has been initiated by broiler breeding companies. Likewise, a recent development in whole-genome selection using dense DNA-markers is also expected to provide effective and powerful tools to reduce DPM occurrence in the future (Petracci *et al.*, 2015).



Figure 3.1. Deep pectoral myopathy (Petracci and Cavani, 2012).

3.2. PSE-like Breast Meat

One of the most frequent challenges to the meat industry associated with the intensive selection for increased muscling is the development of pale, soft and exudative (PSE) meat. The term PSE was originally used to describe a pork product characterized by light colour, flaccid texture, poor water-holding capacity and substantially reduced cooking yield. In swine, a genetic single mutation in the ryanodine receptor of the sarcoplasmic reticulum involved in calcium release has been identified and has been associated with animals that are stress-susceptible and prone to developing PSE meat (Barbut *et al.*, 2008; Picard *et al.*, 2010). With the advent of technologies to identify and eliminate this major cause of extreme cases of PSE, a great reduction in the incidence and severity of PSE has been realized, even if products with poor water holding capacity still exist. Pale, soft and exudative (PSE-like) condition came to light in poultry just a few decades ago (Figure 3.2). Even then, there is no evidence of a link between the development of PSE and genetic mutation (Petracci *et al.*, 2015).

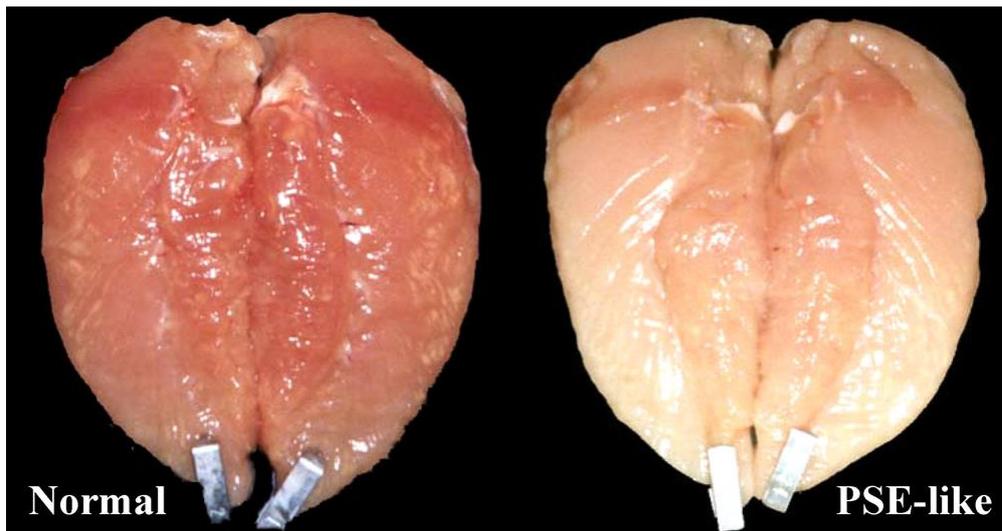


Figure 3.2. Pale, soft and exudative (PSE)-like broiler breast meat (Petracci and Cavani, 2012).

A plethora of studies have been conducted to establish the main causes of PSE-like condition in poultry (Owens *et al.*, 2009; Petracci *et al.*, 2009). Many of which evaluated the role of genetic selection and environmental factors in bringing about PSE-like condition. It has been shown that selection for body weight or muscle development has induced histological and biochemical modifications of the muscle tissue, which can be related with PSE-like condition (Barbut *et al.*, 2008). Several studies conducted demonstrated that modern rapidly growing strains of broilers exhibited a higher incidence of spontaneous or idiopathic myopathy and an increased susceptibility to stress-induced myopathy (reviewed in Petracci and Cavani, 2012). These pathologies are attributable to alterations in intracellular calcium homeostasis and the consequent changes in sarcolemmal integrity and may result from excessive myofiber hypertrophy and inadequate development of support tissues and vascular supply (MacRae *et al.*, 2006, 2007). These authors further added that these myopathies may have profound implications for meat quality and the incidence of specific conditions such as PSE-like meat. Among environmental factors to induce PSE-like meat occurrence, heat stress especially at the end of the growing phase seems to play a key role (Petracci *et al.*, 2010). Studies have pointed out that faster growing birds are more susceptible to heat stress shown by great metabolic heat production, increased body temperature, and mortality. Sandercock *et al.* (2001, 2006) reported that fast growing birds exhibit a reduced thermoregulatory capacity compared with their genetic predecessors and may therefore be more susceptible to heat stress during the pre-slaughter period. This could consequently result in problems including muscle damage, acid-base disturbances, and reduced meat quality. Mujahid *et al.* (2005) demonstrated that acute heat stress increase superoxide free radical production in chicken

skeletal muscle. A condition which is thought to be responsible for the transport stress- and heat stress-induced muscle damage as well as the changes in muscle and meat quality observed in broilers. Thus muscle cell metabolism and alterations in sarcolemmal integrity and tissue structure associated with oxidative damage and myopathy may have profound implications for meat quality and the incidence of specific conditions such as PSE-like meat.

Currently, with the advent of “omics” science Petracchi and Cavani, (2012) observed that there could be more possibilities to further investigate meat quality problems. For instance, today proteomic studies are becoming more and more popular to study the relationship between genome and functional properties of meat. While genome contains information on which genes and alleles are present in the genome, the proteome contains information on which genes are actually being expressed and translated into proteins. Consequently, as Hollung and Veiseth-Kent (2011) put it, understanding the variations and different components of proteome with regard to certain quality or processing parameters will lead to knowledge that can be used in optimizing the process of *rigor mortis*. Post mortem proteomic analyses provide important molecular information on related metabolic pathways and help to identify mechanisms underlying muscle conversion to meat and meat quality development (Picard *et al.*, 2010). Preliminary studies carried out on PSE-like meats using proteomic tools indicated that the process of *rigor mortis* was probably modified in muscles having predominant fast glycolytic metabolism (e.g., *pectoralis major* and *pectoralis minor* muscles) and this could cause modifications in proteolytic enzyme functionalities and/or muscle protein denaturations. In addition, Remignon *et al.* (2008) observed that possible modifications of two glycolytic enzymes: fructose biphosphate aldolase and glyceraldehyde 3-phosphate dehydrogenase could explain the differences in the rate of pH decline between normal and PSE-like breast meat. The authors concluded that proteomics could be used to try to detect, as early as possible animals carrying these modifications that could make them prone to producing poor quality meat. Genomics (nutrigenomics) have also been applied in studies trying to elucidate the effects dietary nutrients could have on gene expression with particular focus on meat quality issues in poultry. For instance, it is well known that supranutritional dietary levels of tocopherols have beneficial effects on oxidation and loss of quality in poultry meat. This can effectively inhibit the development of PSE-like meat thereby improving meat functional properties (Olivio *et al.*, 2001). This is so because the integrity of the cell membrane is thought to influence liquid losses and protection of membranal lipids against lipid oxidation by endogenous vitamin E has been suggested to be the mechanism responsible for the positive influence of dietary vitamin E on the water

holding capacity (Jensen *et al.*, 1998). In support of the above observation, Li *et al.* (2009) using a nutrigenomic approach showed that long-term dietary vitamin E supplementation leads to altered transcription of genes related to lipid metabolism via major signal transduction pathways involving protein kinase C and phosphatidylinositol 3-kinase, thereby improving fatty acid synthesis and composition of body fat. The same authors concluded that vitamin E beneficial effects on lipid stability in muscle and meat quality and fatty acid composition could be related to its influence on the expression of genes controlling lipid metabolism.

3.3. Intramuscular Connective Tissue Defects

Poor cohesiveness of meat is an emerging quality issue in poultry meat due to immaturity of intramuscular connective tissue (IMCT) as a result of the very early slaughter age of modern broiler strains (Petracci and Cavani, 2012). The structural integrity of muscle fibers is maintained by three layers of IMCT:

- (i) the endomysium that surrounds individual skeletal muscle fibers;
- (ii) the perimysium that bundles a group of muscle fibers, and
- (iii) the epimysium that ensheathes the whole muscle (Nishimura, 2010).

IMCT is predominantly composed of cells and extracellular matrix, which is composed of collagen, proteoglycans and glycoproteins. The epimysium is often thick and tough; nevertheless, it is usually separated from cuts of meat therefore plays a minor role in determining meat quality. The IMCT is thus the combined peri- and endomysium depots, even if perimysium constitutes about 90% of total connective tissues in muscles (McCormick, 1999). The strength of IMCT is based on collagen fibrils and there are cross-bridges (cross-links) between the collagen molecule units and also between the collagen molecules. These cross-links determine the physical strength and heat stability of IMCT. The number and stability of cross-links increase with age thereby reducing meat tenderness (Maiorano *et al.*, 2012). In fast-growing birds the collagen is immature with few cross-links resulting in low heat stability. Consequently, meat from these birds is tender, but may turn fragile or even mushy (Puolanne and Voutilainen, 2009).

According to Voutilainen *et al.* (2009) there are two emerging types of defect in commercial poultry meat today:

- ✓ cooked chicken breast meat which is generally fragmented and soft (Figure 3.3); and
- ✓ extremely loose raw turkey breast meat.

The mushy structure of cooked chicken breast meat can be perceived during eating since the need to masticate before swallowing the piece of meat is negligible (Petracci and Cavani, 2012).

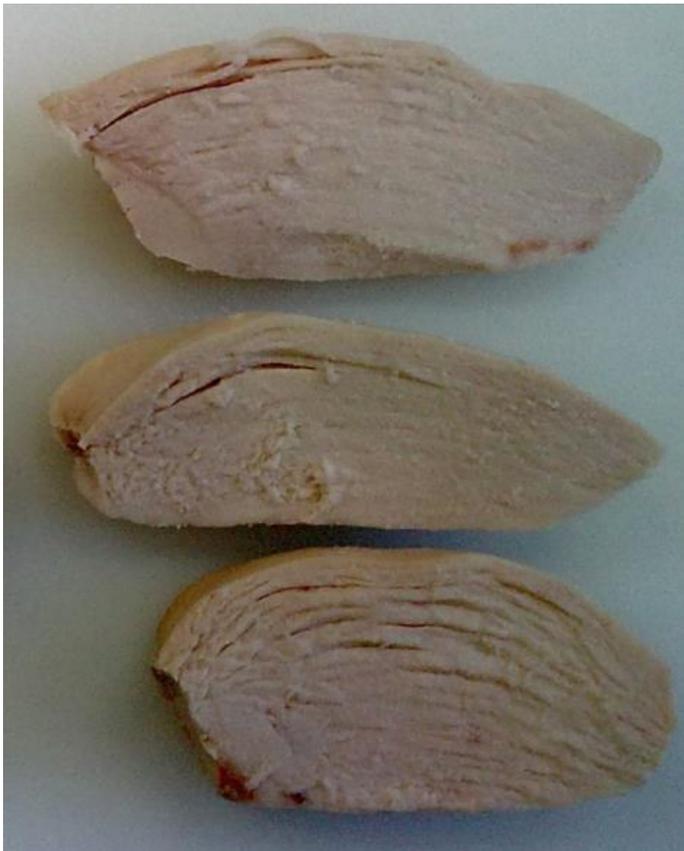


Figure 3.3. Broiler breast meat with poor cohesiveness (Petracci and Cavani, 2012).

During breast muscle development in modern turkey and broiler breeds, an increase in the cross-section of muscle fibers is greater than that in endomysial and perimysial connective tissues (Petracci et al., 2013). This suggests that selection for rapid growth creates muscles that outgrow their life support systems thereby bringing about muscle damage. Hence, final trigger to the disintegration of turkey breast meat could be the formation of large intercellular spaces, as fluid that is released from myofibrils is lost from the muscle fibers post mortem (Petracci and Cavani, 2012). An *et al.* (2010) found that endomysium and perymysium thickness of breast muscles was larger and much smaller, respectively, in fast-growing broilers compared with slow-growing egg-type chickens. This indicates that the growth of

endomysium and perimysium may separately be regulated and this can later translate in poor slicing and fragmentation seen in cooked deli products.

3.4. White striping

This is a new quality issue related to the appearance of white striations in breast muscle fillets in broilers following the directions of muscle fibers as shown in Figure 3.4 (Petracci and Cavani, 2012). Histological observations indicated an increase in degenerative and atrophic fibers in breast fillets affected by white striping. The aetiological causes of white striping are poorly known, although it has been established that several farming factors such as genotype (Lorenzi *et al.*, 2014), sex and growth rate (Kuttappan *et al.*, 2013a) as well as diet and slaughter weight (Kuttappan *et al.*, 2012a) may be involved. While the phenomenon has not been linked to any particular eating attributes of cooked poultry, it does affect the appearance and would possibly lead to rejection of the product. In addition, literature indicates that white striping changes the composition of breast meat and may change nutritional value as well. Kuttappan *et al.* (2012a) found that white-striped fillets had a higher fat content and lower protein content compared with normal fillets. Petracci *et al.* (2014) showed that severe white-striped fillets had higher total energy content than normal fillets and the contribution of energy from fat was increased about three-fold. Furthermore, breast meat affected by white striping has been shown to have an increase in the collagen to total protein ratio, which gives it a lower nutritional value as a result of the low digestibility of collagen and deficiency of some essential amino acids (Petracci *et al.*, 2014; Mudalal *et al.*, 2014). Both moderate and severe white striping is associated with changes in the composition and hence reduced nutritional quality of breast meat. White striping, just like wooden breast abnormality, is also associated with a strong impairment of the technological properties of breast meat (Mudalal *et al.*, 2015). Petracci *et al.* (2013b) reported a dramatically lower water-holding/binding capacity (marinade uptake, cooking loss, and yield) and a softer texture in white-striped meat than normal meat.

In general, breast meat affected by severe white striping is usually used for manufacturing further processed products (e.g. sausages or nuggets) where the chemical composition can be modified during formulation. Fillets which are moderately white striped are marketed for fresh retailing, even though cut-up products may have different nutritional characteristics compared with those reported on the label and with consumer expectations towards poultry meat (e.g. low calories and fat). This means that the cut-up breasts (whole or sliced) can have

somewhat different nutritional characteristics compared with those reported on the label and with consumer expectations towards poultry meat (Petracci *et al.*, 2014).

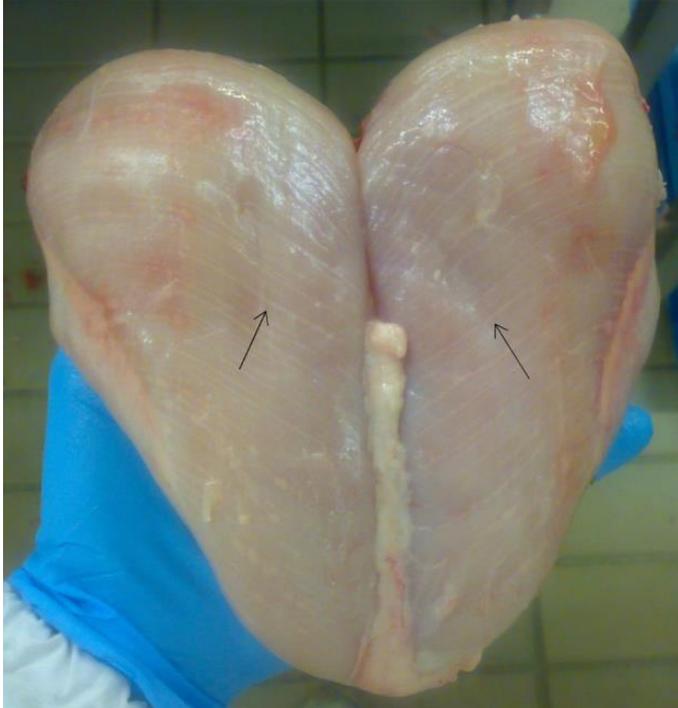


Figure 3.4. Broiler breast meat with “white striping” defect (Petracci and Cavani, 2012).

3.5. Wooden-breast

During the last five years the muscle syndrome wooden breast (WB) has become a serious challenge to the poultry meat industry worldwide. WB is a term for abnormal muscle tissue (a myopathy) in the chicken breast, whereby the muscles are hard, out bulging, pale and often accompanied with white striping (Sihvo *et al.*, 2014). As such, meat with WB is hardened to the touch and exhibit hardened ridges (Chatterjee *et al.*, 2016). Research indicates that WB significantly affects the meat quality attributes of finished products. Mudalal *et al.* (2015) found that raw WB fillets showed lower marinade uptake and higher cooking losses and compression force. Since the appearance of this meat is unpleasant and the functional properties are impaired, severe cases of WB fillets are downgraded and used to manufacture less valuable products. WB represents a significant economic loss for the poultry industry with typical incidences of $5\pm 10\%$ reported in broilers (Wold *et al.*, 2017).

The causes of WB are still not very clear, but are most likely multifactorial where an important part is related to the fast growth of modern broiler chickens (Kuttappan *et al.*, 2016). There is sufficient evidence in literature in support of the fact that histopathological changes in white striped and WB muscles are similar (Kuttappan *et al.*, 2013b; Sihvo *et al.*, 2014; Ferreira *et al.*, 2014), and thus they may have a common aetiology. Histological observations on white-striped meat indicated an increase in degenerative and atrophic fibres associated with loss of cross striations, variation in fibre size, floccular/vacuolar degeneration and lysis of fibres, mild mineralisation, occasional regeneration (nuclear rowing and multinucleated cells), mononuclear cell infiltration, lipidosis and interstitial inflammation and fibrosis (reviewed in Petracci *et al.*, 2015). The histological changes in WB meat showed different levels of polyphasic myodegeneration with regeneration accompanied by accumulation of interstitial connective tissue (fibrosis) that is quite similar to what has been observed for white striping (Sihvo *et al.*, 2014). Similar lesions have also been observed in leg muscles and backs of the carcass (Zimmermann *et al.*, 2012; Kuttappan *et al.*, 2013b).

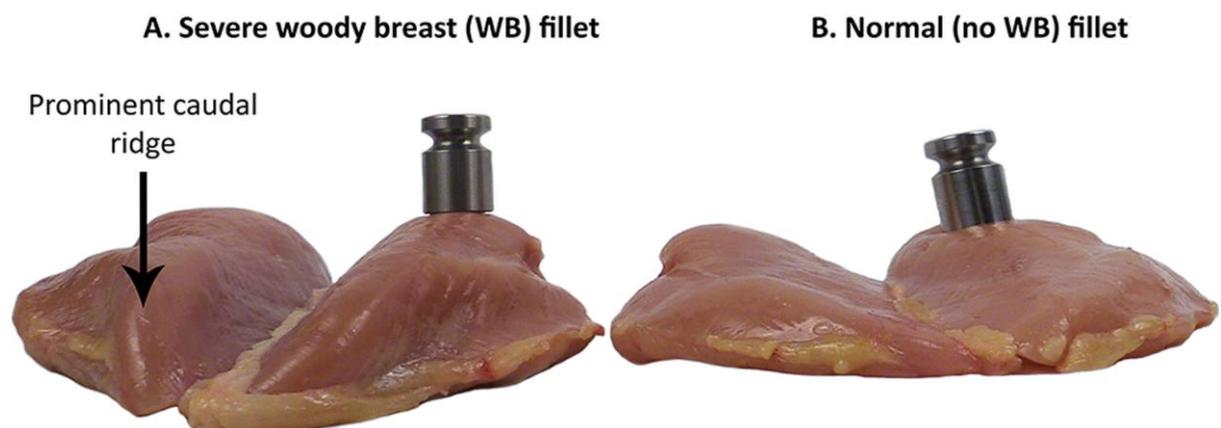


Figure 3.5. Broiler breast meat with “Wooden breast” myopathy (Kuttappan *et al.*, 2016).

Chapter 4

AVIAN INTESTINAL HEALTH AND GUT INTEGRITY

4.1. An Overview of the digestive system in birds

The intestinal tract of a bird is a specialized tube that starts at the beak and ends in the cloaca. The primary function of the gut is the conversion and digestion of food into its basic components for absorption and utilization by the bird. The gut is separated into 5 distinct regions (Figure 4.1); the crop, proventriculus, gizzard, small intestine (duodenum, jejunum, and ileum), and large intestine (caeca, colon and rectum). Each of these regions has a specific role in the digestion process and absorption of nutrients.

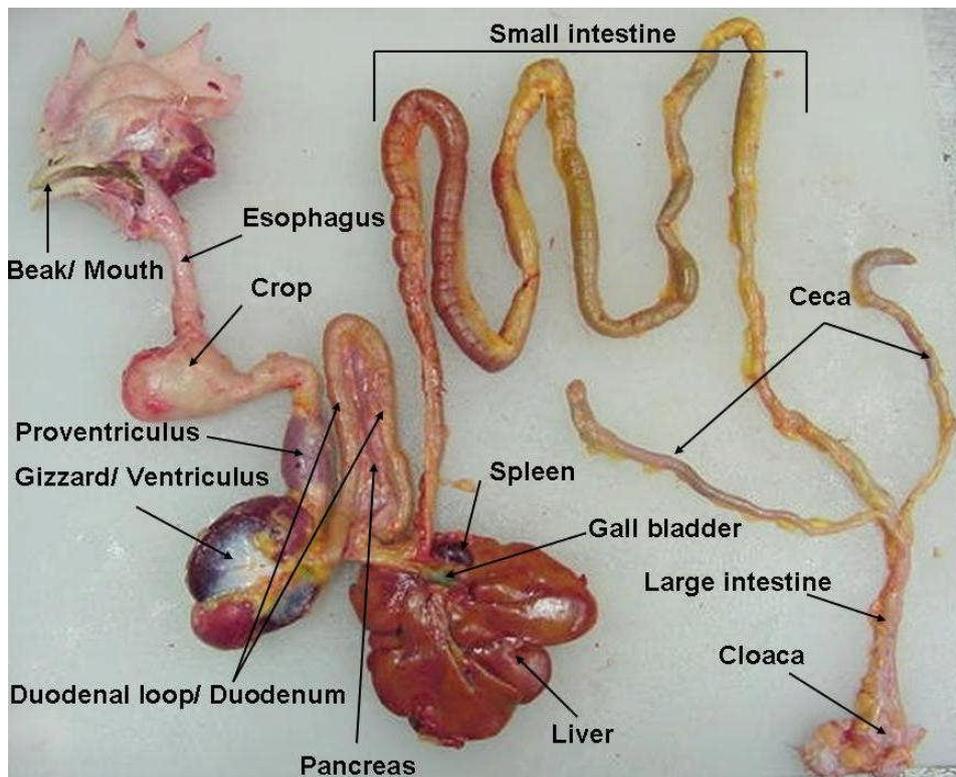


Figure 4.1. The digestive tract of a chicken (www.growelagrovvet.com-accessed 23/6/2017)

4.2. Factors affecting intestinal health and gut integrity

The digestive tract is a major site of exposure to pathogens since the material ingested by the bird can include nutrients, non-nutrients and beneficial as well as potentially harmful micro-organisms. The lumen normally contains feed and its constituents, resident and

transient microbial populations, endogenous nutrients, and secretions from the gastrointestinal (GI) tract and its accessory organs such as the liver, gall bladder, and pancreas. The GI tract must selectively allow the nutrients to cross the intestinal wall into the body while preventing the harmful components of the diet from crossing the intestinal barrier (Korver, 2006). Thus, the gut is not only the major organ for nutrient digestion and absorption, but also works as the first protective mechanism to exogenous pathogens which can colonise and/or enter the host cells and tissues. The GI tract acts as a selective barrier between the tissues of the bird and its luminal environment. This barrier is composed of physical, chemical, immunological, and microbiological components. Intestinal integrity is compromised when the mucus layer is degraded; epithelial cells are effaced or destroyed, the vascular supply is interrupted, or the immune system is compromised. An arsenal of factors associated with diet, infectious disease agents, environment, and management practices can negatively affect the delicate balance among the components of the chicken gut (Hughes, 2005; Yegani and Korver, 2008). This subsequently impairs growth rate and feed conversion efficiency, both of which result in low production performance.

4.2.1. Diet

Nutritional deficiency associated with imbalance in ration formulation, grain engorgement, microbial load in feed among other things affect gut health. The following dietary factors directly or indirectly affect gut health and integrity in poultry.

4.2.1.1. Non-starch Polysaccharides

Although there is a wide range of anti-nutritional compounds present in various feed ingredients including cereals, the major group is the non-starch polysaccharides (NSP). All cereals used in poultry diets contain various levels of NSP such as β -glucans and arabinoxylans (Yegani and Korver, 2008). Choct and Annison (1992) observed that NSPs are generally resistant to the animal's digestive enzymes and therefore tend to create a viscous environment within the intestinal lumen which results in the excretion of sticky droppings soiling the litter. High viscosity of the intestinal contents has been shown to cause digestive and health problems such as decreased digesta passage rate and reduced availability of nutrients. Waldenstedt *et al.* (2000) and Sacranie *et al.* (2012) showed that increased digesta retention time aids bacterial colonization and activity in the small intestine. Barley, wheat,

rye, and oats have high levels of NSP which are known to lead to increased digesta viscosity, decreased digesta passage rate, digestive enzymatic activities and nutrient digestibility, depressed feed conversion efficiency, and growth rate of the birds (Yegani and Korver, 2008). Sometimes this requires supplementation of feeds with exogenous enzymes that have the ability to break down NSP and reduce digesta viscosity, increase digesta passage rate and improve performance, which leads to additional cost of production.

4.2.1.2. Physical Texture and Form of Feed

The physical form of cereal components of feed may affect the morphological and physiological characteristics of the intestinal tract (Yegani and Korver, 2008). Finely ground feed for instance may increase mortality associated with necrotic enteritis especially due to *Clostridium perfringens* and coccidiosis (M'Sadeq *et al.*, 2015). A study by Branton *et al.* (1987) showed an increase in mortality of up to 28.9% in broilers fed finely ground wheat due to a combination of necrotic enteritis and coccidiosis; while coarsely ground wheat reduced mortality by 18.1%. Dietary whole wheat may also improve gut performance by aiding development of the GI tract, particularly the gizzard and also by increasing absorption of dietary nutrients from the lower digestive tract. The relationship between enzyme activities, gut weight and growth performance has been fully elucidated by Hetland *et al.* (2002) where the inclusion of oat hulls in a wheat-based broiler diet increased the gizzard weight, which coincided with a significant improvement (from 97 to 99%) in the digestibility of starch, the most important energy source in broiler diets in the ileum. This was probably due largely to the massive increase in the amount of starch-degrading enzyme, amylase, secreted. In addition, the gizzard bile acid level increased in proportion to the amount of wood shavings retained in the gizzard.

Feeding whole wheat to broiler chickens reduced the numbers of *Salmonella* Typhimurium and *Clostridium perfringens* in the intestinal tract of the birds (Engberg *et al.*, 2004; Bjerrum *et al.*, 2005). Inclusion of whole wheat into the diet increased feed conversion efficiency in some studies (Plavnik *et al.*, 2002), whereas others did not show any positive impact on feed efficiency. Svihus *et al.* (2004) found no significant effects of diets containing whole wheat on body weight gain and feed conversion efficiency, but results showed that nutrients were more efficiently digested and absorbed from these diets compared with diets with ground wheat. Svihus *et al.* (2002) observed that although the gizzard has a remarkably high capacity for processing diets with whole wheat, the average passage rate for a diet through the gizzard does not seem to be affected by the form of the wheat.

Gabriel *et al.* (2003) observed that feeding of whole wheat to broiler chickens, experimentally infected with coccidiosis, enhanced development of *Eimeria tenella* in the ceca. This resulted in a significantly lower weight gain in whole wheat group compared with ground wheat-fed broilers. Conversely, Banfield *et al.* (2002) found no effect of feeding whole wheat on performance of birds before infection with coccidiosis or during recovery from the infection. There were significant increases in activity and size of the gizzard and pancreas in whole wheat-fed birds. This increase in gizzard size is in response to the need to do more grinding to process the whole grains before digestion in the lower parts of the GI tract. Other studies (Banfield *et al.*, 1999; Banfield and Forbes, 2001) have also demonstrated that the inclusion of whole wheat had no significant effect on control of coccidial infections.

On the basis of the literature cited, it can be concluded that when the GI tract is healthy, inclusion of whole wheat into the diet may help to improve digestive tract function, but when the integrity of the intestinal tract is impaired, inclusion of whole wheat may decrease performance of the GI tract.

4.2.2. Infectious Agents

The intestinal tract provides the mechanisms by which the body derives nourishment from its environment while safeguarding the bird by various protective mechanisms. The etiology of an enteric disease is complex, as combinations of viruses, bacteria, and other infectious and non-infectious agents may be involved (Reynolds, 2003). As aforementioned, the primary function of the GI tract is to break down feedstuffs into basic components for transport and absorption for use in maintenance, growth, and production. Any physical, chemical, or biological disturbances of these processes can result in enteric disease (Dekich, 1998). Gastrointestinal infections, such as diarrhea, gut helminth and protozoal infections, directly affect the integrity, morphology, and function of the absorptive mucosa of the intestine possibly resulting in malabsorption. In human, Rodriquez *et al.* (2011) proposed that an important proportion of childhood malnutrition could be linked to impaired intestinal absorptive function resulting from multiple and repeated gastrointestinal infections.

4.2.2.1. Bacterial Infections

Low-grade damage to the intestinal tract by pathogenic bacteria may cause poor feed conversion efficiency and decreased rate of body weight gain in poultry flocks while severe enteric damages by bacterial infections usually result in overt disease with high mortality (Porter, 1998).

4.2.2.2. Parasites

Among internal parasites infesting commercial poultry, protozoa are common causing moderate to severe diseases. Confinement rearing and high-density flocks have increased the exposure to parasitic diseases such as coccidiosis that have short and direct life cycles. In contrast, parasitic diseases with indirect life cycles, such as flukes, many cestodes, and some nematodes, have been practically eliminated (Yegani and Korver, 2008).

Histomoniasis or Blackhead, caused by *Histomonas meleagridis*, is a parasitic disorder of the ceca and liver of many gallinaceous birds, although the turkey appears to be the most susceptible host (McDougald, 1998, 2003c). This disease causes high mortality in turkeys, sometimes approaching 100% of a flock. In chickens, the mortality may be 10 to 20% with high morbidity, although many outbreaks pass unnoticed (McDougald, 2005). The Blackhead disease organism is carried from host to host by eggs of the cecal worm *Heterakis gallinarum* (McDougald, 1998). Lesions of histomoniasis were reported to be more severe in birds with a co-infection with *C. perfringens* as well as *Escherichia coli* (McDougald, 1998, 2003c).

4.2.2.2.1. Coccidiosis

Coccidiosis is a disease of major economic significance in the poultry industry that is caused by several species of the genus *Eimeria*. Many species of coccidia are widespread in countries where poultry are produced on a commercial basis as well as in the backyard production systems. Transmission is dependent on the survival of oocysts of the parasite in moist beddings or soil (McDougald, 1998). The protozoan parasites of the genus *Eimeria* multiply in the intestinal tract and cause tissue damage, which results in mortality, interruption of digestive processes or nutrient absorption, reduced weight gain, and increased susceptibility to other disease agents, notably *Clostridium perfringens* (Broom, 2017). The severity of lesions depends mainly on the extent of exposure or number of oocysts ingested by the host (McDougald, 1998, 2003b; Williams, 2005). The damage to the gut wall reduces the ability of the gut to absorb nutrients resulting in weight loss and diarrhoea. In severe coccidiosis especially where *E. tenella* is involved the damage to the gut wall can be so severe that the bird bleeds into its gut causing blood in the bird's droppings and anaemia characterised by a pale comb and wattles. This gut damage can also disrupt the natural balance of bacteria in the gut thereby allowing harmful bacteria to take over and cross the damaged gut wall causing blood poisoning.

Subclinical coccidiosis can be an important contributing factor in the development of necrotic enteritis in broiler chickens because mucosal damage facilitates the establishment

and multiplication of *C. perfringens* (Al-Sheikhly and Truscott, 1977; McDougald, 2003b; Moore, 2016; Broom, 2017). It has also been shown that certain indigenous bacterial species such as *Streptococcus fecalis*, *E. coli*, *Lactobacillus* species, and *Bacteroides* species play a role in pathology of cecal coccidiosis (Bradley and Radhakrishnan, 1973). Cecal coccidiosis, caused by *E. tenella*, may contribute to increased severity of histomoniasis (Blackhead disease) in chickens (McDougald and Hu, 2001). It has been shown that immunosuppressive diseases or conditions may act in concert with *Eimeria* to produce a more severe coccidiosis. Marek's disease for instance interferes with development of immunity to coccidiosis, and infectious bursal disease may exacerbate coccidiosis (see review by Yegani and Korver, 2008).

4.2.2.3. Viruses

Many viral infections have been associated with enteric disease conditions (Reynolds, 2003). These include rotaviruses (McNulty, 2003), coronaviruses (Guy, 2003), enteroviruses (McNulty and Guy, 2003), adenoviruses (Pierson and Fitzgerald, 2003), astroviruses (Reynolds and Schultz-Cherry, 2003), and reoviruses (Rosenberger, 2003). Typical impacts of viral infections on poultry are depressed daily weight gain, impaired feed efficiency, and decreased flock uniformity (Guy, 1998). Enteric viral infections may occur in birds of all age groups but tend to predominate in young birds (Saif, 2003). The outcome of these infections is usually dependent on other factors such as age and immune status of affected birds, virulence of the involved virus, other infectious agents, nutrition, management practices, and environmental factors (Guy, 1998). Hemorrhagic enteritis is an acute viral disease of turkeys older than 4 weeks of age and is characterized by depression, bloody droppings, and death (Pierson and Fitzgerald, 2003).

Avian reoviruses have been isolated from a variety of tissues in chickens affected by disease conditions such as viral arthritidenosynovitis, stunting syndrome, respiratory disease, enteric disease, immunosuppression, and malabsorption syndrome (Rosenberger, 2003). In young broiler chickens, reoviruses are frequently associated with mortality, viral arthritis, and a general lack of performance including diminished weight gain, poor feed conversions, chronic feed passage problems, uneven growth rate, and reduced marketability.

Malabsorption syndrome in broiler chicks presents a very confusing clinical picture for diagnosticians. In a series of trials conducted by Page *et al.* (1982), an enlargement of the proventriculus and a reduction in the size of the gizzard were the most frequently encountered

postmortem lesions seen. Decreased body weights were also observed in chicks to which reoviruses were orally administered.

4.2.2.4. Toxins

Feedborne toxins can cause enteric disease. Mycotoxins and biogenic amines are among the most common examples of feedborne toxins (Dekich, 1998). The presence of mycotoxins in poultry feed has been identified as a widespread cause of economic losses due to impaired health status and reduced performance (Sklan *et al.*, 2003). Adverse effects on the GI tract are probably the major cause of economic losses resulting from trichothecene mycotoxicoses (Schiefer and Beasley, 1989). Mycotoxins such as T-2 toxin can cause caustic injury to the mucosa, destroying cells on the tips of villi, and affect rapidly dividing crypt epithelium. Histopathology of GI tract lesions due to acute intoxication by purified T-2 toxin is characterized by hemorrhage, necrosis, and inflammation of the intestinal epithelium, which occur before transient shortening of villi and reduction in the mitotic activity in crypt epithelium. Necrosis also occurs in the mucosa of the proventriculus and gizzard (Hoerr, 2003).

Biogenic amines including histamine, cadaverine, putrescine, spermine, and spermidine are present in animal protein products. It has been shown that biogenic amines are involved in the occurrence of malabsorption syndrome, which is characterized by decreased feed efficiency and enlargement of the proventriculus (Stuart *et al.*, 1986). Barnes *et al.* (2001) demonstrated that dietary histidine and cadaverine can cause the pathologies associated with proventriculitis. The action of histidine and cadaverine appeared to be additive or synergistic.

Chapter 5

POULTRY GUT MICROBIOTA, GUT HEALTH AND PERFORMANCE OF BIRDS

5.1. Establishment of the gut microbiota in broiler chicken

Development of the broiler chicken intestinal microbiota starts at the time of hatching. The gastrointestinal tract of poultry comes into contact with exogenous microorganisms immediately after hatching such as with those on the surface of egg shells (normally populated by bacteria from the intestine of the mother). Thereafter it becomes a warm shelter for a very complex microbiota, with over 600 different bacterial species from more than 100 bacterial genera (Torok *et al.*, 2011). In general, the most abundant phylum in the chicken intestinal microbiota is *Firmicutes* followed by two minor phyla, *Proteobacteria* and *Bacteroidetes* (Figure 5.1). In addition, members of phyla *Actinobacteria*, *Tenericutes* (Waite and Taylor, 2014), *Cyanobacteria* and *Fusobacteria* (Qu *et al.*, 2008) can be found though in very low numbers.

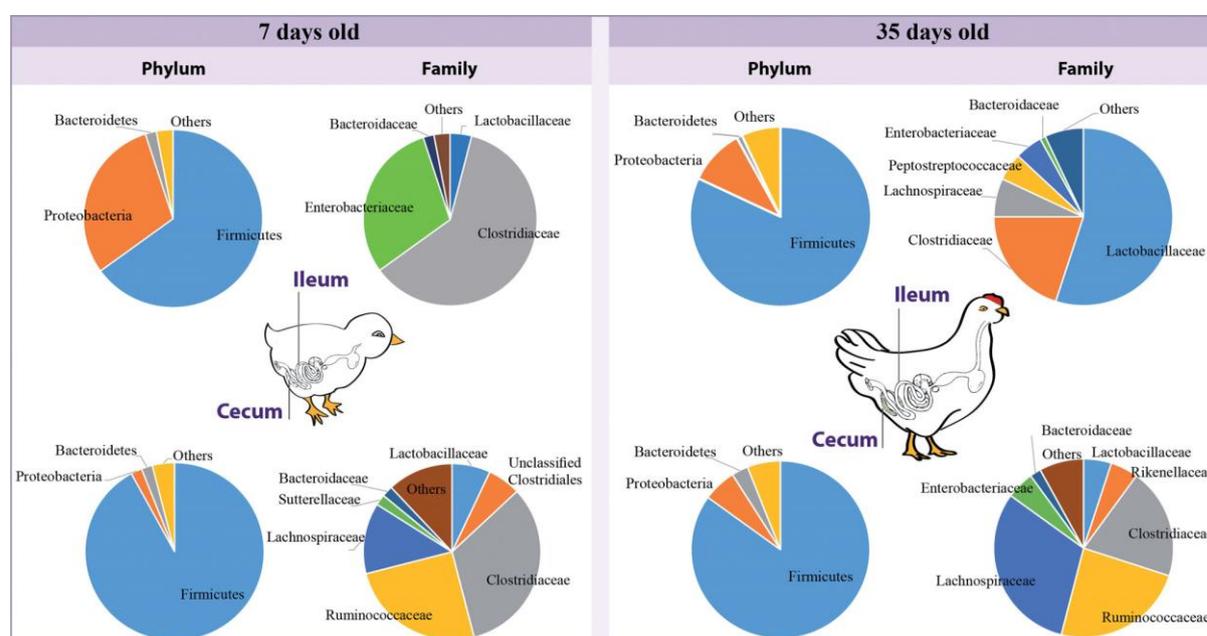


Figure 5.1. The chicken gut microbiome. The graphs provide an overview of the relative abundance of dominant bacterial phyla and families of the broiler chicken ileal (top level) and cecal (bottom level) microbiota at two different ages, 7 and 35 days (Pourabedin and Zhao, 2015).

This makes the microbial inoculum obtained at the early post-hatch or pre-hatch period critical for the establishment of the gut microbial community. This may aid the development

of the immune system and the intestinal microbiota for the entire lifetime of the bird (Rinttilä and Apajalahti, 2013). As the bird grows, this microbiota becomes very diverse until it reaches a relatively stable yet dynamic state. Bacterial communities vary considerably by locations along the GI tract of chickens. Crop, gizzard and duodenum share similar microbiota, dominated by the genus *Lactobacillus*, as high as 99% in some birds (Gong *et al.*, 2007; Sekelja *et al.*, 2012). The highest diversity of *Lactobacillus* was observed in the crop (Gong *et al.*, 2007). The jejunum is also dominated by *Lactobacillus* species, mainly *L. salivarius* and *L. aviarius* (Gong *et al.*, 2007; Feng *et al.*, 2010). The microbial composition of the ileum is more diverse and less stable compared with the duodenum and the jejunum. The ileum is dominated by *Lactobacillus*, *Candidatus Arthromitus*, *Enterococcus*, *Escherichia coli/Shigella* and *Clostridium XI* (Asrore *et al.*, 2015; Pourabedin *et al.*, 2015). The cecum is by far the most densely colonized microbial habitat in chickens and its bacterial diversity is much higher than those in the upper GI tract. The most detailed information regarding chicken gut microbiota is available for the cecum (Pourabedin and Zhao, 2015). The cecum is a key region for bacterial fermentation of non-digestible carbohydrate and a main site for colonization by pathogens. Chickens have two paired ceca, both harboring similar bacterial communities (Stanley *et al.*, 2015). In a study by Gong *et al.* (2007), the cecum was mainly occupied by the *Clostridia* genus followed by genera *Lactobacillus* and *Ruminococcus*. The cecum is also rich in unknown and unclassified bacterial residents (Pourabedin and Zhao, 2015).

Compared with other domestic animals, poultry (i.e. chicken, turkey, and duck) has a shorter gastrointestinal tract and a shorter digesta transit time. This anatomic feature naturally allows for the selection of a completely different intestinal microbial community in poultry compared to other food producing animals. There are extensive interactions of this intestinal microbial community with poultry host and diet, and also interactions among individual gut microbes (reviewed by Deng and Yu, 2014), which have profound effects on poultry nutrition and health, and are therefore of great importance to poultry production.

5.2. Chicken gut microbiota and immune system

Just like in mammals, the immune system of birds is complex and composed of several cells and soluble factors that work together to produce a protective immune response (Yegani and Korver, 2008). It is well established that commensal gut microbiota is important as inducers for the development and maturation of both innate defence mechanisms and adaptive immune responses of chicken (Brisbin *et al.*, 2008). Intestinal microflora plays a

vital role in the modulation of chicken immune responses. For example, it protects the intestine from infections, including different types of *Salmonella*, and it also has a positive effect on the chicken growth rate.

As revealed by studies in mammals, specific commensal bacterial species may also have a vital role in inducing the accumulation of certain immune cell populations in the intestine (Kogut, 2013). For example, bacteria belonging to the phylum Bacteroidetes (i.e., *Bacteroides fragilis*) have been shown to be associated with the development of interleukin-17 (IL-17) producing T-helper cells (Mazmanian *et al.*, 2005). Lactobacilli are a group of commensal bacteria that have long been known for their ability to activate the intestinal immune system and increase the resistance to diseases, in part through the release of low-molecular weight peptides which induce immune activation (Muir *et al.*, 2000).

Furthermore, the bacteria have been reported to produce a wide variety of short chain fatty acids (SCFAs), which are bacteriostatic for a subset of bacterial species either directly or by reducing pH of the intestinal environment, produce bacteriocins with microbicidal or microbiostatic properties and contribute to the colonization resistance against pathogenic microbes by modifying the receptors used by the pathogenic bacteria (Adil and Magray, 2012; Rinttilä and Apajalahti, 2013). Additionally, SCFAs produced by lactic acid bacteria (LAB) favour the renewal and barrier function of the gastrointestinal epithelium (Kogut, 2013).

5.3. Chicken intestinal microbiota and performance

For production animals such as chicken, intestinal health is extremely important. Rinttilä and Apajalahti (2013) noted that when productivity is not threatened by disease, the most important driver of performance is usually the ability of the animal to convert feed into carcass as efficiently as possible. Apart from the defence systems, the importance of intestinal microbiota for the performance of broiler chicken has been the focus of studies for several decades. The intestinal microbiota is composed of both the beneficial bacteria (e.g. gram-positive lactobacilli and bifidobacteria) and harmful (potential pathogenic bacteria such as *Clostridium spp.*, *Salmonella* and *Escherichia*). It is widely accepted that a proper balance between the beneficial and harmful bacteria in the intestine (with at least 85% of total microbiota being good bacteria) is vital for the host's gut health (Choct, 2009). This microbial balance has in the past been sustained by using sub-therapeutic doses of antibiotics as growth promoters in poultry production.

However, with the withdrawal of antibiotics following the ban on their use in poultry production by the EU, the impact of the imbalance may be exacerbated. The review of Kogut (2013) and Rinttilä and Apajalahti (2013), suggest that commensal intestinal bacteria are important in digestion and synthesis of dietary compounds and are involved in the development of gastrointestinal tract. These bacteria also play important roles in the regulation of intestinal epithelial proliferation, host energy metabolism and vitamin synthesis. A host of studies in mammals (reviewed in Brestoff and Artis, 2013) indicate that commensal bacteria contribute to the regulation of digestion by mediating the bile acid synthesis, lipid absorption, amino acid metabolism, vitamin synthesis and short chain fatty acid (SCFA) production.

Most readily digestible dietary carbohydrates are digested and absorbed by the host in the proximal gut, leaving indigestible carbohydrates and residual digestible carbohydrates to bacteria residing in the distal gut. Many intestinal bacteria can hydrolyze indigestible dietary polysaccharides, oligosaccharides, and disaccharides to their compositional sugars, which can then be fermented by intestinal bacteria, yielding short chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate.

The SCFAs can be utilized by the host as energy and carbon source. In birds, such fermentation has been observed in most parts of the gut although more commonly in the cecum, which is known for being densely populated with bacteria (Deng and Yu, 2014). Cecal acetate, propionate, and butyrate are usually not detected in 1-d-old chicks. As the cecal microbiome becomes established, these SCFAs reach high concentrations in about 15 days and remain stable afterwards. In the cecum, SCFAs are absorbed across the epithelium by passive diffusion where they enter a variety of metabolic pathways while others especially butyrate, can serve as an important energy source for intestinal epithelial cells. Additionally, Tellez *et al.* (2006) reported that SCFAs have the ability to regulate intestinal blood flow, stimulate enterocyte growth and proliferation, regulate mucin production, and affect intestinal immune responses as well. All these factors affect production performance in one way or the other. In addition, Lan *et al.* (2005) showed that commensal bacteria influence the activity of digestive enzymes and gut mucosal morphology of chicken.

It should be noted that although the gut microbiota have potential benefits on the digestion of certain dietary components particularly non-digestible carbohydrates, it may have an adverse effect on the utilization of energy by the host (Lan *et al.*, 2005). This is especially the case where dietary energy is supplied in the form of substrates which are easily digestible by the chicken itself. In the latter condition, no/less substrate (non-digestible carbohydrate) is

available for the gut microbiota, resulting in a competition for the substrate between the host and gut microbiota. In this circumstance, the gut microbiota becomes a nutritional “burden” in fast-growing broiler chickens (Yang *et al.*, 2009), where easily digestible feed is usually provided.

5.4. Chicken gut health and production performance

Kabir (2009) observed that the balance of intestinal microbiota is important to promote the healthy gut and maximum growth performance of chickens. The shift of microbiota can affect the morphology of the intestinal wall and induce immune reactions, which can have in turn impact on the energy expenses and growth of the chickens (Humphrey and Klasing, 2003). Likewise, the intestinal colonization by pathogens may induce the immune response that eventually diverts energy and nutrients away from growth to the acute requirement of combating infections (DiAngelo *et al.*, 2009). In chicken, the inflammatory response is important for dealing with microbial infections (Kogut, 2013). However, if left uncontrolled, this immune activation would pose a risk of excessive inflammation and intestinal damage, which may in turn impair the digestive functions of the intestine (Brisbin *et al.*, 2008). Similarly, the excessive inflammation may also cause disturbances in host metabolism (Kogut, 2013). It has been reported that commensal microbiota in the gut plays important roles in the maintenance of intestinal immune homeostasis and prevention of intestinal inflammation (Lan *et al.*, 2005; Belkaid and Hand, 2014). Through their products (SCFAs), the commensal bacteria may exert anti-inflammatory activities, thus preventing the intestinal damage (Brestoff and Artis, 2013). En masse, the balance of intestinal microbiota is crucial for the intestinal homeostasis and healthy/normal functions (digestive and defence) of the gut of chicken.

Chapter 6

MANAGING GUT HEALTH IN BROILER CHICKENS

6.0. Introduction

The efficient conversion of feed into its basic components for optimal nutrient absorption is vital for both broiler and broiler breeder production and welfare. Gut health, an intricate and complex area combining nutrition, microbiology, immunology and physiology, has a key role to play. When gut health is compromised, digestion and nutrient absorption are affected which, in turn, can have a detrimental effect on feed conversion leading to economic loss and a greater susceptibility to disease. Over the past decades, much effort has gone into optimizing the gut microbiota of chickens using dietary interventions. Among them, use of antibiotics at subtherapeutic levels has been the most popular and probably the most effective strategy to enhance feed efficiency and to keep animals healthy. In addition, recent changes in legislation on the use of antibiotics, differing feed requirements and more efficient birds highlight the need for a better understanding of gut function and the possible alternative measures for the maintenance of gut health and the subsequent improvement of performance in chickens.

6.1. Antibiotic growth promoters: effects on gut health and performance

Since the discovery of penicillin by Fleming in 1928, several antibiotics which can be classified based on their molecular targets in bacteria (cell wall, protein synthesis, nucleic acids, folic acid metabolism) have been marketed for the treatment of infectious diseases both in animals and humans. The agents used in the treatment of animals and humans often belong to the same classes of antibiotics having similar modes of action and bacterial cell targets. This interface brings a variety of problems and worries. Bacteria developing resistance to these drugs in animals may be transmitted to humans or spread their mechanisms of resistance, which may eventually be found in human pathogens. Such a situation may lead to the loss of therapeutic efficacy in both veterinary and human medicine. It is evident that antibiotics substantially improve public health. For example, since their discovery about 90 years ago, antibiotics have greatly reduced mortality and morbidity associated with infectious diseases which has increased life expectancy around the world.

In addition to their therapeutic properties, antibiotics are also used in animals for prophylaxis and growth promotion to aid improvement of animal zootechnical parameters. For example, antibiotics such as ceftiofur (a third generation cephalosporin), bacitracin (polypeptide) and virginiamycin (streptogramin) are used in poultry production to prevent and control infections such as respiratory diseases and necrotic enteritis; and to improve food conversion and body weight gain (Diarra and Malouin, 2014). The use of antibiotics as growth promoters was adopted in the 1940s when animals fed dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues showed improved performances (Castanon, 2007). Butaye *et al.* (2003) have estimated that the use of antibiotic growth promoters in animals, through unspecific and not well defined mechanisms, improve body weight by 5–6% and feed efficiency by 3–4%, with the most pronounced effects observed in young animals.

However, the persistent use of antimicrobial agents can change the bacterial environment by eliminating susceptible strains, and only allowing antibiotic resistant bacteria (i.e., those with higher fitness) to survive (O'Brien, 2002). As thus, with persistent usage, antimicrobial agents may modify the intestinal microflora and create a favorable environment for establishment of resistant and pathogenic bacteria by conferring a selective advantage for antibiotic resistant strains (Silbergeld *et al.*, 2008; Dhanani *et al.*, 2015). Studies by Aslam *et al.* (2012) and Johnson *et al.* (2012) demonstrated positive associations between the presence of certain virulence genes and antibiotic resistance determinants. Aslam *et al.* (2012) also showed that poultry meat and products were more likely to carry enterococci that harbour clinically important antimicrobial resistance genes when compared to pork and beef. This finding raises a major public health concern in places where the practice of consuming raw or undercooked poultry meat is relatively common.

The impact of antimicrobial growth promoters on the development of antimicrobial resistant bacteria has been the subject of several reports which led to their eventual ban in the European Union in January, 2006. Also recommendations by the WHO, initiatives taken by the food chain and consumer concerns all point to a widespread and voluntary removal of antibiotic feed additives for animal growth promotion in the long run. Although it is widely acknowledged that the inclusion of AGPs in the diet of livestock increases growth rate, many questions arise on as to whether the benefits outweigh the risks.

The poultry industry has grown and improved in recent years due to the continuous integration of various disciplines for production such as poultry health, nutrition, breeding, husbandry, and knowledge of poultry products. Research in the poultry industry has focused

a lot on genetic selection and control of nutritional and production factors that essentially contribute to muscle growth and improved feed conversion efficiency. For example, in 1928, the average broiler required 112 days and 22 kg of feed to reach 1.7 kg. From 1990, broilers now require about 35–42 days and 4 kg of feed to reach 2 kg (National Research Council, 1999). This improvement could be attributable in part to the use of antibiotics as growth promoters in poultry feed.

In controlled studies evaluating the effects of diet supplementation with bambamycin, penicillin, salinomycin, bacitracin, salinomycin-bacitracin, virginiamycin, chlortetracycline, monensin, and narasin on body weight, feed intake, feed efficiency, and mortality, Diarra *et al.* (2007) and Bonnet *et al.* (2009) found no significant difference between the treatment groups for the overall performance. The authors noted though, that virginiamycin and penicillin improved feed efficiency. Similarly, an experiment conducted by Dumonceaux *et al.* (2006) found that dietary inclusion of virginiamycin increased body weight and improved feed efficiency from days 0 to 15 but that no difference was noted for bird's performance parameters for the remainder of the study. Furthermore, the use of chlortetracycline as a feed supplement has been reported to induce no significant improvement in live body weights or feed conversion efficiencies of 21- and 42-day old chicken (Proudfoot *et al.*, 1988).

Graham *et al.* (2007) evaluated the economic effect of removing antibiotics used for growth promotion in commercial broiler chickens in a non-randomized study in the USA and reported that positive production changes were associated with the use of antibiotic agents. The authors however noted that the benefits were not adequate to offset the cost of antibiotics use. Diarra *et al.* (2007) observed that with modern broiler production practices and improved biosecurity, a broiler body weight of 1.8 kg could be reached by using 3.2 kg of feed in 35 days without addition of any antibiotics in feed. The challenge would however be on controlling enteric pathogens (both microbial and parasitic) which could directly result in production losses.

6.2. Antimicrobial growth promoters and avian gut microflora

The lives of human beings, livestock and poultry are closely associated with microorganisms and the microbiota of their gut plays an important role in their overall health, productivity and well-being (Callaway *et al.*, 2008; Ley *et al.*, 2008). Healthy animals are generally characterised by having a well-functioning intestinal tract. This is fundamental for the efficient conversion of feed for maintenance and for growth or production. The most

important characteristic of a well-functioning intestinal tract is the balance of its bacterial population.

The growth of normal intestinal bacteria varies with the gut environment, and there is an increasing interest in the commensal components of the gut microflora associated with food-producing animals (Yost *et al.*, 2011). This is because it forms the basis of pro- and prebiotics use as growth promoters in poultry. Due to public and possible food safety and environmental health concerns, the monitoring of the changes in the microbiome (microbial genomes) as a function of chicken production practices is imperative. Knowledge of the impacts of antimicrobial agents on the gut microbiome could lead to production practices that improve broiler intestinal health and growth performances.

An arsenal of studies has demonstrated the impact of antimicrobial growth promoters on the chicken gut microflora (Torok *et al.*, 2011; Fung *et al.*, 2013; Singh *et al.*, 2013). For example, pyrosequencing followed by phylogenetic analyses indicated that diet supplementation with penicillin resulted in an elevated proportion of bacteria of the phylum Firmicutes from 58.1 to 91.5% and a decreased proportion of members of the phylum Bacteroidetes from 31.1 to 2.9% in the gut microflora of broilers compared to that observed in broilers fed the control non-supplemented diet (Singh *et al.*, 2013). The use of virginiamycin as a growth promoter was associated with an increased abundance of bacteria in the duodenal loop to proximal ileum, with fewer bacteria affected in the distal regions (ileocecal junction and cecum) (Dumonceaux *et al.*, 2006), indicating that virginiamycin modifies the composition of the chicken intestinal microbiota. Using the 16S rRNA gene-based polymerase chain reaction and denaturing gradient gel electrophoresis profiling, dietary treatment with bacitracin (50mg/kg) altered the composition of the microbiota but did not change its richness (Gong *et al.*, 2008). The authors demonstrated that the impact of bacitracin was particularly obvious in 3-day-old chicks. Lactobacilli were abundant in the cecal microbiota of 3-day-old chicks regardless of the dietary treatment with bacitracin (Gong *et al.*, 2008). Recently, Fung *et al.* (2013) using metagenomic sequencing approaches demonstrated that salinomycin-feeding at a rate of 60 ppm has a profound impact on the dynamics of the chicken ceca microbiome. These authors showed that the salinomycin fed group had an increased abundance of the Elusimicrobia, and a decreased abundance of Chloroflexi, Cyanobacteria, and Synergistetes. For example, the abundance of *Bifidiobacterium* spp. and *Lactobacillus* spp. increased significantly in the salinomycin-fed birds compared to the untreated control group. A functional analysis of environmental gene tags (EGTs) revealed that in the salinomycin-treated birds there was an increased abundance

of the cell wall and capsule, iron acquisition, motility and β -lactamase gene categories while a decrease of multidrug efflux pump EGTs was detected (Fung *et al.*, 2013). According to Lee *et al.* (2011), the decrease of broiler ileal sucrose and maltase activities and increase of ileal mucosal immunoglobulin A (IgA) as well as the increase of *Lactobacillus* counts are among the effects of bacitracin (55ppm) and oxytetracycline (2.5 ppm) that could explain the improvement of feed efficiency in broilers from days 0 to 21.

6.3. Alternatives to AGPs

Antibiotics have been widely used in animal production for decades therapeutically to improve the health and well-being of animals, and prophylactically for the purpose of improving growth rates and feed conversion efficiency (as antimicrobial growth performance promoters, or AGPs). However, due to the emergence of microbes resistant to antibiotics which are used to treat human and animal infections, the European Commission (EC) decided to phase out, and ultimately ban, the marketing and use of antibiotics as growth promoters in feeds in 2006 (EC Regulation No. 1831/2003). This political decision was taken by invoking the precautionary principle: ‘Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation’ (Principle 15 of the Rio Declaration, 1992). On the other hand, in other countries, consumer pressure is pushing the poultry industry to rear animals without AGPs (Dibner and Richards, 2005; Castanon, 2007). AGP removal has led to animal performance problems, feed conversion increases, and a rise in the incidence of certain animal diseases, such as necrotic enteritis (Wierup, 2001; Dibner and Richards, 2005). One disease syndrome that is clearly emerging in the EU broiler industry almost simultaneously with the ban of AGPs is ‘dysbacteriosis’ (Huyghebaert *et al.*, 2011). This is a poorly described condition of the gut that may be synonymous with conditions such as ‘wet litter’, ‘small intestinal bacterial overgrowth’, ‘malabsorption’, and ‘feed passage syndrome’ etc. The common clinical denominator is thinning and ballooning of the small intestine, increased water content of faeces and reduced digestibility of feed with indigested residues visible in the faeces.

The impact of phasing out antibiotic growth promoters in poultry could be abated by the implementation of alternative disease-prevention strategies and management factors, such as alternative husbandry practices in food animal production. Certainly, overall disease and performance problems have been somewhat limited, partly because anticoccidials are still in

use, therapeutic antibiotic use is allowed, and alternatives for AGPs have been empirically used mainly as feed additives.

6.3.1. Characteristics of good AGP alternatives

Ideally, alternatives to antibiotic growth promoters should have the same beneficial effect as AGPs. It is however not totally clear how AGPs exert their beneficial action. The most well-known mechanism proposed is that AGPs have an antibacterial action that favours performance in a number of ways:

1. by reducing the incidence and severity of subclinical infections (George *et al.*, 1982; Brennan *et al.*, 2003);
2. by reducing the microbial use of nutrients (Snyder and Wostmann, 1987);
3. by improving absorption of nutrients because of thinning of the intestinal wall;
4. by reducing the amount of growth-depressing metabolites produced by Gram-positive bacteria (Feighner and Dashkevicz, 1987; Knarreborg *et al.*, 2004). The basis of this mechanistic explanation is that AGPs do not exert growth-promoting effects in germ-free animals (Coates *et al.*, 1963).

The alternatives to antibiotics are needed to maintain the gut health and performance by controlling pathogens and increasing nutrient digestion and absorption. Some of the ways to minimize antibiotics in poultry include use of whole grain cereals, live microbial cultures, use of fermentable sugars and processing/sterilization of feeds. Prominent alternatives in poultry production include organic acids, probiotics, prebiotics, synbiotics, herbal drugs, vitamins, minerals and plant extracts (phytobiotics) etc. (Dhama *et al.*, 2014). The alternatives to AGPs should therefore possess the following attributes:

1. It should improve performance effectively;
2. It should have little therapeutic use in human or veterinary medicine;
3. It should not cause deleterious disturbances of the normal gut flora;
4. It should not be involved with transferable drug resistance;
5. It should not be absorbed from the gut into edible tissue;
6. It should not cause cross-resistance to other antibiotics at actual use level;
7. It should not promote *Salmonella* shedding;
8. It should not be mutagenic or carcinogenic;
9. It should not give rise to environmental pollution;

10. It should be readily biodegradable;
11. It should be non-toxic to the birds and its human handlers.

The following are possible alternatives to AGPs.

6.3.2. Organic acids

Organic acids are widely distributed in nature as normal constituents of plants or animal tissues but can also be formed through microbial fermentation of carbohydrates predominantly in the caeca of poultry (Van Der Wielen *et al.*, 2000). A wide range of organic acids with variable physical and chemical properties exists, of which many are used as drinking water supplements or as feed additives (acidifiers). Many are also available as salts of sodium, potassium or calcium (and/or partially esterified). The advantage of salts over acids is that they are generally odourless and easier to handle in the feed manufacturing process due to their solid and less volatile form (Yadav *et al.*, 2016). They are also less corrosive and more soluble in water.

Organic acids have been shown to have beneficial effects on performance and have in the recent years been proposed as possible alternatives to antimicrobial growth promoters (AGP) as a result of their antimicrobial activity against a wide range of pathogenic and spoilage bacteria owing to their ability to induce pH reduction of the gastrointestinal tract (GIT) environment. Some (e.g. butyric acid) also decrease the incidence of subclinical necrotic enteritis caused by *C. perfringens*, an additional beneficial effect which is highly relevant for the poultry industry (Timbermont, 2009). Organic acids play a number of roles in poultry production and they include lowering the pH of the poultry feed and GIT, improving nutrient utilization, preventing the growth of pathogens among others (reviewed in Yadav *et al.*, 2016). Application in drinking water ensures pathogen reduction and therefore helps in regulating the normal gut flora (Açkgöz *et al.*, 2011; Hamed and Hassan, 2013). The organic acids' ability to acidify the gut environment results in increased intestinal protease enzyme activity which in-turn increases the nutrient digestibility and utilization. This could also be due to the longer acidic digesta retention time in the GIT which provides more time for nutrient digestion (Mayer, 1994). Stabilization of intestinal pH also increases the efficacy of all digestive enzymes.

Furthermore, inhibition of undesirable microbes not only prevents the accumulation of toxic metabolites, but also spares more nutrients available for the host, ensuring higher feed utilization efficiency. Organic acids are used in feed sanitation programme, acting as feed additives and preservatives. By preventing the growth of pathogenic bacteria it delays the

deterioration of feeds and subsequently extends the shelf-life of perishable food ingredients. Organic acids commonly used to reduce the pathogenic microbial load (like *Salmonella* and *Escherichia coli*) include short chain fatty acids, volatile fatty acids and weak carboxylic acids.

Organic acids also reduce the colonization of pathogens on intestinal wall, preventing damage to epithelial cells. Daily application of short chain fatty acids increases epithelial cell proliferation; quickens intestinal repair, increased villous height and in turn increased absorptive capacity. While, medium-chain fatty acids (MCFA) destroy the bacteria by penetrating its phospholipid layer and alters the cell membrane through the formation of pores resulting in leakage of contents (Hermans and De Laet, 2014). It provides an early pathogen barrier for the inhabiting pathogens. Propionic acid is well known for being an effective mold inhibitor (Zha and Cohen, 2014) and its inhibitory effect on feed mycotoxin is well documented. Continuous feeding of propionic acid to chicks reduced *Salmonella gallinarum* count of crop and caecal contents (Van Immerseel *et al.*, 2006). Addition of 0.36% calcium formate also reduced *Salmonella* level both in carcass and caecal samples. Akyurek *et al.* (2011) observed increase in Lactobacilli population and reduction in coliforms and Clostridia in ileum in broilers fed blends of organic acids compared to the antibiotic groups. Similarly, reduction in caecal *Salmonella* through synthesis of antimicrobial peptides in chickens fed with acetate, propionate and butyrate salts has been reported (Sunkara *et al.*, 2011, 2012).

Organic acids cocktail (Hassan *et al.*, 2010; Hamed and Hassan, 2013) is reported to have more synergistic effect with better efficiency compared to antibiotic growth promoters against intestinal pathogens viz. *E. coli*, *Salmonella*. N-heterocyclic dicarboxylic acids and pyridyl-mercapto-thiadiazoles are the new generation organic acid types envisioned as a future broad-spectrum inhibitors of the metallo- β -lactamases (MbLs) which can be used in conjunction with beta lactam antibiotics for counteracting drug resistant serotypes (Abdelrahman, 2016). The availability of calcium especially in egg producing chickens is influenced by the presence of oxalic acid which is present in plant sources. Organic acids also reduce contamination of litter with pathogens and diminish the risk of re-infection, thus reducing the bacterial challenge to poultry.

6.3.2.1. Mechanism of action of organic acids

The mechanism of action of organic acids probably reflects their antibacterial nature. This includes decreasing the pH of drinking water and reducing the buffering capacity of the feed

with subsequent effect on the physiology of the crop and proventriculus (Thompson and Hinton, 1997; Van Immerseel *et al.*, 2006). The ability of organic acids to change from undissociated to the dissociated form depending on the environmental pH enhances their antimicrobial effect. When the acid is in the undissociated form it can freely diffuse through the semi-permeable membrane of the microbe into the cell cytoplasm (Adams and Hall, 1988; Van Immerseel *et al.*, 2006). The organic acid penetrates bacterial cell wall and dissociates into the conjugated base form within bacterial cells leading to a reduction in cellular pH which creates a stressful environment for bacteria by suppressing the bacterial cell enzymes such as decarboxylases and catalases and the nutrient transport systems. This leads to cellular dysfunctions which prevent further replication of the bacterial cells (Mani-Lopez *et al.*, 2012). Other organic acids (e.g. sorbic acid) increase the permeability of the bacterial cell and also interfere with membrane proteins (Abdelrahman, 2016). The efficacy of an acid in inhibiting microbes is thought to be dependent on its pKa value, which is the pH at which half of the acid is dissociated. Organic acids with higher pKa values are known for being more effective antibacterial compounds and their efficacy is generally improved with increasing chain length and degree of unsaturation. It is important however to note that several factors do influence antibacterial activity of organic acids. These include:

1. chemical formula,
2. pKa value of the acid,
3. chemical form (esterified or not, acid, salt, coated or not),
4. molecular weight,
5. the micro-organism related MIC-value of the acid,
6. the nature of the micro-organism,
7. animal species, and
8. the buffering capacity of the feed (as reviewed in Huyghebaert *et al.*, 2011).

Therefore, each acid has its own spectrum of antimicrobial activity related to differences in both specific pH range, membrane structure and in-cell physiology of the microbiota species. The choice of an appropriate acidifier plays an important role in determining its efficacy as an alternative to antibiotics.

6.3.3. Phytobiotics

Phytobiotics represent a wide range of bioactive compounds that can be extracted from various plant sources. In recent years, some interesting and novel applications of phytobiotics in the production and well-being of monogastric animals have emerged. Recent studies

indicate that water-soluble carbohydrate extracts from some plants can be used as potential phytobiotic compounds to modify the gut microflora in broiler chickens (Vidanarachch *et al.*, 2005). Compared with other in-feed antibiotic alternatives, the evaluation of phytobiotics is still in its infancy and their potential use needs to be investigated with broader emphasis. New commercial additives of plant origin, considered to be natural products that consumers would most likely accept, have been proposed to livestock producers. As a result herbs, spices, and various plant extracts have received increased attention as possible antibiotic growth promoter alternatives.

Compared with synthetic antibiotics or inorganic chemicals, plant-derived products are natural, less toxic than antibiotics, and typically residue free. Many are certified by the Food and Drug Administration (FDA) of the United States as Generally Recognized as Safe (GRAS). This therefore makes them ideal candidates for use as feed additives and alternatives to AGP in poultry production. Although the term “phytobiotic” comprises a wide range of substances with respect to biological origin, formulation, chemical description and purity, they can be classified into four groups (Diaz-Sanchez *et al.*, 2015):

- herbs (products from flowering, non-woody and non-persistent plants);
- botanicals (entire or processed parts of a plant, e.g., roots, leaves, bark);
- essential oils (EOs) (hydrodistilled extracts of volatile plant compounds); and
- oleoresins (extracts based on non-aqueous solvents).

Several growth and health promoting properties have been attributed to phytobiotics usage in poultry. These benefits are derived by improving gut health including increasing digestibility (Kroismayr *et al.*, 2008), modifying digestive secretions, and sustaining and improving gut histology (Kreydiyyeh *et al.*, 2003; Jamroz *et al.*, 2003). Furthermore, some phytobiotics stabilize the microbiome, which reduces microbial toxins (Steiner, 2006; Windisch *et al.*, 2008; Perić *et al.*, 2010). This, in turn, reduces inflammation and; therefore, protein production can be allocated to growth as opposed to production of immune modulators (Steiner, 2006; Kroismayr *et al.*, 2008).

The positive effect of phytobiotics is mainly linked to the plant constituents including terpenoids (mono and sesquiterpenes, steroids) phenolics (tannins), glycosides, alkaloids (present as alcohols, aldehydes, ketones, esters, ethers, and lactones) flavonoids, and glucosinolate (Wenk, 2006). For this reason, many herbs and spices can be added to food with the benefit of enhancing organoleptic properties.

Although the precise mechanisms of antimicrobial action of phytobiotics are not clear yet, some mechanisms that have been suggested to be responsible for their beneficial properties include:

- disruption of the cellular membrane of pathogens;
- modification of the surface of the cells affecting the hydrophobicity and, therefore, their virulence capacity;
- stimulating the immune system, specifically activation of lymphocytes, macrophages, and NK cells;
- protecting intestinal mucosa from bacterial pathogens colonization; and
- promoting the growth of beneficial bacteria such as Lactobacilli and Bifidobacteria (Vidanarachchi *et al.*, 2005; Windisch and Kroismayr, 2007).

Among the foodborne pathogens that can be transmitted through the consumption of poultry products, *Salmonella enterica* and *Campylobacter jejuni* are the most common infectious agents (White *et al.*, 1997; Heres *et al.*, 2004). Reducing the colonization of poultry by *Salmonella* Enteritidis and *C. jejuni* in the chicken intestinal tract remains a large challenge. The target of many phytobiotic studies has been to reduce zoonotic pathogens (Reviewed in Diaz-Sanchez *et al.*, 2015), but the information available about the effects and the physiological impact of these active compounds on animal performance is still scarce. It is obvious that, although these compounds may be active against pathogens, they would not be acceptable if production performance were compromised. Table 6.1 summarises a few recent studies on performance parameters in poultry.

In chickens, the primary colonization site of *Salmonella* Enteritidis and *C. jejuni* is the cecum, which can result in horizontal transmission, contamination of egg-shells with feces, and carcass contamination during processing (Stern, 2008; Gantois *et al.*, 2009). Because the cecum is at the posterior end of the gastrointestinal tract, the phytobiotics must retain their activity during transit through the entire gastrointestinal system. Some studies conclude that the antimicrobial property is either reduced or eliminated while moving through the gastrointestinal tract (Kohlert *et al.*, 2002; Meunier *et al.*, 2006). Given the location of *Salmonella* and *Campylobacter*, retention of the antimicrobial is essential for efficacy (Arsi *et al.*, 2014). Because there are several active compounds present in the EOs, elucidating the mechanisms of antimicrobial activity can be difficult (Skandamis *et al.*, 2001; Carson *et al.*, 2002).

One antimicrobial property is attributed to the hydrophobic nature of EOs, which disrupts the bacteria cell membrane (Sikkema *et al.*, 1994). Other nonphenolic components including

functional groups and aromaticity have been demonstrated to have antimicrobial activity (Farag *et al.*, 1989; Bowles and Miller, 1993). Kollanoor-Johny *et al.* (2012) reported that *Salmonella* motility and invasion of avian intestinal epithelial cells were substantially inhibited by trans-cinnamaldehyde and eugenol. Evaluation of gene expression revealed that motility and invasion genes, *motA*, *flhC*, *hilA*, *hilD*, and *invF*, were significantly down regulated.

Table 6.1. Summary of recent studies on the effects of phytobiotics on production performance in poultry

Feed additive	Inclusion Rate	Performance effect	Reference
Cinnamon	200ppm	Improve BW and FCR	(Al-Kassie, 2009)
Grape Seed Extract (GSE)	0.6g kg ⁻¹ 1.8g kg ⁻¹ 3.6g kg ⁻¹	No effects on growth performance	(Brenes and Roura, 2010)
Moringa oleifera leaf	5% 10%	Performance decrease at inclusion levels above 5%	(Olugbemi <i>et al.</i> , 2010)
Bioimin P.E.P 125 poultry	125g/tn	BW improvement 2210±253 gr	(Perić <i>et al.</i> , 2010)
Thymol (Thy)	15gr/tn (thy)	Increase BW by 4.5%	(Tiihonen <i>et al.</i> , 2010)
Green Tea extract	0.1g/kg 0.2g/kg	Increase BW and FE	(Erener <i>et al.</i> , 2011)
Ginger	250 g/100kg 500g/100kg 750g/100kg	No effects on performance	(Mohammed and Yusuf, 2011)
Rosemary EOs Thyme extract	100; 150; 200mg/kg 0.2%; 0.4%; 0.6%	No significant effect on BW/FCR	(Pourmahmoud <i>et al.</i> , 2013)
Rosemary leaf	5.7; 8.6; 11.5g/kg	Rosemary EOs improve LWG and FE	(Yesilbag <i>et al.</i> , 2011)
Enviva EO 101	100 g/tn	Improve BW by 1,924 gr Improve FCR by 1.90	(Amerah <i>et al.</i> , 2012)
<i>Moringa oleifera</i> leaf	25%; 50%; 75%; 100%	Improve feed intake At higher inclusion levels decrease final weight/WG	(Gadzimarayi <i>et al.</i> , 2012)
Rosemary EO Oregano EO EO mixture	50 to 100mg/kg 50 to 100 mg/kg 1,000 mg/kg	Improved BW and FE	(Mathlouthi <i>et al.</i> , 2012)
Copaiba EO	0.30 mL kg ⁻¹ 0.45 mL kg ⁻¹ 0.60 mL kg ⁻¹	Decrease on performance at high inclusion levels	(Aguilar <i>et al.</i> , 2013)
Grape Seed Extract (GSE)	0.025g/kg 0.25g/kg 2.5g/kg 5g/kg	Reduction in BW gain up to 2.5g/kg	(Chamorro <i>et al.</i> , 2013)
Thymol+Carvacrol	60 mg/kg 100 mg/kg 200 mg/kg	Increase ADG (g) by 71.4% Increase G:F (g/Kg) by 601	(Hashemipour <i>et al.</i> , 2013)

Tecnaroma Herbal	100 g/tn;200 g/tn	Improve BW by 3.418 to 3.427 Kg	(Khattack <i>et al.</i> , 2014)
Mix PL	300 g/tn;400 g/tn 500 g/tn	Improve F:G ratio by 1.64 to 1.68	
Marjoram leaf	0.5%;1.0%;1.5%	Improve LBW, BWG, FCR, and FI	(Ali, 2014)

BW = Body Weight; FCR = Feed Conversion Ratio; AFC = Average Feed Conversion; AG = Average gain; FE = Feed efficiency; LWG = Live weight gain; ADG = Average daily gain; G:F = Weight gain/Feed conversion; FI = Feed Intake. Source: modified from Diaz-Sanchez *et al.* (2015).

6.3.4. Exogenous enzymes

The exogenous enzymes include dietary Non-Starch Polysaccharides (NSP) degrading enzymes, proteases and phytase that help in better feed utilization and also reduce on environmental pollution. NSPs in animal feedstuffs are a complex group of components differing widely in chemical composition, physical properties and physiological activity, many of which have negative effects on growth and performance. NSPs include (hemi) celluloses, pectins and oligosaccharides as well as arabinoxylans and beta-glucans. Different cereal types contain variable NSP levels with concomitant differences in chemical composition. For example, maize contains almost exclusively insoluble NSPs, whereas wheat and barley contain NSPs of which the ratio of soluble to insoluble is about 1/6. This ratio is about 3/4 in rye, making this cereal one with particularly high levels of soluble NSPs (Choct, 2002). The mechanism by which NSPs exert their anti-nutritive effects is complex, but their viscous nature is considered a primary cause for their anti-nutritive effect in poultry (Huyghebaert *et al.*, 2011). This is because the increased bulk and viscosity of the intestinal contents decrease the rate of diffusion of substrates and digestive enzymes thereby hindering their effective interaction at the mucosal surface (Choct *et al.*, 1996; Sacranie *et al.*, 2012). NSPs also induce thickening of the mucous layer on the intestinal mucosa (Hedemann *et al.*, 2009) suggesting that the concentrations of soluble NSPs in wheat are inversely correlated with their metabolisable energy (ME_n)-values in broiler chickens (Annison, 1991).

In addition to the direct effect of viscous NSPs on gut physiology and morphology, according to Dänicke *et al.* (1999), there appear to be some indirect effects that could have important implications for the efficient use of nutrients by the chicken. This indirect effect may be related to stimulation of fermentation of NSPs by the gut microbiota (Broom, 2017), leading to volatile fatty acid production (VFA) in the small intestine. Under normal circumstances with low NSP-diets, facultative anaerobes predominate in the chicken small intestine and nearly strict anaerobes make-up the entire caecal microbiota (Bjerrum *et al.*,

2006). But on a NSP rich diet, the VFA-concentration increases mainly in the distal ileal lumen due to excess fermentation combined with a proliferation of the fermentative microflora with a rather limited effect on the activity of the hindgut microbiota. Small intestinal fermentation points to competition with the host for digestible nutrients. Enzyme-free diets containing soluble-NSP rich cereals (wheat) have been shown to induce lymphocyte infiltration in the gut wall and induce apoptosis of epithelial cells much more than cereals such as maize that have low levels of soluble NSPs (Teirlynck *et al.*, 2009). All the aforementioned characteristics of NSPs impact negatively on broiler performance and depress growth rate by encapsulating the nutrients and reducing feed intake. Negative effects of diets with high NSP levels can be partly counterbalanced by adding AGPs (Teirlynck *et al.*, 2009). Without these, supplementing the NSP-rich diet with enzymes results in both a reduction in ileal VFA-concentration and an elevation in caecal VFA-concentration (Choct *et al.*, 1996) as more 'low molecular weight' fermentable material is entering the caecum. Caecal fermentation suggests the conversion of indigestible compounds into readily absorbable VFAs. Dietary NSP-enzymes work by reducing the viscosity of the digesta in the small intestine, so that digesta passage and nutrient digestion rate increase providing less substrate and less time for the fermentation organisms to proliferate. This may restore the normal and efficient endogenous enzymatic digestion of nutrients in the small intestine. The enzymes are partially counterbalancing the adverse effects of soluble NSP on performance (Bedford and Classen, 1992). It is not possible to measure the relative contribution following improved nutrient utilisation or the 'selective' reduction in the microbial population (Smits and Annison, 1996).

However, there is evidence that the consequence of a NSP-mediated reduced rate of digestion is much more radical in the presence of intestinal microbiota due to the degradation of both digestive enzymes and bile salts and colonisation of the absorptive surface area (Smits and Annison, 1996). In the absence of antimicrobial growth promoters, there will be a greater response to enzymes, particularly in less well-digested diets (Elwinger and Teglöf, 1991). Furthermore, NSP degrading enzymes will also reduce the proliferation of pathogenic bacteria such as *Clostridium perfringens* (Jackson *et al.*, 2003). These days all broiler feed contains enzymes such as xylanases and beta-glucanases that breakdown NSPs aimed at overcoming these depressive effects. In addition to NSP degrading enzymes, exogenous proteases help the chicken to utilize the poorly and undigested proteins better (Freitas *et al.*, 2011). On the other hand, phosphorous an important mineral in poultry exists naturally as phytate phosphorous or an organic complex phytic acid in plants which reduces its

availability to the birds. It also affects the availability of other minerals notably, zinc, copper and calcium by forming complexes with them in the gut (Dhama *et al.*, 2014). This means that a greater percentage of the phosphorous naturally present in feed is excreted by the bird leading to environmental pollution. Supplementing poultry diets with exogenous phytase has been reported however to reduce the excretion of phosphorous by up to 50% (Vohra *et al.*, 2006). It thus reduces the need for dietary inorganic phosphorous supplementation (which increases the cost of production) as well as the risk of environmental pollution.

6.3.5. Probiotics

Antibiotics have been routinely used to prevent or control poultry diseases. But due to the emergence of antibiotic-resistant bacteria, the use of antibiotics as growth promoters for poultry production has been banned in Europe and is likely to be so in the near future in other parts of the world mainly due to pressure from the consumers. As a result, there is growing interest in developing alternatives. The use of probiotics, prebiotics, and synbiotics may all be feasible.

Probiotics are the live microbial feed supplements which are used for balancing the microbial population in the intestine through the production of various compounds, competitive exclusion and displacement of pathogen from enterocytes, as well as maintenance of gut pH and thereby improving the health and immune status of the birds (Dhama *et al.*, 2014). Furthermore, they also improve broiler production factors resulting in the production of healthy meat without any drug residues (Alavi *et al.*, 2012). The importance of the gastrointestinal microflora in the health and disease of animals and humans is becoming increasingly recognized. Intake of probiotics should result in the creation of gut microecology conditions that suppress harmful microorganisms in favour of beneficial microorganisms, and ultimately enhance gut health. This is also necessary for a well-functioning and effective digestion of nutrients, resulting in good growth performance. Besides nutrient absorption, the intestine plays an important role as the biggest immune organ of the body. It is hence part of the body's defense system and represents an important barrier against invading pathogens.

The idea that intestinal bacteria could play a role in maintenance of gut health was first shown by Metchnikoff in 1907, when he studied lactic acid bacteria in fermented milk products and their use in increasing longevity and maintenance of youthful vigour in humans. A plethora of studies later confirmed that the gastrointestinal microflora of the host is actually

responsible for the natural resistance of animals against infections (Fuller, 1989; Anandakumar and Lakshminarayan, 1997; Bengmark, 1998).

Probiotic and competitive exclusion approaches have been used as one method to control endemic and zoonotic agents in poultry. In traditional terms, competitive exclusion in poultry has implied the use of naturally occurring intestinal microorganisms in chicks and poults that were ready to be placed in brooder house. Nurmi and Rantala (1973) and Rantala and Nurmi (1973) first applied the concept when they attempted to control a severe outbreak of *Salmonella infantis* in Finnish broiler flocks. In their studies, it was determined that very low challenge doses of *Salmonella* (1 to 10 cells into the crop) were sufficient to initiate salmonellosis in chickens. Additionally, they found that it was during the 1st week post-hatch that the chicks were most susceptible to *Salmonella* infections. Use of a *Lactobacillus* strain did not produce protection, and this forced them to evaluate an unmanipulated population of intestinal bacteria from adult chickens that were resistant to *S. infantis*. On oral administration of this undefined mixed culture, adult-type resistance to *Salmonella* was achieved. This procedure later became known as the Nurmi or competitive exclusion concept. The competitive exclusion approach of inoculating day-old chicks with an adult microflora successfully demonstrates the impact of the intestinal microbiota on intestinal function and disease resistance (Kabir, 2009). Although competitive exclusion fits the definition of probiotics, the competitive exclusion approach instantaneously provides the chick with an adult intestinal microbiota instead of adding one or a few bacterial species to an established microbial population. Inoculating day-old chicks with competitive exclusion cultures or more classical probiotics serves as a nice model for determining the modes of action and efficacy of these microorganisms. Because of the susceptibility of day-old chicks to infection, this practice is also of commercial importance. By using this model, a number of probiotics (reviewed in Kabir, 2009; Dhama *et al.*, 2014) have been shown to reduce colonization and shedding of *Salmonella* and *Campylobacter*. Schneitz (2005) observed that competitive exclusion is a very effective measure to protect newly hatched chicks, turkey poults, quails and pheasants and possibly other game birds, too, against *Salmonella* and other enteropathogens.

Young animals acquire microflora from the environment shortly after birth. As they age, the microflora stabilizes in the intestinal environment and equilibrium is reached between the host's beneficial and harmful microbes (Gibson and Roberfroid, 1995; Dhama *et al.*, 2014). Under normal conditions, animals pick up their microflora from adult animals and from the environment very quickly, but this is unlikely under conditions of modern animal production

where chicks are not allowed access to the hens. Commercial broiler chicks hatch in extremely clean conditions and don't have contact with adult animals. Then they are transferred in houses previously cleaned and disinfected. For them, to build up and establish a well-balanced microflora naturally is difficult. During that time, the chicks are not protected against the colonization with pathogenic microbes. For the chicks, it is crucial to develop a protecting microflora as early and fast as possible, which can be supported by the application of probiotics. Probiotic products can be sprayed onto the chicks already in the hatchery or be applied via the drinking water during the first days of life. They provide conditions in the chicks' intestines that favour the colonization by beneficial microbes. Therefore supplementing microbes that contribute to proper microbial balance in feed or *in ovo* would boost the host's ability to establish a proper microbial population in its gut and beneficially affect the host by improving the properties of the indigenous GI microbiota (Kumar *et al.*, 2011). This is the basis of the use of probiotics as alternatives to AGP in poultry production.

Besides establishing a balance in the gut microflora, they:

- specifically generate antibacterial substances (such as bacteriocins or colicins, lactoferin, hydroperoxide, lactoperoxidase),
- compete with pathogens for nutrients,
- modulate immune response,
- compete with pathogenic bacteria through CE for adhesion receptors to the epithelium,
- probiotics have also been found to improve digestion and utilization of nutrients.

Not only that, they help in metabolism of minerals and synthesis of vitamins which are responsible for proper growth and metabolism. These can neutralize toxins released by pathogenic bacteria by releasing anti-enterotoxin (such as acidolin, acidophilin and lactin) and have also been proved to bind mycotoxins present in feed (reviewed in Dhama *et al.*, 2014).

In broiler nutrition, probiotic species belonging to *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* have been reported to have beneficial effects on broiler performance (Tortuero, 1973; Owings *et al.*, 1990; Jin *et al.*, 1998; Zulkifli *et al.*, 2000; Kalavathy *et al.*, 2003; Kabir *et al.*, 2004; Gil De Los Santos *et al.*, 2005; Mountzouris *et al.*, 2007), modulation of intestinal microflora and pathogen inhibition (Mountzouris *et al.*, 2007; Giannenas *et al.*, 2012; Tellez *et al.*, 2012; Dhama *et al.*, 2013a,b), and immunomodulation (Zulkifli *et al.*, 2000; Dalloul *et al.*, 2003; Kabir *et al.*, 2004; Koenen *et al.*, 2004).

6.3.5.1. Mechanisms of Action

Several mechanisms have been proposed to explain the effects of probiotics and it is likely that the positive results reported in the different animal studies are due to a combination of some, if not all, of these. They include:

- i. maintaining normal intestinal microflora by competitive exclusion and antagonism;
- ii. altering metabolism by increasing digestive enzyme activity and decreasing bacterial enzyme activity and ammonia production;
- iii. improving feed intake and digestion; and
- iv. stimulating and modulating the immune system

(Kabir, 2009; Dhama *et al.*, 2014; Yadav *et al.*, 2016).

The efficacy of probiotics however depends on a number of factors which includes the physiological state of the bird, type and concentration of probiotics strain, persistence in intestine, ability to survive during feed processing and compatibility with natural microbiota of the intestine (Chaucheyras-Durand and Durand, 2010; Yadav *et al.*, 2016). An ideal probiotics strain should be resistant to acid, bile salts and digestive enzymes. It should also be able to multiply rapidly so as to produce microbial population required for producing the desirable effect. Furthermore, the strain used should not impart antibiotic resistance into the intestinal microflora (Dhama *et al.*, 2011; Mookiah *et al.*, 2014).

Overall the following are the benefits of probiotics in general terms:

- Improves gut health by upholding a desired equilibrium in its microbial population and reducing incidences of diarrhea;
- Inhibits growth of pathogens thereby reducing mortality;
- Results in better feed conversion efficiency;
- Improves growth rate and body weight gain;
- Improves the digestive enzymes and in turn nutrient absorption;
- Reduces circulating cholesterol level through regulation of lipid metabolism;
- Enhances efficacy of vaccines;
- Plays important role in fast detoxification of mycotoxins;
- Reduce stress associated with administration of antibiotics, temperature, vaccination, transportation etc.;
- Synthesis of the B complex vitamins;
- Improves litter quality *via.* enteric and also litter ammonia production;

- Enhances the intestinal short chain fatty acids which could alter the microbial composition in gut;
- Leaves no residues on products (i.e. meat and eggs);
- Decreases environmental pollution

(Mookiah *et al.*, 2014; Yadav *et al.*, 2016).

A latest approach in probiotics feeding especially in poultry is the *in ovo* injection of probiotic culture. As the newly hatched chick will have a sterile gastro-intestinal tract, so it harbors the microflora when they are exposed to various microbes in the environment on its arrival to its rearing house. Colonization in chicks takes place after hatching (Amit-Romach *et al.*, 2004) but presence of few numbers of microbes in their intestine during pre-natal stage itself was reported by Pedroso (2009) and Bohorquez (2010).

A plethora of scientific reports show that feeding of probiotics in birds reduces the impact of various stress conditions. It is well established that newly hatched chicks are usually exposed to different types of stressors like hatching, sexing, vaccination, dehydration, starvation, transport, etc. Various *in ovo* injection studies have shown that embryonic administration of essential amino acids, minerals, carbohydrates, fatty acids reduced the impact of these stress and enhanced growth performance in broilers. Hence, the administration of probiotic culture in *in ovo* could also be of help in overcoming stress from various factors during early life. In an experiment in broilers, *in ovo* injection with a combination of probiotic organisms at 17.5 days of incubation significantly reduced *Salmonella* counts in the intestine (de Oliveira, 2014). Overall, the development of a probiotic product comprising microorganisms selected for their competitive exclusion potential seems a promising approach to fulfil both the objectives for food safety and enhanced broiler performance.

6.3.6. Prebiotics

Manipulation of the gut microbial composition using prebiotic supplementation has been the subject of many investigations in the recent years. Prebiotics are certain non-digestive feed components that benefit the host by selectively accelerating growth rate and/or proliferation of one or more of a limited number of bacteria in the colon of host so that the health of the gut can be improved (Gibson and Roberfroid, 1995). These provide the substrate to the beneficial intestinal microorganisms. In a comprehensive review by Gaggia *et al.* (2010), several bacterial culture-based studies have reported higher abundance of lactobacilli and bifidobacteria in the gut microbiota of chickens fed prebiotic supplemented diets. The

main function associated with prebiotics include alteration of GI microflora, immune stimulation, preventing colon cancer and reducing pathogen invasion, reduction of cholesterol and odor compounds (Cummings and Macfarlane, 2002), improve gut health through intestinal microbial balance, promotion of enzyme reaction, reduction in ammonia and phenol products and ultimately reducing production cost (Ghiyasi *et al.*, 2007; Khksar *et al.*, 2008; Peric *et al.*, 2009).

Non-digestible carbohydrates (oligo and polysaccharides), some peptides, proteins and certain lipids (both esters and ethers) are candidate prebiotics. The predominant prebiotics tried in chickens however are glucooligosaccharides (GOS), fructooligosaccharides (FOS), mannanoligosaccharides (MOS), stachyose and oligochitosan (Jiang *et al.*, 2006). Lactose disaccharides mainly composed of glucose and galactose, have prebiotic effect in chickens since chickens lack the enzyme lactase. Therefore, the lactose enters the lower segment of the intestine and caeca undigested, where they are hydrolysed by microbial activity. Fructooligosaccharides (FOS) and its longer chain version, inulin, are among the most studied prebiotics in humans and animals. FOS are natural linear polymers, up to 10 monomeric, of β -(2-1)-linked fructosyl units, terminated by one glucose residue. FOS are not hydrolyzed by mammalian or avian digestive enzymes and thereby reach the colon undigested, allowing fermentation by gut microbiota (Roberfroid *et al.* 2010).

Some attributes of a good prebiotic include:

- (i) it should neither be hydrolysed nor absorbed in the upper part of the gastrointestinal tract;
- (ii) must benefit host's health by improving colonic microbiota composition;
- (iii) induce systemic effects to enhance health of the host;
- (iv) be palatable as a feed ingredient and;
- (v) be easy to process in large scale.
- (vi) should have a known structure which can be documented

(Roberfroid *et al.*, 1998; Gaggia *et al.*, 2010; Hajati & Rezaei, 2010; Dhama *et al.*, 2014; Yadav *et al.*, 2016).

Addition of prebiotics to poultry diets can minimize the use of antibiotics ultimately reducing bacterial drug resistance. Furthermore, use of prebiotics in poultry diet can reduce colonization of pathogens such as *Escherichia coli*, *Vibrio cholera*, *S. Typhimurium*, *S. Enteritidis* etc. Supplementation of oligosaccharides reduced total viable counts in meat and caecum (reviewed in Gaggia *et al.*, 2010). Similarly, addition of MOS to the diet of broilers reduced the severity of the infection due to either *Eimeria tenella* alone (Elmusharaf *et al.*,

2006) or a mixture of *E. acervulina*, *E. maxima* and *E. tenella* (Elmusharaf *et al.*, 2007). Prebiotics also promotes the growth of *Bifidobacteria* and *Lactobacillus* and reduces the harmful intestinal pathogens (Dhama *et al.*, 2007). Thus, prebiotics can be used as one of the alternatives of antibiotics with an aim to improve poultry health and performance through alteration of intestinal microbial population and stimulating immune system by pathogen reduction, however, more studies are needed to elucidate exact role and mode of action as single component or in combination. The presence of microfloral population in gastrointestinal tract influences the growth and immune system in chickens. Prebiotics are well known for their ability to enhance the establishment of good microbes (Gibson, 1999; Van Loo *et al.*, 1999; Bednarczyk *et al.*, 2016) but they are also involved in altering the innate immune response through binding with receptors, promotes endocytosis, cytokines and chemokines (Di Bartholomeo *et al.*, 2013).

It has also been shown that prebiotics particularly MOS have greater influences in birds subjected to pathogens or environmental stresses. In *E. coli* challenged, transport-stressed turkey poults, yeast extracts supplemented (1 g/kg) diet increased the number and oxidative burst activity of heterophils, and enhanced disease resistance (Huff *et al.* 2010). In a study with broiler chickens kept under suboptimal environmental conditions, MOS (1 g/kg) increased cecal bacterial diversity, and promoted growth of *Lactobacillus* and *Bifidobacterium* species in the cecum (Pourabedin *et al.* 2014). Whole yeast cell wall supplementation (2 g/kg) also decreased a coccidial infection-induced increase in the cecal *E. coli* and *Salmonella* colonization (Shanmugasundaram *et al.* 2013). It is hypothesised that mannose-containing carbohydrates bind with pathogen lectins and prevent its attachment to the epithelial surface. Mannose-bound pathogens therefore pass through the GI tract without colonization.

Inulin, an FOS is widely used as prebiotic in both human as well as in animals. Even though, they are indigestible in the intestinal tract but serve as a substrate for the growth of *Bifidobacteria* (Niness, 1999; Kelly, 2008). Inulin also promotes the production of secretory immunoglobulin A (SIgA) at ileum (Nakamura *et al.*, 2004) and increases the immunity against invading bacteria in the gut (Buddington *et al.*, 2002). Kim *et al.* (2011) indicated that FOS (2.5 g/kg diet) increased the population of *Lactobacillus*, whereas it restricted the growth of *C. perfringens* and *E. coli* in broilers. In the same study, FOS treatment increased the ileal *Lactobacillus* diversity (Kim *et al.*, 2011). In an *in vitro* study, Babu *et al.* (2012) investigated the influence of FOS inulin on the ability of the chicken macrophage-like HD11 cell line to phagocytose and kill *S. Enteritidis*. They found that prebiotic treated cells had

significantly fewer viable intracellular *S. Enteritidis* than the untreated cells, and this effect was linked to reduced IL-1 β -associated macrophage cell death. In contrast, there are pieces of evidence suggesting that some pathogenic *E. coli* strains can metabolize FOS. A gene cluster, called the *fos* locus, has been identified in the genome of avian extraintestinal *E. coli* (ExPEC) that encodes proteins involved in FOS metabolism (Schouler *et al.*, 2009; Porcheron *et al.*, 2011). The products of the gene cluster provided a strong growth advantage for the ExPEC strains to colonize the chicken intestine (Porcheron *et al.*, 2012). If this occurs, then the growth of undesirable bacteria can suppress the beneficial effects provided by probiotic-mediated utilization of FOS (Pourabedin and Zhao, 2015).

6.3.6.1. Mechanism of action of prebiotics

Details on how prebiotics exert beneficial effects on their host are not very clear since they tend to be indirect through supporting the growth and proliferation of selected useful bacterial species in the GIT. Various potential mechanisms have been proposed for health benefits of prebiotic-mediated changes in the gut microbiota (summarized in Figure 6.2; Pourabedin and Zhao, 2015). Briefly, prebiotics are metabolized by the gut commensal microbiota. The gut microbiota can ferment prebiotics into SFCA, mainly acetate, propionate and butyrate. SCFA then lower the luminal pH, provide energy sources for epithelial cells and have profound effects on inflammation modulators and metabolic regulations. A well-balanced bacterial community can also improve intestinal mucosal structure. Some bacterial strains produce antimicrobial factors or stimulate the immune system by signalling dendritic cells. Oligosaccharides and monosaccharides can reduce pathogen colonization by blocking the receptor sites used by pathogens for attachment to the epithelial cell surface. In a nutshell, prebiotics work by:

- ✓ competitive exclusion of pathogens (Callaway *et al.* 2008);
- ✓ production of antimicrobial factors (Chen *et al.* 2007, Munoz *et al.* 2012);
- ✓ stimulation of host adaptive immune system (Babu *et al.* 2012; Yitbarek *et al.* 2012);
- ✓ improving gut morphological structure (Chee *et al.* 2010; Pourabedin *et al.* 2014);

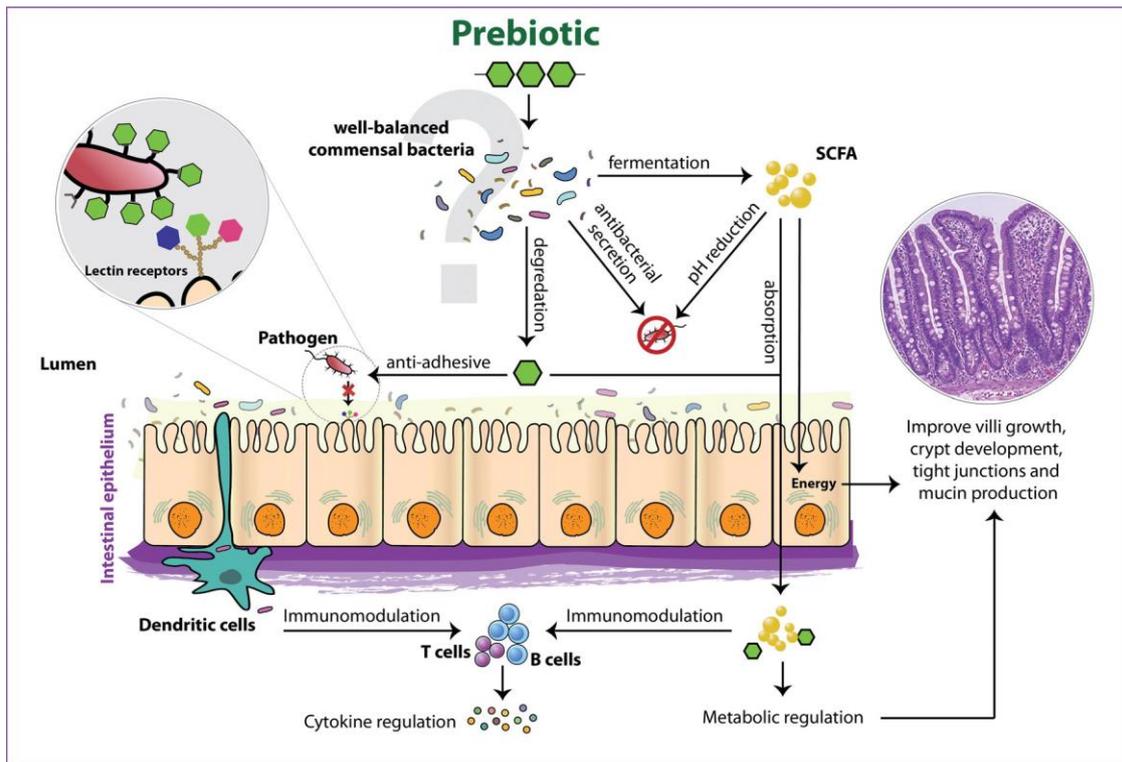


Figure 6.2 Potential mechanisms of action of prebiotics (Pourabedin and Zhao, 2015)

6.3.7. Synbiotics

The mixture of probiotics and prebiotics which provides the live culture and feeding them for better survival in the bird's intestinal tract (Yang *et al.*, 2009; Gaggia *et al.*, 2010) constitute synbiotics. Fructo-oligosaccharides and bifidobacteria, and lactitol and lactobacilli are the commonly known combinations of pro- and prebiotics for use as synbiotics (Yang *et al.*, 2009). The supplementation of prebiotics which ensure growth of probiotics is called synbiotics (Huyghebaert *et al.*, 2011). The supplementation of both probiotics and prebiotics could improve the survival and persistence of the useful organism in the gut of birds as specific substrate is available for fermentation (Yang *et al.*, 2009; Adil and Magray, 2012). Synbiotics were effective in improving the growth (Abdel-Raheem *et al.*, 2012; Mookiah *et al.*, 2014; Tavaniello *et al.*, 2017) and some carcass traits (Maiorano *et al.*, 2017) of broilers.

Feeding of synbiotics in broiler chicken was found to have beneficial effect on intestinal morphology and nutrient absorption leading to enhanced performance (Awad *et al.*, 2008; Hassanpour *et al.*, 2013). Very few studies have reported the optimal benefits of synbiotics in poultry (Li *et al.*, 2008). Much attention has to be paid to finding out the best combination of pro and prebiotics and the subsequent evaluation of their synergistic effects for use as potential synbiotics to ensure maintenance of proper health. An investigation by Madej *et al.*

(2015) and Madej and Bednarczyk (2016) in broilers revealed that *in ovo* administration of inulin (prebiotic) along with *Lactobacillus* organism altered the development of various immune organs. Slawinska *et al.* (2014a) reported that *in ovo* administration of synbiotics into the developing chicken embryo is an effective way to provide stimulus for the immune organs of the growing chickens. According to the authors (Slawinska *et al.* 2014a), *Lactococcus lactis* probiotics survived in the chicken guts throughout their lifespan. The authors also observed synergistic effects of the RFO prebiotic and *Lactococcus lactis* subsp. *cremoris* IBB SC1 on the development of the immune organs, i.e. bursa of Fabricius (in meat-type chickens) and spleen (in general-purpose chickens) as well as on lymphocyte proliferation in the thymus in both chicken genotypes studied. It was therefore concluded that the *in ovo* administration of selected synbiotics is a promising approach in chicken immune system enhancement, as it combines merits of prebiotics and probiotics and by early administration into the embryo, supports development of their immune organs.

On the other hand, studies on the effects of prebiotics and synbiotics on broiler performance and the few data on meat quality traits have yielded inconsistent results. This could probably be explained by the variation in the experimental conditions, dosage and type of pre- or synbiotics used, genotype of birds used among other factors.

Chapter 7

IN OVO TECHNOLOGY

7.1. Application of *In Ovo* technology in poultry production

Holding chicks post-hatch without feeds and water for long hours is a common practice in the poultry industry. However, studies assessing the impacts of this practice on growth and development post-hatch have yielded interest in finding ways to aid poult nutrition before placement in brooder farms. For instance, Uni *et al.* (1998) revealed that early fasting clearly delays gut maturation, affecting the development of mucosal morphology and intestinal enzyme activity and Noy and Sklan (1998, 2001) showed that early feeding could stimulate gastrointestinal motility and use of yolk sac nutrients which are necessary for growth. Similarly, Careghi *et al.* (2005) observed higher weight gain in broilers fed immediately after hatching compared to the held chicks. The authors also found that late hatching benefitted more from the early access to feed. It has also been observed that early inoculation of a young chick with the native microbiota of a healthy adult bird can facilitate the development of an early GIT microbiota, thereby, leading to enhanced intestinal immunity as well as improved growth performance (Crhanova *et al.*, 2011; Roto *et al.*, 2016). This is the basis of extensive research on the application of *in ovo* technology in the poultry industry.

The *in ovo* method of delivery of bioactive substances and supplements to the chicken's embryo, provides an alternative means to both compensate for the starvation period that newly hatched chicks undergo and aid early establishment of a healthy GIT microbiota prior to exposure to pathogens. Numerous researches on *in ovo* technology show positive results regarding the supply of important bioactive substances that aid enteric development and digestion of nutrients in chicks (Ferket, 2012; Gholami *et al.* 2015). The main aim of pre-hatch feeding is to equip the embryo with the nutrients necessary to continue intestinal development post-hatch at or close to the same rate as pre-hatch. Uni and Ferket (2003) observed that supplying the embryo with exogenous nutrients would allow the GIT to develop the structures and functionality to properly digest and absorb nutrients immediately when exogenous nutritional supplementation is provided after hatch. These nutrients, along with the yolk sac reserves, can contribute not only to maintaining the systems and metabolism already established but also to continuing growth, development, and proper nutritional status (Noy and Sklan, 1998). Numerous studies have been conducted to

investigate the efficacy of *in ovo* delivery of various bioactive substances in poultry, including nutrient supplements (reviewed in Roto *et al.*, 2016; Table 7.1).

Use of *in ovo* feeding could enhance the nutritional status of the embryo and also hatched chicks resulting in overall improvement in production performance. Uni and others (2004; 2005) indicated muscle improvement and immune development, breast meat yield and improved health status. Similarly, Foye *et al.* (2007) showed enhanced jejunal nutrient uptake, increase in activity of the intestinal enzymes and post hatch growth by feeding birds *in ovo*. Likewise, Gholami and colleagues (2015) reported improved hatching weight and final weight of chickens, as well as lower FCR and abdominal fat percentage after *in ovo* administration of betaine and choline in broiler chickens. When Smirnov *et al.* (2006) inoculated eggs with carbohydrates (maltose, sucrose, and dextrose), the results showed that the additional energy source enhanced the development of goblet cells and increased the villi surface area in the intestines. While other studies (Uni *et al.*, 2005, Foye *et al.*, 2006) on *in ovo* delivery of carbohydrates indicated increased body weight and increased liver glucose at hatch.

There has been interest in identifying substances that may enhance the development or response of the immune system at an earlier stage (Roto *et al.*, 2016). Furthermore, stimulating the immune system and taking prophylactic measures rather than having to use therapeutic dosages is superior from a food safety and public health viewpoint. Additionally, improving the immune response of immature chicks is crucial for survival and performance to market age. Some experiments to improve immunocompetence via *in ovo* injection of vitamins, amino acids, and carbohydrates have also been attempted. The results showed beneficial effects on antibody and macrophage response, immunomodulation, and humoral and cellular immunity (Gore and Qureshi, 1997; Kidd, 2004; Bhanja and Mandal, 2005; Bhanja *et al.*, 2015). Additionally, injections of antibodies and antibiotics have been attempted (McReynolds *et al.*, 2000; Kim *et al.*, 2007). Increased antibody residues in both the yolk sac and blood serum were observed as a result of *in ovo* injection of antibiotics on the 18th day into the amnion. This was associated with reduced establishment of a competitive exclusion culture when embryonated eggs were supplied with commercial competitive exclusion culture isolated from cecal microbiota of healthy adult chickens (Foye *et al.*, 2006).

In ovo technology was first used in poultry research to deliver Marek's disease (MD) vaccine at about the 18th day of incubation with good results (Sharma and Burmester, 1982). *In ovo* vaccination against Marek's disease was established as a reliable method to ward off

infection due to exposure to the Marek's disease virus as early as the 1980s after realizing that post-hatch vaccinated flocks occasionally experienced extensive mortality as a result of infection with the MD virus. One of the most probable reasons was that the post-hatch vaccinated birds were exposed to MD prematurely, allowing insufficient time for the young chicks to build immunity following vaccination (Roto *et al.*, 2016). Sharma and Burmester (1982), recognizing the ability of late-stage embryos and fetuses to support immune responses to viral and bacterial antigens, used the *in ovo* injection for the MD vaccine in embryonic chickens. They observed significantly better protective indices with embryonic stage vaccination regardless of day of *in ovo* injection between the 16th to 20th days of incubation. According to the same authors, the 18th day of incubation gave the greatest protection from vaccination compared to those vaccinated at hatch ($P < 0.05$), with no significant effect on hatchability (Sharma and Burmester, 1982). Subsequent work demonstrated that *in ovo* administration of Marek's disease (MD) vaccines HVT (Sharma and Burmester, 1982; Sharma and Witter, 1983), SB-1 (Sharma and Witter, 1983), and CVI988 (Zhang and Sharma, 2001) to late-stage chicken embryos was safe and would induce earlier immunity than post-hatch administration (Sharma, 1984; Zhang and Sharma, 2001). The concept of *in ovo* MD vaccination was moved from the laboratory to the field by the development of an automated *in ovo* injection machine (Sarma *et al.*, 1995). Commercial *in ovo* vaccination began in the United States in late 1992 and is routinely practiced in many commercial broiler hatcheries in more than 30 countries today (Avakian *et al.*, 2002).

The success with *in ovo* vaccination marked the beginning of extensive research with various bioactive substances such as nutrients, hormones, and immunostimulants. However, Roto *et al.* (2016) observed that the only uncertainty of the method has been with optimization of delivery with reference to age, volume, location of injection, as well as other factors such as stress caused to the embryo by disruption of the internal environment or osmotic balance, and insufficient evaluation for the optimal individual or mixed substances for injection or their appropriate concentrations for delivery. The process and technique used to administer *in ovo* bioactive substance is critical to achieving the desired outcome. There have been several patents for automated delivery at different sites of injection, with different biological substances and supplements as well as the age of injection and the method of automation (Roto *et al.*, 2016). According to Wakenell *et al.* (2002), there are five regions for *in ovo* injection in the late stage of embryonic development (Figure 7.1). These are the air cell, the allantoic membrane, the amniotic fluid, the yolk, and the embryo's body. The authors evaluated the consequences of *in ovo* injection of the MD vaccine to eggs on the 17th

and 18th days of incubation at various locations. The results indicated that injection of the vaccine into the amniotic fluid or the embryo's body gave the greatest protection efficacy (90%), whereas injection of the vaccine into either the air cell or allantoic fluid resulted in less than 50% protection. The authors concluded that the precision in the depth of the injection is crucial for instance, if the needle does not go deep enough into the egg, the result is the dispersion of the vaccine to the air cell or allantoic fluid, while injecting the needle too deep may cause trauma to the embryo. On the other hand, Uni and Ferket (2003) noted that the most ideal injection site and time for delivering nutrients were the late term embryonic development stage and the amniotic fluid respectively. The authors added that this was because the embryo consumes the amniotic fluid and its contents find their way into the intestine. As such, substances administered into this region get to be consumed along with the amniotic fluid and are therefore presented to the enteric tissues. Al-Murrani (1982) first experimented with supplementation of amino acids to the yolk sac at the 7th day of incubation. Results indicated that the embryo did not use the protein until late-stage embryonic development to gain weight and they carried the additional weight through market age.

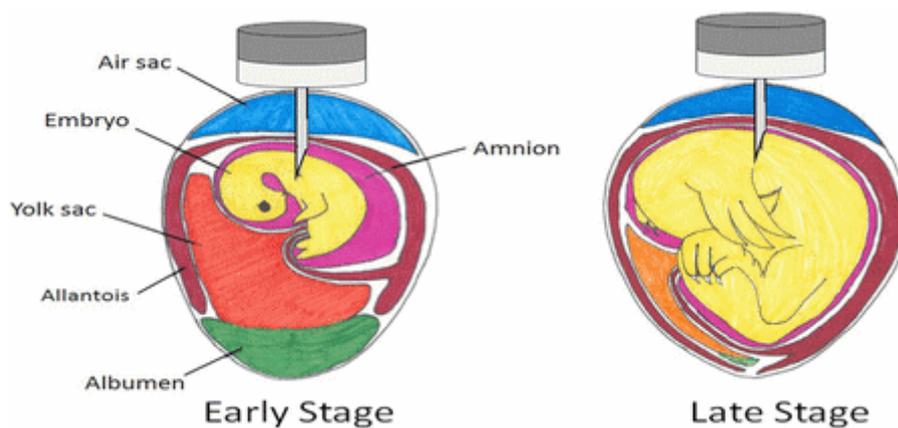


Figure 7.1 Possible *in ovo* injection sites at early and late stages of incubation.
(Roto *et al.*, 2016).

Table 7.1 Summary of some of the effects of *in ovo* delivered bioactive substances in chicken embryos at various locations and times of incubation

Bioactive substance	Reference	Stage of incubation	Target	Results
Carbohydrates	Zhai <i>et al.</i> , 2011a,b; Uni <i>et al.</i> , 2005; Dos Santos <i>et al.</i> , 2010; Smirnov <i>et al.</i> , 2008	Late stage	Amniotic fluid	Trophic effects on small intestine and effects on goblet cell activity; effects on embryonic metabolism and body weight
Amino acid	Bakayaraj <i>et al.</i> , 2012; Ohta <i>et al.</i> , 1999; Zhai <i>et al.</i> , 2008	Early and late	Amniotic fluid; yolk sac; air cell	Effects on chick to egg ratio, body weight, bursal and thymus weight; effects on body weight in relation to location and day of injection; effects on feed intake, feed conversion ratio and immune response.
Hormones	Liu <i>et al.</i> , 2013; Hargis <i>et al.</i> , 1989; Moore <i>et al.</i> , 1994	Early and late stage	Albumen	Effects on muscle content; effects on body weight, skeletal growth, feed efficiency and adipose tissue development.
Pre- pro- and synbiotics	De-Oliveira <i>et al.</i> , 2014; Villaluenga <i>et al.</i> , 2004; Maiorano <i>et al.</i> , 2012; Pruszynski-Oszmalek <i>et al.</i> , 2015	Early stage	Air cell, amnion	Effects on muscle fibers and histology; effects on Salmonella colonization; effects on body weight gain and pancreatic enzyme activity, effects on Bifidobacteria count in faces
Protein (antibodies)	Kim <i>et al.</i> , 2007; McReynolds <i>et al.</i> , 2000	Late and early	Yolk sac, albumen, amniotic fluid	Effects on body weight and muscle mass varied among injection sites; effects on antibiotic residue detection
Immuno-stimulants	Gore and Qureshi, 1997; Taghavi <i>et al.</i> , 2008; McGruder <i>et al.</i> , 1995	Late stage	Amniotic fluid	Effects on <i>in vitro</i> bactericidal activity of heterophils and protection against Salmonella invasion; effect on macrophage and antibody response

^aDay of incubation, early stage = 0–12 days; late stage = 13–21 days of incubation (Roto *et al.*, 2016).

7.2. Delivery of Prebiotics *in ovo*

Experimentation into the *in ovo* injection of prebiotics and synbiotics is a fairly recent area of poultry research. The rationale of distributing prebiotics to a developing embryo is driven by the recognition that activities of both substances work toward improving GIT health. Previous researches (Tako *et al.*, 2004; Uni and Ferket, 2004; Uni *et al.*, 2005; Smirnov *et al.*, 2006) have shown that the *in ovo* injection of a mixture of carbohydrates dissolved in saline, sucrose (a monosaccharide), maltose (a disaccharide), and dextrin (a polysaccharide) with or without β -hydroxy- β -methylbutyrate (HMB, a leucine metabolite) improved embryonic intestinal development with a subsequent increase in the body weight of chicks at hatch. Prebiotics and/or synbiotics have been shown to alter GI microflora, alter the immune system, prevent colon cancer, reduce pathogen invasion and reduce cholesterol and odour

compounds (Hajati and Rezaei, 2010). However, the major challenge has been related to the efficiency of administration of prebiotics under fully controlled conditions. It is well established that for prebiotics and synbiotics to be effective, they have to be administered to the animals as early as possible (Slawinska *et al.*, 2014a). In addition, the effect of confounding variables such as water quality, and environmental factors should be minimized in order to achieve the desired results. To exclude some of these factors, *in ovo* procedure of delivering these substances directly into the air chamber of chicken embryo has been developed and patented (Gulewicz and Bednarczyk, Polish patent Nb. 197726). Nevertheless, results from studies on the effects of *in ovo* delivered prebiotics and synbiotics have been inconsistent. In a study by Maiorano *et al.* (2012) *in ovo* injection of synbiotics at 12 days of incubation to the air cell had little influence on carcass weight and pectoral muscle percentage. While Bednarczyk *et al.* (2011, 2016) showed that *in ovo* delivered prebitics (RFOs) significantly increase body weight and feed conversion ratio. Similarly, Pruszynska-Oszmalek *et al.* (2015) evaluated the effects of *in ovo* injection of prebiotics (inulin and Bit²os) and synbiotics (inulin with *Lactococcus lactis*) into the air cell at 12 days in chickens. The authors reported that synbiotics had no significant impact on the feed conversion ratio while prebiotics significantly increased the final body weight. Additionally, the delivery of both synbiotics and prebiotics increased the activities of the pancreatic enzymes amylase, lipase, and hydrolase (Pruszynska-Oszmalek *et al.*, 2015). These enzymes are involved in the digestion of food; thus, it is probable that the increased activity is beneficial to the newly hatched chicks in their transition from endogenous to exogenous nutrients (Pruszynska-Oszmalek *et al.*, 2015).

Considerable attention has been given to the effect on immune response and activity with regards to *in ovo* delivery of prebiotics and/or synbiotics. Madej and Bednarczyk (2015) showed that *in ovo* injection of synbiotics stimulate gut-associated lymphoid tissue (GALT: Peyer's patches, cecal tonsils, Meckel's diverticulum, and esophageal and pyloric tonsils) colonization by T cells than prebiotics alone. Furthermore, the *in ovo* injection of synbiotics to the air cell at 12 days was shown by Slawinska *et al.* (2014) to stimulate the development of immune organs (bursa of Fabricius and spleen) as well as increase proliferation of lymphocytes in the thymus. Stimulation of synthesis of immunoglobulins has also been demonstrated with the *in ovo* injection of prebiotics and synbiotics at 12 days of incubation to the air cell (Madej *et al.*, 2015). It has also been confirmed that a single *in ovo* injection with prebiotics into 12 day old chicken embryo leads to an increase in the number of bifidobacteria at the time of hatch ensuring the long-term maintenance of a high level of

bifidobacteria in the intestinal tract in chickens experimentally (Villaluenga *et al.*, 2004; Pilarski *et al.*, 2005; Pruszyńska-Oszmalek *et al.*, 2015; Bednarczyk *et al.*, 2016) and under field condition (Bednarczyk *et al.*, 2009, 2011). This observation suggests that the application of prebiotics to the birds in feed can be successfully replaced by injecting these compounds *in ovo* in very low doses.

Research conducted evaluating the dosages of prebiotic preparations injected *in ovo* demonstrated that the dose of the bioactive substance given is crucial to achieving the desired results in chicken. Whereas Villaluenga *et al.* (2004) observed increased numbers of bifidobacteria associated with increased dosage of prebiotic mixtures of various oligosaccharides, the increased dosages were also negatively associated with hatchability and embryo weight. Bednarczyk *et al.*, (2011) also found a significantly lower hatchability percentage in *in ovo* treated group with 1.9 mg of RFO compared to the control group. The authors could not clearly explain the decline in hatchability percentage of the injected embryos since different factors affect the hatchability of *in ovo* injected embryos (i.e. site of injection, features and doses of injected substances and technology of incubation among others).

To that end, it was deemed necessary to dissociate the influence of prebiotics dose level on hatchability from other factors. In a subsequent experiment, Bednarczyk *et al.* (2016) indicated that the optimal dose of DiNovo (DN) and Bit²os (BI) prebiotics for *in ovo* delivery that did not reduce chicks' hatchability, were 0.88 mg/embryo (DN) and 3.5 mg/embryo (BI) respectively. The same authors also confirmed that a single *in ovo* injection of prebiotics at the appropriate dosage can successfully replace prolonged in-water supplementation post-hatching.

PART TWO

AIM OF THE THESIS

The digestive tract of animals harbours a great number of living and metabolizing microorganisms (microbiota), that not only influence physiological functions of the host but are also considered fundamental for a proper development of several vital traits, including immune system (Sekirov *et al.*, 2010). In poultry farming the intestinal microbiota and the “gut health” are topical subjects, especially since the ban on therapeutic use of antibiotics to avoid the onset of antibiotic resistance and safeguard consumer health.

Probiotics, prebiotics and synbiotics are one of the proposed solutions, as alternatives to AGPs, to prevent enteric disease and increase performance in poultry. To be effective, these compounds have to be administered to the animals under fully controlled conditions and as early as possible. An innovative method for introducing bioactive substances into chickens is the *in ovo* injection into eggs intended for hatching (reviewed in chapter 7; Figure 1). This technique is based on the introduction -on the appropriate day of embryonic development- of bioactive substances into the air chamber of the egg or directly into the developing embryo and it has been validated under temperate climatic conditions (Bednarczyk *et al.*, 2011, 2016; Maiorano *et al.*, 2016, 2017).

This method allows for a precise and uniform delivery of the bioactive substance to all embryos at an early stage of development, which unifies the effects of prebiotics across the flock and ensures proper development of gut microflora in all chicks. Studies conducted in the temperate climatic condition have already revealed that *in ovo* injection of prebiotics and probiotics into the air cell during embryogenesis improves egg hatchability (Pilarski *et al.*, 2005) and modulate the optimal composition of the chicken’s microbiota, fully developed at hatching (Bednarczyk *et al.*, 2011; Maiorano *et al.*, 2012, Slawinska *et al.*, 2014a,b). These effects are reportedly stable throughout the chicken’s lifespan, influencing metabolic and immune responses of the host, and produce an improvement in performance production and meat quality.

However, *in ovo* administration was validated only for meat-type chickens under temperate climatic condition, not taking into account the richness of the poultry biodiversity and climatic variation. Thus, in this study it was hypothesized that chickens of different production types (broiler and indigenous chickens) have different reaction to microbiome stimulation by *in ovo* delivery of prebiotics under different climatic conditions.

Objectives

The aim of the study was to evaluate the efficacy of prebiotics delivered *in ovo* on egg hatchability, production performance, and health and meat quality of Ross 308 broiler and Kuroiler chickens reared under tropical and temperate climatic conditions, respectively.

The specific objectives were:

- a) To assess the effect of prebiotics delivered *in ovo* on egg hatchability;
- b) To investigate the effect of prebiotics delivered *in ovo* on productive and meat quality traits in Ross broiler and Kuroiler chickens;
- c) To investigate the efficacy of prebiotics delivered *in ovo* on gut health of Kuroilers under tropical climatic condition in the face of a natural coccidiosis challenge.

PART 3: RESEARCH WORK

Chapter 8

EXPERIMENT 1

Efficacy of *in ovo* delivered prebiotics on health, performance and meat quality of Ross 308 broiler chickens reared under temperate climatic conditions

8.1 Introduction

As aforementioned, pro-, pre- and synbiotics are important candidates for the replacement of antibiotic growth promoters in poultry nutrition and production. The *in ovo* administration approach has been validated and found to be a better option for the delivery of the bioactives to birds early in life. Nevertheless, results from studies on the effects of *in ovo* delivered prebiotics and synbiotics are not exhaustive. For instance, in a study by Maiorano *et al.* (2012) *in ovo* injection of prebiotics and synbiotics at 12 days of incubation to the air cell had little influence on carcass weight and pectoral muscle percentage. But the latest study by the same authors found beneficial effects of the treatment on performance and carcass traits (Maiorano *et al.*, 2017). While Bednarczyk *et al.* (2011, 2016) showed that *in ovo* delivered prebiotics (RFOs) significantly increase body weight and feed conversion ratio. Similarly, Pruszynska-Oszmalek *et al.* (2015) evaluated the effects of *in ovo* injection of prebiotics (inulin and Bi²os) and synbiotics (inulin with *Lactococcus lactis*) into the air cell at 12 days in chickens. The authors reported that prebiotics and synbiotics had no significant impact on the feed conversion ratio; while, final body weight was higher in birds that received *in ovo* Bi²os (prebiotic) and inulin + *Lactococcus lactis* ssp. *Lactis* (synbiotic). Additionally, the delivery of prebiotics and synbiotic increased the activities of the pancreatic enzymes amylase, lipase, and hydrolase (Pruszynska-Oszmalek *et al.*, 2015). This may explain the positive effect of additives on body weight observed.

The aim of the first experiment was to re-evaluate the efficacy of a commercial prebiotic (Bi²os[®], a trans-galactooligosaccharides - GOS) delivered *in ovo*, on growth performance, carcass traits, fatty acid composition and meat oxidative stability in Ross 308 broiler chickens reared under the temperate climatic condition.

8.2 Materials and methods

8.2.1 *Birds and experimental design*

To date, inulin derived from *Dahlia tubers* was considered a gold standard in the prebiotic applications. But, development in the field achieved during continuous search for scientific excellence led to the discovery of second generation prebiotics. For instance, a study by my research group at the University of Molise, Italy and its partners in Poland aimed at developing, testing and launching of prebiotics that would combine beneficial properties on gastrointestinal tract as well as the health of the animals highlighted second generation galactooligosaccharide (GOS) prebiotics as a very efficient product with extremely beneficial properties up on *in ovo* injection in broilers. Based on the above finding, this study used a non-digestive trans-galactooligosaccharides (**GOS**) from milk lactose digested with *Bifidobacterium bifidum* NCIMB 41171, injected *in ovo* to assess the effects of prebiotics under temperate climatic conditions. The injected dose had been optimized by Bednarczyk *et al.* (2016).

The eggs used in this experiment were from Ross 308 broiler (meat-type) chickens. Eggs were incubated in a small broiler hatchery (Avicola Fanelli, Riccia, Campobasso, Italy). On day 12 of incubation, prior to the injection, the eggs were candled to select only the ones containing viable embryos. The 300 eggs that had viable embryos were randomly divided into three experimental groups: prebiotic group (**BI**) was injected with 200 μL of physiological saline solution containing GOS at a dose of 3.5 mg/embryo, saline group (**S**) was injected with 200 μL of physiological saline solution, and control group (**C**) not injected. The eggs were injected on day 12 of embryonic incubation when the allantochorion is completely developed and highly vascularized (Villaluenga *et al.*, 2004). After injection, each hole was sealed with natural glue and egg incubation was continued until hatching (Figure 8.1).

At hatching, the number of healthy chicks was scored for each experimental group. Chicks were sexed, vaccinated against Marek disease and Coccidiosis and 120 males (40 chicks/group) were grown to 14 day of age in collony cages in an environmentally controlled poultry house, with temperature ranging between 30 and 32°C and relative humidity between 65 and 70%. At 15 d of age, healthy male chicks were transferred to the experimental farm located in Bonefro (at 628 m above sea level) in Molise region, Italy. Birds were grown to 42 days of age in in floor pens (n = 4 replicate pens, 10 birds in each pen) fitted with the solid floor, with outdoor access, in a poultry research house that provided good husbandry conditions (e.g. stocking density, litter, ventilation). Animal handling followed the

recommendations of European Union directive 86/609/EEC. Chickens were fed *ad libitum* starter and grower-finisher diets that were formulated to contain adequate nutrient levels and had free access to water (Table 8.1). Along the rearing period, chickens were weighed and counted within each pen weekly.



Figure 8.1. Manual *in ovo* injection and sealing with natural glue.

Table 8.1. Composition and nutritional value of diet

	Period	
	1-21 d	21-41 d
Ingredients (%)		
Corn	22	31.6
Wheat	19.5	15
Soybean meal	31.5	25
Wheat middlings	13	15
Corn gluten	10	10
Soybean oil	1.32	1.1
Calcium Carbonate	1.2	1
Dicalcium Phosphate	0.5	0.5
NaCl	0.2	0.2
Sodium bicarbonate	0.1	0.1
Vitamin-mineral premix 1 ¹	0.3	-
Vitamin-mineral premix 2 ²	-	0.3
Phytase	0.1	0.1
Coccidiostat	0.1	0
Color additives	0.1	0.1
Methionine	0.08	-
<i>Calculated nutritional value of the diet (%)</i>		
Protein	24.00	21.00
Lipid	4.50	4.50
Crude fiber	4.50	4.00
Ash	7.00	6.00
Lysine, %	1.10	1.00
Methionine, %	0.35	0.30
Calcium, %	1.30	1.10
Available P, %	0.70	0.60
Sodium, %	0.15	0.20

¹Supplied per kilogram of diet: vitamin A, 12,000 IU; vitamin D3, 3,600 IU; vitamin E, 50.1 mg; vitamin B1, 3 mg; vitamin B12, 0.04 mg; vitamin B2, 6 mg; vitamin B6, 3.99 mg; CuSO₄ 5H₂O (Cu, 10mg), 38.26mg; Ca(IO₃)₂ (I, 1.50mg), 2.31mg; FeCO₃ (Fe, 45mg), 93.15mg; MnO (Mn, 36mg), 46.44mg; MnSO₄ (Mn, 35mg), 110.88mg; Na₂SeO₃ (Se, 0mg), 0.43mg; ZnO (Zn, 51mg), 63.24mg.

²Supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D3, 3,000 IU; vitamin E, 41.68 mg; vitamin B1, 2.90 mg; vitamin B12, 0.03 mg; vitamin B2, 5 mg; vitamin B6, 3.33 mg; CuSO₄ 5H₂O (Cu, 8mg), 32.72mg; Ca(IO₃)₂ (I, 1.25mg), 1.93mg; Fe₂O₃ (Fe, 560mg), 800.8mg; FeCO₃ (Fe, 38mg), 77.63mg; MnO (Mn, 30mg), 38.70mg; MnSO₄ (Mn, 30mg), 92.40mg; Na₂SeO₃ (Se, 0mg), 0.36mg; ZnO (Zn, 43mg), 52.7mg.

8.2.2 Slaughter survey

At 42 d of age, 20 randomly chosen birds per treatment, of similar estimated body weight, were individually weighed and slaughtered. Hot carcass weight was recorded and carcass yield was calculated. The breast muscle (including *pectoralis major* and *pectoralis minor*), leg muscle (including thigh and drumstick), wings and back+neck were removed from the carcass and then weighed. The weight percentages of breast and leg muscles, wings and back+neck were calculated as a percentage of eviscerated carcass weight.

Pectoral muscle pH was measured 24 hours *post-mortem* on the upper part of the left-side breast fillet using a portable pH Meter (FiveGo, Mettler-Toledo AG, Schwerzenbach, Switzerland).

At the same time, colour coordinates (lightness, L*; redness, a*; yellowness, b*) were measured on the bone-side surface of left-side breast fillet using a Chroma Meter CR-300 (Minolta Corporation, Italia s.r.l., Milano).

WHC, expressed as expressible juice, was measured on pectoral muscle 24 h after chilling using the press method described by Grau and Hamm (1957). Afterwards, pectoral muscles were vacuum packaged and stored (-18°C) until meat quality analyses.

8.2.3 Fatty acid analysis

The fatty acid composition of intramuscular fat samples was determined after chloroform-methanol extraction (Folch et al., 1957), and fatty acids were determined as methyl esters (FAME), using a gas chromatograph ThermoQuest TRACE 2000 (SACtm-5 column 3000cm \times 0.25mm, Supelco, USA). Helium was used as the carrier gas at a flow rate of 1.5 mL/min with constant flow compensation. GC inlets were held at a temperature of 240 $^{\circ}\text{C}$, and the detector was maintained at a temperature of 250 $^{\circ}\text{C}$. The oven temperature was programmed from 150 $^{\circ}\text{C}$, and followed by a ramp at a rate of 5 $^{\circ}\text{C}/\text{min}$ till 240 $^{\circ}\text{C}$ with a final hold of 15 min (the total analysis time was 33.00 min). The individual FA peaks were identified by comparison of retention times with those of FAME authentic standards run under the same operating conditions. Results were expressed as percentage of the total FA identified. To assess the nutritional implications, the n-6/n-3 FA ratio and the PUFA/SFA (P/S) ratio were calculated. Moreover, in order to evaluate the risk of atherosclerosis and the potential aggregation of blood platelets, respectively the atherogenic index (AI) and the thrombogenic index (TI) were calculated, according to the formulas suggested by Ulbricht and Southgate (1991).

8.2.4. Determination of meat oxidative stability by TBARS assay

The TBARS assay measures malondialdehydes (MDA) present in the sample, as well as malondialdehydes generated from lipid hydro peroxides by the hydrolytic conditions of the reaction. TBARS was measured using the colorimetric method of Vyncke (1970). Briefly, 5 g of meat were homogenized (Ika® Ultraturax T25) with 15 ml of extraction solution (7.5% trichloroacetic acid, 0.1% propylgallate, 0.1% EDTA). The mixture was then filtered using plastic funnels fitted with Whatman 1 (ø 70mm) filter paper. After which 5 ml of the filtered solution were mixed with 5 ml of 0.02M solution of 2-thiobarbituric acid (TBA) and incubated at 100°C for 40 min. The absorbance was measured by spectrophotometer (V-730, Jasco Co., Ltd., Tokyo, Japan) at 532 nm. The calibration curve was prepared by using a dilution of tetraethoxypropane (TEP) instead of MDA, considering that 1M of TEP corresponds to 1M of MDA. The value of TBARS was expressed as mg MDA/kg of raw meat.

8.2.5. Statistical analyses

Data were analysed by one-way ANOVA using the SPSS package (SPSS, 2010). The multi-comparison Scheffe's test was used to separate the differences among the mean for statistical significance ($P < 0.05$).

8.3 Results

8.3.1 Performance and meat quality/carcass traits

Hatchability, ranging from 90% to 91.2%, was similar among experimental groups (Fig. 8.2). Mortality of the chickens during this study was very low (2%) and not dependent on the substance injected *in ovo*.

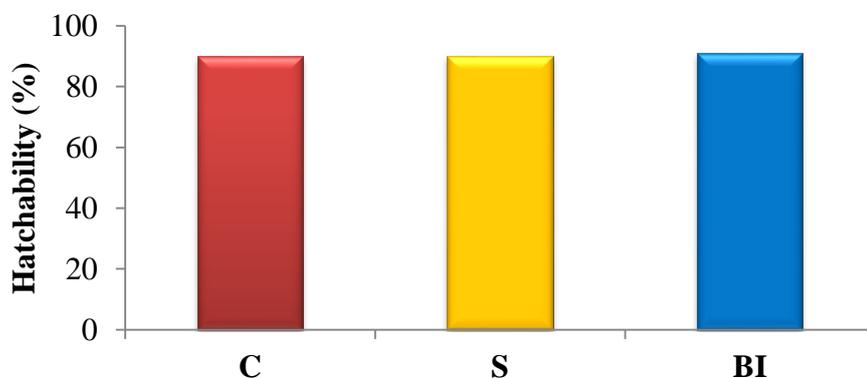


Figure 8.2. Effect of *in ovo* injection on hatchability scores (C = control; S = saline; BI = Bi²tos)

Effect of *in ovo* prebiotic administration on growth performance is presented in Table 8.2. Initial body weights were the same among all experimental groups ($P > 0.05$). In the period from week one to three, BI group showed a significantly higher weight gain ($P < 0.01$) in comparison to the C (+2.5 %) and S (+1.9 %) groups. While for the entire rearing period (week 1 - 6), both BI and S groups showed higher BWG in comparison to the C group (+7.5% and 6.8%, respectively; $P < 0.05$).

Table 8.2. Growth performance of Ross broiler chickens

	Group ¹			SEM	<i>P</i> -value
	C	S	BI		
Initial weight (g)	40.37	39.89	40.54	0.13	0.093
Body weight gain (g)					
1-3 week	701.79 ^B	706.16 ^B	719.30 ^A	1.49	0.001
1-6 week	2246.53 ^b	2399.58 ^a	2415.11 ^a	14.40	0.028

¹Group: C = Control, uninjected eggs; S = Saline, *in ovo* injection of physiological saline; BI = Bi²tos.

^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$).

^{A,B}Means within a row lacking a common superscript differ ($P < 0.01$).

At slaughter, 20 chickens per group of similar estimated body weight were randomly selected. Chickens from BI group were significantly heavier ($P < 0.01$) at slaughter than those from C group, but similar to those of S group (Table 8.3). Similarly, carcass, leg and back+neck weights were higher ($P < 0.05$) in S and BI groups as compared to C. However, no significant differences among experimental groups ($P > 0.05$) were found for carcass yield, main commercial cut yields (breast, legs and wings) and back+neck yield.

Table 8.3. Slaughter traits of Ross broiler chickens

	Group ¹			SEM	P-value
	C	S	BI		
Final body weight (g)	2232.75 ^b	2439.10 ^a	2412.25 ^a	23.26	0.001
Carcass weight (g)	1544.00 ^b	1700.25 ^a	1661.50 ^a	18.30	0.001
Carcass yield (%)	69.14	69.66	68.89	0.27	0.501
Breast weight (g)	494.00	518.00	527.75	7.98	0.323
Breast yield (%)	31.86	30.51	31.79	0.33	0.171
Leg weight (g)	445.00 ^b	479.00 ^a	480.50 ^a	5.21	0.143
Legs yield (%)	28.85	28.25	28.93	0.25	0.335
Wings weight (g)	148.25 ^B	166.75 ^A	158.25	1.96	0.470
Wings yield (%)	9.62	9.83	9.54	0.11	0.471
Back+neck weight (g)	441.50 ^B	485.75 ^A	486.40 ^A	5.97	0.003
Back+neck yield (%)	28.68	28.57	29.29	0.23	0.407

¹Group: C = Control, uninjected eggs; S = Saline, *in ovo* injection of physiological saline; BI = Bi²tos.

^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$).

^{A,B}Means within a row lacking a common superscript differ ($P < 0.01$).

As reported in Table 8.4, pH₂₄, colour (L*, a*, b*) and WHC were not negatively affected by the treatment ($P > 0.05$).

Table 8.4. Physico-chemical properties of breast muscle of Ross broiler chickens

	Group ¹			SEM	P-value
	C	S	BI		
pH ₂₄	6.08	6.14	6.09	0.02	0.296
Color 24 h					
L*	42.99	44.87	45.56	0.58	0.176
a*	3.53	3.45	3.54	0.13	0.959
b*	6.45	6.00	6.40	0.24	0.718
WHC	11.92	11.73	11.37	0.26	0.677

¹Group: C = Control, uninjected eggs; S = Saline, *in ovo* injection of physiological saline; BI = Bi²tos.

8.3.2 Total lipid and fatty acid composition

Effects of the treatment on total lipid and fatty acid composition of breast muscles are presented in Table 8.5. Total lipid content was higher ($P < 0.01$) in BI group compared to C group, with intermediate values for S group ($P > 0.05$). The treatment did not affect total amounts of saturated fatty acids (SFA) and individual SFA content of meat. The most concentrated SFA in all experimental groups were palmitic acids (C16:0; 23.45 – 24.95%) followed by stearic acid (C18:0; 9.49 – 10.21%).

No effect of treatment was observed in the composition of monounsaturated fatty acids (MUFA), consequently, total MUFA contents were the same in all experimental groups ($P > 0.05$). Quantitatively, oleic acid (C18:1n 9) was the most abundant MUFA recorded in the study (24.49 – 25.53%).

In ovo administration of prebiotic lowered (-2.6%; $P < 0.05$) the total poly unsaturated fatty acid (PUFA) content compared to the S group. The total n-6 and n-3 PUFA contents of meat from BI group birds were also significantly lower than those of the S group ($P < 0.05$). For individual fatty acid, only docosahexanoic acid (C22: 6n 3) was affected by the treatment being lower in the BI group in comparison with the S group (-0.31%; $P < 0.05$) and C group ($P > 0.05$). Regardless of the treatment, the most abundant PUFA were linoleic (C18:2n 6; 25.13– 25.73%) and arachidonic acids (C20:4n 6; 5.22 – 6.44%).

Regarding selected fatty acid indices, n-6/n-3 ratio was significantly higher in the prebiotic treated group (+16.2; $P < 0.05$) than in S group. In addition, P/S was relatively lower in BI group compared to S and C ($P = 0.071$). The treatment had no effect on the other nutritional indices (atherogenic and thrombogenic).

Table 8.5. Total lipid content (%) and fatty acid composition (% of total fatty acids) of breast muscle from broiler chickens

	Group ¹			SEM	<i>P</i> -value
	C	S	BI		
Total lipid	1.24 ^B	1.40	1.85 ^A	0.09	0.006
<i>Fatty acid</i>					
C 14:0	0.42	0.42	0.49	0.02	0.112
C 14:1	0.06	0.06	0.08	0.00	0.128
C 16:0	23.45	23.56	24.95	0.36	0.151
C 16:1 n-7	2.25	2.28	2.71	0.11	0.175
C 18:0	10.21	9.86	9.49	0.23	0.475
C 18:1 cis9	25.53	24.49	25.46	0.42	0.554
C 18:1 cis11	2.40	2.72	2.81	0.09	0.123
C 18:2 n-6	25.69	25.73	25.13	0.28	0.633
C 18:3 n-6	0.19	0.18	0.19	0.01	0.562
C 18:3 n-3	1.20	1.18	1.18	0.05	0.986
C20:0	0.04	0.04	0.04	0.00	0.228
C 20:1 n-9	0.17	0.17	0.17	0.00	0.873
C 20:2 n-6	0.54	0.56	0.46	0.02	0.176
C 20:3 n-3	0.06	0.08	0.07	0.01	0.231
C 20:4 n-6	5.90	6.44	5.22	0.29	0.224
C 20:5 n-3	0.13	0.16	0.14	0.01	0.235
C 22:4 n-6	0.25	0.29	0.19	0.02	0.154
C 22:5 n-3	0.78	0.89	0.65	0.04	0.072
C 22:6 n-3	0.72	0.88 ^a	0.57 ^b	0.05	0.038
<i>Partial sum</i>					
ΣSFA	34.11	33.89	34.97	0.44	0.570
ΣMUFA	30.42	29.72	31.23	0.46	0.421
ΣPUFA	35.47	36.40 ^a	33.80 ^b	0.40	0.017
Σn-6	32.57	33.20 ^a	31.19 ^b	0.32	0.024
Σn-3	2.90	3.20 ^a	2.62 ^b	0.09	0.014
<i>Nutritional index</i>					
n-6/n-3	11.38	10.44 ^b	12.13 ^a	0.25	0.018
P/S	1.05	1.08	0.97	0.02	0.071
Atherogenic index	0.38	0.38	0.42	0.01	0.148
Thrombogenic index	0.85	0.83	0.90	0.02	0.271

¹ Group: C = Control, uninjected eggs; S = Saline, *in ovo* injection of physiological saline; BI = Bi²tos.

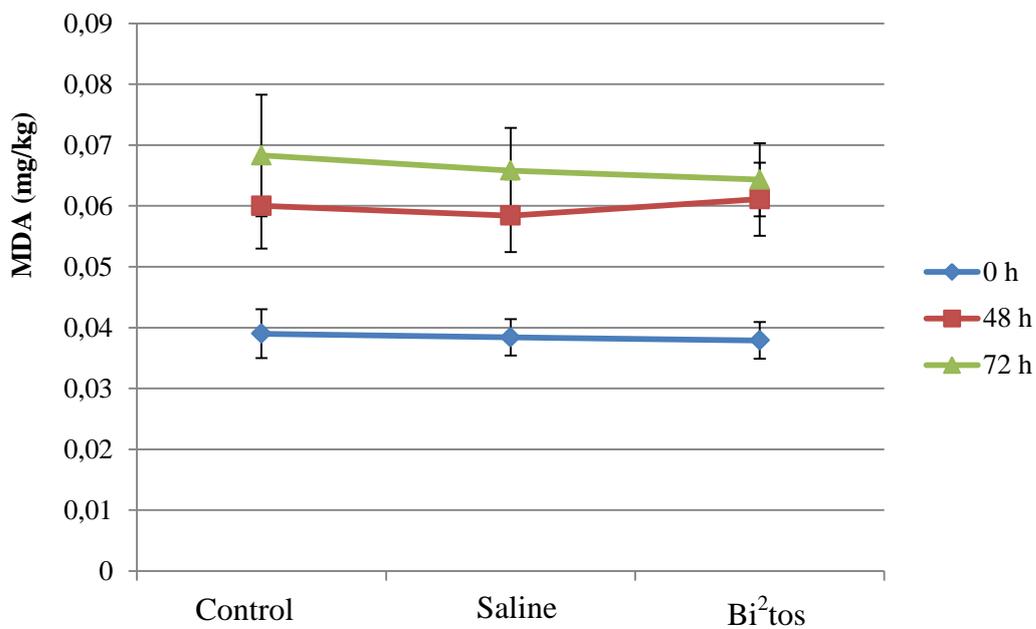
^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$).

^{A,B}Means within a row lacking a common superscript differ ($P < 0.01$).

8.3.3 Meat oxidative stability

The effects of the treatment on meat oxidative stability are presented in Figures 8.3a,b. TBARs values were almost the same among experimental groups throughout the storage period though was slightly lower in BI compared to the control group after 72 hours of storage (Figure 8.2a; $P > 0.05$). In all experimental groups lipid oxidation increased significantly from time 0 to 72 hours of storage ($P < 0.01$). While for the control group, significant increase was observed only from time 0 to 48 hours of storage ($P < 0.05$). The TBARs values were highest for meat stored for 72 hours at 4°C in all experimental groups ($P < 0.01$).

a



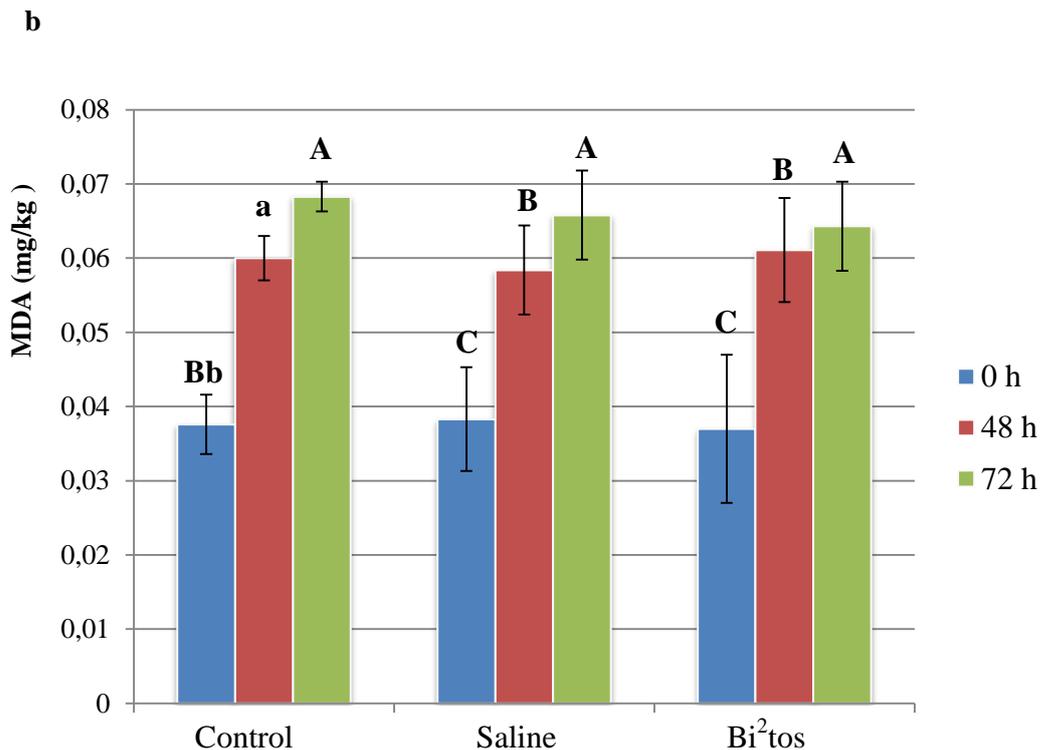


Figure 8.3. Effect of prebiotic treatment on TBA reactive substances values (mg MDA/Kg of meat) of breast muscle (a) and lipid oxidation development of breast muscle within each treatment (b), during 3 days of storage at 4°C (means ± SE). A, B, C: $P < 0.01$; a, b: $P < 0.05$.

8.4 Discussion

8.4.1 Performance and meat quality/carcass traits

Results from the study showed a satisfactorily high hatchability (> 90%) though similar among experimental groups. The improvement in growth performance in birds after prebiotic treatment has previously been reported in broiler chickens (Wang *et al.*, 2015; Bednarczyk *et al.*, 2016; Maiorano and Bednarczyk, 2016). In the present study, prebiotics improved BWG throughout the rearing period. In contrast, other studies observed significant positive changes on production performance only in the later phases of growth (Wang *et al.*, 2016). Kim *et al.* (2011) found no significant difference in performance among treatments in the first 2 weeks of experimenting with different prebiotics at various dosages and conventional antibiotic. Similarly, Dizaji *et al.* (2012) reported improved body weight only from 42 days of age in inulin in-feed supplemented birds. While Hanning *et al.* (2012) showed that prebiotic GOS has positive effects on growth performance in Naked Neck chickens only from week 4 to 6 but lowered BW in Cornish White Rock cross chickens at the 6th week of rearing. In a related

study, Maiorano *et al.* (2017) found only marginal improvements in BW in broilers treated with prebiotics (DiNovo and Bi²tos), reared under commercial conditions. On the other hand, some studies have shown no positive effects of prebiotics on growth performance in broilers (Baurhoo *et al.*, 2009; Alzueta *et al.*, 2010; Housmand *et al.*, 2012; Salehimanesh *et al.*, 2016). These contradictory responses of chickens to prebiotics may be attributable to several factors that influence the efficacy of prebiotics in broiler feeding. Some of these factors are product type, inclusion level, diet type, animal characteristics, husbandry hygiene, and environmental stress conditions (Patterson and Burkholder, 2003; Velasco *et al.*, 2012). For example, Orban *et al.* (1997) noted that more beneficial effects of prebiotics are observed under suboptimal experimental conditions. And Hooge (2004) reported that broilers fed diets supplemented with a prebiotic or antibiotic had better performance than the control group under disease or crowding stresses, whereas under conditions of little stress, growth performance was similar in the control, antibiotic, and prebiotic groups.

In the present study, prebiotics (Bi²tos) significantly improved carcass weight compared to the control group. This observation is in agreement with Maiorano *et al.* (2017) who found a significant increase in carcass weight and in contrast, a higher carcass yield in the prebiotics group. In an earlier study by the same group, Maiorano *et al.* (2012) observed only minimal improvement on carcass traits for broilers treated with raffinose family oligosaccharides (RFO) prebiotics. Carcass yield in the present study which ranged from 68.89% to 69.66% were similar to results regarding carcass yield reported by Sirri *et al.* (2010) for fast growing broiler chickens. However, different findings are reported in literature (Pelicano *et al.*, 2005; Pilarski *et al.*, 2005; Sarangi *et al.*, 2016) for broilers treated with pre- and synbiotics slaughtered at 42 days of age (74.39 – 80.18%). While, Perryman *et al.* (2013) found values of carcass yield ranging from 70.5 - 72.2 % for Ross broiler chickens fed control or low oligosaccharide soybean meal based diets, slaughtered at 40 days of age. In line with the current study, Pilarski *et al.* (2005) reported no significant differences of different prebiotics on breast muscle and leg yields in broilers. Furthermore, Pelicano *et al.* (2005) reported no significant differences between MOS treated group and control for carcass, breast, legs, back, wings yields and IMF in 42 day old broilers. Likewise, Hanning *et al.* (2012) found no significant differences on breast and wing yields in broilers fed three different types of prebiotics and slaughtered at 56 days. Breast, leg, back and wing yields obtained in the current study were in agreement with reported values by Pelicano *et al.* (2005) and Perryman *et al.* (2013). The carcass traits of birds are affected by the genotype, sex, age, and nutritional

content of the ration used especially during the growing period among other factors. These discrepancies among studies could be attributed to the variation in the above factors.

Colour, pH and WHC are important indicators of the technological quality of meat that directly or indirectly affect consumer satisfaction with the final product. For instance, poor WHC in raw poultry meat results in diminished visual appeal and inferior palatability traits for consumers as well as reduced ingredient retention, protein functionality, and product yields for processors (Bowker and Zhuang, 2015). On the other hand, pH influences the microbiological shelf life of meat as well as its WHC and colour. In line with previous work (Pelicano *et al.*, 2005; Maiorano *et al.*, 2012) on prebiotics in chickens, WHC, colour and meat pH were unaffected by the treatment with prebiotics, indicating that prebiotics (Bi²tos) have no negative effects on broiler meat. Conversely, Park and Park (2011) found higher WHC values in meat from broilers fed 250 g/ton of feed oligosaccharides (inuloprebiotics) compared with the control. The detected values of ultimate pH (6.08 - 6.14) were a little bit higher than that reported in literature (5.82 – 5.87, Maiorano *et al.*, 2012; 5.67 – 5.99, Nazim *et al.*, 2017) for broiler chickens treated with gut modulating bioactives. The ultimate pH values obtained in this study are in the same range with those reported by Glamoclija *et al.* (2015) for Ross broiler chickens slaughtered at 42 days of age. The high pH values obtained here could be due to minimal stress that the birds may have sustained prior to slaughter which could have resulted in depletion of glycogen in the muscle (Dadgar *et al.*, 2012). Since glycogen is the substrate for lactate production in muscle, the less glycogen that is present at harvest, the less lactate is produced after harvest, and subsequently, the less the pH will decline in postmortem muscle.

Furthermore, it is widely accepted that a high rate of decline in post mortem pH coupled with a high muscle temperature usually associated with stress or intensive physical activity shortly prior to slaughter, causes denaturation of muscle proteins resulting in reduced WHC in poultry (Listrat *et al.*, 2016) and vice versa. A large extent of pH decrease reduces the net electric charge of muscle proteins (Fernandez *et al.*, 2002; Huff-Lonergan and Lonergan, 2005). This decrease in net protein charge results in diminished WHC due to the availability of fewer charged protein sites for binding water and because the lack of repulsive charges allows muscle proteins to become more closely packed, which forces more of the immobilized water into the free water compartment. A very low WHC results in PSE-like meat (Petracci *et al.*, 2015). While, very high WHC gives rise to dark, firm and dry meat product (Huff-Lonergan and Lonergan, 2005). In the current study, even though the pH

values were generally high, no negative effect was observed on WHC, thus, no meat quality defect was detected.

Much as the treatment did not affect meat colour; the lightness (L*) values (42.99 – 45.56) obtained in the current study were considerably lower than those previously reported for broiler meat (51 – 60.3, Castellini et al., 2006; Park and Park, 2011; Dal Bosco et al., 2014). Fletcher (1999) indicated a strong negative correlation between lightness values and muscle pH of meat. Castellini et al. (2006) also found a negative correlation between meat pH and colour in Ross broilers.

8.4.2 Effects of the treatment on total lipid and fatty acid composition of breast muscles

Intramuscular fat (IMF) which has an influence on meat eating quality notably juiciness and tenderness, was higher in prebiotic group compared to the other experimental groups. This is in agreement with the report of Maiorano *et al.* (2017) who found a slightly higher value of IMF in commercial broilers treated with prebiotic (Bi²tos) *in ovo* compared to the control and DiNovo groups. Prebiotics modulate lipid metabolism indirectly through the effects of short chain fatty acids (Propionate, acetate and butyrate) produced as a result of microbial fermentation in the gut (Delzenne *et al.*, 2008). For example, acetate enters the hepatocyte, where it is activated mainly by cytosolic acetyl coenzyme A synthase 2 and enters the lipogenesis and cholesterologenesis pathways. This has been proposed as the rationale behind the hypercholesterolemic/hyperlipogenic effect of those prebiotics whose fermentation in the colon result in enhanced acetate not propionate production. Conversely, it has been shown that propionate contributes to a decrease in lipogenesis and cholesterologenesis *in vitro*, in rats by acting as a competitive inhibitor of the protein controlling the entrance of acetate into liver cells. According to Delzenne *et al.* (2008), the possible effects of the prebiotic on total lipid and fatty acid composition most likely depend on the ratio of propionate to acetate resulting from its fermentation reaching the liver cells. Established prebiotics, such as fructooligosaccharides and galactooligosaccharides, which support the growth of Bifidobacteria, mainly mediate acetate production (Puertollano *et al.*, 2014). This probably explains why in the present study IMF was significantly higher in the prebiotic group. The content of IMF found in this study is however, consistent with the results reported in literature for Ross broiler chickens (Slakova *et al.*, 2009; Dal Bosco *et al.*, 2014) on normal basal diet slaughtered from d 40 to 81 d though slightly lower than those reported by Maiorano *et al.* (2017) who found IMF percentages ranging from 1.83 to 2.30 for broilers

slaughtered at 42 d; however, the method of analysis used from Maiorano *et al.* (2017) was different from that used in this study.

In line with results of previous work on prebiotics, reported by Maiorano and Bednarczyk (2016), the treatment did not affect total SFA and MUFA contents of meat in the present study. In contrast, Velasco *et al.* (2010) reported a significantly lower MUFA amounts in inulin treated sunflower oil fed birds compared to the control but just as in this study, showed no significant effect of the treatment on SFA content. The total SFA amounts obtained in this study are in agreement with the finding of Narciso-Gaytan *et al.* (2011) in Cobb X Ross but were comparatively lower than those reported by Dal-Bosco *et al.* (2014) for Ross broiler chickens. Conversely, total MUFA contents were higher in this study compared to the results obtained by Dal Bosco *et al.* (2014). The inconsistencies observed among studies can be explained by variations in factors such as diet, genotype and slaughter age that are known to affect lipid metabolism in birds.

Regardless of the treatment, the most concentrated fatty acids were linoleic (C18:2n 6) followed by oleic acid (C18:1n 9), palmitic (C16:0) and stearic (C18:0) acids in descending order of abundance. This observation corroborates previously reported results on broiler chickens of different genotypes and slaughter ages (Sirri *et al.*, 2010; Dal Bosco *et al.*, 2012; Boschetti *et al.*, 2016). For instance, the values obtained in this study are within the range reported by Popova *et al.* [(2016; C16:0 (25.74 - 28.93%), C18:0 (9.50 – 13.37%), C18:1 (19.87 – 25.15%), C18:2n 6 (17.29 – 21.12%) and C20:4n 6 (7.54 – 15.10%)] for intensively reared broilers slaughtered at 9 and 18 weeks respectively.

Nutritionally, the constituent fatty acids in dietary fats influence their biological roles in the human body. Linoleic acid and oleic acid which in this study were the most abundant are 2 major fatty acids in dietary fat and in plasma triglycerides. Linoleic acid, (18:2n 6), an essential fatty acid, is converted into arachidonic acid, (20:4n 6), which is an important precursor, via the cyclooxygenase pathway, of inflammatory eicosanoids such as prostaglandins and hydroxyeicosatetraenoic acids. Thus, linoleic acid has been implicated in the upregulation of inflammatory responses (Reaven *et al.*, 1993). On the other hand, oleic acid plays a significant role in the human diet because it improves insulin sensitivity, reduces the hyperglycaemia, promotes β -cell proliferation and favorably mediates plasma lipids, reducing both LDL cholesterol and the triglycerides (Kuna and Achinna, 2013). In fact, oleic acid molecules in the cis configuration were reported to exert the most potent antiapoptotic effect against β -cells among the MUFAs, whereas SFAs promote apoptosis and increase total cholesterol (Dhayal *et al.*, 2008). For example, palmitic acid is associated with increase in

total serum cholesterol in human. Stearic acid on the other hand is generally considered to be a neutral fatty acid because it has been shown to have no net impact on the plasmatic level of either LDL or HDL cholesterol in humans (Yu *et al.*, 1995). According to D'Alessandro *et al.* (2012) the effect of stearic acid on the plasmatic level of either LDL or HDL cholesterol is mainly due to its reduced digestibility and easy desaturation into oleic acid.

The treatment had only minimal effects on individual polyunsaturated fatty acids (PUFA) composition of broiler meat but significantly lowered the total n-6 and n-3 fatty acid resulting in lower total PUFA content in the BI group. In contrast, Maiorano and Bednarczyk (2016) reported a slightly higher amount of PUFA ($P > 0.05$) in prebiotics (Bi²tos and DiNovo[®]) groups compared to the control. In the same vein, Velasco *et al.* (2010) reported higher amounts of total PUFA in inulin treated sunflower oil fed birds compared to the control. Overall, the total PUFA values (33.80 – 36.40) obtained in the current study are in agreement with previously reported values for broiler chickens (Castellini *et al.*, 2006) although slightly higher than those reported by Narciso-Gaytan *et al.* (2011) and Dal Bosco *et al.* (2012, 2014).

In the current study, the lower total n-3 PUFA contents of meat resulted in higher n-6/n-3 ratio in the prebiotic group. This observation is inconsistent with the finding reported in Maiorano and Bednarczyk (2016) where prebiotics had no significant effects on the n-6/n-3 ratio and total omega 6 and omega 3 fatty acids. Much as there is limited information in meat science literature on the effects of prebiotics on fatty acid composition with which to compare these results, the discrepancies among the few studies could be due to the interaction of prebiotics with other factors which directly or indirectly affect lipid metabolism in birds such as diet, genotype, slaughter age and the type of prebiotic used among others. For example, in the study of Velasco *et al.* (2010), in birds fed the sunflower oil diets, inulin caused a linear increase in PUFA content and in PUFA:SFA and UFA:SFA ratios, mainly due to a linear decrease in C16:0 content and a linear increase in C18:2n-6 content. But the same effects were not detected in inulin treated birds fed palm oil diets (Velasco *et al.*, 2010) suggesting that the potential effect of the prebiotic on intramuscular lipid composition probably depends on the degree of saturation of dietary fat source.

The ratios of omega 6 to omega 3 (n-6/n-3: 10.44 to 12.13) obtained in the current study are within the range reported in literature for Ross 308 breast muscles (Dal Bosco *et al.*, 2012, 2014). For instance, in the studies of Dal Bosco and colleagues, n-6/n-3 ratios reported for Ross broiler chickens ranged from 10.40 to 18.19 (Dal Bosco *et al.*, 2012, 2014). Generally, poultry meat is characterized by the highest n-6/n-3 ratio compared to meat from other animal species, essentially due to the higher amount of n-6 fatty acids than muscles of

the others (Rule *et al.*, 2002; Wood *et al.*, 2003). It is well established that linoleic acid is the predominant essential fatty acid in poultry and as a result the n-6 PUFA are the primary products found in tissue lipids. Thus, the ratio n-6/n-3 in poultry meat is well away from the ideal value of 1 and above the recommended maximum of 4. However, the fatty acid profile of poultry meat can be altered by inclusion of n-3 fatty acid in the diet of the birds resulting in variation in results reported from different studies in broilers (Velasco *et al.*, 2010; Dal Bosco *et al.*, 2014; Boschetti *et al.*, 2016; Maiorano and Bednarczyk, 2016). A plethora of studies has shown that concentrations of n-3 and n-6 PUFAs, especially beneficial EPA and DHA, in chicken muscles increase due to dietary enrichment with oils/meals rich in C18:3n-6 and C18:3n-3 fatty acids (Ponte *et al.*, 2008; Haug *et al.*, 2010). In a study by Mandal *et al.* (2014), lowering the dietary n-6/n-3 fatty acid ratios by feeding linseed oil to birds decreased n-6/n-3 ratio and increased beneficial EPA and DHA by 4 to 7-folds in chicken thigh and breast muscles respectively. The authors concluded that altering the n-6/n-3 fatty acid ratios in the diets may augment the concentrations of long chain n-3 PUFA and modulate the n-6/n-3 ratio in chicken meat resulting in healthy meat products without affecting growth performance.

With reference to the effect of the treatment on other fatty acid indices, atherogenic and thrombogenic indices were the same in all experimental groups while P/S, was slightly lower in BI group. Across treatments, P/S values (0.97 – 1.08) obtained in this study were slightly higher than the recommended value of 0.4 – 0.7, mainly due to the greater contribution of linoleic acid though consistent with reported values for broilers in literature. Compared with muscles of the other species, chicken breast has higher linoleic and arachidonic acids and relatively lower stearic acid, which are largely responsible for the high P/S ratios observed in broilers (Rule *et al.*, 2002; Wood *et al.*, 2003). It is important to note that a high P/S ratio is nutritionally desirable since it reduces the risk of cardiovascular problems and other diseases.

In addition, the values of atherogenic index obtained (0.38 – 0.42) in this study though not significantly different among treatments, were generally lower than those reported in literature while the thrombogenic index values (0.83 - 0.90) were consistent with previous reports in broilers (Castellini *et al.*, 2006; Popova *et al.*, 2016). These indices, calculated according to the formulas suggested by Ulbricht and Southgate (1991), take into account the different effects, which the single fatty acid might have on human health and, in particular, on the probability of increasing the incidence of pathogenic phenomena, such as atheroma and/or thrombus formation (Ulbricht and Southgate, 1991).

8.4.3 Effect of the treatment on meat oxidative stability

In the current study, treatment with prebiotic Bi²tos did not have a marked influence on meat oxidative stability except for the slightly lower value obtained after 72 hours of aerobic display. This observation is inconsistent with finding of Maiorano *et al.* (2017) with Bi²tos on broiler chickens where prebiotic treatment resulted in an increase in lipid oxidation of meat with storage time. This variation in the results could be related to the effects of the treatment on fatty acid composition. It is worth noting that among meat macronutrients, the lipid fraction is the most susceptible to modifications which are the major causes of chicken meat quality deterioration and the subsequent reduction of shelf life of meat and meat products (Funaro *et al.*, 2014). Prebiotics can alter lipid metabolism (Letexier *et al.*, 2003; Delzenne *et al.*, 2008) and enhance the polyunsaturated:saturated fatty acids ratio in chicken meat (Zhou *et al.*, 2009; Velasco *et al.*, 2010) with benefits to human health. Whereas this modification is nutritionally desirable, it could increase susceptibility of meat to oxidation.

In the current study as already mentioned before, prebiotics lowered the PUFA content of meat while in the previous studies (Maiorano and Bednarczyk, 2016; Maiorano *et al.*, 2017) the same treatment had the tendency to increase PUFA content of broiler meat which probably explains the increase in lipid oxidation reported by the authors. Dietary PUFAs are susceptible to oxidation both during processing and storage. Lipid oxidation is known to increase as the level of unsaturation increases making PUFAs more readily oxidizable (Yun and Surh, 2012) thus, resulting in lower oxidative stability of meat. In the study of Grau *et al.* (2001), meat from birds with unsaturated diets (linseed oil-fed) had the highest PUFA content and also the highest TBARS values (600 vs 1600 µg MDA/Kg of meat) even when the birds were supplemented with antioxidants. Likewise, Narciso-Gaytan *et al.* (2011) found TBARS values ranging from 0.5 – 3.5 mg MDA/Kg in cooked meat from Cobb × Ross broilers enriched with n-3 and conjugated linoleic acid (CLA) fatty acids in diet. The authors noted from the results that, as the proportions of MUFA and PUFA in chicken meat increased, the susceptibility of meat to lipid oxidation also increased over the storage time with meat from broilers fed menhaden fish oil or flaxseed oil being more susceptible to lipid oxidation than were their counterparts from the CLA treatment (3.5 vs 2.0 mg/kg). Similarly, in organic production system where PUFA content of meat is generally enhanced, Castellini *et al.* (2006) reported very high TBARS values ranging from 2 – 3.8 mg MDA/Kg of meat in Ross broiler chickens measured from 0 to 96 hours of storage.

Lipid and pigment oxidation in meat impact negatively on purchase decisions of consumers, resulting in substantial economic losses. In the present study, prebiotic treatment

had no negative effect on meat oxidative stability as evidenced by the lower TBARS values obtained. Furthermore, regardless of the treatment, the TBARS values obtained (0.039 – 0.069 mg MDA/Kg of meat) in this study were generally lower than those previously reported for Ross broiler chickens (Grau *et al.*, 2001; Castellini *et al.*, 2006; Dal Bosco *et al.*, 2014; Maiorano *et al.*, 2017) under more or less the same storage condition.

8.5 Conclusions

The results obtained from this study clearly proved that the *in ovo* prebiotic administration increased body weight gain in Ross 308 broiler chickens throughout the rearing period with remarkable differences being observed mainly in the starter period (1- 3 weeks). This might be explained by the early supplementation of prebiotic in chicken embryos, which is associated with a selective stimulation of beneficial gut microbiota, especially bifidobacteria and lactobacteria, as previously reported in related studies. Slaughter traits were also positively affected by *in ovo* prebiotic administration; while, no negative effects on meat quality traits and oxidative stability of breast muscle were found. In general, the effect of prebiotic on chicken growth performance could be attributed to the better health status of chickens. The treatment however lowered the omega 6 and omega 3 PUFA contents of meat resulting in higher n-6/n-3 ratio.

Chapter 9

EXPERIMENT 2

Efficacy of *in ovo* delivered prebiotics on gut health, performance and meat quality of Kuroiler chickens reared under tropical climatic conditions

9.1 Introduction

Incorporation of immunobiotics, particularly lactic acid bacteria could be useful as immunomodulators to stimulate the gut-associated immune system in neonatal chicks, and thereby protect them from disease without decreasing growth performance as a possible substitution of antibiotics (Sato *et al.*, 2009). Studies have indicated that prebiotics could serve the same purpose by selectively stimulating the growth and proliferation of some useful bacteria and modulating the immune system in birds (Slawinska *et al.*, 2014a). For instance, addition of MOS to the diet of broilers reduced the severity of the infection due to either *E. tenella* alone (Elmusharaf *et al.*, 2006) or a mixture of *E. acervulina*, *E. maxima* and *E. tenella* (Elmusharaf *et al.*, 2007).

In Uganda just like in any other developing country, poultry production forms an integral part of the economy with many socio-economic and cultural values attached to the birds. However, one of the major challenges to improved poultry production is the prevalence of poultry diseases that threaten the intensification of production. Among these are gastrointestinal parasitic diseases notably, coccidiosis. Coccidiosis is a disease caused by parasites of the genus *Eimeria* and phylum Apicomplexa with a complex life cycle, affecting mainly the intestinal tract of many species of birds. It is of great economic significance in farm animals, especially chickens. The economic significance of coccidiosis is attributed to decreased animal production (higher feed conversion, growth depression and increased mortality) and the costs involved in treatment and prevention. Pathogenesis entails *Eimeria* invading the intestinal cells as part of the life cycle. The resulting intestinal damage impairs nutrients digestion and absorption, gut barrier function, and ultimately leads to bacterial infections particularly necrotic enteritis (Chapman, 2014). The global annual costs inflicted by coccidiosis to commercial poultry have been estimated at 2 billion € (Peek and Landman, 2011).

The use of anti-coccidial feed additives during past 50 years has played a major role in the growth of the poultry industry facilitating increased availability of high quality,

affordable poultry products to the consumers. However, some degree of resistance to all anti-coccidial drugs, including ionophores which are now the mainstay of coccidiosis control has been reported (Chapman, 1994, 1998; Chapman and Jeffers, 2014; Tewari and Maharana, 2011). Concerns over the development of resistant *Eimeria* species to existing anti-coccidial drugs and restrictive use of antibiotics to control secondary bacterial infections further stresses the urgent need to explore alternative strategies for maintaining intestinal functionality in chickens.

The suggestion that vaccination be combined with chemotherapy is not new, as it has been used for long in the poultry industry but efforts have not been made to develop an integrated control program by adopting other alternatives as well. Plant, bacterial, and other substances claimed to alleviate GIT infections such as coccidiosis either directly or indirectly by improving health and immune status have been evaluated individually. The efficacy of prebiotics has mainly been evaluated under fully controlled disease free experimental conditions. So far, there is limited data available on integration of these strategies into one performance enhancement and or disease (coccidiosis) control programme. In this experiment, it was hypothesised that prebiotics perform differently under field condition in the tropics where management and environmental factors predispose birds to enteric diseases. Thus, the aim of the experiment was to investigate the efficacy of prebiotics, antibiotic-chick formula and a combination of the two on growth performance, carcass traits and gut health in the face of a natural coccidiosis infection in Kuroiler chickens reared under field condition in Uganda.

9.2 Materials and methods

9.2.1 Biological materials

To evaluate the efficacy of prebiotics delivered *in ovo* on productive and meat quality traits and gut health under tropical climatic conditions, eggs from Kuroiler chickens were incubated. At the 12th day of incubation eggs were candled and 150 eggs with viable embryos were randomly divided into two groups. Of these, 75 eggs were injected with 0.2 ml of Bi²tos (3.5mg/embryo BI, Clasado Ltd, Sliema, Malta) a non-digestive trans-galacto oligosaccharides (GOS) from lactose digested with *Bifidobacterium bifidum* NCIMB 41171 (as in experiment 1); and 75 eggs were left uninjected as control. Immediately after injection, all holes in the eggs were sealed with natural glue and the egg incubation was continued until hatching.

At hatching, one day old chicks were vaccinated against Marek's disease and transferred to the experimental farm as required where they were reared in a brooder house for 21 days before being transferred to the littered floor pens with rice husk (Figure 9.2). Hatched chicks (Figure 9.1) from each of the two experimental groups above were further randomly divided into two groups: one group received antibiotic chick formula (poltricin with oxytetracycline at a dose of 1g/litre of drinking water for 7 days) while the other was left without the antibiotic chick formula. Thus, giving rise to four experimental groups: Control (C), Antibiotic (A), Bitos (B) and Bitos + Antibiotics (AB). The birds were reared in a local poultry farm in Gulu District where coccidiosis infection was previously confirmed by field veterinarians (personal communication with the District Veterinary Officer). Routine hygiene practices such as fumigation and litter changing were observed as recommended. All birds were reared under semi-intensive confined system for a period of 18 weeks. Chickens were fed *ad libitum* starter, grower and finisher diets (Table 9.1) and were vaccinated as required. The birds had constant access to water and feed. Body weights were taken per pen on a weekly basis and also faecal samples were collected for parasitological analysis to check for possible infection with enteric parasites.



Figure 9.1 Hatched chicks.



Figure 9.2 Littered floor rearing pens.

Table 9.1 Composition of feed mixtures

Component (%)	Starter (0 - 6 weeks)	Grower (6 - 14 weeks)	Finisher (14 - 18 weeks)
Maize bran	52.36	64.72	60.6
Sunflower cake	20.94	19.42	18.2
Fish meal	20.94	7.12	18.2
Lime	0	6.27	0
Magic protein ¹	1.05	1.29	0
NaCl	0.52	0.65	0.6
Mineral-vitamin premix ²	0.52	0.52	0.6
Calcium	2.09	0	0.6
Bovonite (Sodium bentonite)	1.57	0	0.6
Meat booster ³	0	0	0.6

¹Provided per kg of diet: plant protein, 34%; animal protein, 14%; natural minerals, 47.7%; trace elements, 1.8%; vitamins, 2.5%.

²provided per kg of diet: vitamin A, 13 000 IU; vitamin D3, 4000 IU; vitamin E, 80 IU; vitamin K, 3mg; riboflavin, 6.0mg; pantothenic acid, 6.0 mg; niacin, 20 mg; vitamin B6 2mg; folic acid, 0.5mg; biotin, 0.10 mg; thiamine, 2.5 mg; vitamin B12 20mg; Mn, 120 mg; Zn, 90 mg; Fe, 30 mg; Cu, 10mg; I, 1.5mg; Se, 0.2mg; antioxidants, 100mg.

³Provided per kg of diet: metabolizable energy, 11.9%; crude protein, 35.7%; lysine, 1.3%; methionine, 2.03%; tryptophan, 7.3%; crude fibre, 3.7%; threonine, 13.6%; calcium, 02.04; phosphorus, 15.3% and traces of sodium, potassium, xylanase, amylase-protease, antioxidant.

9.2.2 Slaughter survey

At the age of 18 weeks, a total of 24 birds were randomly selected (12/Sex and 6 from each experimental group), weighed and slaughtered. After evisceration, the hot carcass weight was recorded, and carcass yield was calculated. In addition, the breast muscle, legs, wings and back + neck were removed from all carcasses and their percentages based on hot carcass weight were calculated.

After 24 h of refrigeration, the Pectoral muscle (PM) pH was measured, using a portable pH meter equipped with a penetrating glass electrode.

In addition, water holding capacity was determined using the filter paper press method as in experiment 1. PM were collected and analysed for fatty acid composition.

9.2.3 Fatty acid analysis

Lipid extraction from pectoral muscle samples was performed by the method of Folch *et al.* (1957). The extracted lipids were esterified and analysed by gas chromatography (GC/FID). Briefly, 1 μ l of the esterified extract was injected onto a 30m x 0.32mm x 0.5 μ m solgel wax column with polyethylene-glycol (PEG) as the stationary phase and helium gas at 20 psi as the mobile phase. The column was mounted in a GC/FID (Varian chrompack CP-3800). The injector temperature was 260°C. The temperature of the column was kept at 50°C for 5 min after injection and thereafter increased to 180°C at a rate of 20°C/min, followed by an increase of 2°C/min to 200°C, held for 11 minutes and then finally ramped to 250°C at 2°C/min, held for 2.5 minutes. The individual fatty acid peaks were identified by comparison of retention times with those of known standard FAME mixture run under the same operating condition. Quantification of the esters was achieved by integration of the peaks using interactive graphics software, with the relative amount of each fatty acid ester in each sample being expressed as a percentage of all the esters in the sample. To assess the nutritional implications, the n-6 fatty acids/n-3 fatty acids and the PUFA/SFA ratios were also calculated.

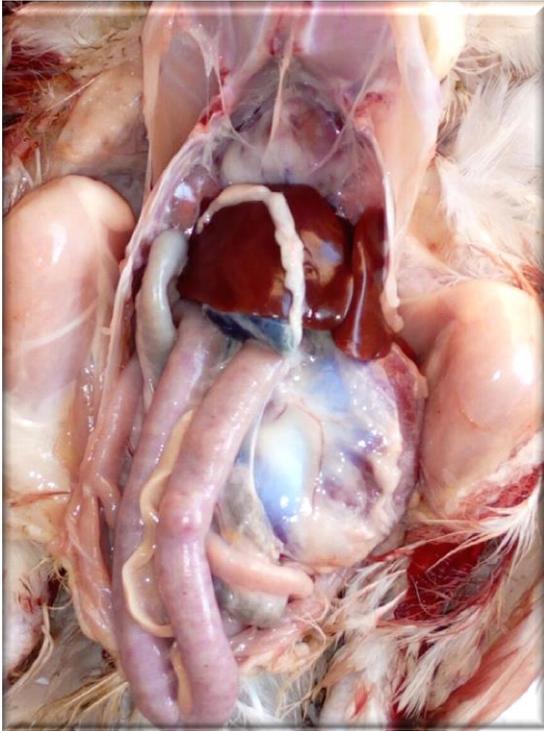
9.2.4 Parasitological analyses

The numbers of oocysts in faeces were determined in samples collected from each pen during the rearing period from week 4. For each pen, fresh excreta samples were collected from every corner of the pen and from the center of the pen and were kept in separate airtight plastic bags. The modified McMaster counting chamber techniques of Hodgson (1970) was used. Briefly, 10% (w/v) faeces suspension in a salt solution (151 g NaCl mixed into 1 L of

water) was prepared. After shaking thoroughly to obtain a homogenous mixture, 1 ml of the suspension was mixed with 9 ml of a salt solution (131 g of NaCl mixed into 1 L of water). Then, the suspension was pipetted into a McMaster chamber and the number of oocysts was counted and expressed per gram of faeces as described by Peek and Landman (2003).

9.2.4.1 Lesion scoring

On weeks 12 (on the day of starting therapeutic treatment with Amprolium) and 18 (at the end of the experimental period) 3 and 6 birds per treatment, respectively, were randomly selected and coccidial intestinal lesion (Figure 9.3) scored. The 0-4 lesion scoring system of Johnson and Reid (1970) was used. The areas scored were the upper, middle and the caecal regions of the intestine, which are the natural predilection sites for *Eimeria spp.* of veterinary significance in poultry, considering that in nature mix infection with two or three *Eimeria spp.* is not uncommon. Based upon severity of the lesions, a score of 0 (no lesions), 1 (mild lesions), 2 (moderate lesions), 3 (severe lesions), or 4 (extremely severe lesions) is recorded for each chicken. The severity of coccidial lesions was scored while the investigator was blinded to treatment modality.



Lesions on the upper part of the small intestine



An open section of the intestine with lesions

Figure 9.3 Characteristic coccidial lesions (personal photos).

9.2.3 Statistical analysis

Statistical analyses of the data were performed using SPSS (SPSS, 2010). Data on *in vivo* performance were analyzed by one-way ANOVA where treatment was the main factor. Data on slaughter traits and meat quality characteristics were evaluated by ANOVA, using a 4 x 2 factorial design. The model included treatment and sex and their interaction as fixed effects and individual animal as a random effect. Scheffé's test was applied to compare the mean values among the experimental groups at the 5% level of significance. For *in vivo* performance, the pen was considered as the experimental unit. For slaughter traits and meat quality, the individual bird was considered as the experimental unit.

9.3 Results

9.3.1 *In vivo* performance of Kuroiler chickens reared under tropical climatic condition

Overall hatchability was 62.67%; for the BI-group was 65.33% while for the C-group, only 60% of the eggs hatched, mainly due to the poor managerial condition at the hatchery. The effects of the treatment on growth performance of Kuroilers are presented in Table 9.2.

In the first three weeks, the treatment did not have any significant effect on body weight. At 6 weeks of age, the B group was the heaviest of all the other groups ($P < 0.05$ and $P < 0.01$); no significant differences were observed among the other groups. While at 9 and also 15 weeks no significant differences were observed among the treatment groups; but on week 12 the B group had a lower weight compared to the other groups ($P < 0.05$ and $P < 0.01$). On the other hand, AB group was the heaviest of all experimental groups ($P < 0.01$) at the end of the experiment. Likewise, groups C and B were significantly heavier than A ($P < 0.05$) but lighter than AB ($P < 0.01$) by week 18.

Table 9.2 *In vivo* performance of Kuroiler chicken

Age (weeks)	Body Weight (g)				SEM	<i>P</i> -value
	C	A	B	AB		
0	30.0	30.05	30.25	30.10	-	-
3	130.87	116.13	117.47	124.73	2.26	0.039
6	288.60 ^B	309.10 ^b	362.83 ^{Aa}	291.57 ^B	9.78	0.001
9	577.97	617.47	615.70	593.37	7.32	0.161
12	928.53 ^A	913.73 ^a	841.87 ^{Bb}	933.43 ^A	12.15	0.002
15	1254.70	1317.90	1237.60	1350.30	23.83	0.325
18	1753.60 ^{Ba}	1699.70 ^{Bb}	1753.47 ^{Ba}	1825.03 ^A	14.02	0.000

Treatment: C= Control; A = Antibiotics; B = Bitos; AB = Antibiotics + Bitos;

^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$).

^{A,B}Means within a row lacking a common superscript differ ($P < 0.01$).

Effect of the treatment on average daily weight gain is presented in Table 9.3. For the period from 0 to 3 weeks, C chickens had higher ($P < 0.05$) ADG compared to B group; while no significant differences ($P > 0.05$) were found for A and AB groups. Conversely, from 3 to 6 weeks, the prebiotic group showed the highest ($P < 0.01$ and $P < 0.05$) ADG compared to the rest of the treatment groups but it was significantly lower ($P < 0.01$ and $P < 0.05$) than the other groups for the period 9 to 12 weeks. Just like with body weight, ADG did not differ ($P > 0.05$) among treatments for the period 6 to 9 weeks and 12 to 15 weeks, as well as 15 to 18 weeks.

Table 9.3 Average daily weight gain of Kuroiler chickens reared under tropical climatic condition

Age (weeks)	Average daily gain (g)				SEM	P-value
	C	A	B	AB		
0-3	80.83 ^a	66.30 ^{ab}	60.83 ^b	66.60 ^{ab}	2.79	0.035
3-6	157.73 ^B	192.97 ^b	245.37 ^{Aa}	166.83 ^B	10.88	0.000
6-9	289.27	308.37	252.87	301.80	8.17	0.040
9-12	350.57 ^A	296.30 ^a	226.17 ^{Bb}	340.07 ^A	15.64	0.000
12-15	326.17	404.10	395.73	416.87	22.15	0.531
15-18	498.90	381.80	515.87	474.73	25.29	0.254

Treatment: C= Control; A = Antibiotics; B = Bitos; AB = Antibiotics + Bitos;

^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$).

^{A,B}Means within a row lacking a common superscript differ ($P < 0.01$).

As shown in Table 9.4, the control birds generally excreted more oocysts compared to the experimental groups ($P < 0.05$ and $P < 0.01$) throughout the observation period. The highest amount of oocysts shed were observed on week 12 in all experimental groups but were higher in the C and A groups in comparison with the B and AB groups ($P < 0.05$). However, the B and AB groups started showing clinical signs of the disease by week 11, barely two weeks from the first appearance of oocysts in their excreta.

Table 9.4 Oocyt excretion (thousands) per gram of excreta (OPG) in naturally infected Kuroilers

Age (week)	Treatment (OPG in '000s)				SEM	P-Value
	C	A	B	AB		
6	0.009	0.005	0.000	0.000	0.003	0.281
7	0.027 ^a	0.017	0.000 ^b	0.000 ^b	0.003	0.019
8	0.070 ^a	0.016	0.000 ^b	0.000 ^b	0.006	0.015
9	1.143 ^A	0.508	0.039 ^{Bb}	0.018 ^B	0.034	0.000
10	1.373 ^{Aa}	0.786 ^{Bb}	0.466 ^B	0.473 ^B	0.049	0.001
11	82.871 ^a	72.115	47.701 ^b	47.534 ^b	3.252	0.010
12	31.026 ^a	29.810 ^a	18.585 ^b	18.590 ^b	1.095	0.005
13	15.694 ^A	10.301 ^{Ba}	6.628 ^{Bb}	7.518 ^B	0.339	0.000
14	0.910	0.097	0.016	0.046	0.102	0.040

Treatment: C= Control; A = Antibiotics; B = Bitos; AB = Antibiotics + Bitos;

^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$).

^{A,B}Means within a row lacking a common superscript differ ($P < 0.01$).

The effect of the treatment on intestinal lesion score is shown in Table 9.5. The C birds showed higher intestinal lesion scores both at week 12 ($P > 0.05$) and week 18 ($P < 0.01$) compared to the B and AB groups. While the scores for the A group were generally intermediate between C and B or AB ($P > 0.05$).

Table 9.5 Intestinal lesion score in Kuroiler chickens naturally infected with *Eimeria spp.*

Age (week)	Treatment				SEM	P-Value
	C	A	B	AB		
12	2.67	2.00	1.00	0.67	0.373	0.283
18	1.67 ^A	0.67	0.17 ^B	0.17 ^B	0.132	0.002

Treatment: C= Control; A = Antibiotics; B = Bitos; AB = Antibiotics + Bitos; ^{A,B}Means within a row lacking a common superscript differ ($P < 0.01$).

9.3.2 Slaughter traits, pH and WHC of Kuroiler chickens reared under tropical climatic condition

Effects of the treatment on slaughter traits, pH and water holding capacity are presented in Table 9.6. Slaughter weight was not significantly different among treatments. Conversely, the B group had a slightly higher carcass weight compared to the rest of the treatments ($P = 0.027$).

Breast weight was not affected ($P > 0.05$) by treatment. Differently, breast yield was generally higher in all treatment groups compared to the C, however significant differences were found only with AB group (+6.0%; $P < 0.05$). Leg weight was not significantly different among experimental groups but tended to be higher in B and AB groups ($P = 0.055$). Also the leg yield was not different ($P > 0.05$) among the 4 groups but tended to be higher in treated groups compared to C group ($P = 0.079$). Wings weight was higher in B and AB groups ($P < 0.05$) compared to the control, A group showed intermediate value ($P > 0.05$). Wings yield was higher in AB group compared to the C group ($P < 0.05$), intermediate values were observed in A and B groups ($P > 0.05$). Back + neck weight and yield were not significantly affected by the treatment even though weight was slightly higher in B and AB groups ($P = 0.053$).

Regarding the effect of sex on the studied traits, as expected, males were heavier than females at slaughter ($P < 0.01$) and had higher carcass, leg, wings, back + neck weights ($P < 0.01$) and breast ($P < 0.05$) weight. While carcass, breast, leg, wings and back + neck yields were not influenced by sex ($P > 0.05$).

pH and WHC values were similar ($P > 0.05$) among experimental groups and between the two sexes.

Significant interaction between treatment x sex ($P < 0.05$) was observed for breast yield and wings weight.

Table 9.6 Slaughter traits, pH₂₄ and WHC of Kuroiler chicken reared under tropical climatic condition

TRAITS	TREATMENT (T)				SEX (S)		SEM	P-value		
	C	A	B	AB	F	M		T	S	T x S
Animal, N	6	6	6	6	12	12				
Body weight	1758.33	1700.00	1783.33	1650.00	1458.33	1987.50	26.19	0.307	0.000	0.724
Carcass weight (g)	1085.33	1013.17	1180.17	1012.83	904.50	1241.25	19.90	0.027	0.000	0.156
Carcass yield (%)	61.87	59.88	65.87	61.27	61.97	62.48	0.98	0.206	0.799	0.504
Breast weight (g)	215.83	237.00	261.00	255.00	214.83	269.58	9.88	0.395	0.014	0.240
Breast yield (%)	19.38 ^b	23.98 ^{ab}	22.43 ^{ab}	25.40 ^a	23.96	21.64	0.68	0.036	0.106	0.048
Leg weight (g)	262.83	265.50	314.17	322.67	233.75	348.83	8.90	0.055	0.000	0.385
Leg yield (%)	24.08	26.40	26.83	31.65	26.25	28.23	0.96	0.079	0.319	0.459
Wings weight (g)	91.33 ^b	107.67 ^{ab}	123.00 ^a	122.83 ^a	98.17	124.25	2.99	0.005	0.000	0.028
Wings yield (%)	8.57 ^b	10.90 ^{ab}	10.52 ^{ab}	12.17 ^a	11.06	10.01	0.37	0.023	0.172	0.167
Back+neck weight (g)	229.00	197.67	261.50	248.33	194.25	274.00	7.78	0.053	0.000	0.404
Back+neck yield (%)	21.25	19.47	22.08	24.80	21.80	22.00	0.74	0.123	0.894	0.744
pH ₂₄	5.70	5.71	5.67	5.65	5.67	5.68	0.01	0.246	0.490	0.185
WHC (%)	10.24	9.85	11.05	9.47	10.15	10.15	0.45	0.637	0.999	0.831

Sex: F = Female; M = Male. Treatment: C= Control; A = Antibiotics; B = Bitos; AB = Antibiotics + Bitos;

^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$).

9.3.3 Effects of *in ovo* delivered prebiotics on intramuscular fat content and fatty acid composition of Kuroiler chickens reared under tropical climatic condition

Total fatty acid composition of breast muscle of Kuroilers of the different treatment groups is presented in Table 9.7. Total amount of saturated fatty acids (SFA; ranging from 35.59 to 38.47 %,.) and single SFA, were not affected ($P > 0.05$) by the treatments. The most abundant SFA was palmitic acid (C16:0, ranging from 21.31 to 24.00 %) followed by stearic acid (C18:0, ranging 3.92 to 5.90 %) and several other SFA (C12:0, C14:0, C15:0, C20:0, C22:0) with lower amount (similar or less than one percent).

The total amount of monounsaturated fatty acids (MUFA), ranging from 24.15 to 27.57 %, was affected ($P = 0.012$) by the treatment; in general, breast muscles of the B group as well as those of the AB group had lower amounts of MUFA compared to C and A groups. This trend was linked to the amount of oleic acid (C 18:1 n-9), the most abundant MUFA, which was lower ($P < 0.01$) in B (19.52 %) and AB (18.27 %) groups compared to C (24.73) and A (24.44 %) groups. No significant differences were found for other MUFA (C14:1, C15:1, C16:1, C17:1, C24:1).

Meat from the B group displayed a higher (+ 3.72 %) amount of total polyunsaturated fatty acids (PUFA) compared to the control group ($P < 0.05$). The B group also had slightly higher ($P = 0.096$) amount of PUFA compared to the A group, while no significant difference was observed between the B and AB groups ($P > 0.05$). Total amount of n-3 PUFA (C18:3, C20:3, C20:5, C22:5) was higher in B compared to the control ($P < 0.05$) group, while, A and AB had intermediate values ($P = 0.09$). Total n-6 PUFA (C18:2, C18:3, C20:4) were not statistically different among treatment groups.

With reference to selected fatty acid ratios, the ratio of PUFA to SFA (P/S) was not significantly different among experimental groups. On the other hand, the n-6/n-3 ratio was lower in B ($P < 0.01$) and AB ($P < 0.05$) groups compared to C and A groups.

The effect of sex was observed only on a few fatty acids. Total MUFA was higher ($P < 0.05$) in males compared to the females; while PUFA were higher in females than males. The rest of the fatty acids and fatty acid ratios were not affected by sex of the birds. There were significant treatments x sex interaction effects on some individual fatty acids and total n-3 ($P < 0.05$) as well as n-6/n-3 ratio ($P < 0.01$).

Table 9.7 Total Fatty acid composition and nutritional indices of breast muscles from Kuroiler chickens

Animal, N	Treatment (T)				Sex (S)		SEM	P-value		
	C	A	B	AB	F	M		T	S	T x S
	6	6	6	6	12	12				
IMF (%)	1.90	2.27	2.00	1.98	2.11	1.96	0.07	0.346	0.146	0.044
∑SFA (%)	36.65	35.59	35.80	38.47	36.78	36.47	0.522	0.231	0.767	0.787
∑MUFA (%)	27.57	27.51	24.71	24.15	24.89	27.07	0.405	0.012	0.016	0.131
∑PUFA (%)	35.78 ^b	36.91 ^{ab}	39.50 ^a	37.38 ^{ab}	38.32	36.46	0.334	0.009	0.013	0.309
PUFA/SFA	0.98	1.05	1.11	0.97	1.05	1.00	0.024	0.182	0.360	0.874
∑n-3 (%)	10.02 ^b	11.35 ^{ab}	15.12 ^a	13.51 ^{ab}	12.62	12.39	0.489	0.010	0.819	0.022
∑n-6 (%)	25.76	25.56	24.38	23.87	25.70	24.07	0.625	0.156	0.661	0.100
n6/n3	2.67 ^{Aa}	2.35 ^{Aa}	1.61 ^B	1.90 ^b	2.03	1.94	0.078	0.000	0.093	0.002

Sex: F = Female; M = Male. Treatment: C= Control; A = Antibiotics; B = Bitos; AB = Antibiotics + Bitos.

IMF = intramuscular fat; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

^{a,b}Means within a row lacking a common superscript differ ($P < 0.05$). ^{A,B}Means within a row lacking a common superscript differ ($P < 0.01$).

9.4 Discussion

9.4.1 Performance and gut health

Results of the experiment regarding body weight (BW) and body weight gain (BWG) indicated a significant increase in the prebiotics group compared with control at week 6 of rearing. Generally, all treatment groups showed higher BW and BWG at this time of the experiment compared to the control. In the first three weeks of age, there was no significant difference among treatments for BW. This is in line with Hooge *et al.* (2003) and Dizaji *et al.* (2012) who observed significant differences on performance traits only from 49 and 42 days of age, respectively. On the other hand, Bednarczyk *et al.* (2016) reported improvements in performance for prebiotic-treated birds only in the first 21 days of age in broiler chickens. Considering that in this study, slow growing birds were used unlike in their study, probably the effect of genotype and differing managerial and environmental factors could explain the variation in the obtained results. For instance, Hanning *et al.* (2012) assessed growth performance in two breeds of chickens (naked neck, a slow growing breed and Cornish White Rock cross broilers) in response to three types of prebiotics (Plum fibres, Galactooligosaccharides (GOS) and Fructooligosaccharides, FOS). Positive effects of GOS were observed only from week 4 to week 6 and no significant difference was recorded earlier than the 4th week or on the 8th week (end of experiment) for Naked Neck birds. While for the Cornish White Rock cross broilers no significant differences were observed but instead the same treatment lowered BW at week 6 compared to other treatments and the control. On the other hand, the authors found no difference between the group that received FOS and the control throughout the experimental period while plum showed significantly higher BW compared to the control only on week 8 in both chicken breeds (Hanning *et al.*, 2012).

In the present study, on week 12 BW as well as BWG of the prebiotic group were lower than those of the other experimental groups. This coincided with a high *Eimeria* oocyst count from the excreta of the same group. Rizvi and Anjum, (2000) noted that stress due to Parasitic infection causes a decrease in body weight gain and feed consumption and also affects the immune system of birds. However, the response of the birds in the prebiotic group to treatment with anticoccidial drug was generally better than the control and antibiotics groups as shown by the immediate improvement in BW and BWG and a reduction on oocyst count following the commencement of treatment. Also the lowest lesion scores were observed in the prebiotic treated groups. Barberis *et al.* (2015) observed better health condition and production performances in birds of the group that received prebiotic-

anticoccidial combination in which a decrease in coccidia replication and lesion scores were found in association with low oocysts shedding kinetics and a stimulated immune system (increased lymphoid organs weights, indices and a bigger number of lymphocytes) in *Eimeria* experimentally infected birds. The authors concluded that prebiotics enhanced the effectiveness of anticoccidial drugs in the control of coccidia infections in broiler chickens. The protective effect of prebiotics against coccidiosis is thought to be related to the inhibition of asexual schizonts' development following the stimulation of local immune mechanisms (Elmusharaf *et al.*, 2006).

However, several hypotheses have been put forward to explain the beneficial effect of prebiotics in avian coccidiosis control. It is believed that these products have the ability to simultaneously stimulate cellular immune response which plays a major role in controlling intestinal parasitism and local production of secretory antibodies during natural exposure to *Eimeria* spp. (Gomez-Verduzco *et al.*, 2009). The bursa of fabricius, spleen, thymus and lymphoid tissue associated to the intestine (cecal tonsils) are known to be major actors in the immune response against intestinal pathogens (Lillehoj and Lillehoj, 2000). Nollet *et al.* (2007) reported that prebiotics (MOS) augment vaccination thereby increasing the resistance of birds to coccidia infection. The inclusion of prebiotics has been shown to enhance the cellular and humoral immune responses (Li *et al.*, 2007; Slawinska *et al.*, 2014a,b) in broilers. Furthermore, they protect the intestinal mucosa against inflammatory reactions induced by pathogens or toxins by increasing the villi length and facilitating their regeneration (Gao *et al.*, 2008). Other prebiotics can compete with sporozoites for binding sites on intestinal epithelial cells and thereby reduce the adhesion and the subsequent proliferation of parasites (Shoaf *et al.*, 2006).

In this study, no *Eimeria* oocysts were observed in faecal samples obtained from the prebiotic and prebiotic + antibiotic pens in the first 8 weeks of rearing unlike in the controls where scanty oocysts could be seen in faeces from week 5 of age. On the other hand, the prebiotic-treated birds started showing clinical signs of the infection barely 2 weeks after the first few oocysts were observed in faecal samples. The many defense mechanisms involved in the protection from coccidian infection could probably explain the above observation. Most likely the control birds had gained some sort of immunity in the course of the early exposure to the infection compared to the treatment group. Antibodies (IgA, IgG and IgM) production begins shortly after natural infection (Lillehoj and Lillehoj, 2000) or vaccination (Ayaz *et al.*, 2008) with an efficacious protection of intestinal mucosa and a significant reduction in clinical signs' severity and mortality rates. It is however important to note that this immunity

does not protect the bird for its entire life time. Furthermore, cellular immune response supported by the T lymphocytes enhances resistance to coccidiosis (Pakandl *et al.*, 2008) and cytokines intensify the level of this protection. The increase in Interferon gamma levels (IFN- γ) is particularly associated with protective immunity against coccidiosis (Barberis *et al.*, 2015). Since prebiotics have been reported to have immuno-modulatory effects on birds (Slawinska *et al.*, 2014), this probably explains the delay in the birds of the prebiotic-treated groups from acquiring infection as early as the controls under the same rearing condition of the experiment.

Final body weights were significantly higher for the prebiotic and prebiotic + antibiotic group and these chickens were heavier than the others. This further confirms the result of Hooge *et al.* (2003) who found the highest final BW in the group that received a combination of feed additives (antibiotics and prebiotics). These results further support the hypothesis that dietary prebiotics promote growth in chickens owing to their ability to strongly bind the pathogenic bacteria and decoy pathogens away from the intestinal lining (Maiorano *et al.*, 2012; Bednarczyk *et al.*, 2016) and therefore could be used either as alternatives to AGP or in combination with AGPs.

9.4.2 Slaughter and carcass traits

Slaughter, carcass and breast weights, were not significantly affected by treatment although the prebiotic group had a slightly higher carcass weight (+94.84 g). While, breast yield, leg and wings weights as well as yield were higher in all treatment groups compared to the control but highest in prebiotic + antibiotic group. This is contrary to the results reported in Maiorano *et al.* (2017) but consistent in part with Maiorano *et al.* (2012) who found no significant differences in slaughter weight, carcass weight, carcass yield and feed conversion ratio but reported a slightly higher pectoral muscle percentage in prebiotics compared to the control group. Conversely, Pilarski *et al.* (2005) reported no significant differences of different prebiotics on final BW, carcass, breast muscle and leg weights in broilers. Likewise, Hanning *et al.* (2012) found no significant differences on breast and wing yields in broilers fed three different types of prebiotics.

The significant interaction between the studied factors for breast yield had shown that meat of females from the control group had lower breast yields while females from the experimental groups (A, B, AB) had higher breast yields compared to the males. For wings weight, meat from males was heavier than those of females in all groups except the antibiotic group.

Overall, the pH values and carcass yield obtained in this study although not different among treatments agree with the findings of An *et al.* (2010) and Sirri *et al.* (2011) for slow and medium growing strains of birds slaughtered at more or less the same age. The pH values obtained in this study are however lower than those reported by Eleroglu *et al.* (2013; 5.88 – 6.24) for birds slaughtered at 14 weeks of age.

Sexual dimorphism was clearly evident in this study with males displaying better carcass traits. This is in agreement with the findings of Maiorano *et al.* (2017) with Bi²tos and DiNovo in commercial poultry where males were heavier (+14.6%, $P < 0.01$), had higher carcass and breast weights ($P < 0.01$) but similar breast muscle yield ($P > 0.05$). A finding indicating higher live body and carcass weights in male chickens is in agreement with literature (Shafey *et al.*, 2013; Benyi *et al.*, 2015; Motsepe *et al.*, 2016). These differences can be explained by the metabolic differences between sexes. In a study by Zdunczyk *et al.* (2014) in laying hens, supplementation with RFO in blue lupin seed meal at 20% promoted daily egg productivity accompanied by a decrease in BW and FI suggesting a close link between reproductive activity and growth performance.

9.4.3 Intramuscular fat content and fatty acid composition of Kuroiler breast muscle

Intramuscular fat (IMF) content plays a key role in various quality attributes of meat, positively influencing sensory quality traits, such as taste, juiciness and flavour. IMF content varies among species, breeds and also between muscle types in the same breed. Other factors are involved in the variation of IMF content in animals, including gender, age and feeding. IMF in breast muscle of chickens is generally lower (1% to 2%) compared to other species, such as cattle, lamb and pigs, due to the fact that the breast muscle of chickens is composed mainly of type IIb glycolytic fibres with lower lipid content (Hocquette *et al.*, 2010). In the present study, IMF ranged from 1.90 to 2.27 % which is in line with the report of Maiorano *et al.* (2017).

Overall, in this study, total PUFA contents were higher while MUFA amounts were lower than those reported in some studies on chickens (Kral *et al.*, 2013; Velasco *et al.*, 2010) but consistent with those reported from studies on slow and/ or medium growing older chickens (Sirri *et al.*, 2010, 2011; Dal Bosco *et al.*, 2012; Boschetti *et al.*, 2016; Popova *et al.*, 2016). The lower concentration of MUFA was mainly related to the lower content of oleic acid (C18:1) whose values are known to decrease tremendously with increasing age of the birds

(Popova *et al.*, 2016). Also the total amount of SFA is consistent with that reported from Sirri *et al.* (2010) in slow-growing chickens.

Nutritionally, the fatty acid profile observed in this study is generally favourable health wise since omega-3 polyunsaturated fatty acids (n-3 PUFAs) have for long been associated with potential health benefits in chronic disease prevention. Short-chain n-3 PUFAs are widely distributed in plant oil such as flaxseed and soybean oil, while long-chain n-3 PUFAs are usually found in marine products such as fish oil. Although short-chain n-3 PUFAs are more common and less expensive, the potential health benefits of n-3 PUFAs are known to be related to long-chain n-3 PUFAs only (Chen *et al.*, 2017). Compelling data from epidemiological and interventional studies have demonstrated an inverse correlation between long-chain n-3 PUFAs and risk of some chronic diseases, including cardiovascular diseases (Dolecek and Granditis, 1991; Nestel, 2001), myocardial infarction (Von Schacky and Harris, 2007; Das, 2016), bronchial asthma and different types of cancer (reviewed in Chen *et al.*, 2017). As Haug *et al.* (2010) put it, increasing the long-chain *omega-3* fatty acids intake through the ordinary diet is a better strategy than relying on supplements. Furthermore, when these fatty acids come from animal foods rather than as purified dietary supplements, they are ingested along with antioxidant nutrients that are important for prevention of peroxidation *in vivo*, such as selenium, glutathione (plus glutathione precursor amino acids), etc. These nutrients with antioxidant properties protecting against tissue damage caused by ischemia and reperfusion may themselves have important antimutagenic, anticarcinogenic, and anti-inflammatory properties and may very likely synergize with many of the protective effects of long-chain *omega-3* fatty acids (Haug *et al.*, 2010). The results of the present study confirm the importance of poultry meat in meeting the dietary requirements of n-3 PUFA in human.

The results on fatty acid composition of Kuroiler chickens showed a marked difference on the proportion of a few fatty acids in the breast muscle between males and females. In particular, males had higher total MUFA amount compared to female Kuroilers while females displayed higher total PUFA amounts. In this study, the birds were slaughtered at 18 weeks of age when the females were starting to lay eggs. These observations are therefore probably due to differences in lipid metabolism mainly associated with hormonal changes that occur with the commencement of egg production in females. In fact, several sex-related differences may be explained by the physiological changes in metabolism in female birds due to egg laying. Scholtz *et al.* (2009) reported that during the laying period, the hepatic synthesis of triglycerides, phospholipids, and cholesterol is generally increased. According to Dal Bosco *et al.* (2012) who obtained similar results in a study of the effect of genotype and

slaughter age on fatty acid composition in egg-line and meat-type birds, laying hens seem to have a higher efficiency in long chain fatty acid deposition compared to meat-type chickens. Alessandri *et al.* (2012) noted that the elongation of the fatty acids is partly affected by the oestrogen level which apparently rises when the hens approach or start egg laying, declining slowing by the laying sequence.

Regarding the effects of the treatment, research on the effects of prebiotics on meat nutritional quality (fatty acid composition) is fairly recent, so generally there is limited information in literature with which to compare the results of this study. Meat from the prebiotic group displayed higher amount of total PUFA compared to the control group while MUFA was lower in the prebiotic and prebiotic + antibiotic groups compared to the control and antibiotic groups. Similar results were obtained by Velasco *et al.* (2010) with inulin a fructan prebiotic when administered together with sunflower oil in broiler chickens. In contrast, Maiorano and Bednarczyk (2016) reported that the prebiotics had no significant effect on total MUFA content of broiler meat, but in line with the present study described a slightly higher amount of PUFA in prebiotics group compared to the control. The discrepancies in the above findings could be explained by the variation in diet, sex, genotype and most importantly slaughter age of the experimental birds. Popova *et al.* (2016) showed that in a conventional system under controlled conditions, the age at slaughter is of immense importance when assessing the potential of birds to produce high quality meat in regard to its fatty acid profile. The authors noted that while effect of the line was limited to differences in some individual fatty acids and related indices of lipid metabolism mainly in breast, age induced significant changes in the fatty acid composition in both thigh and breast muscles. Total SFA amount was similar in all experimental groups in this study. This is in agreement with previous work on prebiotics (Velasco *et al.*, 2010; Maiorano and Bednarczyk, 2016).

Regarding the selected fatty acid ratios, only the ratio of n-6 to n-3 was significantly different among experimental groups with lower ($P < 0.01$) values for prebiotic and prebiotic + antibiotic groups compared to the control. Generally, the obtained data showed a particularly lower n-6 to n-3 ratio across the experimental groups due to the higher incidence of n-3 fatty acids, probably due to the inclusion of n-3 fatty acids into the diet administered to the birds and slaughter age. Although the values of the n-6/n-3 ratio obtained in this study were generally lower than those reported in literature, Popova *et al.* (2016) showed that n-6/n-3 ratio decreases and P/S ratio tends to increase with age in slow growing lines of chickens. The authors obtained 9.08 and 8.48 for La Belle and White Plymouth Rock respectively slaughtered at 18 weeks versus 12.69 and 15.01 at week 9 respectively from PM. Likewise,

Dal Bosco *et al.* (2012, 2014) found values ranging from 6.23 to 8.32 for different lines of slow growing birds slaughtered at 81 days (\approx 12 weeks). In a related study, Sirri *et al.* (2011) reported an n-6/n-3 ratio of 4.34 in slow growing chickens slaughtered at 96 days (\approx 14 weeks). Whereas the report of Sirri *et al.* (2011) was based on data from birds raised in organic system, the authors noted that pasture intake had no significant impact on fatty acid composition suggesting that the lower n-6 to n-3 ratio is most likely associated with age at slaughter and genotype of the birds used. The ratios of P/S, n-6/n-3 PUFA, are widely used to evaluate the nutritional value of fat. The above result is of particular importance from a nutritional point of view, because the obtained values fall within the recommended ideal value of 1 and the maximum value of 4 (Enser *et al.*, 2001).

The P/S ratios found in this study (0.98 – 1.11) though not different significantly among treatment groups, are quite similar to those reported for older birds (Popova *et al.*, 2016) but lower than those reported by Eleroglu *et al.* (2013; 2.91 – 3.25) and Boschetti *et al.* (2016; 2.01 – 2.23). Contrarily, Velasco *et al.* (2010) found higher PUFA to SFA (P/S) ratio in the prebiotics with sunflower oil group compared to the control. Likewise, Maiorano and Bednarczyk (2016) reported a significantly higher value in the prebiotics group compared to control. The obtained values of P/S ratios in this study are a little higher than the recommended value of 0.4 – 0.7, even if it is lower than values of other meat species (Wood *et al.*, 2003).

Significant treatments x sex interactions were observed for total n-3 fatty acids, which were higher in females in control and antibiotic groups while in prebiotic and prebiotic + antibiotic groups, meat from males displayed higher contents of n-3 fatty acids compared to the females. The n-6/n-3 ratio was higher in males in all groups except in prebiotic + antibiotic group.

9.5 Conclusions

To the best of our knowledge, this is the first trial of *in ovo* delivery of prebiotics under field condition in Uganda, if not the tropics at large. The results obtained from this study clearly proved that the *in ovo* prebiotic administration with or without antibiotic chick formula increased chicken body weight from the 3rd to 6th week of the rearing period even though the final body weight was significantly higher in the prebiotic + antibiotic group only. Results of the study also showed that prebiotics protected Kuroiler chickens from intestinal parasites in general and coccidia in particular in the first 56 days of age and tended to have a synergistic effect with anticoccidial drug in the management of the disease post-infection in the field.

Slaughter traits and fatty acid profile were also positively affected by *in ovo* prebiotic administration with or without antibiotic chick formula. Males had significantly higher slaughter weights and better carcass traits compared to females, except for WHC, pH and IMF that were not affected by sex.

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