RED OSIER DOGWOOD EXTRACTS AS ALTERNATIVES TO IN-FEED ANTIBIOTICS IN BROILER CHICKENS

By

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ABSTRACT

This thesis was conducted to investigate the efficacy of red osier dogwood extracts (**RDE**) as alternatives to in-feed antibiotics on the growth performance, digestive and absorptive functions, intestinal microbiota and meat quality in broiler chickens. A total of 320 1-day-old Cobb 500 chicks with an initial weight of 48.3 ± 3.3 g were assigned to 4 dietary treatments fed in 3 phases and provided in mash form for 46 days. The treatments were:1) negative control (NC) corn-soybean basal diet, 2) positive control (PC) basal diet and 30 ppm avilamycin, 3) basal diet supplemented with 1000 ppm RDE (RDE1) 4) basal diet with 3000 ppm RDE (RDE2). Each treatment was assigned 8 replicate pens, where n=80 per treatment and raised on floor pens of sizes 4,808.5 sq. inches with straw as bedding. Growth performance data was recorded for 41 days. Sampling of heart, liver, spleen, intestinal segments and digesta was conducted on day 45. Breast meat was collected on day 46 in a commercial slaughterhouse. The results showed that the RDE had no effect on the average daily gain, average daily feed intake, feed conversion ratio and relative organ weight of broiler chickens. The RDE2 treatment reduced the mortality rate during the whole experimental period, suggesting that RDE has the ability to improve livability. The results also showed that RDE1 and RDE2 supplementation altered the diversity of ileal and cecal microbiota and RDE2 increased the ileal digestibility of crude fat and amino acids and nutrient transporters mRNA gene expressions and the villus: crypt ratio of jejunum, while RDE1 and RDE2 reduced the mRNA expression of tight junction proteins, suggesting that the RDE supplementation improved the intestinal environment and functions. There was no significant difference among the treatment means of white striping and woody breast scores and their incidence. Moreover, meat quality showed no difference in the pH, drip loss, myofibrillar fragmentation index, shear force, lipid peroxidation and cook traits. However, a high dosage of RDE (3 g/kg) reduced

redness when compared to the negative control group. In conclusion, RDE may be used as potential

alternatives to in-feed antibiotics in broiler chickens.

DEDICATION

I would like to dedicate this thesis to my parents Ben Mogire and Janet Moige Mogire.

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FOREWORD

Part of this thesis was presented as a poster presentation at the Annual Poultry Science Meeting in Montreal, Canada in July 2019. The reference format in this thesis is according to the Poultry Science journal. This thesis is written in a manuscript format prepared during my MSc. (Animal Science) program as listed below:

1. Mogire, M.*, Choi, J.*, Adewole, D.*, Yang, C.*, Lu, P.*, Liu, S.*, Rodas-Gonzalez, A., YANG, C. Effects of Red Osier Dogwood extracts as alternatives to in-feed antibiotics on growth performance, gut health and meat quality in broiler chickens (Poster presentation). 2019 Poultry Science Association Annual Meeting, Montreal, Canada, July 15-19, 2019.

2. Yang, C.*, Choi J.*, Mogire, M.*, Rodas-Gonzalez, A., Diarra, M., Wang Q., Gong J., YANG, C. Effect of encapsulated citral and cinnamaldehyde on gut health and meat quality of broiler chickens (Poster presentation). 2019 Poultry Science Association Annual Meeting, Montreal, Canada, July 15-19, 2019.

3. Yang, C.*, Choi, J.*, Mogire, M.*, Rodas-Gonzalez, A., Diarra, M., Wang, Q., Gong, J., YANG, C. Improvement of gut health and meat quality of broiler chickens by encapsulated citral and cinnamaldehyde (Poster presentation). 2019 ASAS-CSAS Annual Meeting & Trade Show, Austin, United States, July 2019.

4. Lu, P.*, Mogire, M*., Choi, J.*, Adewole, D.*, Liu, S.*, Yang, C.*, Rodas-Gonzalez, A., YANG, C. (2019). Effect of protease as an alternative to in-feed antibiotics on growth performance, gut health and meat quality in broiler chickens. Journal of Animal Science, under review.

5. Mogire, M*., Choi, J.*, Adewole, D.*, Liu, S.*, Yang, C.*, Lu, P.*, Rodas-Gonzalez, A., Yang, C. (2019). Effect of Red Osier Dogwood extracts as an alternative to in-feed antibiotics on growth performance, gut health and meat quality in broiler chickens. Animal Nutrition, Under preparation.

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LIST OF ABBREVIATIONS

ADFI	Average daily feed intake
ADG	Average daily gain
AGP	Antibiotic growth promoters
AID	Apparent ileal digestibility
AME_n	Apparent metabolizable energy
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
B_0AT1	Broad neutral amino acid transporter 1
BHA	Butylated hydroxyanisole
BW	Body weight
CAT1	Cationic amino acid transporter 1
CCAC	Canadian council on animal care
CCR	Caloric conversion ratio
CD	Crypt depth
CDH1	Cadherin 1
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CIE	Commission internationale de l'eclairage
CLDN1	Claudin 1
CO_2	Carbon dioxide
ConA	Concanavalin
CP	Crude protein
CRD	Completely randomized design
EAAC1	Excitatory amino acid transporter 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

EGTA	Ethylene glycol tetra acetic acid
ERK1	Extracellular signal-regulated kinase 1
FCR	Feed conversion ratio
GSH-Px	Glutathione peroxidase
IκBα	I-kappa-B alpha
IκBKE	I-kappa-B kinase epsilon
IL-6	Interleukin 6
JNK1	c-Jun N-terminal kinase 1
KCl	Potassium chloride
KPO$_4$	Potassium phosphate
LED	Light-emitting diodes
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MFI	Myofibrillar fragmentation index
MgCl$_2$	Magnesium chloride
Mj/Kg	Megajoules per kilogram
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide hydrogen
NaN$_3$	Sodium azide
NE$_P$	Net energy for production
NF-κB	Nuclear factor kappa
$_o$C	Degrees celsius
ODC	Ornithine decarboxylase
OECD	Organisation for economic co-operation and development
OH	Hydroxyl
OTU	Operational taxonomic units
PBMC	Peripheral blood mononuclear cell

PCoA	Principal component analysis
PCR	Polymerase chain reaction
PepT1	Peptide transporter 1
PRSS3	Serine protease 3
PVC	Polyvinyl chloride film
RDE	Red osier dogwood extracts
REDOX	Oxidation-reduction
RNA	Ribonucleic acid
ROD	Red osier dogwood
ROS	Reactive oxygen species
SGLT1	Sodium-dependent glucose co-transporter 1
SOD	Super oxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TLR-3	Toll-like receptor 3
TME_n	True metabolizable energy
TNF-α	Tumor necrosis factor alpha
VCR	Villus height to crypt depth ratio
VH	Villus height
WB	Woody breast
WBSF	Warner bratzler shear force
WS	White striping
xCT1	Cysteine/glutamate transporter 1
ZO-1	Zonula occludens 1
PSE	Pale soft exudative
DFD	Dark firm and dry
NO	Nitric oxide

TCA   Tricarboxylic acid cycle

1.0 CHAPTER 1 GENERAL INTRODUCTION

Intensive livestock farming has incorporated improvements in genetics, management, and nutrition aimed at meeting the high demand for animal protein. Poultry meat, for instance, has seen an increase in consumption and demand of poultry meat parts due to the nutritional value, ease to prepare and affordable for middle-class homes (Petracci et al., 2014; OECD, 2020). The use of antibiotic growth promoters (**AGP**) has been practiced in livestock agriculture since 1940 (Castanon, 2007). The use of AGP has been previously reported to improve production by increasing feed efficiency, body weight and meat yield in broiler chickens (Miles et al., 2006). Additionally, the use of AGP promotes healthy gut in broilers by reducing the population of pathogenic bacteria in the gut (Dibner and Richards, 2005; Mehdi et al., 2018). However, the use of AGP in livestock agriculture has had several challenges, first of all is that commercial livestock farming has been reported to be one of the contributors to the development of antibiotic resistance in previously susceptible bacteria (DiMarzio et al., 2013). Besides, AGP usage has an environmental impact with excretion of metabolized or unmetabolized forms of antibiotics affecting microbial biodiversity when poultry litter is used as manure in fields (Carvalho and Santos, 2016). Lastly, concern by consumers over AGP use and food safety has contributed to bans and restrictions in the use of AGP (Wideman et al., 2016). As of December 2018, Canada restricted the use of medically important antibiotics in all livestock (ANAC, 2020).

Due to these concerns, the search for alternative compounds to AGP has been intensified (Morgan, 2017). Antibiotic alternatives such as antimicrobial peptides (Daneshmand et al., 2019), probiotics (Alfaig et al., 2013), prebiotics (Bednarczyk et al., 2016), essential oils (Renault, 2012), organic acids (Nguyen et al., 2018) and plant extracts (Hernandez et al., 2004) have been utilized with positive results in broiler chickens production. Plant extracts have gained a lot of interest due to remarkable abilities to improve the growth performance (Barreto et al., 2008), meat quality (Bera et al., 2019), gut microbiota (Cueva et al., 2010) and intestinal digestive and absorptive function (Hafeez et al., 2016; Ren et al., 2018). Some examples of plant extracts used in broiler chicken production include polyphenolic compounds such as phenolic acids (Branciari et al., 2017), flavonoids (Suzuki and Hara, 2011) and tannins (Huang et al., 2018).

Cornus stolonifera commonly known as red osier dogwood (**ROD**) is an ornamental shrub that grows naturally in boggy areas of Canada and North America and produces flowers and white berries (Renault, 2012). ROD plant contains polyphenolic compounds with varying concentrations depending on seasons with the highest concentration being is 220 mg/g from the air-dried leaves of the plant (Isaak et al., 2013). The polyphenols present in ROD include ellagic and gallic acids, quercetin, rutin and high concentrations of anthocyanins (Obomsawin, 2001; Bate-Smith et al., 1975; Isaak et al., 2013). ROD has been observed to mitigate ruminal acidosis and improve protein efficiency in situ when the leaves, bark and stem were fed to beef

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heifers (Gomaa et al., 2018). Ground ROD has improved the intestinal histomorphology and attenuated oxidative stress in weaned piglets which have been challenged with *Escherichia coli* K88+/F4 (Jayaraman et al., 2018; Koo et al., 2018). However, unchallenged weaned piglets fed with ROD aqueous extracts reduced the body weight and average daily gain (ADG) in the first 14 days with no impact on apparent total tract digestibility (Lee et al., 2018). This may be attributed to the bitter taste associated with plant extracts which affects palatability and feed intake as piglets are sensitive to the bitter taste. Recent *in vitro* study using red osier dogwood extracts (**RDE**) and rutin demonstrated the ability of the extracts to mitigate inflammation and oxidative stress induced by TNF-α, hydrogen peroxide and lipopolysaccharide (**LPS**) in Caco-2 cells (Jiang et al., 2019; Liu et al., 2019; Yang et al., 2019). However, there has been no study published evaluating the effects of the RDE on broiler chickens. It is important to determine whether the *in vitro* results can be replicated *in vivo*. Further, it is crucial to determine whether the results observed in piglets are the same in broiler chickens. Additionally, it would be of interest to evaluate whether RDE can have an effect on meat quality when supplied in the diet.

2.0   CHAPTER 2 LITERATURE REVIEW

Plants possess a variety of bioactive chemical compounds known as phytochemicals. These phytochemicals can be extracted from plants and applied in different forms in animal feed such as plant extracts (Forte et al., 2018), essential oils (Branquinho et al., 2017) or ground plants (Jayaraman et al., 2018) in livestock animals production. Phytochemicals are secondary metabolic products that vary in structure and include alkaloids, sulfur-containing phytochemicals, terpenoids, carotenoids and polyphenols (Bravo, 1998). They play a significant role in plants such as protection from pathogens, insects and environmental factors. In addition, phytochemicals have beneficial effects in the physiological, metabolic and immune functions of humans (Vattem et al., 2005; Kawabata et al., 2015), rodents and farm animals (Berard et al., 2009; Qin and Hou, 2017).   There have been multiple hypotheses argued to be the reason for these effects on poultry health, performance and carcass quality is due to the polyphenolic compounds antimicrobial (Cueva et al., 2010; Lou et al., 2012), anti-inflammatory (Varmuzova et al., 2015; Jiang et al., 2019) and antioxidative (Duffy and Power, 2001; Goli et al., 2005) properties. Dietary polyphenols have received tremendous attention among animal nutritionists, feed scientists, farmers and consumers. Applications of polyphenolic compounds in poultry production have been observed to improve growth performance (Hafeez et al., 2016), digestive functions (Hernandez et al., 2004), immunity, overall bird health (Vossen et al., 2011; Alipour et

al., 2015; Mwale and Masika, 2015) and quality of poultry products (Choi et al., 2010; Farahat et al., 2017).

This review will, therefore, attempt to explain the mode of actions and effect of polyphenolic compounds on broiler health, growth performance, digestive and absorptive function, immunity and meat quality when fed to broiler chickens.

2.1 CLASSIFICATION AND CHEMICAL STRUCTURE OF POLYPHENOLS

Polyphenols are a class of phytochemicals that are generally involved in the plant's defense against ultraviolet radiation or aggression by pathogens and pests (Agati et al., 2013). Polyphenols exist in dietary plants, such as fruits, vegetables, herbs, spices and tea (Bravo, 1998; Tang et al., 2001). The diversity and wide distribution of polyphenols in plants necessitate that polyphenolic compounds be classified into groups for identification. Polyphenols have been categorized according to their source of origin, function, and chemical structure. In this review, polyphenolic classifications will be done focusing on the chemical structures of compounds. Polyphenols are abundant in plants with over 8000 types of compounds describe to date (Barbieri et al., 2017) and can be broadly further categorized into 3 main classes with several subclasses as follows: 1) phenolic acids such as stilbenes and lignins; 2) flavonoids such as quercetin, kaempferol, catechins, and anthocyanins; 3) other polyphenols such as resveratrol, ellagic acid and curcumin. The basic structure of polyphenolic compounds contains at least one hydroxyl group attached to an aromatic ring. Most compounds have more than

one hydroxyl group and more than two aromatic rings. Their structural diversity ranges from simple acids to complex polymers bound to polysaccharides (Bravo, 1998) organic and carboxylic acids, lipids and amines (Pietta, 2000). It is from these variations that different classes of polyphenols such as flavonoids and tannins are derived.

2.1.1 Phenolic Acids

These are simple phenols with one aromatic ring and can be divided into cinnamic and benzoic acids (Bravo, 1998). Cinnamic acids have a C_3-C_6 structure while benzoic acids are composed of a C_6-C_1 structure (Rice-Evans et al., 1996; Balasundram et al., 2006). Examples of cinnamic acids include caffeic acid while benzoic acids include gallic acid commonly found in herbs and shrubs such as the red osier dogwood (**Fig 2.1**) (Weerakkody et al., 2010; Isaak et al., 2013).
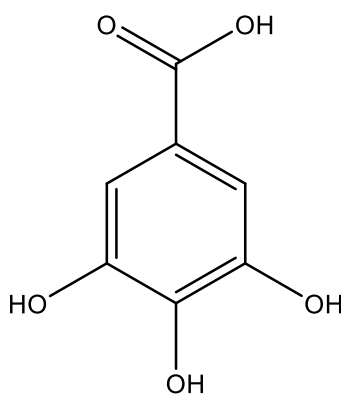
Figure 2.1. Gallic acid structure.

2.1.2 Flavonoids

The largest class of polyphenols are flavonoids. These are low molecular weight compounds with a C_6-C_3-C_6 structure and are composed of 3 rings; one heterocyclic and two aromatic rings as shown in **Fig. 2.2**. Flavonoids can be further classified into

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different subclasses according to variations in aromatic rings 1 and 2. For instance, glycosylation or sulfation in either of the two aromatic rings results in the formation of different compounds (Hunter, 1995; Pietta, 2000; Spencer et al. 2003). These subclasses include flavonols, flavones, flavanones and flavanols (**Fig. 2.2**).
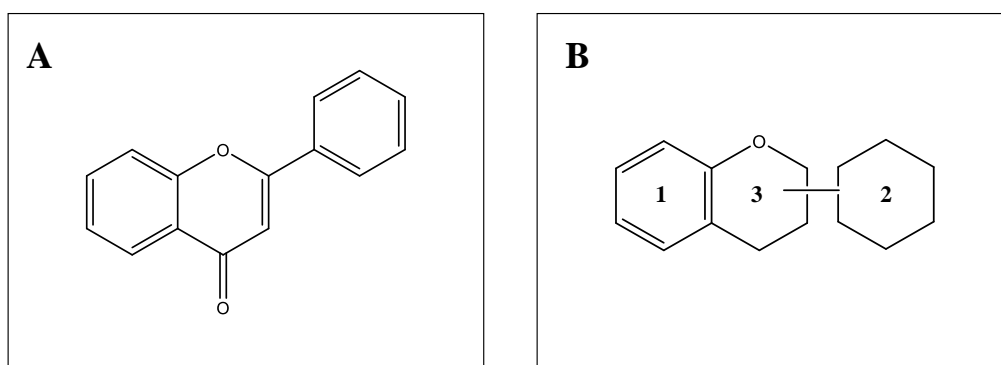
Figure 2.2. The structure of flavone backbone (A) and basic flavonoid backbone (B).

Flavones and flavanones have an o-glycosides and c-glycosides present in their structure. However, c-glycosides are more common in flavones and these sugars are not cleaved by acid hydrolysis (Cushnie and Lamb, 2005). Flavanones such as naringenin and espirengen are mostly present in citrus fruits. Flavanols, also known as catechins, occur as o-glycosides and isoflavones are mostly present in legumes. Flavanols are one of the most commonly studied flavonoids due to their high antioxidant activity and include the compound quercetin (Pietta, 2000; Seal, 2016). Members of this subclass have the distinct characteristic structural modifications of basic flavonoid structure that increase their antioxidant activity. The structure is made up of a dihydroxyl group in ring 2, a C_2-C_3 double bond and a 4-oxo group in the heterocyclic ring as shown in **Fig. 2.3** (Agati et al., 2013; Ferreira et al., 2015).
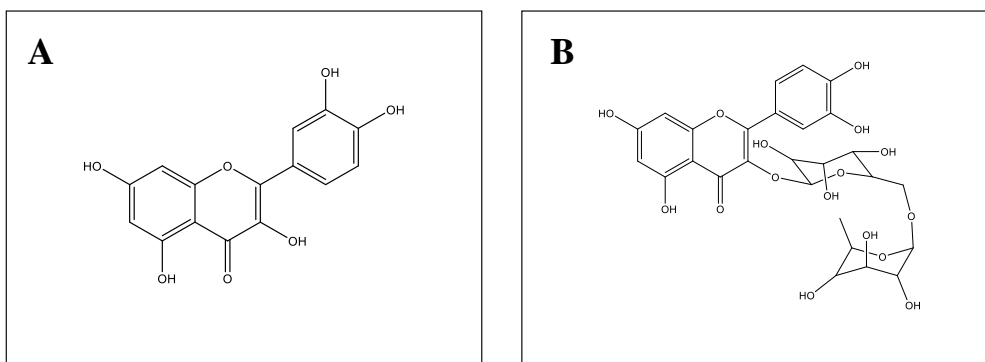
Figure 2.3. Structure of quercetin (A) and rutin (B).

2.1.3 Others

Apart from phenolic acids and flavonoids, the polyphenol group contains other compounds such as tannins and their derivatives. Tannins are complex, highly hydroxylated, high molecular weight compounds that form insoluble complexes with proteins and carbohydrates (Bravo, 1998). Tannins are capable of forming high molecular weight compounds through oxidative condensation. They can be further divided into two subgroups; hydrolysable and condensed tannins (Balasundram et al., 2006). Hydrolysable tannins can be digested by acids, alkali, and water in the presence of an enzyme to yield an acid and alcohol. Examples include ellagitannin (derived from hexahydroxy diphenic acid a dimeric condensation product of gallic acid), gallotannins (gallic acid), and tannic acids. Condensed tannins on the other hand are high molecular weight compounds formed by oxidative condensation or enzymatic polymerization composed of the core structure being flavan-3-ol, flavan-3,4-diol or a leucoanthocyanidin molecule. Low molecular weight tannins are soluble in organic acids, but high molecular weight condensed, and hydrolyzable tannins are insoluble

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(Balasundram et al., 2006; Pietta, 2000). The structural activity of tannins is their ability to form an insoluble matrix with the protein collagen present in hides.

2.2 CHEMICAL STRUCTURE AND FUNCTIONAL RELATIONSHIP OF POLYPHENOLS

Polyphenols have been described to have antioxidative, antimicrobial and anti-inflammatory properties (Madrigal-Carballo et al., 2009; Cueva et al., 2010; Kawabata et al., 2015). This functional ability is linked to their chemical and molecular structures such as their electronic properties, lipophilic and hydrophilic components, topology and steric effects (Shoaib et al., 2017). The degree of lipophilicity, for example, is important in the antioxidative capacity of phenols as it facilitates entry into cells, the site of free radicals attack, and enables interaction of polyphenols with different cells and cellular biomolecules such as proteins and enzymes (Mellou et al., 2005).

2.2.1   Antioxidant Potential

Research using plant extracts has shown that polyphenolic compounds present in plants are able to improve the oxidative stability and antioxidant status both in cultured cells and in poultry studies. This function of polyphenols is achieved either independently, by polyphenols functioning as radical scavengers, or by synergistic effects between compounds and the endogenous cellular antioxidative system (Alía et al., 2003; Brenes et al., 2008; Yang et al., 2019). Antioxidant activities of polyphenols are based on their molecular structure such as the presence of hydroxyl groups and double bonds that donate hydrogen and electrons (**Fig. 2.4.,** Branciari et al., 2017).

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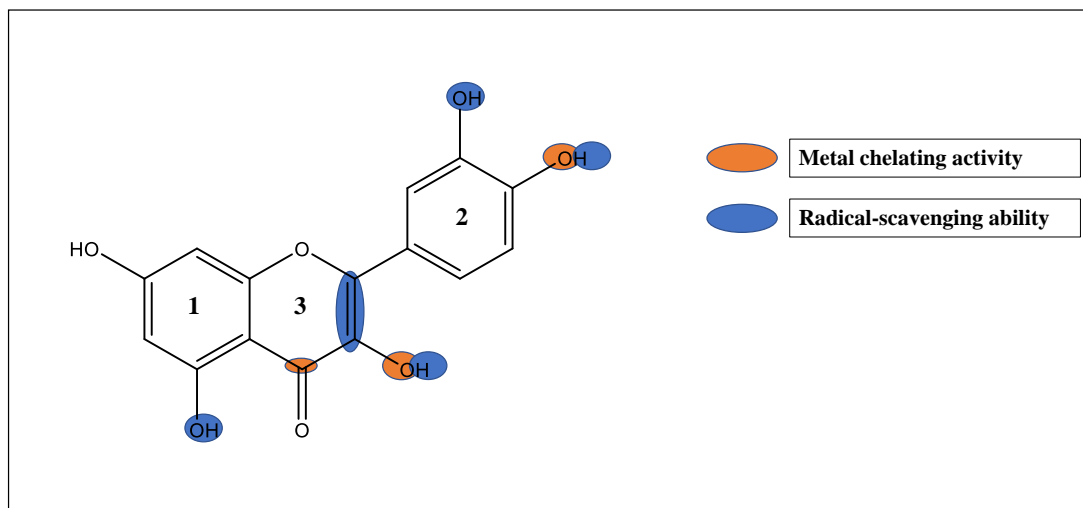
Figure 2.4. Quercetin skeletal structure showing the antioxidant structure-activity relationship.

There are several mechanisms by which polyphenols can exert an antioxidative effect in cells independent of the cellular antioxidative mechanism. The compounds can act as scavengers of reactive oxygen species, free radicals and chelate metal ions (Rice-Evans et al., 1996; Spencer et al., 2003, 2004). As an antioxidant, polyphenol scavenges for free radicals by donating electrons and absorbs oxygen radicals. This effect is made possible due to the structural feature that allows for electron delocalization such as the presence of C_2-C_3 double bonds and 4-oxo group (Ferreira et al., 2015). The hydroxyl groups of polyphenols donate hydrogen molecules to the reducing agents in REDOX reactions, effectively terminating the free radicals (Madrigal-Carballo et al., 2009). Hydroxyl groups in the 3', 4' and 5' positions contribute to the function as an antioxidant by scavenging for free radicals (Seeram and Nair, 2002). In addition to the neutralization of reactive oxygen and nitrogen species, polyphenols can bind to metals and terminate reactions that result in oxidation. The metal chelating activity of polyphenols is attributed to an ortho-dihydroxyl structure. The presence of a double

bond in the 4-oxo group allows for electron delocalization and provides a binding site for trace metals (Pietta, 2000; Seeram and Nair, 2002).

The antioxidative capacity of compounds is dependent on two factors; 1.) the presence and position of the hydroxyl groups in relation to the carboxyl groups and 2.) the number of hydroxyl groups present (Balasundram et al., 2006). For example, hydroxycinnamic acids have a greater antioxidative capacity compared to some hydroxybenzoic acids such as mono and dihydroxy benzoic acids despite being smaller in size (Rice-Evans et al., 1996). This is because hydroxycinnamic acids have an increased hydrogen donating capability compared to hydroxybenzoic acids. The hydrogen donating abilities of hydroxycinnamic acid groups are increased by the presence of the -CH=CH-COOH group.

Another mechanism of action against oxidative stress by polyphenols is based on the synergistic activity of compounds with the endogenous enzymatic antioxidant system (Brenes et al., 2008). There are mechanisms by which the cell maintains its oxidative balance. This is mainly through the use of antioxidative enzymes such as superoxide dismutase (**SOD**), catalase and glutathione peroxidase (**GSH-Px**). Polyphenols have been reported to increase the activity of glutathione peroxidase by increasing mRNA transcription of antioxidant enzymes and posttranslational modifications (Alía et al., 2003; Puiggròs et al., 2005). Another study reported that extracts from the red osier dogwood plant increased the gene expression of SOD, GSH-Px and hemeoxygenase-1 (**HO-1**) enzymes (Yang et al., 2019). It is important to note

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that polyphenols, under certain conditions, can act as pro-oxidants producing free radicals such as superoxide and hydrogen peroxide that induce nucleic acid damage or mutagenesis, induce apoptosis in cells and initiates lipid peroxidation (Dorman and Hiltunen, 2011; Eghbaliferiz and Iranshahi et al., 2016). Alteration of cellular homeostases such as an increase in pH (high alkaline environments), depletion of cellular GSH, presence of oxygen molecules and increased concentration of transition metals such as iron and copper contribute to the pro-oxidant activity of polyphenolic compounds (Zhou and Elias, 2012; Chen et al., 2012).

2.2.2 Anti-inflammatory Activity

Polyphenols such as quercetin, kaempferol, apigenin and hesperidin are among the compounds that demonstrate an anti-inflammatory capacity in animals (Harborne and Williams, 2000). The mechanism of action by which plant extracts achieve an anti-inflammatory effect involves one or two phases, affecting both the vascular and cellular phases involved in inflammatory reactions. Plant extracts have been reported to downregulate the mRNA expression of pro-inflammatory cytokines such as interleukin (**IL**)-8 in the case of red osier dogwood extracts (Jiang et al., 2019). Similarly, an *in vitro* study by Yang et al. (2019) found that polyphenolic compounds downregulated the levels of IL-8, IL-6 and tumour Necrosis Factor alpha (**TNF- α**) using red osier dogwood extracts in Caco-2 cells subjected to hydrogen peroxide treatment. Polyphenols can also affect the cellular phase by inhibiting prostaglandin production and migration of neutrophils to the site of injury (Iwamoto et al., 2015; Branquinho et

al., 2017). This effect is related to the structural modifications such as the presence of alkyl groups in the side chains, a methoxy group in the aromatic and benzodioxole ring (Durant-Archibold et al., 2018).

2.2.3 Antimicrobial Activity

A microorganism is ubiquitous in nature and can act as symbionts, commensals or pathogens to the host environment. Microbes include fungi, bacteria, protozoal organisms and viruses. Research studies have focused on the ability of polyphenols to have static or cidal effect on pathogenic microorganisms (Harrop et al., 1989). Polyphenolic compounds have demonstrated wide and varied results in their antimicrobial effects against fungi, bacteria and viruses (Song et al., 2011; Kawabata et al., 2015; Yang et al., 2017).

The antifungal activity of flavonoids is associated with the hydroxyl groups of C_5 and C_7 of ring 1, the integrity of the C_2-C_3 double bond and the presence of the OH groups at C_4 and C_3 all play a role in the inhibitory action of flavonoids on spore development in fungi (Cushnie and Lamb, 2005; Yang et al., 2017).

The antibacterial property of polyphenols is described as broad-spectrum as this has been observed to be against both Gram-positive and Gram-negative bacteria (Harrop et al., 1989). The essential oil cinnamon oil has been reported to have antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, phenolic acids from berries inhibited the growth of *Salmonella* and the flavonoid quercetin has been reported to have anti-staphylococcal activities (Puupponen-Pimiä et al., 2001;

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Hirai et al., 2010; Zhang et al., 2016). This functional property of polyphenols is modulated by one or a combination of multiple mechanisms including; modification of bacterial DNA structure, inhibition of DNA and RNA synthesis, interference with energy metabolism, inactivating enzymes and cell membrane destruction (Randhir et al., 2004; Nohynek et al., 2006; Lou et al., 2012).

Polyphenols inhibit the growth of bacteria by interfering with the genetic structure and process of DNA and RNA formation. One such effect is the ability of polyphenols to inhibit bacterial enzyme DNA gyrase (Barbieri et al., 2017). DNA gyrase is an enzyme involved in nucleic acid synthesis and has multiple binding sites for ATP and nucleic bases. Polyphenolic compounds compete for the ATPase binding site on the enzyme gyrase thereby inhibiting bacterial DNA synthesis. Secondly, polyphenols can interfere with the nucleic acid synthesis by forming hydrogen bonds with the nucleic acid bases of bacteria making them unavailable for RNA and DNA synthesis (Cushnie and Lamb, 2005). Another mechanism by which polyphenols interfere with bacterial genetic information has been observed in the gram-negative bacteria *E. coli.* Here, polyphenols promote excessive DNA cleavage through the enzymatic system known as topoisomerase IV dependent DNA cleavage. Regulation of topoisomerase IV is important for cell survival thus this leads to induction of SOS and growth inhibition in bacterial cells (Bernard et al., 1997).

Additionally, polyphenols can interfere with cell membrane structure, thereby leading to cell membrane destruction. This is accomplished by disrupting the cell

membrane fluidity, thus interfering with the hydrophilic and hydrophobic parts of the inner and outer membrane layers (Tsuchiya and Iinuma, 2000). Structural features of polyphenolic compounds such as; the number and substitutions of OH groups in the B ring and degree of unsaturation of the C_2-C_3 bond are involved in the interference of bacterial energy production and consumption (Agullo et al., 1997). For example, flavonoids can interfere with mitochondria's intermediary metabolism pathways (Williams et al., 2004). Flavonoids inhibit energy production in bacterial mitochondria by inactivating enzyme NADH cytochrome c reductase, which is involved in the electron chain transport of metabolic respiration (Haraguchi et al., 1998).

2.3 APPLICATIONS OF PLANT EXTRACTS IN BROILER CHICKENS

Due to the beneficial properties observed in polyphenolic compounds and the knowledge that has been acquired on the mechanisms of action, several phytochemicals from plants have been applied in broiler chicken production. Studies have investigated the use of herbs and spices extracts such as thyme (thymol), oregano (carvacrol) and cinnamon (cinnamaldehyde) (Du et al., 2016, 2016; Wei et al., 2017), ground whole plants or plant organs (Giannenas et al., 2018), polyphenolic extracts such as quercetin (Medina et al., 1999; Goliomytis et al., 2014; Mazur-Kuśnirek et al., 2019) and other plant extracts (Hernandez et al., 2004; Khalaji et al., 2011; Vase-Khavari et al., 2019).

2.3.1   Effect of Plant Extracts on Production Performance

Broiler chickens' growth performance is measured by parameters such as body weight gain, feed intake and feed efficiency (feed conversion ratio). However, growth

performance is affected by multiple factors such as immunity, nutrient digestion, absorption and utilization, and gut health and these factors will be discussed in detail below.

Cinnamon (cinnamaldehyde), oregano (carvacrol) and capsicum oleoresin (capsaicin) are three of the most studied plant extracts that have been applied to *in vivo* experiments. In 21-day old broilers, a plant extract mixture containing 3% cinnamaldehyde, 5% carvacrol and 2% capsicum extracts demonstrated a 14.5% improvement in weight gain and 9.8% improvement in feed efficiency compared to the control diet (Bravo et al., 2014). Similarly, increasing concentrations of thymol and carvacrol mixture were reported to have a linear increase in average daily gain (**ADG**) and reduction of gain to feed ratio in ages 10, 24 and 42 days (Hashemipour et al., 2013). In a different study, 200 ppm of essential oils (cinnamon, oregano and pepper extracts) were compared with 5000 ppm *Labiatae* extract and there was an observed improvement in weight gain of 14-21-day old chicken fed with the *Labiatae* extract compared to non-supplemented and essential oils fed groups while antibiotic supplemented broilers showed faster gain in 28-35 days old (Hernandez et al., 2004). Similarly, broilers supplemented fermented *Ginkgo biloba* leaves had improved weight gain, while black cumin seeds supplied in the diet increased body weight and reduced feed conversion ratio (**FCR**) (Khalaji et al., 2011; Cao et al., 2012). The results above demonstrated the positive effects of plant extracts on broiler production performance, however, there have been some contradicting results reported in other studies.

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Plant extracts have been reported to have, either no effect or negative effects on production performance. Khalaji et al., (2011) reported a decrease in feed intake in 1-21 days old chicks when the diet was supplemented with *Artemisia leaves* and *Camellia L.* plant extracts at 300ppm and 500 ppm concentrations. However, 500 ppm *Camellia L.* plant extracts reduced weight gain in 21, 35 and 41-day old broilers. Further, broiler diets supplemented with *Moringa oleifera* crushed leaf had a linear and quadratic reduction in body weight, weight gain and increased FCR (Cui et al., 2018). Experimental studies utilizing grape seed and rosemary leaf extracts have reported that the extracts have no effect on production performance parameters (Yesilbag et al., 2011; Farahat et al., 2017).

In contrast, negative effects have been reported and they may be attributed to a variety of factors such as plant extracts effects on feed palatability and nutrient bioavailability. Compounds present in plant extracts can reduce the palatability of feed due to the bitter taste thereby reducing intake as observed in quercetin supplementation (Goliomytis et al., 2014). In addition, polyphenolic compounds can form complexes with nutrients such as amino acids and trace minerals thus reducing bioavailability and digestibility of nutrients (Allen et al., 1997; Huang et al., 2018). On the other hand, the lack of any positive or negative effects on growth performance in some studies may be attributed to the large variation in broiler breeds selected for studies, management practices, diets and compounds present in the plant extracts used. In addition, this observed effect can be attributed to the lack of an immunological or environmental

challenge to the broilers during the experimental period (Allen et al., 1997; Istiqomah et al., 2013; Habibi et al., 2014; Arczewska-Wlosek and Swiatkiewicz, 2015).

2.3.2 Effect of Plant Extracts on Intestinal Development and Function

The broiler digestive system is composed of the crop, gizzard, proventriculus and the intestines which play different roles in the digestion. In broilers, the crop functions as a temporary storage organ where feed is moistened and digested by exogenous enzymes (Chaplin et al., 1992). On the other hand, the gizzard and proventriculus function as the true stomach involved in mechanical and chemical digestion of feed by enzymes and gastric juices (Svihus, 2014). The small intestines are an important digestive organ that is separated into three regions, namely the duodenum, jejunum and ileum. In addition to digestion, the jejunum and ileum are responsible for nutrient absorption from the lumen by nutrient transporters. Therefore, changes in the morphology of the jejunum and ileum can affect nutrient digestion and absorption (Kroghdahl, 1985).

Nutrient transporters are specific and vary in affinity and location with apical and basolateral transporters identified. Apical nutrient transporters related to protein digestion and absorption include peptide transporter (**PepT1)** and neutral amino acid transporters **(B$_0$AT1)** with basolateral transporters such as cationic amino acid transporters (**CAT).** Essential amino acids are largely absorbed from the lumen by **(B$_0$AT1),** lysine (cationic amino acid) by B$_{0+}$AT, and peptides by PepT1 (Taylor and Poncet, 2012**).** Glucose is mainly absorbed through Na-dependent glucose cotransporter

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(**SGLT1**) which accounts for 80% of all absorbed glucose (Roder, 2014; Gorboulev et al., 2012).

The extent of nutrient digestibility in broiler chickens is influenced by the quality of the feed supplied and the digestive capacity of the broiler which is determined by proper development and function of digestive organs. Plant extracts have demonstrated the potential to affect the digestive ability of broilers by improving their digestive morphology (Cao et al., 2012), enzyme production (Hashemipour et al., 2013), nutrient absorption (Gilbert et al., 2007) and nutrient utilization. However, no reports have associated plant extracts with improvements in feed quality.

An improvement in the digestive surface maximizes the contact of feed with digestive enzymes and increases feed digestibility (Brenes et al., 2008; Abolfathi et al., 2019). Zhang et al. (2015) reported the ability of fermented *Ginkgo biloba* leaves to increase the duodenum and jejunum villus height and decrease the jejunum crypt depth of broilers small intestines in a linear fashion. This effect of plant extracts has been observed in other studies that have reported increased villus height, decreased crypt depth and increased villus crypt ratio (Karthivashan et al., 2015; Samuel et al., 2017; Cui et al., 2018). The intestinal digestive surface can be altered by one or a combination of different mechanisms which includes increasing intestinal cell proliferation and improving intestinal health which reduces villi cell apoptosis and cell shedding (Bilić-Šobot et al., 2016; Sabino et al., 2018). Losso et al. (2004) reported that ellagitannins' can increase cell proliferation by 20%. The increase in cell proliferation is brought

about by increasing cell numbers at each cycle. In addition, plant extracts can promote the selective growth of normal cells devoid of genetic damage when treated with plant extracts *in vitro* (Brus et al., 2018). Polyphenolic extracts can also increase the expression of epidermal growth factor (**EGF**), epidermal growth factor receptor (**EGFR**) and ornithine decarboxylase (**ODC**) genes involved in cell proliferation, differentiation, growth and tissue repair in heat-stressed broilers (Xiong et al., 2016). Both *in vitro* and *in vivo* results from the studies above demonstrated that plant extracts can increase cell proliferation and enterocyte health by preventing genetically damaged cell development and promoting enterocyte cell development and injury repair mechanisms. In addition to improving broiler digestive capacity, plant extracts can reduce the feed passage rate in the intestines thereby increasing enzyme-feed interaction and feed digestibility (Branciari et al., 2017).

An improvement in digestibility of nutrients may be as a result of an increase in the concentration, activity and affinity of digestive enzymes (Hashemipour et al., 2013), improved digestive surface (Abolfathi et al. 2019), and optimum digesta passage rate (Branciari et al., 2017). Kamel et al. (2001) demonstrated that plant extracts have the capacity to increase appetite and enzyme production in brush borders, thereby improving feed intake and feed digestion. Similarly, Hashemipour et al. (2013) observed an increased secretion of pancreatic trypsin, lipase and protease activity in 24-day old broilers which were fed with thymol and carvacrol mixture. However, plant extracts have been reported to lack a stimulatory effect on pancreatic or peptic protein

digestion (Rawel et al., 2002). The mechanism by which plant extracts increase enzyme production and activity is not well understood. However, Kim et al. (2010) demonstrated that plant extracts can alter the expression levels of genes related to metabolism, signal transduction and immunity. For example, capsicum extract can increase intestinal and pancreatic lipase activity, hepatic lipid oxidation and hepatic lipogenesis effectively reducing lipid accumulation in the liver. On the other hand, carvacrol upregulates the expression of protein metabolic genes such as protease, selenoprotease X, serine protease 3 (**PRSS3**).

Plant extracts can also improve absorption by upregulating the genes involved in nutrient transport, although the research on this effect is scarce and the mechanism of action is not yet clear. There was an observed increase in mRNA gene expression of Na-dependent glucose cotransporter (**SGLT1**) and peptide transporter (**PepT1**) when broilers were supplemented with a feed additive blend in a challenge condition (Santos et al., 2019). On the contrary, chia seed soluble extracts, rich in caffeic and ferulic acid, had no effect on SGLT1 gene expression (Silva et al., 2019).

Plant extracts have been reported to significantly increase crude fat digestibility when fed to broilers chickens (Bravo et al., 2014; Pirgozliev et al., 2015). Fat is a high energy source, an improvement in fat digestibility increases the energy available for broiler growth and provides essential fatty acids required in the diet. Bravo et al., (2014) observed a significant difference in fat digestibility when broilers were fed a plant extract mixture. The study also reported a tendency to decrease total heat production

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while increasing total energy retained in the carcass of broilers fed with the treatments. The reduction in energy losses through heat production shifts focus from maintenance functions to growth and production in broilers. This improved nutrient utilization is further demonstrated by an increase in net energy for production (NE_P) in the study. Similarly, Pirgozliev et al., (2015) observed a 10.2% increase in fat digestibility. In addition, the study reported an improvement in caloric conversion ratio (**CCR**) with an averaged 1.3MJ/Kg decrease in energy required for each kg of growth. There were no differences in nitrogen-corrected apparent metabolizable energy (AME_n) digestibility observed in the study leading to the conclusion that the plant extract mixture improved nutrient utilization by improving CCR. *Ginkgo biloba* leaves and leaves extracts have been reported to significantly increase the nitrogen-corrected true metabolizable energy (TME_n), crude protein (**CP**), amino acids and dry matter digestibility in a linear and quadratic manner when compared to basal diets without plant extracts (Ren et al., 2018). Similarly, Hernandez et al., (2004) observed an increase in the ileal digestibility of starch and crude fiber and apparent fecal digestibility of fat, dry matter and crude protein in essential oils and *Labiatae* treated diets.

2.3.3 Effect of Plant Extracts on Intestinal Microbiota and Barrier Function

Gut health is important in the overall broiler "immunity", nutrient utilization and growth performance. Gut integrity, barrier and intestinal microbiota contribute to improve gut health and optimal digestive function of the broiler.

Escherichia coli (**E. coli)** is an opportunistic pathogen in broiler chickens' intestines and is affected by microbial balance while Salmonella is an asymptomatic pathogen that reduces broiler performance (Hsu et al., 2016). Studies by different authors have reported a reduction in both coliform and salmonella counts, with an effect on cecal and intestinal *E. coli* populations due to the effects of extracts from plants such as *Camelia L* and gingko leaves supplemented in the diet (Khalaji et al., 2011; Zhang et al., 2015; Vase-Khavari et al., 2019). Although the exact mechanism of action of plant extracts against pathogenic bacteria is not well elucidated, some knowledge is available. For instance, the *Scutellaria* plant was found to form metal complexes with iron which reduced iron availability and modified electron acceptors resulting in an iron deficiency for pathogens. However, reduced iron availability for pathogens resulted in the promotion of the growth of beneficial microorganisms (Varmuzova et al., 2015). Plant extracts supplementation has been reported to increase beneficial bacteria in the intestinal tract such as *Faebacillus*, *Lactobacillus*, *Bifidobacterium* and *Clostridium* cluster III and XIV (Zhao et al., 2013; Silva et al., 2019; Vase-Khavari et al., 2019). In addition, plant extracts containing 5% cinnamaldehyde, 3% carvacrol and 2% capsaicin can also reduce the total intestinal bacterial population as observed in a study that reported less proliferation of bacteria by the observed decrease in microbial sialic acid excretion (Pirgozliev et al., 2015).

Modulation of gut barrier function is important in order to prevent intestinal leakage and pathogenesis. Plant extracts can improve gut barrier function by increasing

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mRNA expression of tight junction protein and promote assembly of tight junction proteins (Suzuki and Hara, 2009; Zou et al. 2016). Polyphenolic compounds in a feed additive blend were observed to increase the mRNA levels of Claudin 1 (**CLDN1**) and Zonula occluden 1 (**ZO-1**) (Santos et al., 2019) while PFA increased relative expression of ZO-2, CLDN5 and occludins (**OCLN**) mRNA gene expression (Paraskeuas and Mountzouris, 2019).

2.3.4 Effect of Plant Extracts on Immunity

Aside from improving gut health in broiler chickens, bioactive compounds of plants and their extracts have been reported to have stimulatory activity on the overall immune system. The compounds have been observed to modulate both the innate and adaptive immune systems (Farahat et al., 2017; El-Deep et al., 2019).

The mechanism by which plant extracts such as *Moringa olifera* and phenolic plant extract modulate the innate immune system is by stimulating anti-inflammatory cytokines such as IL-6, I-kappa-B-alpha (**IκBα**), I-kappa-B kinase epsilon (**IκBKE**) and toll-like receptor 3 (**TLR-3**) which suppress the expression of pro-inflammatory cytokines TNFα and NF-kB activation (Sabino et al., 2018; El-Deep et al., 2019; Santos et al., 2019). Alternatively, plant extracts contain compounds that may induce the production of nitric oxide by macrophages thereby activating the innate immune system (Vossen et al., 2011). Another indirect mechanism of plant extract on broiler health is through the improvement of broiler chickens' body oxidative status (Habibi et al., 2014; Wang et al., 2016; Branciari et al., 2017). One such example is observed when there is

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an increase in the relative heart weight in broilers supplemented with quercetin that may be able to reduce metabolic disorders associated with reduced relative heart weight (Goliomytis et al., 2014). The reduced relative weight of the heart is associated with the development of ascites and sudden death syndrome in broilers due to reduced function of the heart and less oxygen supplied to the tissues (Rance et al., 2002).

Immunological challenges have been applied to study the broiler's adaptive immune response to antigens. When broiler chickens were challenged with sheep red blood cells (**SRBC**), the broilers showed an increased production of immunoglobulins in black cumin seeds and essential oil supplemented groups compared to non-supplemented broiler groups (Khalaji et al., 2011; Hashemipour et al., 2013). The immunostimulatory effect on adaptive immunity was also observed in response to vaccination against the New Castle Disease where overall antibody tiers were increased when broilers were supplemented with grape seed extracts (Farahat et al., 2017). The exact mechanism of adaptive immune system stimulation is not clear but can be speculated from other studies. One potential mechanism is by the effect of plant extracts on the size and activity of immune-related organs such as the spleen, liver and bursa such as early peripheral blood mononuclear cells' (**PBMC**) proliferation which causes an increase in the relative weight of immune-related organs (Song et al., 2011). In addition, plant extracts may have compounds that can act as lymphokines such as concanavalin A (**ConA**) and lipopolysaccharide (**LPS**) from *Pinus radiata* bark which can stimulate activation and differentiation of adaptive immune cells (Park et al., 2011).

2.3.5 Effect of Plant Extracts on Meat Quality

Meat quality is an important aspect of broiler chicken production as this affects meat processing ability and purchasing power (Wideman et al., 2016). Appearance and nutritional value of meat are important factors that determine meat value and are affected by physicochemical factors that occur both pre and post-mortem (Boulianne and King, 1998; Mancini and Hunt, 2005). Post-mortem muscle biochemistry is altered by the absence of oxygen for respiration. As a result, ATP is depleted leading to the production of lactic acid that lowers muscle pH and affects physiochemical parameters such as tenderness, meat color and water holding capacity (**Fig. 2.5,** Bowker et al., 2000).

Rosemary extracts and herbal mixtures were reported to lower the pH_{45}, pH ultimate and day 3 pH of broiler breast and thigh meat when they were added in feed with no changes in drip loss (Jang et al., 2008; Yesilbag et al., 2011). In contrast, *Ginkgo biloba* leaves extracts increased the pH of broiler meat while simultaneously decreasing the drip loss percentages (Cao et al., 2012).

Meat tenderness is an important attribute affecting the palatability and value of poultry products (O'Sullivan and Kerry, 2009; An et al., 2010; Li et al., 2014). However, few studies have been published reporting the effects of bioactive compounds in plants on broiler meat physicochemical attributes such as tenderness. Studies have reported that plant extracts can improve meat tenderness by decreasing the shear force value and cook loss percentage when broilers are fed diets containing marigold and

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Ginkgo biloba leaves extracts (Cao et al., 2012; Wang et al., 2016). In contrast, other studies did not observe any effect of plant extracts on shear force and cook loss values (Alfaig et al., 2013; Istiqomah et al., 2013). The biochemical mechanism of action by which plant extracts alter physicochemical properties of meat is still not clear.

Research conducted using plant extracts have reported different results on meat color. For instance, broiler chickens fed oregano extracts had an increase yellowness values (Young et al., 2003), however, oregano extracts showed a contradictory result regarding lightness, where a study indicated decreased lightness (**L***) values (Jang et al., 2008), while another study using oregano aqueous extracts observed no difference on meat color among treatments (Forte et al., 2018). Similarly, some other studies have reported no effects from plant extracts such as rosemary, ginkgo biloba leaves and dietary saponins on meat color (Yesilbag et al., 2011; Cao et al., 2012; Bera et al., 2019). In contrast, *Moringa olifera* extracts increased meat lightness values (Karthivashan et al., 2015), another study using supplementation of commercial phytogenic additives observed reduced yellowness values of meat (Orlowski et al., 2018) while plant extracts such as marigold flower have been observed to increase the redness value when fed to broiler chickens (Wang et al., 2016). The increased redness (**a***) values while using plant extracts has been reported to be an effect of reduction in metmyoglobin oxidation in meat (Suman and Joseph, 2013; Zhang et al., 2015; Wan et al., 2018).

Plant extracts such as saponins and phenolic compounds have been reported to improve the storage stability of meat thus extending the shelf life by exerting antioxidative effects against lipid peroxidation and protein oxidation (Cui et al., 2018; Gomathi et al., 2018; Bera et al., 2019). The bioactive compounds in plant extracts have been observed to reduce malondialdehyde (**MDA**) values, increase the total superoxide dismutase (**T-SOD**) and antioxidant capacity (**T-AOC**) of broiler meat when plant extracts (rosemary, gingko biloba, oregano and marigold) are supplemented in broiler diets (Jang et al., 2008; Yesilbag et al., 2011; Cao et al., 2012; Wang et al., 2016). A reduction of lipid peroxidation on intramuscular fat and improvement on the redox balance in broilers may be as a result of two mechanisms: 1) Plant extracts effects on intramuscular fat deposition and concentration and 2) direct antioxidative capacity of plant extracts on reactive oxygen species present in the muscle (Botsoglou et al., 2003; Symeon et al., 2014). Several studies have been published reporting the reduction of MDA levels of meat by a reduction in the rate of lipid peroxidation due to the antioxidative effects of plant extracts such as quercetin (Tang et al., 2001; Jang et al., 2008; Goliomytis et al., 2014). Plant extracts improve the oxidative stability of the muscle by stimulating endogenous antioxidant systems or supplementing the cellular antioxidant systems (Brus et al., 2018). There was an observed increase in the mRNA expression of SOD1 and SOD2 enzymes through multiple pathways such as the mitogen-activated protein kinase (**MAPK**) pathway with increased expression of extracellular signal-regulated kinase 1 and 2 (**ERK1**, **ERK2**) and c-jun N-terminal

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kinase 1 (**JNK1**) genes (Orlowski et al., 2018). Alternatively, Schiavone et al., (2007) reported that silymarin extracts improved the oxidative stability of breast and thigh meat by reducing the lipid concentration in the meat.

Recently, the quality of broiler breast meat has focused on visual myopathies, white striping (**WS**) and woody breast (**WB**) meat. WS is characterized by the occurrence of fat deposits within the muscle visualized as white lines running parallel to the muscle fibers in raw meat (Petracci et al., 2014). WB is a myopathy characterized by tough muscles located in the caudal region of pectoralis major muscle of broiler chickens and in severe situations can be seen as a 'ridge-like bulge' (Kuttappan et al., 2013). WS and WB have multiple etiology that contribute to myopathic lesions such as genetic selection for fast growth, hypoxia, oxidative stress, fiber type, inflammation and alteration of calcium- signaling pathways as described in **Fig. 2.6** (Macrae et al., 2006; Kuttappan et al., 2013, 2016; Livingstone et al., 2019). Currently, there is limited information about the effects of plant extracts on these pathologies; particularly using RDE.

2.3.6 Challenges and Opportunities

Plant extracts have demonstrated the ability to improve performance, livability, immunity, nutrient utilization and efficiency in broiler chickens. However, it is important to perform further studies to evaluate the most beneficial and cost-effective form of administration, bioactive compounds, effective dosage and mechanism of action which is not well understood. The positive results displaying antioxidant activity

in vitro by polyphenols may be altered *in vivo* as a result of the metabolism of the bioactive compounds. Bioavailability is another concern as intracellular uptake of flavonoids and their metabolized forms depend on the cell type. In quercetin, for example, metabolism and addition of methyl structures change its uptake by cells or the ability to penetrate cells. (Spencer et al., 2003, 2004; Williams et al. 2004).

Additionally, plant extracts can also improve meat color, tenderness and storage stability. However, apart from cook loss and shear force, there have been no studies that have evaluated the effect of plant extracts on other tenderness measurements such as myofibrillar fragmentation index. Taking all these factors into consideration the impact of plant extracts on the physiochemical properties of broiler meat has not been properly elucidated.
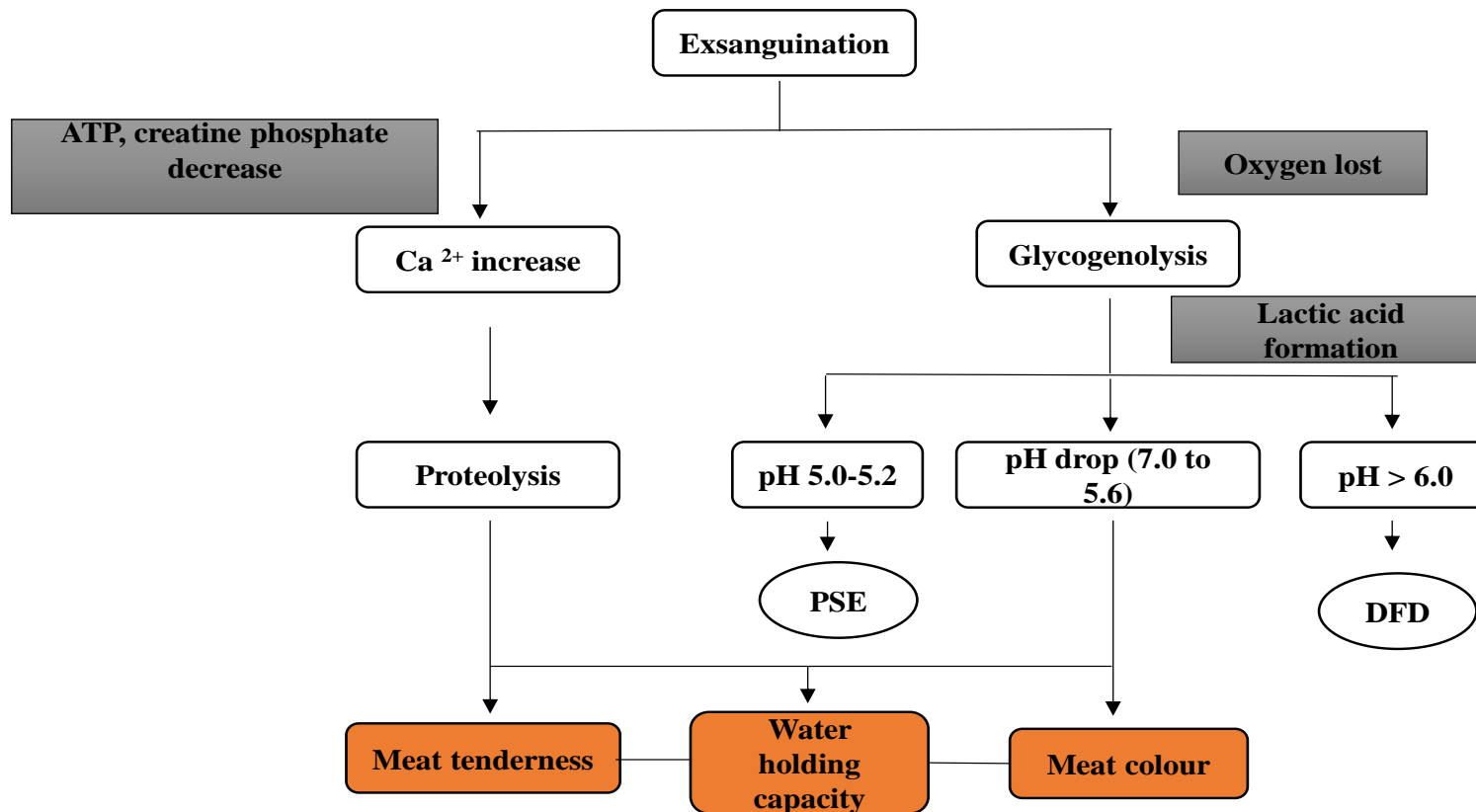
Figure 2.5. Biochemical changes from muscle to meat after slaughter.

Note: Pale soft exudative (PSE), dark firm dry (DFD), adenosine triphosphate (ATP).
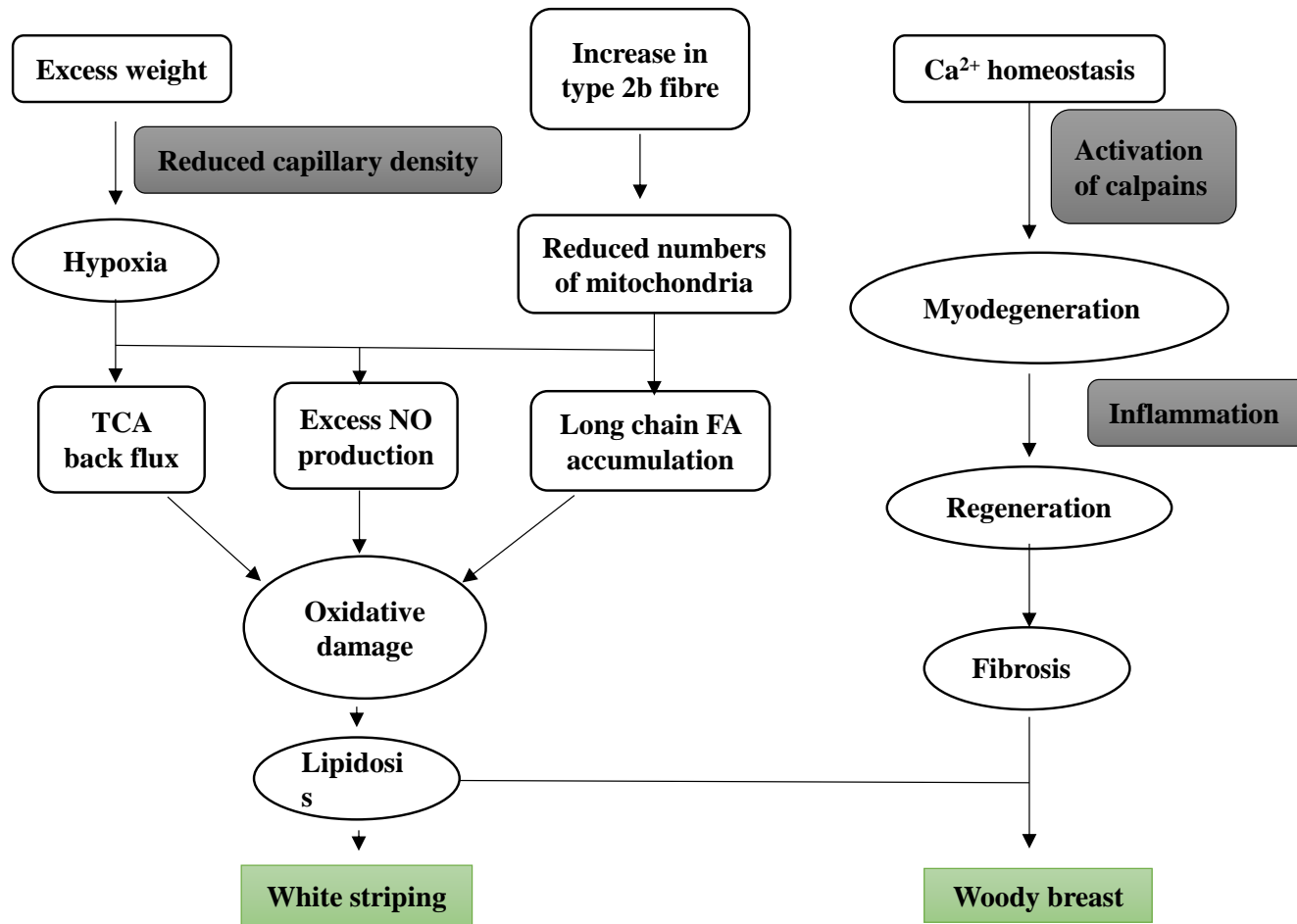
Figure 2.6. Pathways and metabolites involved in WS and WB incidence.

Note: Tricarboxylic acid cycle (TCA), fatty acid (FA), nitric oxide (NO).

3.0 CHAPTER 3 HYPOTHESIS AND OBJECTIVES

3.1 HYPOTHESIS

The following hypothesis was tested out in this thesis:

1. Red Osier Dogwood extracts (RDE) can be used as alternatives to in-feed antibiotics to improve growth performance, meat quality and gut health in broiler chickens.

3.2 OBJECTIVES

The overall objective was to determine the effect of RDE on the growth, gut health and meat quality of broiler chickens throughout the production period. The specific objectives were as follows:

1. To compare the low and high dosage of RDE with antibiotic and no-antibiotic groups and determine the differences and treatment effects on growth performance (ADG, ADFI, FCR and mortality);

2. To evaluate the effect of RDE on gut microbiome (ileal and cecal microbiome), gene expression of nutrient transporters and tight junction protein in intestinal segments, nutrient digestion and intestinal morphology (duodenum, jejunum and ileum);

3. To determine the effect of RDE on the incidence of white striping and woody breast on broiler breast meat; and

4. To analyze the effect of RDE on the appearance and physicochemical parameters of broiler breast meat.

4.0 CHAPTER 4

EFFECTS OF RED OSIER DOGWOOD EXTRACTS AS AN ALTERNATIVE TO IN-FEED ANTIBIOTICS ON GROWTH PERFORMANCE, INTESTINAL DIGESTIVE AND ABSORPTIVE FUNCTION, MICROBIOTA AND MEAT QUALITY IN BROILER CHICKENS

4.1 ABSTRACT

This study evaluated the efficacy of red osier dogwood extracts (**RDE**) as an alternative to in-feed antibiotics on performance, intestinal morphology, nutrient transporters, apparent ileal digestibility and meat quality of broiler chickens. A total of 320 1-day-old Cobb 500 chicks with an initial weight of 48.3 ± 3.3 g per pen were assigned to 4 dietary treatments provided in 3 phases for 46 days. The treatments were fed as mash diets and include :1) negative control (**NC**) corn-soybean basal diet, 2) positive control (**PC**) basal diet and 30 ppm avilamycin, 3) basal diet supplemented with 1000 ppm RDE (**RDE1**) 4) basal diet with 3000 ppm RDE (**RDE2**). Growth performance was measured from days 1-41, samples for heart, liver, spleen, intestinal segments and digesta were collected on day 45 while breast meat was collected on day 46 in a commercial slaughterhouse. There were no significant differences in average daily gain, feed conversion ratio average daily feed intake and organ weights among treatments ($P > 0.05$). Intestinal morphology showed reduced jejunal crypt depth in RDE1 and increased villus: crypt ratio in groups treated either RDE1 or RDE2 ($P <$

0.05). Cationic amino acid transporter mRNA expression was decreased ($P < 0.05$) in RDE and PC treatments while peptide and neutral amino acid transporter were highly expressed ($P < 0.05$) in broilers fed RDE2 treatment compared to NC. There were no differences in the mRNA expression of the high-affinity glutamate transporter, cysteine transporter 1 and sodium-dependent glucose co-transporter 1 ($P > 0.05$). Apparent ileal digestibility of crude fat was increased in RDE2 and PC compared to NC while amino acid digestibility was greater in RDE1, RDE2 and PC groups ($P < 0.05$). Relative abundance of Proteobacteria ($P = 0.08$) and *Oscillospira* ($P = 0.09$) tended to be greater in RDE2 while unidentified genus *Christensenallacea* tended to be greater in PC ($P = 0.07$). RDE2 had an increased relative abundance in the genus *Turicibacter* compared to PC, NC and RDE1 ($P < 0.05$). Alpha diversity was not significantly different among treatments while PC showed a tendency in beta diversity of the cecum ($P = 0.09$) and RDE2 was significantly different in the ileum compared to NC, PC, RDE1 ($P < 0.05$). Cecum and ileum bacteria differed between communities in all treatments both dominant and non-dominant species ($P = 0.001$). RDE1, RDE2 and PC treatments showed decreased ZO-1 and CDH1 abundance compared to NC ($P < 0.05$). Meat quality showed no difference in white stripping and woody breast scores, pH, drip loss, myofibrillar fragmentation index, shear force, lipid peroxidation and cook traits ($P > 0.05$). However, RDE2 treatment showed reduced redness compared to the negative control ($P < 0.05$). In conclusion, RDE had no effect on growth performance, improved

the intestinal health and function of broiler chickens and had no detrimental effects on

meat quality.

Key words: red osier dogwood, broiler chicken, growth performance, digestibility,

nutrient transporter, meat quality

4.2 INTRODUCTION

There is an increasing demand in the world for protein, and poultry meat products have become more popular. For instance, the Organisation for Economic Co-operation and Development statistics estimate that consumption of poultry meat will increase from the current 30.66 kg/capita in 2020 to 31.88 kg/capita by 2028 (OECD library, 2019). As demand increases, intensive farming practices such as the use of AGP have been employed to improve production in commercial livestock agriculture (Castanon, 2018). The exact mechanism by which AGP increase livability and efficiency is not clearly understood but has been explained to be partly as a result of interactions with gut microbiota (Dibner and Richards, 2005; Mehdi et al., 2018). Despite the beneficial effects of AGP, subtherapeutic dosages of antibiotics in broiler production could carry the development of antibiotic resistance in bacteria (Threlfall, 2002; DiMarzio et al., 2013; Antunes et al., 2016), which has shifted consumer preferences for chickens raised without antibiotics. Those issues have contributed to restrictions in preventative use of antibiotics (Kuttappan et al., 2012; Petracci et al., 2014; Wideman et al., 2016), and they have been banned in the European Union since 2006 and in Canada as of December 2018 (Maron et al., 2013; Mehdi et al., 2018). Therefore, it has become imperative to search for alternative products that can improve the performance and health of broiler chickens. Recently, there has been a lot of interest in the use of plants and their secondary metabolites as feed additives to improve animal health and productivity (Wallace et al., 2010). The use of aqueous and organic solvents

extracts from plants such as terpenoids, saponins, polyphenols, alkaloids, essential oils and organic acids, have been employed as alternatives to AGP due to their antimicrobial, antioxidative and anti-inflammatory properties (Zdunczyk et al., 2010; Tholl, 2015; Barbieri et al., 2017). Red osier dogwood's leaves, stem, berries and flowers are rich in polyphenolic compounds including quercetin, rutin, kaempferol, gallic and ellagic acid and various cyanidin- glycosides (Balasundram et al., 2006; Isaak et al., 2013). The polyphenolic compounds present in red osier dogwood extracts (RDE) have been demonstrated to have antimicrobial (Nohynek et al., 2006; Hirai et al., 2010; Song et al., 2011), antioxidative and anti-inflammatory potentials (Seeram and Nair, 2002; Isaak et al., 2013). Further, RDE has been demonstrated to improve piglet performance and gut health by its antioxidant capacity (Jayaraman et al., 2018; Koo et al., 2018). To our knowledge, there have been no studies conducted using RDE in broiler chickens. These findings offer RDE an opportunity to be used as a potential antibiotic alternative in broiler chickens.

Due to the proven beneficial effects of polyphenolic compounds present in RDE, we hypothesized that RDE can improve growth performance, digestive function and meat quality of broiler chickens. Therefore, the objective of this study was to evaluate the effects of RDE in low and high concentrations on growth performance, organ weights, intestinal morphology, nutrient digestibility, nutrient transporter, microbiota, tight junction proteins and meat quality in broiler chickens.

4.3 MATERIALS AND METHODS

4.3.1   Red Osier Dogwood and Antibiotics

Red osier dogwood plant was cultivated and obtained from Swan River Manitoba and extracted and distilled by Red Dogwood Enterprises (Swan River, Manitoba, Canada) to obtain the red osier dogwood extract powder.

4.3.2   Animal Trial and Growth Performance

The broiler chickens were raised according to animal use protocol (F18-024) which was approved by the University of Manitoba Animal Care Protocol Management and Review Committee. The birds were cared for and handled in accordance with the guidelines of the Canadian Council on Animal Care (**CCAC**, 2009).

A total of 320 1-day-old Cobb500 male broiler chicks were distributed in a single room containing 32 floor pens with dimensions of 81.5 inches × 59 inches = 4,808.5 square inches and a depth of 4 inches of straw as bedding (8 pens per treatment, 10 birds per pen) based on body weight (**BW**, 48.3 ± 3.3 g) and raised for 46 days. Broilers had access to *ad libitum* feed and water, feed was provided as mash diets in trough feeders (day 1-5) and hanging metal poultry feeders (day 6 to end of trial). Water pots (double wall metal poultry fount 5 gallon) were used during starter phase and transitioned to water cups (6.5 inches in diameter and 2.25 inches deep). The broilers were fed the 4 treatments during the entire experimental period, additives were added at the expense of energy ingredient corn and all diets were prepared and mixed at the University of Manitoba feed mill (Glenlea Research Station, MB, Canada). Treatments

included a negative control (corn-soybean based diet; **NC**), positive control with basal diet + 30 ppm avilamycin (**PC;** Surmax 100 Premix, avilamycin premix 100 g/kg Elanco Canada Co. Ltd. Guelph, Ontario, Canada), **RDE1** (basal diet + 0.1% RDE) and **RDE2** (basal diet + 0.3% RDE). The diets were formulated in phases according to the genetic breeding company's guideline (Cobb - Vantress); starter (day 1-14), grower (15-28) and finisher (29-46) as shown in **Table 4.1**. The temperature was maintained at 31°C for day 1 then decreased by 1°C every 3 days up to day 7. From day 7-14 temperature was maintained at 28 °C, reduced to 25 °C from day 21-28, reduced to 24 °C from day 28-35 then decreased further to 22 °C from day 35 to day 41 and maintained at 21°C up to the end of the trial (days 42-46). The lighting program during the trial was as follows: 24L:0D from 0 to 3 d, 22L:2D from 4 to 7 d and 18L:6D in the period from 8 to 39 d and 23L:1D in the period of 40 to 46 days respectively to simulate commercial broiler rearing.

Feed intake and BW were recorded and calculated at the end of each phase (day 15, 29, 41) BW and feed intake were used to calculate average daily gain (**ADG**), average daily feed intake (**ADFI**) and feed conversion ratio (**FCR**). Mortality was monitored daily. During experimental period 5, 11, 10 and 4 broilers in the NC, PC, RDE1 and RDE2 treatment groups respectively were culled due to leg problems.

Table 4.1. Diet formulation and nutrient levels of the basal diet (g/kg, as fed basis unless otherwise stated)

Ingredients	Starter	Grower	Finisher
Maize	522.29	529.38	566.00
Soybean meal	305.00	261.00	225.00
Corn gluten meal	35.00	35.00	35.00
Wheat	25.00	30.00	30.00
Canola meal	25.00	30.00	30.00
Soybean oil	22.60	43.80	45.80
Corn DDGS	20.00	30.00	30.00
Limestone	15.00	13.00	13.00
Vitamin premix[1]	10.00	10.00	10.00
BIOFOS[2]	9.00	7.00	5.00
Mineral premix[3]	5.00	5.00	5.00
99% DL-methionine	2.65	2.37	2.05
Lysine-HCl	2.25	2.46	2.31
Threonine	0.71	0.49	0.34
Xylanase 8000G	0.20	0.20	0.20
Phytase 5000G	0.30	0.30	0.30
Calculated composition			
AME, kcal/kg	3000.00	3150.00	3200.00
CP, %	22.30	20.80	19.40
Ca, %	0.86	0.74	0.70
Total P, %	0.60	0.54	0.52
SID Lys, %	1.18	1.10	1.00

SID Met, %	0.60	0.55	0.50
SID Thr, %	0.78	0.74	0.67

[1]Provided per kilogram of diet: vitamin A, 8,255 IU; vitamin D3, 3,000 IU; vitamin E, 30 IU; vitamin B12, 0.013 mg; vitamin K3, 2.0 mg; niacin, 41.2 mg; choline, 1300.5 mg; folic acid, 1.0 mg; biotin, 0.25 mg; pyridoxine, 4.0 mg; thiamine, 4.0 mg; calcium pantothenic acid, 11.0 mg; riboflavin, 6.0 mg.

[2] BIOFOS = 21% Monocalcium phosphate

[3]Provided per kilogram of diet: manganese, 70.0 mg; zinc, 80.0 mg; iron, 80.0 mg; iodine, 0.5 mg; copper, 10 mg; selenium, 0.3 mg.
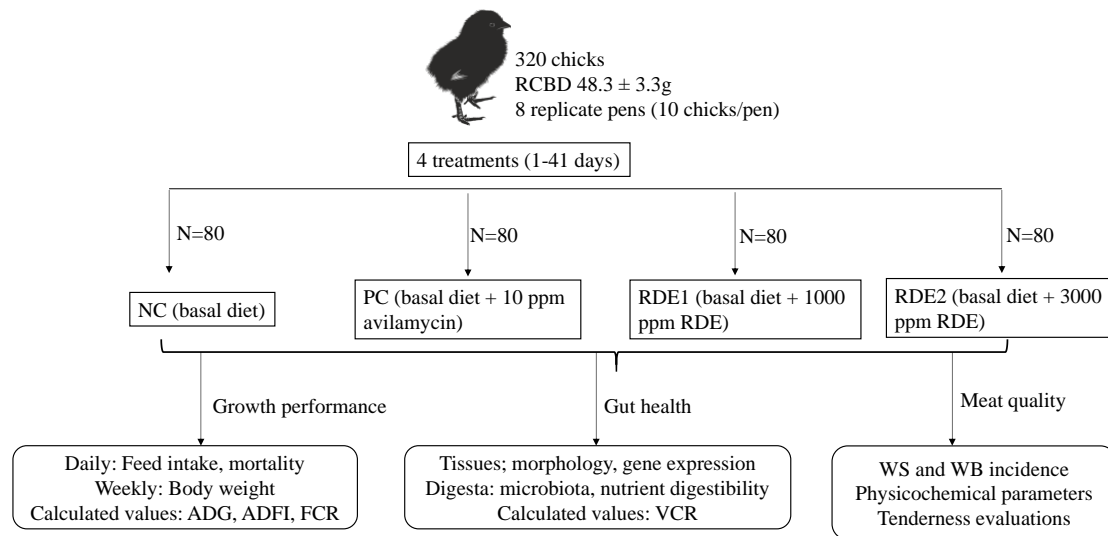
Figure 4.1. Summary of animal trial and sample collection

4.3.3 Sampling and Sample Processing

4.3.3.1 Tissue and digesta

At d 45, one bird per replicate pen was randomly selected (8 birds per treatment in total), euthanasia performed using a CO_2 chamber and confirmed with cervical dislocation and the body weight measured. The heart, bursa, spleen and liver were obtained from broilers and weighed for evaluation of relative organ weight. Jejunal segments were obtained, rinsed with cold PBS, frozen in liquid nitrogen and then stored at -80°C for gene expression analysis. Duodenal (from the pylorus to the distal portion

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of the duodenum loop), jejunal (from the distal part of the duodenum loop to Meckel's diverticulum) and ileal segments (from Meckel's diverticulum to cecum openings) were obtained, flushed with cold PBS and fixed in 10% neutral buffered formalin for intestinal morphological analysis. Ileal digesta was collected and stored at -20_o C for digestibility studies. Ileum and cecum digesta were aseptically collected, frozen in liquid nitrogen then stored at -80_o C for microbiota analysis.

4.3.3.2 Slaughtering and Breast Sample Collection

Upon completion of the trial, broiler chickens at 46 days of age were caught and placed in plastic crates for transportation to a commercial slaughterhouse for processing after 6 hours fasting period. The carcasses were cleaned and chilled in still ice water bath for 2-4 hours, skinned and deboned to obtain the breast muscle (*pectoralis major*) for white striping (**WS**) and woody breast (**WB**) scoring and meat quality analysis.

After WS and WB scoring, samples were placed in labelled plastic bags, stored in ice and transported to the lab. In the lab at 2 °C and 24 h post-mortem, the samples (**A**; left and **B**; right breasts) were further subdivided to obtain five sub-samples that were used for meat quality analyses (**Fig. 4.8**). Starting from the right breast, the first subsample was cut from the cranial section ($8 \times 4 \times 2$ cm) and used to assess ultimate pH and myofibrillar fragmentation index (MFI). A rectangular cut ($8 \times 4 \times 3$ cm_3, mid-region) was excised from the cranial and caudal parts for cooking traits and Warner-Bratzler shear force. In addition, a raw cylindrical meat cut (caudal region) 2.5 cm diameter $\times$ 6 cm length, was obtained for gravimetric water holding capacity analysis

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(drip loss). From the left breast, the caudal portion ($8 \times 4 \times 2$ cm$_3$) was excised for initial lipid oxidation analysis and the rest of the breast was used for retail color and purge loss evaluations. Except for retail color and purge loss evaluation samples, the samples were stored at -40 °C.

4.3.4 Laboratory Analysis

4.3.4.1 Intestinal Morphology

The tissues were dehydrated with alcohol, fixed with paraffin and embedded in wax. 6 μm sections were mounted onto slides deparaffinized in xylene, rehydrated then stained with the Hemoxylin and eosin dyes for histological analysis. Carl Zeiss MicroImaging microscope equipped with a morphometric software system (Carl Zeiss Ltd., Göttingen, Germany) was used to capture the images of the intestinal lumen. Eight to twelve well developed villi were selected from all parts of the lumen avoiding bias by labeling each slide using numbers instead of treatments and used to measure villus height (**VH**)-from the tip of the villus to the villus-crypt junction and crypt depth (**CD**)-from the villus base to the bottom of the crypt and calculate VH to CD ratio (**VCR**).

4.3.4.1 Gene Expression

The samples were suspended in lysis solution, RNA was extracted from tissues pulverized in liquid nitrogen and suspended in lysis buffer using the RNAqueous® Kit (AM1912) per the manufacturer's instructions. Extracted RNA was stored at -80 $_o$ C and used for cDNA synthesis using the iscript cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada) with the master mix containing 4 μl 5× iScript mix, 1 μl of

iScript R– transcriptase and 5 µl water. cDNA was stored at -20 C and used for Real Time-PCR. RT- PCR was performed using the IQ™ SYBR® Green Supermix (Bio-Rad Mississauga, ON, Canada) using 6 µL of cDNA samples, 10 µL supermix, 2 µL water and 1 µL forward and reverse primers each as shown in Table 3. The thermal cycle was set as; 95° C for 3 min, 40 cycles at 95° C for 20 s, 60° C for 30 s and 72° C for 30 s and the data was analyzed using the 2 delta delta method after normalization against the reference gene β-actin (Livak and Schmittgen, 2001).

Table 4.2. Primer sequences for Real-time quantitative PCR analysis

Gene	Genbank accession number		Primer sequences (5′-3′)	Product size
β-actin	NM_205518.1	FP	AATGGCTCCGGTATGTGCAA	112 bp
		RP	GGCCCATACCAACCATCACA	
B₀AT1	XM_419056	FP	GCTCTACAGTGTTTGGAACCC	111 bp
		RP	AAACTAGGCACACCAGCGAT	
CAT1	NM_001145490.1	FP	AACTGGGTTTCTGCCAGAGG	122 bp
		RP	AACCCATGATGCAGGTGGAG	
EAAC1	XM_424930.5	FP	GATTGTTCTGAGCGCTGTCG	115 bp
		RP	ACCAAAGGCATCTCCCAAG	
PepT1	NM_204365	FP	CTTTGGCTACCCCTTGAGCA	127 bp
		RP	AAAGTTGTCATCCCACCGCA	
SGLT1	NM_001293240.1	FP	ATGCTGCGGACATCTCTGTT	117 bp
		RP	TCCGTCCAGCCAGAAAGAAT	
XCT	XM_426289.5	FP	TGAGCTGGGAACGTGCATTA	115 bp
		RP	AGGGCGAATAACCAGCAGTT	

ZO-1	XM_015278981.1	FP	TATGCACAAGGAGGTCAGCC	97 bp
		RP	TTGGCCGAAGCATTCCATCT	
CDH1	NM_001039258.2	FP	GGCAAGCCGTTTACCACATC	110 bp
		RP	ATAATCCAGGCCCTTGGCTGT	
claudin1	NM_001013611.2	FP	GGTATGGCAACAGAGTGGCT	91 bp
		RP	CAGCCAATGAAGAGGGCTGA	

4.3.4.2 Apparent Ileal Nutrient Digestibility (AID)

Broilers finisher diet was mixed with 0.3% chromic oxide as an indigestible marker from days 42-45. Frozen ileal digesta was used for analysis of crude fat, crude protein, dry matter and amino acids digestibility. The chromium extraction method was used for analysis of dry matter according to the AOAC (2000) method (procedure # 934.01). Approximately 0.9 - 1.1g of diet and in-house standards were weighed with 0.25 - 0.35g used for analysis of digesta samples. Samples were dried for 12 hours set at 600 °C and digested with acid and chromium content determined using an inductively coupled plasma spectrometer (Varian Inc., Palo Alto, CA, USA).

Crude protein (**CP**) was estimated using total nitrogen content measured with a Leco NS 2000 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI, USA) and CP calculated as Nitrogen $\times$ 6.25. Amino acid digestibility was measured using acid hydrolysis method AOAC (2006; procedure # 994.12) followed by oxidation of methionine and cysteine with performic acid (AOAC, 2006; procedure # 985.18) and alkaline hydrolysis of tryptophan with barium hydroxide octahydrate Commission

Directive (2000). Amino acids content was evaluated using the amino acid analyzer (SYKAM, Germany). Nutrient digestibility was calculated using the following formula:

Nutrient Digestibility (%) = {1-(NF*CD)/(ND*CF)} *100

Where; NF = nutrient concentration in digesta,

CD = chromium concentration in diet,

ND = nutrient concentration in diet,

CF = chromium concentration in digesta.

4.3.4.3 Microbial Analysis

Genomic DNA was extracted from ileum and cecum digesta using the QIAamp® DNA Stool Mini Kit (QIAGEN, Toronto, Canada) according to the manufacturer's instructions. The quality and quantity of DNA were evaluated using spectrophotometry (NanoDrop-1000, Thermo Fisher Scientific, Waltham, UK) and agarose gel electrophoresis then stored at -20 °C. Sequencing libraries of the 16S ribosomal RNA gene (**rRNA**) were prepared according to the Illumina 16S Metagenomic Sequencing Library Preparation Guide Rev. B and sequenced on a MiSeq instrument (Illumina). Briefly, a 444-bp fragment spanning the V3-V4 hypervariable region was amplified with specific primers using 2×KAPA HiFi HotStart ReadyMix (VWR, CA89125-042) and purified with AMPure XP beads (Beckma Coulter A63880). Unique 8-bp dual indexes were added by PCR using the Nextera XT Index Kit (Illumina Inc., FC-131-1002), and PCR products were cleaned up with AMPure XP beads. Samples were pooled together at equimolar concentrations and sequenced using

a 600-cycle v3 reagent kit (Illumina, MS-102-3003). Sequenced data were analyzed by Quantitative Insights Into Microbial Ecology (QIIME, version 2.0; Caporaso et al., 2010). Paired-end reads were joined with fastq-join and quality filtered and demultiplexed in QIIME. The reads were clustered at 97% sequence identity (similar to the species level) using uclust (Edgar, 2010), and operational taxonomy units (**OTU**s) were picked against the Greengenes database (gg_otus_13_8) using an open-reference approach (DeSantis et al., 2006). The taxonomic assignment of the sequences was performed using the uclust consensus taxonomy assigner. Taxa that could not be assigned were presented as 'unclassified' using the highest taxonomic level that could be assigned to them. The sequences were aligned against the Greengenes core set with PyNast, and a phylogenetic tree was constructed with FastTree.

Alpha-diversity metrics were then calculated by QIIME, and a beta diversity distance matrix based on Bray Curtis dissimilarity, Jaccard index, weighted and unweighted UniFrac metric was calculated and used for principal co-ordinate analysis (PCoA).

4.3.4.4 White Striping and Woody Breast Incidences

WS and WB incidences were evaluated visually and scored by one person at the processing plant to minimize variation. The visual myopathies were recorded as the presence of either WS or WB and severity scored. Breast fillets were manually palpated on both the right and left breast to evaluate WB incidence and scored on a 4-point scale as follows: normal (fillets are flexible throughout; 0), mild (hard in the cranial region

but flexible; 1), moderate (hard throughout but flexible in mid to caudal region; 2) and severe (the whole fillet is extremely hard and rigid; 3) (Tijare et al., 2016). WS was visually scored on a 3-point scale as: normal (no distinct white lines; 0), moderate (visible white lines <1 mm thick; 1) and severe (large white lines 1-2 mm thick; 2) (Kuttappan et al., 2012).

4.3.4.5 Meat pH, Color and Drip Loss Evaluations

The dorsal part of the muscle was used to measure pH using a portable waterproof meat pH meter with a penetrating metal rode (HI 99163 Hanna instrument ™ Carrollton, Texas, USA) calibrated with pH 4.01 and pH 7 solutions. The breast was split into a butterfly while avoiding contact on the exposed surface, plated on a Styrofoam tray (foam meat tray, 8.25 × 5.75 × 1 cm, Pack All Manufacturing Inc, Rockland, ON, Canada) with a soaking pad and covered with oxygen-permeable polyvinyl chloride film (PVC; 037242 PUR Value Polyvinylchloride Standard Meat Films, AGL, Richmond Hill, Ontario, Canada) for retail display analysis. The trays were placed on a retail display cabinet (Model MI, Hussmann Corporation, Canada) at 3 °C under LED lighting (light-emitting diodes; Acuity Brands Dimmable Rigid 30-LED Light Strip Board HTG S7 - 94v-0 – 4000k) with an intensity of 1240 lx. The trays' positions were rotated randomly every 24 hours to account for temperature and lighting differences on the machine. The pH and objective color were recorded at the beginning and at the end of the retail display period (96 hours). The objective color was measured at 3 locations on the meat surface using the Konica Minolta (Chroma Meter

CR-410 Minolta Canada Inc., Mississauga, Ontario, Canada) using illuminant D65, 10°

standard observer angles and 2.54 cm aperture (Commission Internationale de

l'eclairage, 1978) that was blanked against a white tile. CIE measurements L*

(lightness), a* (redness) and b* (yellowness) were recorded. At the end of the display

period and after color evaluation, the breast fillets were removed from the retail cabinet,

weight and pH were measured again, and vacuum packaged (6" × 10" FlairPak Vacuum

Pouch, Flair Flexible Packaging Corporation, Canada/USA) and frozen at -40°C for

subsequent lipid oxidation analysis.

Drip loss was evaluated according to the method by Remignon et al. (1996) with

some modifications. Briefly, approximately 2.5 cm diameter x 6 cm length of breast

muscle was obtained from the caudal part and suspended perpendicularly in a flat-

bottomed volumetric flask, ensuring the tissue did not touch the flask surfaces and

sealed airtight. The flasks were placed at 2 $^\circ$C for 48 hours and the initial and final

weight used to calculate drip loss percentage using the following equation;

Drip loss % = [(initial weight-final weight)/initial weight] *100.

4.3.4.6 Lipid Peroxidation

The breast muscle malondialdehyde (MDA) levels were evaluated using the

thiobarbituric acid reactive substances (TBARS) method to evaluate lipid peroxidation

from 2-time points according to Buege and Aust (1978). Breast meat sample weighing

10 g was suspended in 30 mL of cold deionized distilled water and homogenized for 60

seconds (Osterizer 10 speed blender). The homogenate was transferred to 50 mL

conical centrifuge tubes and centrifuged for 10 minutes at 1850 x g at 4 °C (Thermo

Scientific™ Sorvall™ RC6 plus). Afterwards, 2 mL of the supernatant was carefully

transferred into 15 mL conical centrifuge tubes in duplicates and mixed with 4 mL

TCA/TBA solution (15% TCA and 20Mm TBA- MW 144.15) and 100μL of 10 %

butylated hydroxyanisole (BHA) by vortex. The tubes were placed in a boiling hot

water bath (100 °C) for 15 minutes and afterwards transferred to an ice water bath for

10 minutes. The mixture was centrifuged at 1850 x g, for 10 minutes at 4 °C and

supernatant carefully dispensed into 1.8 mL cuvettes for absorbance measurements read

at 531 nm using a spectrophotometer machine (GENESYS 30 visible

spectrophotometer Thermo Scientific™).

4.3.4.7 Cooking Traits and Shear Force

Samples were thawed overnight at 2 $_o$C, weighed then placed in sandwich bags

and cooked in a water bath set at 85° C to an internal temperature of between 75 °C –

78 °C. A thermometer was inserted in the thickest part of the breast meat to monitor the

temperature. Samples were drained and left to cool down at room temperature for 2

hours, lightly dried with paper towels and the weight was recorded to calculate cook

loss. Thus, cooking time and cook loss were indicated as cooking traits.

Cooked samples were kept in the cold room (2 °C) overnight and tempered to

room temperature (20 °C) for 30 minutes before Warner Bratzler Shear Force (WBSF)

measurement. Five rectangular strips of dimensions 1 cm wide, 1 cm thick and 2-4 cm

long were obtained along the fibre axis. The analyzer was loaded with a 10 kg loading

cell and the weight was calibrated with a 2 kg weight, the return speed was set at 10 mm/sec. The strips were placed with fibres perpendicular to the blades of WBSF analyzer (TA-XT Plus, Texture Technologies) for shredding, two shear values were recorded from each strip.

4.3.4.8 Myofibrillar Fragmentation Index

For myofibrillar fragmentation index analysis (MFI), 4 g of breast meat was minced using a knife in a cold room and suspended in 40 mL of cold MFI buffer (100 mM KCl, 20 mM KPO4, 2 mM MgCl2, 2 mM EGTA, 1 mM NaN3, pH 7.0) and homogenized for 30 seconds (Osterizer 10 speed blender). The homogenate was centrifuged at 1000 x g for 15 minutes at 2 °C (Sorvall™ RC6 plus Thermo Scientific™). The supernatant was discarded, and the pellet and fat cap resuspended in 40 mL cold MFI buffer using a stirring glass rod then centrifuged again. The supernatant and fat cap were discarded, and the pellet resuspended in 10 mL cold MFI buffer and mixed by vortexing. The mixture was poured through a strainer and the suspension obtained for protein estimation using BSA and 4 mL of biuret reagent absorbance was read at 540 nm using a spectrophotometer (GENESYS 30 visible spectrophotometer Thermo Scientific™). The suspension was then diluted to a final volume and concentration of 8 mL and 0.5 mg protein/mL respectively, vortexed and the absorbance read at 540 nm using a spectrophotometer (Thermo Scientific™ GENESYS™ 30 visible spectrophotometer). The MFI value was obtained using the equation MFI = 200*Absorbance (Culler et al., 1978).

4.3.5   Statistical Analysis

This experiment was designed as a completely randomized design and each pen was considered an experimental unit. Therefore, the variation associated with dietary treatment was considered a fixed effect while replicates as a random effect. Statistical analysis of all data was performed using SAS 9.4 (SAS Institute, Cary NC, 2012). The data values were subjected to a normality test by using PROC UNIVARIATE procedure. Using normal plot options and Shapiro–Wilk statistics, outliers were detected and discarded to satisfy the normal distribution assumption. Performance, intestinal parameters and meat quality data were analyzed using the MIXED model procedures of SAS version 9.4 (SAS, 2012). Least squares mean were separated (F test, $P < 0.05$) using the adjusted Tukey–Kramer's multiple comparison test. The degrees of freedom in the denominator was adjusted using the Kenward-Roger procedure. Chi-square analysis (Fisher's exact test) was analyzed by PROC FREQ to analyze the difference in the distribution of severity scores in WS and WB and mortality. Differences were considered significant at $P < 0.05$, and trends ($0.05 \leq P \leq 0.10$) were also presented.

4.4 RESULTS

4.4.1 Growth Performance

There were no differences observed among treatments on performance parameters. The ADG, ADFI and FCR were similar between treatments in all phases and during the whole trial ($P > 0.05$). However, PC tended to have a decrease FCR in grower phase ($P = 0.07$) as shown in **Table 4.3**.

Table 4.3. Effect of non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets on growth performance.

Items[1]	Treatments[2]				SEM[3]	*P*-value
	NC	PC	RDE1	RDE2		
Starter (d 1-14)						
ADG, g	33.32	32.34	34.14	35.18	0.45	0.126
ADFI, g	44.99	46.07	47.98	47.54	0.52	0.128
FCR, g/g	1.35	1.43	1.41	1.35	0.02	0.286
Grower (d 15-28)						
ADG, g	88.18	91.64	88.13	86.93	1.14	0.519
ADFI, g	118.64	119.03	120.21	118.79	1.31	0.977
FCR, g/g	1.35	1.30	1.37	1.37	0.01	0.07
Finisher (d 29-41)						
ADG, g	116.54	118.09	121.69	119.08	2.02	0.849
ADFI, g	185.56	182.63	185.81	192.69	2.43	0.531
FCR, g/g	1.60	1.55	1.54	1.62	0.01	0.115
Whole phase (d 1-41)						
ADG, g	76.57	77.88	78.43	77.46	0.90	0.862
ADFI, g	110.06	108.87	111.63	114.56	1.18	0.203
FCR, g/g	1.44	1.40	1.43	1.48	0.01	0.113
Mortality (%)	3.75	2.5	1.25	1.25	-	0.027

[1]ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio.

4.4.2   Relative Organ Weight and Intestinal Morphology

The feeding treatments did not affect the relative weight of the heart, liver, bursa

and spleen ($P > 0.05$) as observed in **Table 4.4.**

Table 4.4. Effect of non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets on relative organ weight of 45-days old broiler chickens

Items	Treatments[1]				SEM[2]	P-value
	NC	PC	RDE1	RDE2		
Heart	0.42	0.42	0.42	0.45	0.01	0.185
Liver	2.25	2.16	2.21	2.12	0.06	0.81
Bursa	0.15	0.14	0.16	0.14	0.01	0.525
Spleen	0.079	0.084	0.085	0.087	0.01	0.779

1NC, negative control (non-antibiotics diet); PC, positive control (diets with antibiotics); RDE1, diets containing 0.1% red osier dogwood extracts; RDE2, diets containing 0.3% red osier dogwood extracts.
2SEM, Pooled standard error of mean

The CD, VH and VCR of duodenum and ilea were similar across treatments (P

> 0.05). However, at the jejunal level, RDE2 had lower CD (μm) compared to NC ($P <$

0.05). In addition, both RDE treatments presented greater jejunal VCR (μm/μm)

compared to PC and NC ($P < 0.05$) as shown in **Fig. 4.2**.

Figure 4.2. The effect of non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets on the villus height (A), crypt depth (B) and the villus height/crypt depth (C) of broiler chickens at day 45.

Note: NC, negative control (basal diet); PC, positive control (basal diet with 30 ppm avilamycin); RDE1, basal diet with 0.1% red osier dogwood extracts; RDE2, basal diet with 0.3% red osier dogwood extracts. All data reported are treatment means ± standard error. Different letters in the same figure indicate significant differences among treatments ($P < 0.05$).

4.4.3 Apparent Ileal Digestibility

Regarding apparent ileal digestibility, there was no significant difference in AID of dry matter ($P > 0.05$) and crude protein digestibility tended to differ ($P = 0.07$), conversely, RDE2 and PC had increased crude fat digestibility compared to NC and RDE1 ($P < 0.05$, **Fig. 4.3**). The AID of all amino acids was increased in RDE1, RDE2 and PC treatments compared to NC ($P < 0.01$), however, Pro and Ser showed decreased ($P < 0.01$) digestibility in RDE1 treatment compared to RDE2 as observed in **Table 4.5.**
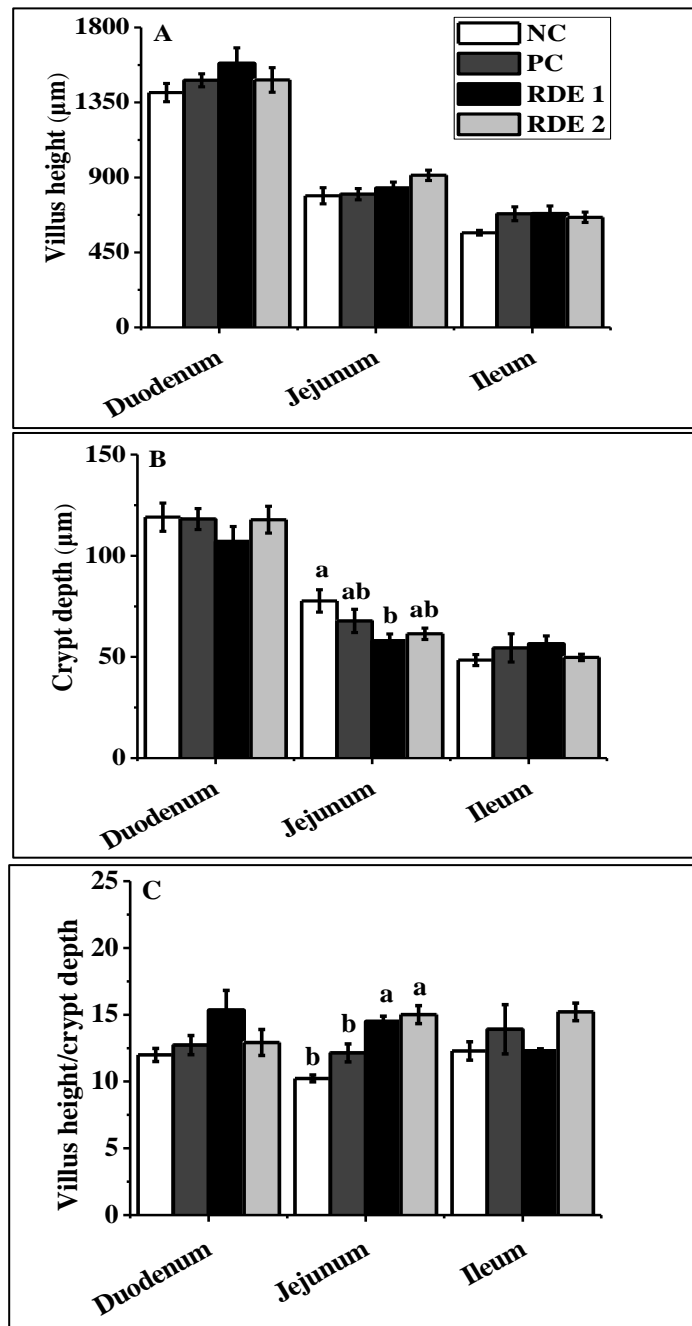
Table 4.5. Effect of non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets on apparent ileal AA digestibility of 45-days old broiler chickens.

Item	Treatments[1]				SEM[2]	P value
	NC	PC	RDE1	RDE2		
Ala	77.53b	85.32a	83.36a	85.60a	1.01	< 0.01
Arg	77.99b	87.65a	86.00a	87.98a	1.10	< 0.01
Asp	73.77b	79.66a	77.25b	81.51a	1.02	< 0.01
Cys	69.64b	79.81a	76.97a	79.31a	1.42	< 0.01
Glu	81.94b	87.58a	86.29a	87.67a	0.83	< 0.01
Gly	70.68b	78.28a	75.38b	80.05a	1.18	< 0.01
His	53.45b	64.26a	60.65ab	63.05a	1.34	< 0.01
Ile	61.11b	81.33a	79.52a	83.87a	1.96	< 0.01
Leu	77.61b	85.96a	85.06a	86.43a	1.05	< 0.01
Lys	81.24b	88.35a	84.22b	86.55a	0.94	< 0.01
Met	89.28b	92.40a	91.90a	93.50a	0.51	< 0.01
Phe	75.47b	85.64a	83.28a	86.04a	1.16	< 0.01
Pro	79.23c	84.19ab	82.83bc	84.58a	0.82	< 0.01
Ser	75.10c	81.33ab	78.22bc	82.14a	1.01	< 0.01
Thr	65.45b	76.97a	74.28a	78.99a	1.17	< 0.01
Trp	77.87b	81.32ab	83.25a	80.77ab	0.84	< 0.01
Tyr	72.33b	85.18a	83.11a	87.00a	1.40	< 0.01
Val	61.62b	79.99a	77.70a	82.02a	1.79	< 0.01

Table 4.6. Chemical composition and amino acid profile of finisher diet (%).

Items	NC	PC	RDE1	RDE2
Dry matter	90.36	89.61	89.64	89.71
Crude protein	19.69	19.60	19.61	19.64
Crude Fat	6.85	6.78	6.81	6.76
Arginine	1.09	1.08	1.09	1.08
Histidine	0.56	0.54	0.53	0.55
Isoleucine	0.63	0.63	0.64	0.69
Leucine	1.64	1.64	1.63	1.64
Lysine	1.23	1.22	1.20	1.22
Methionine	0.61	0.61	0.61	0.61
Phenylalanine	0.80	0.82	0.82	0.82
Threonine	0.68	0.67	0.67	0.69
Tryptophan	0.19	0.18	0.18	0.18
Valine	0.76	0.74	0.77	0.75
Alanine	1.19	1.17	1.19	1.18
Aspartic acid	1.56	1.55	1.55	1.60
Cysteine	0.32	0.33	0.36	0.33
Glutamic acid	3.13	3.19	3.11	3.13
Glycine	0.64	0.65	0.65	0.65
Proline	1.12	1.12	1.13	1.13
Serine	0.92	0.93	0.95	0.94
Tyrosine	0.59	0.58	0.58	0.60

Figure 4.3. The effect of non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets on the apparent ileal digestibility.

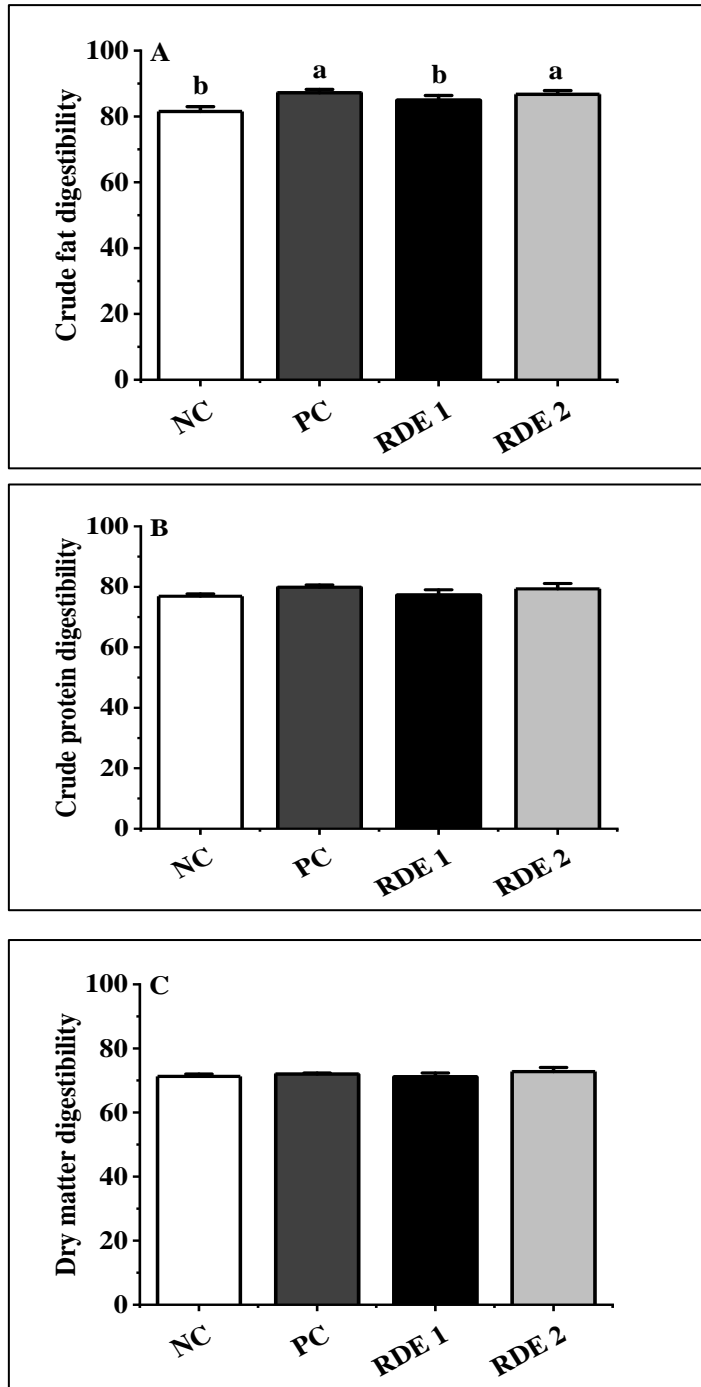**Note:** Crude fat (A), crude protein (B) and dry matter (C) of broiler chickens at day 45. NC, negative control (basal diet); PC, positive control (basal diet with 30 ppm avilamycin); RDE1, basal diet with 0.1% red osier dogwood extracts; RDE2, basal diet with 0.3% red osier dogwood extracts. All data reported are treatment means ± standard error. Different letters in the same figure indicate significant differences among treatments ($P < 0.05$).

4.4.4 Gene Expression

Regarding mRNA expression, RDE2 presented an increased abundance of nutrient transporters PepT1 and B$_0$AT1 compared to PC and RDE1 ($P < 0.05$); while RDE1, RDE2 and PC treatments showed reduced expression levels in CAT1 than NC ($P < 0.05$). No significant differences were observed in mRNA abundance of EAAC1, xCT1 and SGLT1 ($P > 0.05$, **Fig. 4.4**).

On tight junction proteins gene expression, RDE1, RDE2 and PC treatments showed decreased ZO-1 and CDH1 abundance compared to NC ($P < 0.05$). However, no differences were observed in mRNA abundance of CLDN1 among treatments ($P > 0.05$, **Fig. 4.5**).

Figure 4.4. The effect of **non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets** on mRNA abundance of nutrient transporters.

Note: B₀AT1 (A), CAT1 (B); EAAC1 (C); PepT1 (D); SGLT1 (E) and XCT (F) gene expression of broiler chickens at day 45. NC, negative control (basal diet); PC, positive control (basal diet with 30 ppm avilamycin); RDE1, basal diet with 0.1% red osier dogwood extracts; RDE2, basal diet with 0.3% red osier dogwood extracts. All data reported are treatment means ± standard error. Different letters in the same figure indicate significant differences among treatments ($P < 0.05$).

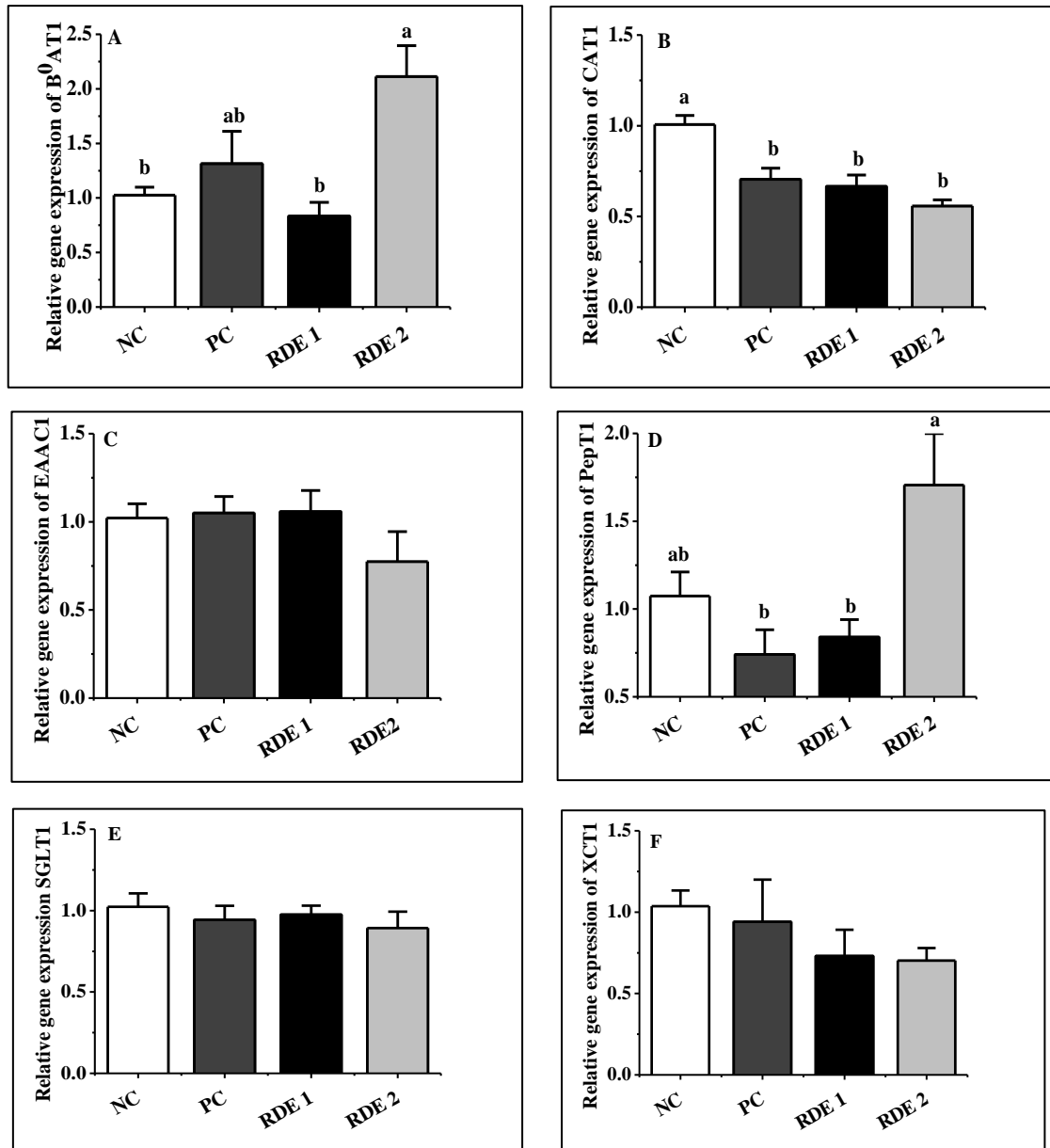**Figure 4.5. The effect non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets on tight junction proteins genes.**

Note: Z0-1 (A), CDH1 (B) and CLDN1 (C) of broiler chickens at day 45. NC, negative control (basal diet); PC, positive control (basal diet with 30 ppm avilamycin); RDE1, basal diet with 0.1% red osier dogwood extract; RDE2, basal diet with 0.3% red osier

dogwood extract. All data reported are treatment means ± standard error. Different letters in the same figure indicates significant differences among treatments ($P < 0.05$).
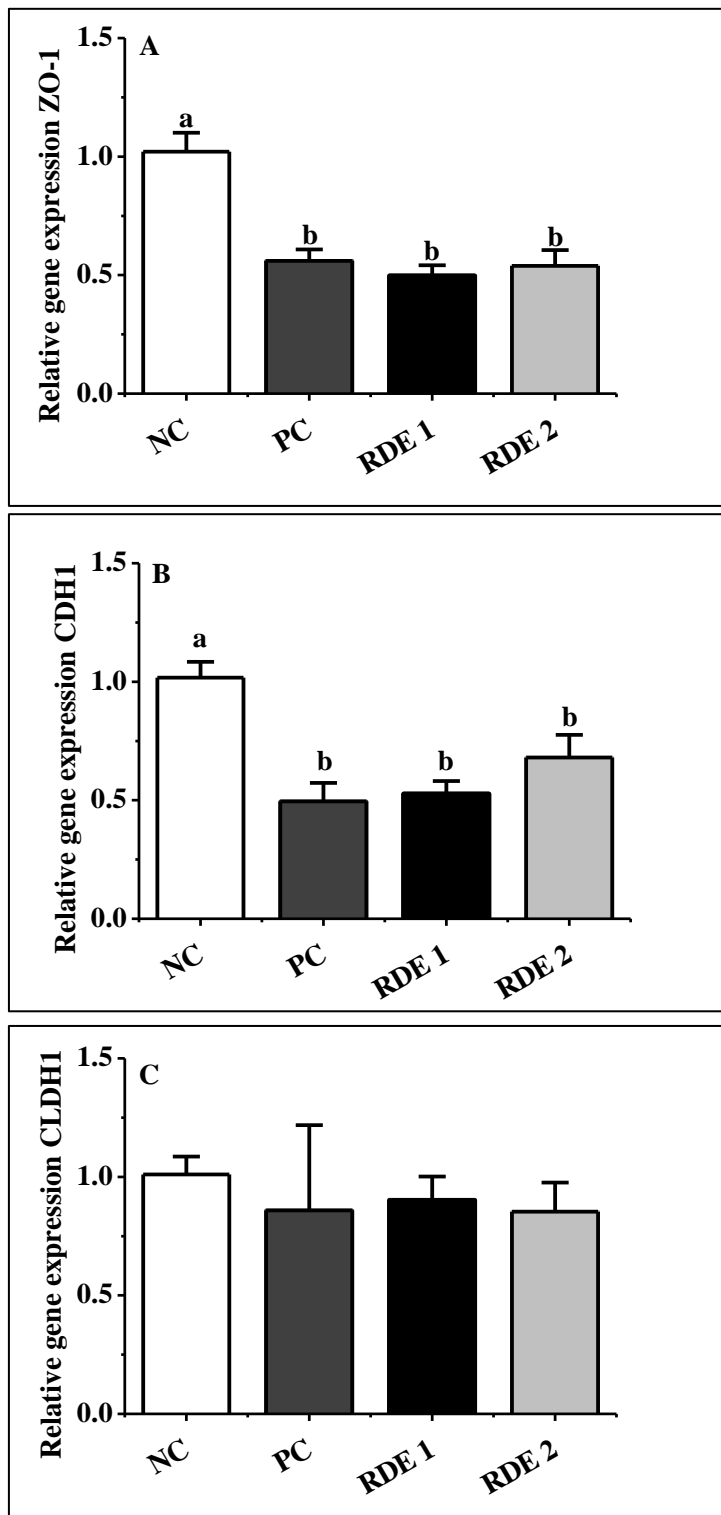
4.4.5   Intestinal Microbiota

The relative abundance of the dominant phyla and genera are shown in **Fig. 4.6**. Firmicutes, Bacteroidetes and Proteobacteria were the dominant phyla. In the ileum, RDE2 showed a tendency to have greater Proteobacteria abundance when compared to PC ($P = 0.08$) and significantly increased abundance of *Turibacter* spp. when compared to all other treatments ($P < 0.05$). PC tended to have an increased abundance of unidentified genus of family *Christensenallacea* compared to all other treatments ($P = 0.07$). In the cecum, PC had increased abundance in unidentified genus compared to NC and RDE1 ($P < 0.05$) and genus *Oscillospira* showed a tendency to increase in RDE2 ($P = 0.09$).

On analysis of composition, treatments had different relative abundance in taxa (Bacteroidetes, Cyanobacteria *Lachnospiraceae, Ruminococacea* and *Clostridiacea*, *Turicibacter, Oscillospira).* Ileal bacterial composition was altered by treatments in the order level (Turicibacterales, Bacillales and Clostridiales) and species level.

Treatments did not influence within community diversity, richness and evenness at both ileum and cecum ($P > 0.05$).

On beta diversity, there was a highly significant spatial effect (between the ileum and cecum digesta) on bacterial communities observed on all indexes tested ($P = 0.001$, **Fig 4.7**.) In the cecum, PC and RDE2 had a significant difference in the unweighted UniFrac index ($P < 0.05$) and showed a tendency to differ in the Jaccard

index ($P = 0.09$). In the ileum, there was a significant difference between treatments RDE2 with PC, NC and RDE1 ($P < 0.05$) on the Bray Curtis dissimilarity and Jaccard indexes. RDE2 also tended to differ with PC, NC and RDE1 on the unweighted and weighted UniFrac indexes ($P = 0.1$).
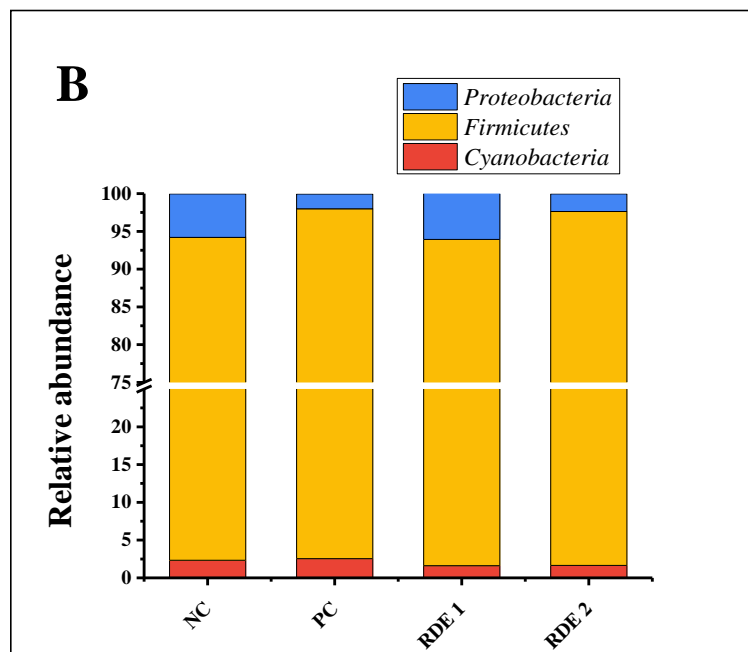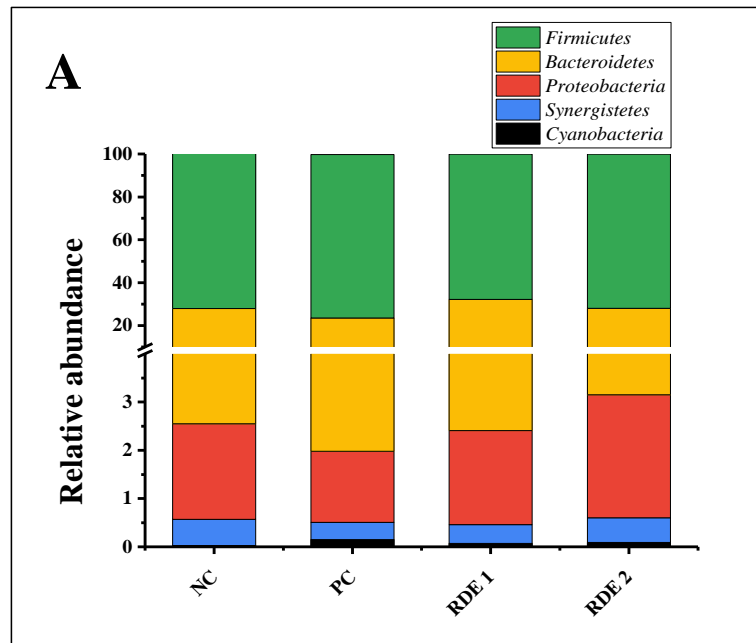
Figure 4.6. Relative abundances (%) of bacterial phyla of intestinal digesta in non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets.

Note: Cecum digesta (A) and ileum digesta (B) of 45 days old broiler chickens. NC, negative control (basal diet); PC, positive control (basal diet with 30 ppm avilamycin); RDE1, a basal diet with 0.1% red osier dogwood extract; RDE2, a basal diet with 0.3% red osier dogwood extract. All data reported are treatment means.
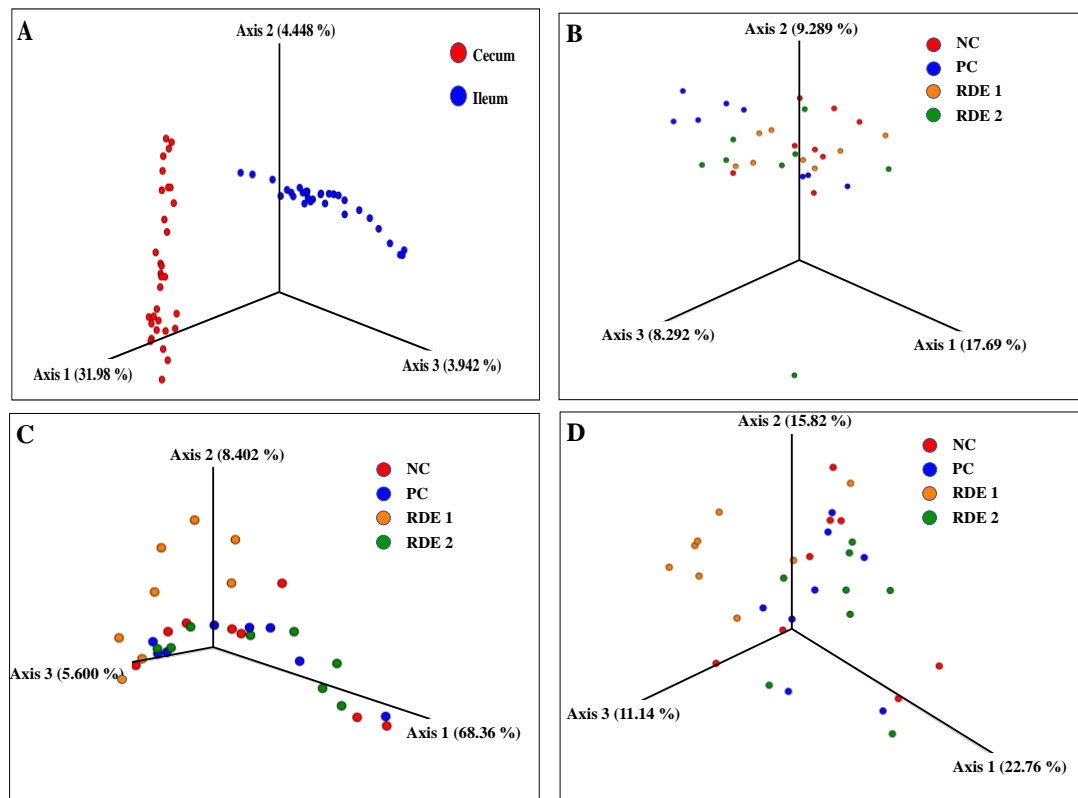
Figure 4.7. PCoA plots showing beta diversity distribution of non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets.

Note: Cecum digesta and ileum digesta of 45 days old broiler chickens Based on Bray–Curtis dissimilarity of ileum (blue) and cecum (red) samples based (A), unweighted UniFrac of cecum samples (B), Bray–Curtis dissimilarity of ileum samples diversity based on (C) and 3D plot of ileum samples diversity based on Jaccard index (D). NC, negative control (basal diet); PC, positive control (basal diet with 30 ppm avilamycin); RDE1, a basal diet with 0.1% red osier dogwood extract; RDE2, a basal diet with 0.3% red osier dogwood extract.

4.4.6 White Striping and Woody Breast Incidences

There was no significant difference among treatment on WS and WB scores and

their incidence on chicken breast (**Table 4.6**, $P > 0.05$).

Table 4.7. Effect of non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets on WS and WB incidence in breast meat of 46-days old broiler chickens

Items[1]	Treatments[2]				SEM[3]	P-value
	NC	PC	RDE1	RDE2		
WS scores	0.19	0.32	0.24	0.21	0.05	0.42
White striping % (n)						
Normal	95.24 (20)	90.91(20)	96.55(28)	100 (28)	-	0.43
Moderate	4.76 (1)	9.09 (2)	3.45 (1)	0.00 (0)	-	
WB score	0.08	0.27	0.28	0.14	0.07	0.20
Woody breast % (n)						
Normal	100(21)	86.30(19)	89.66(26)	96.43(27)	-	0.39
Mild	0.00(0)	13.64(3)	6.90(2)	3.57(1)	-	
Moderate	0.00(0)	0.00(0)	3.45(1)	0.00(0)	-	
Myopathy % n	4.76 (1)	13.64 (5)	10.34 (4)	3.57 (1)	-	0.57

[1]WS score, white striping score; WB score, woody breast score; n, counts; myopathy, either WS or WB present

[2]NC, negative control (non-antibiotics diet); PC, positive control (diets with antibiotics); RDE1, diets containing 0.1% red osier dogwood; RDE2, diets containing 0.3% red osier dogwood.

[3]SEM, Pooled standard error of mean

4.4.7 Meat Quality

Meat quality attributes such as lightness, yellowness, pH, drip loss, cook loss, WBSF, TBARS, and MFI were not affected by the feeding treatments ($P > 0.05$). RDE2 presented reduced redness values compared to NC (**Table 4.7**, $P < 0.05$); while NC and RDE1 treatments presented intermediate values ($P > 0.05$).

Table 4.8. Effect of non-supplemented, red-osier dogwood extracts and antibiotic supplemented diets on breast meat quality of 46-days old broiler chickens

Items[1]	Treatments[2]				SEM[3]	P-value
	NC	PC	RDE1	RDE2		
pH	5.78	5.9	5.79	5.91	0.05	0.51
Purge loss (%)	1.34	1.26	1.09	0.8	0.35	0.52
L*	58.68	59.86	59.3	60	0.57	0.33
a*	11.52 a	10.91ab	10.91ab	10.50b	0.24	0.04
b*	18.7	19.07	17.46	18.57	0.46	0.11
Cooking loss (g)	31.46	34.82	31.39	33.07	2.27	0.53
Cooking loss (%)	23.11	23.63	22.81	22.68	0.66	0.75
Cooking time (min)	37.62	38.13	36.31	36.95	1.32	0.43
WBSF (kg)	1.59	1.34	1.89	1.72	0.16	0.10
TBARS	0.165	0.172	0.127	0.169	0.22	0.67
MFI	45.71	46.4	45.24	46.92	0.89	0.99

[a, b] Different letters within the same row indicates significant difference
[1] L*, lightness; a*, redness; b*, yellowness; WBSF, Warner-Bratzler Shear Force; TBARS, thiobarbituric acid reactive substances
[2] NC, negative control (non-antibiotics diet); PC, positive control (diets with antibiotics); RDE1, diets containing 0.1% red osier dogwood; RDE2, diets containing 0.3% red osier dogwood.
[3] SEM, Pooled standard error of mean

4.5 DISCUSSION

Red dogwood extracts are a mixture of polyphenolic compounds extracted from the red osier dogwood plant. The extracts are rich in rutin, quercetin, kaempferol, gallic and ellagic acids with highest concentrations reported at 220 mg/g (Obomsawin, 2001; Bate-Smith et al., 1975; Isaak et al., 2013).

In this study, the results showed no differences in growth performance among treatment groups. Similar results on BW and feed intake have been reported in studies using other kinds of plant extracts such as grape seed extracts, a strong antioxidant due to the high concentration of polyphenolic compounds, at concentrations between 125 - 2000 ppm and dietary rosemary leaves, an aromatic plant with high antioxidant activity due to the presence of phenolic compounds, at 8600 ppm and 11500 ppm respectively (Yesilbag et al., 2011; Farahat et al., 2017). In contrast, studies have reported improvements in performance parameters such as weight gain and feed intake in 21-day old broilers after supplementation with 5000 ppm *Labiatae* extracts and a 100 ppm mixture of carvacrol, cinnamaldehyde and capsicum oleoresin which have compounds with antimicrobial activity (Hernandez et al., 2004; Bravo et al., 2014; Pirgozliev et al., 2014). In addition, Goliomytis et al., (2014) observed a reduction in the feed conversion ratio of broiler chickens fed with a quercetin supplemented diet. This contrast in performance and the lack of a statistically significant difference may be attributed to a combination of factors such as nutrition, management and hygiene, in the current study, this is highlighted by similar performance between antibiotic- free (NC) and antibiotic-

supplemented (PC) groups. It is important to note that PC had decreased FCR and increased ADG when compared to NC although the difference was not statistically significant. The experimental facility and environment may not have presented a challenging condition to the broilers making it difficult to observe big differences in performance. Research has shown that immunological and environmental stress increases the likelihood of showing beneficial effects of plant extracts, in this study all the broilers were in good health (Allen et al., 1997).

In the current study, the treatment RDE2 showed reduced mortality rate during the whole experimental period (1.25%), which is in agreement with previous studies using 5000 ppm *Labiatae* extracts, 8000 ppm amaranth seeds and 3000 ppm sea buckthorn and 3000 ppm chokeberry pomace that improved livability to 100%. Amaranth seed and choke berry pomace are rich in antioxidant compounds, amaranth contains, flavonoids such as quercetin, tocopherols and squalene while choke berry pomace 10.58g /100g of phenolic compounds (Hernandez et al., 2004; Orczewska-Dudek et al., 2018). The bioactive compounds present in plants are thought to have an immunomodulatory effect by preventing inflammation, improving oxidative stability and exerting antibacterial activity (Mishra et al., 2008; Orczewska-Dudek et al., 2018). The antioxidative effect of polyphenolic compounds such as rutin and quercetin present in RDE may be the mechanism by which plant extracts reduced mortality in broiler chickens. However, this mechanism cannot be confirmed in the current study as no

differences were observed in relative organ weights of immune-related organs such as liver, bursa and spleen and the oxidative status of the broilers was not assessed.

The VH, CD and VCR are morphological parameters that indicate the digestive and absorptive capacity of the small intestines (Mekbungwan et al., 2002). An increase in the VH length increases the digestive surface and a decrease in CD is an indicator of improved gut health due to less shedding at the villi tips thus reducing enterocytes cell turnover and CD (Nabuurs et al., 1993; Iji et al., 2001). The decrease in jejunal CD and increase in VCR in the present study was also observed by previous studies utilizing 50-100 ppm of gallic acid and 500 ppm and 1000 ppm of elecampane rhizome extracts rich in rutin and gallic-acid equivalents in broiler chickens (Samuel et al., 2017; Abolfathi et al., 2019). Intestinal morphology is linked with intestinal health and has been associated with intestinal microbiota balance and gut oxidative status of broiler chickens (Wu et al., 2016; Abolfathi et al., 2019). An increase in VCR and decrease in crypt depth may be a result of the anti-inflammatory and antioxidative effect of polyphenolic compounds in RDE that exert a protective effect on intestinal enterocytes. Polyphenols from olive mill wastewater have been reported to have protective effects on intestinal enterocytes by exerting anti-inflammatory and antioxidant activities (Sabino et al., 2018). Additionally, polyphenolic compounds such as ellagitannins have been reported to increase cell proliferation by 20% by increasing the cell numbers at each stage of mitotic cycle (Losso et al., 2004). Pathogens and their toxic metabolites can cause damage to intestinal enterocytes resulting in cell death and increased villus

shedding thereby reducing VH and increasing CD (Oviedo-Rondón, 2019). Plant extracts can reduce pathogenic bacteria in the gut such as *Escherichia coli* and *Clostridium perfringens* by increasing the population of beneficial bacteria (for instance, *Lactobacillus* spp.) resulting in competitive exclusion of bacterial pathogens and direct bactericidal or bacteriostatic effects on pathogens (Barbieri et al., 2017; Giannenas et al., 2018; Abolfathi et al., 2019). Therefore, the improvement of VCR and decreased CD in the current study may be due to a combination of factors such as the balance of intestinal microbiota, proliferative effects and protective effects on the intestinal environment brought about by the antimicrobial, antioxidative and anti-inflammatory effects of polyphenolic compounds in RDE.

Apparent ileal CP and DM digestibility did not differ among treatment groups in the current study in agreement with previous researches on 1500 - 2500 ppm of natural chestnut rich in hydrolysable tannins and 15000 - 60000ppm of polyphenol-rich grape pomace extracts (Brenes et al., 2008; Schiavone et al., 2008). However, greater apparent ileal amino acid digestibility observed in supplemented groups was similar to amino acid digestibility of 21 and 41-days old broiler chickens in previous studies utilizing quercetin and kaempferol rich *Gingko biloba* leaves and leaves extracts with significant differences observed at the lowest concentrations of 2000 ppm and 4000 ppm respectively (Ren et al., 2018). Digestive functions such as enzyme activities and digestive surface can affect the digestibility of nutrients. (Kamel, 2001; Jamroz et al., 2005) reported the ability of phytogenic feed additives to increase enzyme production

in brush borders and increase liver lipase activity. Although the enzyme activity was not investigated in this study, we investigated and observed improved intestinal health and improved morphology observed by higher VCR that translated to greater digestibility of amino acids. The increased digestibility of crude fat in this study may be explained by the activity of plants and plant extracts on enzymes and microbiota. Hashemipour et al. 2013; Platel and Srinivasan (2001) reported that herbs spices and essential oils can stimulate the secretion of bile acids and increase the activity of pancreatic and brush border enzymes. In addition, increased microbial populations are reported to reduce fat digestibility due to their hydrolytic action on bile acids (Smith et al., 1998). Therefore, the increased crude fat digestibility may also be attributed to the antimicrobial action of RDE on intestinal microbiota. However, despite the increased AID of amino acid and crude fat, this effect was not translated to improved growth performance this might be due to the low concentration of crude fat (4.5%) supplied in the diet making the difference in digestibility insufficient to impact performance (Bravo et al., 2014). Further, although there was an increase in the AID of amino acids, this effect was not observed in growth performance due to the imbalance of amino acids in the diet supplied by crude protein or possibly a reduction in nitrogen retention brought about by increased catabolism and excretion of amino acids and phenolic-amino acid conjugates absorbed (Morris et al., 1999; Oduguwa et al., 2007).

Amino acid absorption in the small intestines can take place as either free form or as a di- or tri-peptide. PepT1 transporter is a proton-dependent transporter and is the

only known intestinal peptide transporter with broad specificity. B_0AT1 is a neutral amino acid transporter while CAT1 is cationic amino acid transporter. B_0AT1 and CAT1 (basolateral amino acid transporter) are responsible for the transportation of methionine and threonine, lysine and arginine respectively (Hatzoglou et al., 2004; Poncet and Taylor 2013). Nutrient transporter gene expression can be altered by phytogenic feed additives containing phenolic plant extracts although the research is scarce (Santos et al., 2019). In this study, amino acid transporters PepT1 and B_0AT1 were highly expressed in RDE treated groups while CAT1 was decreased in expression compared to the control group. To our knowledge, this study is the first published report of its kind evaluating plant extracts effects on nutrient transporters in broiler chickens under non-challenge conditions, therefore, the mechanism of action is not clear. Previous studies have reported that the up-regulation of nutrient transporters is correlated to increased levels of circulating amino acids and improved amino acid digestibility (Yu et al., 2017; Osmanyan et al. 2018). Therefore, the observed improvement in digestive surface and amino acid digestibility (see discussions above) may have contributed to the up-regulation of amino acid transporters in response to an increase in amino acid digestibility and concentration. It is well known that overabundance of cationic amino acids in enterocytes can cause a downregulation of CAT1 in order to maintain homeostatic levels of plasma amino acid concentrations (Hatzoglou et al., 2004). In this study, the observed decrease in mRNA levels of CAT1

in jejunum may have been as a result of increased amino acid concentration in the enterocytes correlated with an upregulation of PepT1 transporter gene.

The intestinal lumen barrier is composed of tight junction proteins from the occludin (**ZO**-1, **OCLN** 2, 3) and claudin (**CLDN**) families. This segment allows selective absorption and prevents pathogens and toxins from passing through to the blood (Anderson and Van Itallie, 2009). CDH1 and ZO-1 mRNA levels were decreased in RDE treated groups relative to NC, but expression levels were similar to PC similar to previous studies using *Allium hookeri* and carvacrol essential oils (Lee et al., 2017; Liu et al., 2018). An upregulation of barrier function protein is a potential indicator of increased cellular damage and repair. Challenge studies in broilers and piglets treated with plant extracts observed an upregulation in tight junction proteins mRNA expression. This upregulation was concluded to be a repair mechanism after intestinal barrier was destroyed by pathogens and toxins (Liu et al., 2014; Varasteh et al., 2015; Wei et al., 2017). Improved intestinal health is a result of reduced cell damage due to a reduction of pathogens, toxins and pro-oxidants (Santos et al., 2019). The low expression in the tight junction protein in RDE treated groups in the current study may be a resultant effect of the anti-inflammatory, antioxidative and antimicrobial effects that promote optimal intestinal health.

Microbiota of the broiler intestinal segments differ in species richness, diversity and structure with the cecum showing the highest abundance and most diverse communities (Shang et al., 2018). In this study, the cecum had a greater number of

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observed OTUs with 7 phyla identified at > 0.1% relative abundance. Firmicutes, Proteobacteria and Bacteroidetes were the three most dominant phyla and members of genus *Oscillospira* and *Ruminococcus* were the dominant species at genera level. The observed difference between the ileum and cecum microbiota richness and diversity is similar to previous studies using green tea, mulberry extracts and avilamycin (Choi et al. 2018; Chen et al. 2019). The cecum has been reported to be more diverse than the ileum having concentrations of up to 10_{12} CFU/mL of cultivable bacteria in contrast to the ileum's $10_8 – 10_9$ CFU/mL (Wielen et al., 2002; Witzig et al., 2015). In contrast to the cecum microbiota, the dominant phyla in the ileum were Firmicutes, Proteobacteria and Cyanobacteria with mean values of relative abundance ranging between 91% - 96% for Firmicutes and 2% - 6% for Proteobacteria. The increase in Firmicutes to Bacteroidetes ratio (**F/B**) in RDE2 in this study is similar to results reported in previous studies utilizing a commercial plant extract with carvacrol and thymol and blueberry and blackberry pomace (Salaheen et al., 2017; Zhu et al., 2019). A greater F/B ratio is correlated to improved performance in the body weight and energy efficiency of broilers (Singh et al., 2012). In this study, the observed increase in the F/B ratio was attributed to the decrease in Bacteroidetes in the ileum of broiler chickens treated with RDE2.

Microbiota studies in broiler chickens using plant extracts have largely focused on microbial counts of beneficial and pathogenic bacteria (Varmuzova et al., 2015; Abolfathi et al., 2019), therefore, research on plant extracts effects on the microbial

diversity, structure and composition of intestinal microbiota is limited. The objective of this study was to evaluate whether dietary supplementation with RDE can alter the diversity and composition of ileum and cecum luminal microbiota. Similar to previous studies conducted on cecum contents using grape pomace supplementation and avilamycin treatments the plant extracts and antibiotics did not alter the relative abundance or community diversity and richness of microbiota (Choi et al., 2018; Herrero-Encinas et al., 2020). However, the abundance of *Oscillospira* species was increased in broilers supplemented by RDE. *Oscillospira* is involved in the production of butyrate a fatty acid that improves intestinal morphology and immunity (Biasato et al., 2018). In contrast, tannin supplementation altered luminal cecum microbiota by an observed increase in the members of *Ruminococcaceae* and *Lachnospiraceae* families (Díaz Carrasco et al., 2018). Additionally, Bacitracin and virginiamycin were observed to have a significant impact on cecal bacterial composition (Lu et al., 2008; Pourabedin et al., 2015). Growth promoters have been described to have a major impact on the foregut microbial communities compared to the hindgut due to the relative stability of microbiota in the ceca as confirmed by this study (Betancourt et al., 2014; Huang et al., 2018).

In this study, members of bacterial taxa Turicibacterales, Bacillales and Clostridiales of Firmicutes phylum were increased by treatment in the ileum. Unsurprisingly, NC tended to have the highest abundance of *Staphylococcus* species although the numbers observed were low. Genus *Staphylococcus* contains pathogens

such as *Staphylococcus aureus*, a pathogen in broilers that causes depressed performance (Devriese, 1980). *Turicibacter* was reported to affect broiler physiology and feeding behaviour in broilers with restricted feed intake or challenged with *Salmonella* (Oh et al., 2017; Siegerstetter et al., 2017). Members of this taxa have been reported to deconjugate primary bile acids and are decreased in the increased concentration of bile acids in mice (Kemis et al., 2013). In the current study, the ileum of RDE treated broilers had increased abundance in members of the genus *Turicibacter*.

The occurrence of white striping and woody breast affect consumer preferences, nutrient composition and further processing abilities of meat (Mudalal, et al., 2015; Petracci et al., 2014). WS and WB are caused by different factors that trigger hypoxic conditions and affects muscle metabolic processes of the TCA cycle, fatty acid oxidation, arginine and taurine metabolism contributing to oxidative stress and inflammation (Boerboom et al., 2018). Previous studies have demonstrated the ability of rutin and RDE to decrease LPS-induced inflammation *in vitro* by preventing the production of IL-8 and the expression of pro-inflammatory cytokines in Caco- 2 and C2C12 cells that initiate inflammatory reactions (Jiang et al., 2019; Liu et al., 2019). In addition, RDE has been reported to have the antioxidative ability *in vitro* by upregulating expression of hemeoxygenase-1 (**HO-1**), superoxide dismutase (**SOD**), and glutathione peroxidase (**GSH-Px**) enzymes in Caco-2 cells involved in endogenous antioxidant activity effectively mitigating oxidative stress (Yang et al., 2019). These results suggest that the supplementation of the antioxidant compound rutin or rutin-

containing plant extracts (e.g., RDE) may present a promising approach to control WS and WB in broiler chickens. However, in the current study, no statistically significant differences among treatment mean scores of WS and WB and their incidence of chicken breast were observed. However, the supplementation of RDE tended to show numerical reductions in their incidence. These numerical reductions may be associated with the antioxidative and anti-inflammatory activity of RDE. Another potential cause of WS and WB is a reduction in protein synthesis rate and an increase in protein breakdown rate and lipidosis are associated with oxidative stress and inflammation (Vignale et al., 2016; Baldi et al., 2017). Future studies are needed to investigate the effects of RDE on nutrient concentration such as nitrogen retention, protein and lipid profiles, oxidative stress and inflammation in breast muscle of broiler chickens.

Several studies have indicated no significant effects of plant extracts treatments namely cinnamon oil at 0.5 ppm to 1.0 ppm and commercial phytogenic compounds including a bacteriostatic herbal growth promotor at 5500 ppm on meat quality traits of broiler breast meat such as pH and lipid oxidation (Symeon et al., 2014; Orlowski et al., 2018), thyme essential oils at 500 ppm on cook loss, and 3000 ppm of antioxidant-rich garlic powder on shear force values (Choi et al., 2010; Alfaig et al., 2013) similar to the results observed in the current study. Regarding objective color traits, Young et al. (2003) and Wang et al. (2016) reported an increase in redness and yellowness values with oregano at 3000 ppm and marigold extracts at a concentration of 750 ppm respectively while Yesilbag et al. (2011) reported no differences in objective color

values using rosemary leaves supplementation with increasing concentrations of up to 11,500 ppm. However, in the current study, the 0.3% RDE supplementation treatment reduced redness values but there was no difference in other color traits. These differences in results may be due to different plant extracts used in the experiments, marigold and oregano contains lutein and other pigment-containing carotenoids while RDE has flavonoids and other phenolic compounds. Additionally, no differences were observed between redness values between the NC and 0.1 % RDE treatments. The results suggest that 0.1 % of RDE did not affect meat quality characteristics. It is important to mention that *in vivo* studies investigating plant extracts effects on meat quality largely concentrate on essential oil and known herbs and spices used to treat or marinate meat. For this reason, the studies discussed above may have similar antioxidative effects to RDE although the bioactive compounds differ.

In conclusion, the supplementation of RDE improved the digestibility of crude fat and amino acids, intestinal morphology and the mRNA levels of nutrient transporter in the present study. Additionally, RDE altered the ileum and cecum luminal microbiota composition and diversity between communities. Although RDE did not significantly reduce the incidence of WS and WB, the supplementation of RDE tended to show a numerically reduction in their incidence. Moreover, the supplementation of RDE reduced the mortality rate and produced a limited effect on meat quality. Therefore, RDE may be used as a potential alternative to in-feed antibiotics in broiler chickens in combination with other strategies such as nutrition, management and hygeine.
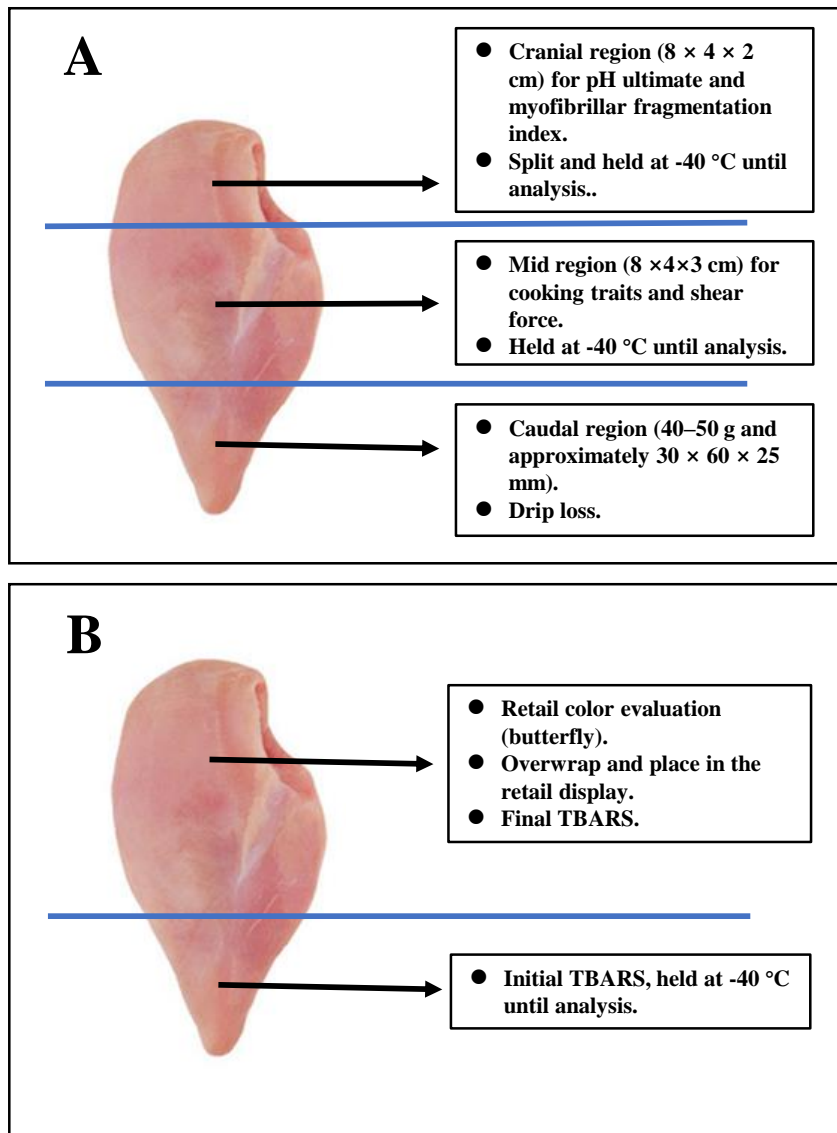
Figure 4.8 (A and B) Subsampling of alternating left and right breast fillet for physical and chemical analysis of meat quality.

5.0 CHAPTER 5 GENERAL DISCUSSION AND CONCLUSIONS

5.1 GENERAL DISCUSSION

The use of antibiotics in the production of broiler chickens is aimed at improving the efficiency of production with a focus on reducing inputs while maximizing output. Antibiotics have been utilized with significant improvements in body weight, feed conversion ratio (Waldroup et al., 1986; Kumar et al., 2018) and meat yield recorded (Singh et al., 2008). However, recent legislation restricts antibiotic use in livestock production necessitates the search for antibiotic alternatives such as the aqueous extracts of the red osier dogwood plant.

In the current study, the aqueous extracts from the red osier dogwood plant were used as a replacement for in-feed antibiotics in broiler chickens' production in day-old chicks from the starter phase to market. The study compared antibiotic-free and antibiotic supplemented (avilamycin) controls with low and high levels of RDE supplemented groups. Interestingly, RDE supplemented groups had an observable decrease in the mortality rate when compared to avilamycin treated group. While the antibiotic may have improved the immunity and health of the broiler, there were increased cull rates in this group due to leg problems. Fast-growing broilers have been observed to have problems with the shank as the bone is not able to support all the weight of the broiler chicken (Kapell et al., 2012). With this as a preliminary study on performance parameters, it would be beneficial to further investigate the effect of the extracts on production performance under an immunological or environmental or pathogen challenge.

Intestinal morphology, nutrient digestibility and transporter studies demonstrate that RDE can improve the digestion and absorption capacity of the broiler. The rate of nutrient disappearance from the intestinal lumen was observed to be greater in RDE groups as the

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expression of nutrient transporters and the digestibility of crude fat and amino acid was highest in these groups. In addition, the increase in VCR indicated that RDE optimized the digestive and absorptive function of intestines in the broiler. Further analysis should be done to evaluate RDE effects on the activity and affinity of digestive enzymes in order to better understand nutrient digestion by this extract. Digestibility should also be analyzed at each phase to understand whether the treatments alter digestibility depending on age. Furthermore, research on the mode of action by which RDE increased VCR and nutrient transporters is required too. Gut barrier function can be further investigated using techniques such as western blotting to determine the protein abundance of tight junction proteins and Ussing chamber to measure gut permeability. Intestinal microbiota in the broiler gut is important to animal production, gut health and overall immunity. The microbial diversity and composition and its alteration have a direct and indirect impact on the intestinal morphological structure, nutrient digestion and absorption, energy efficiency and reduction of pathogenic bacteria population (Diàz Carrasco et al., 2019). The increase in F/B ratio observed in RDE treatments may be one of the explanations for increased digestion of amino acids and crude fat observed. Additionally, the improvement in intestinal morphology observed may be linked to the increase in the abundance of butyrate-producing *Oscillospira* species in addition to a greater F/B ratio. The interrelationships between intestinal microbiota and function can be confirmed by further studies involving metagenomics to elucidate functional differences brought about by alterations in the microbiota composition and diversity.

Meat quality affects the monetary value of meat due to its impact on consumer preference (Petracci et al. 2014). The quality of meat is affected by various pre and post-mortem factors. Pre-mortem factors such as diet have been previously manipulated to alter the quality of meat. The fast growth attributed to the use of AGP in diets has been linked to be the main cause of WS and WB incidence (Kuttappan et al. 2013). Red color was affected by RDE; however, appearance of the product should be evaluated by consumer to determine their acceptability. In this study RDE had no effect of physicochemical parameters of meat, additionally, the treatment reduced the number of broiler breast fillet with WS and WB incidence. As this is the first study evaluating RDE effects on meat quality, future research should be conducted to determine whether these results can be replicated in commercial conditions and determine. It is important to further evaluate the nutrient composition of the meat to determine nutrient composition and polyphenolic content of the meat. Additionally, the metabolic form of polyphenolic compounds should be investigated as this can further explain their bioavailability and mechanism of action. Further, a sensory evaluation can be done to evaluate whether RDE alters the taste of broiler meat. A study can also be conducted to determine the effect of RDE on meat quality when extracts are applied directly into the meat (injection enhancement) to meat.

5.2 GENERAL CONCLUSION

In conclusion, dietary inclusion of RDE had no effects on the growth performance and relative organ weight of broiler chickens. However, the RDE supplementation had several beneficial effects including reduced mortality rate, increased diversity of ileal and cecal

microbiota, increased ileal digestibility of crude fat and amino acids, enhanced mRNA expression of tight junction proteins and nutrient transporters, and improved villus: crypt ratio of jejunum. Moreover, the RDE supplementation had no effect on the incidence of WS/WB and meat quality. Based on the results obtained in this research, it is concluded that RDE supplementation can have some positive influences on birds and can be used to replace in-feed antibiotics in broiler chickens.

6.0   CHAPTER 6 FUTURE DIRECTIONS

Future directions include:

1. To evaluate the efficacy of RDE as alternatives to antibiotics by examining its effect on gut health and development, immune responses, and oxidation of broiler chickens challenged with *Salmonella Enteritidis* or *Campylobacter jejuni* or lipopolysaccharide (LPS) ;

2. To analyze the mechanism and pathways involved in regulating gene expression of nutrient transporters in broiler chickens;

3. To evaluate the bioactive metabolites in meat using metabolomics and proximate analysis of nutrient composition of meat in broiler chickens;

4. To investigate the effects of RDE supplementation on the sensory evaluation of breast meat in broiler chickens; and

5. To evaluate the effects of RDE supplementation on egg production performance, egg quality, sensory evaluation, and antioxidative parameters of laying hens.

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