NUTRITIONAL AND PHYSIOLOGICAL ASPECTS OF THE USE OF RESISTANT STARCH AS FUNCTIONAL FIBER IN BROILER CHICKENS' DIETS

by

OLUSEYIFUNMI IYABO WURAOLA

(Under the Direction of Oluyinka Abiona Olukosi)

ABSTRACT

This research series assessed the optimum resistant starch (RS) nutrition in broiler chickens with emphasis on growth performance and gut health metrics as influenced by different RS sources, dietary concentrations, and feeding durations. It provided an understanding of the relationship between the metabolic products or metabolites from starch digestion and fermentation, growth, disease resistance, and tolerance responses. The first study evaluated how sources and levels of RS may influence the growth performance and nutrient utilization in broiler chickens using gut health metrics, digestion metabolites and gene expression as indices. The factors differentially modulated the nutrient digestion and energy utilization based on their botanical origins and dietary concentrations with consequences on ileal oligosaccharides and caecal SCFA profiles. Study two explored the effects of RS feeding lengths (7, 14, and 21 days) and dietary RS levels on growth performance and nutrient digestibility. RS feeding length did not significantly impact overall growth or nutrient digestibility but improved feed conversion ratio (FCR), apparent ileal digestible energy (AIDE), and metabolizable energy (ME) were observed with 35 g/kg high-amylose corn starch (HCS) outperforming 25 g/kg raw potato starch (RPS). The third study investigated the influence of optimal nutrition of resistant starch (RS) on intestinal integrity,

immune response, growth performance, and broiler resistance to *Eimeria* challenge. The inclusion of 25 g/kg RPS and 50 g/kg HCS improved broiler growth performance during early phases, with 50 g/kg HCS yielding better results in the prepatent infection phase. *Eimeria* infection adversely affected growth performance during acute to compensatory phases. The apparent ileal digestibility (AID) of essential amino acids (AA) was also depressed by infection except for Trp, whereas RS diets improved the AID of Trp and Cys. These experiments provided significant insights on the use of RS to promote growth performance, disease resistance, and gut health, offering applicable strategies for the poultry industry.

INDEX WORDS: Resistant starch, Growth performance, Gut health, Nutrient utilization, *Eimeria spp*.

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DEDICATION

To Paxton, Lucas and Lois Oluseyifunmi, your love and presence brought light to every day. I am endlessly grateful for each of you, and I dedicate this work to your bright futures.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

The world population growth and rising incomes have resulted in increased demand for food. Therefore, annual meat production is expected to grow by over 200 million tons to reach a total of 470 million tons in 2050 (FAO, 2009). Poultry with short generation intervals present the best option for increasing meat supply, but the necessity to raise poultry without antibiotics has provided the impetus for finding alternative approaches to help maintain flock health (FAO, 2009; Barker, 2021). Growing evidence indicates that optimizing host intestinal health and the gastrointestinal microbial ecosystem are the major factors influencing overall bird health and resilience to disease challenges (Kogut and Arsenault, 2016).

In addition to existing nutrition-based approaches for improving gut health, functional carbohydrates, such as resistant starches (RS), offer a promising nutritional approach for improving gut health in humans and animals due to their ability to selectively modulate intestinal ecology and immune system (Tan et al., 2021). Resistant starches are functional starch molecules that escape digestion in the proximal intestine and are fermented by the microorganisms in the distal part to produce metabolites such as short-chain fatty acids (SCFA), which are beneficial for gut health (Landon et al., 2012).

RS modulates nutrient digestion and gut microbial community primarily by acting as a prebiotic. The SCFA produced from microbial fermentation of RS serve as the primary energy source for colonocytes. The reduction of distal luminal pH by increased SCFA production favors the proliferation of beneficial gut bacteria along with SCFA-producing ones, such as

Bifidobacteria and *Lactobacilli,* which in turn produce more SCFA, thereby supporting the metabolism of enterocytes and colonocytes and consequently the overall gut homeostasis (Giuberti et al., 2015). Evidence of improved distal gut morphometrics has been reported in ducks fed dietary raw potato starch (Qin et al., 2020).

Aside from serving as the primary source of energy for colonocytes, SCFA exert their regulatory effects on metabolism through their activity on histone deacetylase (HDAC) and G protein-coupled receptors (GPRs), namely GPR43(FFAR2) and GPR41 (FFAR3). FFAR2 are expressed in immune cells, enteroendocrine cells and adipocytes with propionate as its most effective stimulator whereas, FFAR3 are highly expressed in adipose tissue, spleen and colon (Liu *et al.*, 2021). They modulate a variety of intestinal inflammatory responses through different pathways. For instance, they regulate mTOR and promote the differentiation of T-cells into T-reg and Th cells through inhibition of histone deacetylases (HDACs), which play a crucial role in anti-inflammatory response (Liu *et al.,* 2021). Propionate and butyrate could activate FFAR2, FFAR3, or GPR109A or inhibit histone deacetylase (HDACs) to reduce nitric oxide synthase or the production of pro-inflammatory cytokines, acting as anti-inflammatory factors. They may also inhibit HDAC without going through FFAR3 and FFAR 2 (Chang et al., 2014; Sun et al., 2018; Zou et al., 2019; Liu et al., 2021). However, acetate cannot act as an inhibitor of histone deacetylase (Zheng et al., 2015).

Butyrate, being a histone deacetylase inhibitor, can inhibit NF-kB activation, which can downregulate the expression of pro-inflammatory cytokines in broilers, whereas acetate seems to be more involved in the production of pro-inflammatory cytokines and chemokines by the activation of GPR41 or GPR43 (Liu et al., 2021). The depression in the production of pro-

inflammatory cytokines through inhibition of HDAC on immune cells and mononuclear cells by SCFA protects against gut inflammation

Therefore, feeding of dietary RS to broiler chickens during an enteric challenge as reported in weaned pigs and ducks may be beneficial as SCFA produced by microbial fermentation of RS may alter microbial composition and modulate immune response (Yi et al., 2023; Zhang et al, 2022; Dobranowski and Stintzi, 2021). Some gut bacteria have been shown to stimulate anti-coccidial antibodies and enhance immune response. The existence of Lactobacillus at various locations in the gastrointestinal tract enhances nutrition utilization, impedes the colonization of the intestines, and reduces the intestinal lesions resulting from invasion by *E. tenella* (Madlala et al., 2021). They also hinder the secretion of siderophores, restricting iron availability for parasite invasion (Madlala et al., 2021).

However, resistant starches belonging to the same group may behave differently based on the type of crystallinity exhibited and different botanical origins (Giuberti et al., 2015). The crystallinity pattern influences the degree of resistance to pancreatic amylase, which ultimately determines the digestibility of the starches and the oligosaccharide profile in the distal digestive tract fermentation (Macfarlane and Macfarlane, 2003; Martinez-Puig et al., 2003). The metabolites in the digesta may alter the trajectory of the digestion process as well as cause a shift in gut microbiota (Choct et al., 1999; Dittoe et al., 2022). The final digesta oligosaccharide profile dictates the degree of bacteria penetration and the rate of depolymerization influencing their availability for bacterial fermentation. This fermentability also determine the final microbial composition as well as the profile of the SCFA produced. (Giuberti et al., 2015; Macfarlane and Macfarlane, 2003; Martinez-Puig et al., 2003).

For instance, *Firmicutes* such as *Faecalibacterium prausnitzii* and *Roseburia sp.* produce high amounts of butyrate, whereas *Bacteroidetes* produce high levels of acetate and propionate (DenBesten et al., 2013). Furthermore, the SCFA produced from RS fermentation may also act by modulating the metabolites in systemic circulation and regulate the expression of genes that are associated with gut development and health (Regassa and Nyachoti 2018). Additional factors such as physiological conditions of animals, age, RS sources, concentrations, species of animal, and feeding lengths could influence the use of RS as functional fiber in broiler chicken diets. Therefore, the experiments reported in this dissertation examine how the impacts of site and extent of starch digestion, sources, and levels of resistant starches can interact to influence the growth performance and intestinal environment in broiler chickens. In addition, the existing results showed high variability due to different RS sources and levels which could be feeding-duration dependent, among other factors. Therefore, one of the reported studies focused on the impact of duration of feeding resistant starches on their physiological and gut health effects. Lastly, feeding dietary RS to broiler chickens during the enteric challenge may be beneficial, as reported in humans, rodents, ducks, and weaned pigs, due to their numerous benefits as a functional fiber. Consequently, the last experiment in the series examined how resistant starches influence broiler chickens' response during an enteric disease challenge model.

CHAPTER 2

2. LITERATURE REVIEW[1]

[1] Oluseyifunmi, I. W., & Olukosi, O. A. To be submitted to *Animals*.

ABSTRACT

Resistant starch (RS) offers a promising nutritional approach for improving gut health in poultry. The fermentation of RS in the distal gastrointestinal tract produces microbial metabolites such as SCFA which selectively modulate gut microbial community and nutrient digestion. The individual SCFA are linked with different bacteria groups and taxa. The bacteria, in turn, may increase the production of SCFA. Different RS and concentrations in diets may yield variable quantities and profiles of SCFA under distinct physiological conditions and in different species of animals. These SCFA regulate cytokine production via activation of G protein-coupled receptors (GPRs), GPR43(FFAR2), and GPR41 (FFAR3) and or inhibiting histone deacetylase (HDAC) in immune and mononuclear cells, playing significant roles in shaping the gut and bone health in poultry, thereby modulating the immune system which is pertinent during intestinal inflammation.

KEYWORDS: Resistant starch, Gut health, Bone health, Cytokines, Short chain fatty acids

2.1 INTRODUCTION

The world population growth and rising incomes have resulted in increased demand for food, therefore annual meat production is expected to grow by over 200 million tons to reach a total of 470 million tons in 2050 (FAO, 2009). Poultry with short generation interval present the best option for increasing meat supply but the necessity to raise poultry without antibiotics has provided the impetus for finding alternative approaches to help maintain flock health (FAO, 2009; Barker, 2021). Growing evidence indicates that optimizing host intestinal health and the gastrointestinal microbial ecosystem are the major factors influencing overall bird health and resilience to disease challenges (Kogut and Arsenault, 2016).

In addition to existing nutrition-based approaches for improving gut health, functional carbohydrates, such as resistant starches (RS), offer a promising nutritional approach for improving gut health in humans and animals due to their ability to selectively modulate intestinal ecology and immune system (Tan et al., 2021). Resistant starches are functional starch molecules that escape digestion in the proximal intestine and are fermented by the microorganisms in the distal part to produce metabolites such as short-chain fatty acids (SCFA) which are beneficial for gut health (Landon et al., 2012).

RS modulates nutrient digestion and gut microbial community, primarily by acting as a prebiotic. The reduced luminal pH in the distal part of the intestine facilitated by increased SCFA favors the proliferation of beneficial gut bacteria along with SCFA-producing ones which in turn produce more SCFA, thereby supporting the overall gut homeostasis (Giuberti et al., 2015). The SCFA produced from microbial fermentation of RS serve as the primary source of energy for colonocytes and have anti-inflammatory properties. They elicit trophic effects, such as increasing

tight junction protein production, epithelial cell turnover, and mucus secretion (Giuberti et al., 2015; Liu et al., 2021).

Resistant starches also initiate metabolic changes by altering genes that are involved in lipid oxidation, energy expenditure, and insulin sensitivity (Li and Ma, 2024). Dietary RS consumption modulates neural signaling, via activation of free-fatty acid receptors through SCFA, which triggers the release of satiety hormones and at the same time modulate immune responses. Physiological conditions of animals (lactation, gestation, disease, and growing), underlying gut microbiota, species, age, different sources of RS, concentrations and interaction with other dietary components influence the functionality of RS in modulating gut homeostasis and metabolism (Macfarlane and Macfarlane, 2003; Suarez-Belloch et al., 2013; Chen et al., 2024).

The physiochemical properties, crystalline structure, and susceptibility to amylolytic enzymes of different RS further determine the structure and the amount of oligosaccharide profile that reaches the distal GIT as well their subsequent accessibility to microbial activity in the ceca (Devi et al., 2009; Giuberti et al., 2015). These dictate the individual profile and total SCFA produced in the ceca, and each SCFA vary in their ability to regulate immune-modulatory metabolites (cytokines) through inhibiting histone deacetylase (HDAC) and or activating G protein-coupled receptors (GPRs), namely GPR43(FFAR2) and GPR41 (FFAR3) (Liu et al., 2021).

Despite the existing evidence of improved gut and overall animal health associated with dietary RS consumption, there are few studies that focus on impacts of RS supplementation in poultry or its interaction with gut-bone pathways via modulation of immune system. This review discusses the functional role of RS with special emphasis on poultry.

2.2 DEFINITION OF RESISTANT STARCH

Following the presentation of the RS concept by Englyst and coworkers in 1982, it was discovered that starch enclosed within plant cells along with a particular partially gelatinized starch granules showed resistance to full digestion in the proximal gut, but instead undergo microbial fermentation in the distal gastrointestinal tract (GIT). As a result, the definition of RS was broadened to encompass starch and its degradation end products that enter the large intestine to be fermented by the resident microbes (Englyst et al., 1996).

RS, as defined by Tharanathan (2002) and Fuentes-Zaragoza et al. (2010) represents the portion of starch that remains unhydrolyzed into D-glucose in the small intestine for up to 120 minutes following ingestion but instead undergoes fermentation in the colon. Recently, resistant starches were redefined based on their observed effects on the composition of the intestinal microbiota at the phylum level as types that promotes the growth of specific phyla of bacteria, including *Bacteroidetes, Firmicutes, and Actinobacteria* (Li, and Hu, 2022).

2.3 CLASSIFICATION OF RESISTANT STARCHES

There are various forms of RS with varying degree of hydrolysis and structure. RS are therefore classified based on different criteria. RS was previously classified on the basis of sources and factors affecting their resistance to digestion as: RS1 found in grains, enclosed in plant cell walls and resist enzymatic digestion, RS2 as naturally occurring in foods like raw potatoes, green bananas with some requiring higher temperature to gelatinize, RS3 forms when cooked starchy foods cool, undergo gelatinization and crystallizes in the process, RS4 formed from chemical modifications, and RS5 which consists of complexes formed from combination of starch and lipids (Tan et al., 2021; Kong et al., 2003).

Most bacterial fermentation occurs in the proximal colon, microbial metabolites drop from proximal to distal colon, with SCFA concentrations in the distal colon around 40 % lower than the proximal colon. A slow and steady RS fermentation rates are important in enhancing SCFA intestinal barrier functions. Based on fermentability and specific site of fermentation within the large intestine, resistant starches were classified as:

1. Rapidly fermentable RS: This denotes the starch components that undergo prompt fermentation upon entering the proximal colon.
2. Slowly fermentable RS: These are RS that undergo a gradual fermentation process from the proximal colon to the distal colon.
3. Unfermentable RS: These refer to the RS that cannot undergo fermentation within the colon and are subsequently excreted by the animal (Li and Hu, 2022).

2.4 SOURCES OF RESISTANT STARCH

Starches with high amylose content form the primary source of resistant starch production. Starches from tubers and legumes (potato, red kidney bean, yellow peas, and Chinese chestnut) with larger average starch granule sizes are good sources of RS as they are more resistant to enzymatic hydrolysis during digestion. Other examples of RS sources include high-amylose corn, rice starches, normal rice, normal corn, brown lentil, hull-less barley, oat, rice, sorghum, triticale, wheat, tapioca starch, green banana, and others (Tan et al., 2021; Jiang et al., 2020).

2.5 STARCH DIGESTION IN CHICKENS

Many factors influence starch digestibility in animals. These include the starch structure, physiochemical properties, amylose to amylopectin ratio, age, species and physiological status of the animals. In addition, the various feed milling processes, including heating, pelleting, flaking, extrusion, and expansion have great impact on starch digestion, which is influenced by the distinct

botanical origins of different starches (Alsaffar, 2011). The enzymatic breakdown of starch in avian species, particularly chickens, is crucial for their nutrient assimilation. Chickens have a large capacity to secrete pancreatic amylolytic enzymes, which allows them to digest starch better than most non-ruminant animals. Broiler chickens have more than 96% total tract starch digestibility at 3 days old, and this reduces from days 5 to 7 but returns to normal at day 14. Generally, starch digestibility declines as fast-growing broiler chickens advance in age (Svihus, 2014; Zelenka and Ceresnakova, 2005; Lehrner and Malacinski, 1975).

After ingestion, the feed travels down the crop, where starch hydrolysis begins (over 20%). Three distinct Lactobacillus strains in the chicken crop exhibit the enzymatic capability of producing amylase, supporting microbial pre-digestion of starch within the crop. The avian gastric region consists of two compartments: the proventriculus and the ventriculus. The proventriculus (glandular stomach) secretes gastric juices, including HCl, pepsinogen, and mucus, whereas the ventriculus or gizzard (muscular stomach) has a surface texture that aids in grinding and mixing, thereby increasing the surface area of feed to digestive fluids.

Chickens do not have salivary α-amylase activity, so the enzymatic digestion of starch starts in the luminal chamber of the jejunum, where most starch digestion takes place due to its high glucoamylase and α-amylase activities, but declines towards the ileum (Noy and Sklan, 1995; Osman, 1982; Moran, 1982; Svihus et al., 2005). Starch molecules are therefore hydrolylzed into maltose, maltotriose, short-branched oligosaccharides, and α-limit dextrin. Brush-border dextrinase, maltase-glucoamylase, and sucrase-isomaltase further hydrolyze disaccharides and oligosaccharides into monosaccharides. The epithelial lining absorbs the monosaccharides and glucose, which is the final product of starch digestion and is transported by Na/glucose cotransporter 1 (SGLT1) through active transport against an electrochemical gradient across the

apical membrane of the enterocytes and the facilitated-diffusion glucose transporter 2 (GLUT2) facilitates the passive exit at the basolateral membrane, allowing glucose to enter the extracellular medium near the blood capillaries (Roy et al., 2006).

2.6 FERMENTATION OF RESISTANT STARCH AND ASSOCIATED BENEFITS

The ceca are the largest sites for microbial fermentation in poultry. RS constitutes one of the major energy sources for gut microbiota in animals. After starch digestion in the small intestine, the portion that resists enzymatic hydrolysis (RS) undergoes microbial fermentation in the ceca, producing SCFA, carbon dioxide, hydrogen, and methane. Most of the SCFA produced include acetate, propionate, butyrate, and a small quantity of valerate, isovalerate, and isobutyrate (Deehan et al., 2020). Increased production of SCFA lowers the cecal and ileal luminal pH and changes digesta pH, creating an acidic environment not conducive to the proliferation of pathogenic microbes (Roy et al., 2006).

The SCFA produced favors the proliferation of beneficial bacteria, such as *Bifidobacteria* and *Lactobacilli*, which in turn stimulate SCFA synthesis. *Firmicutes* such as *Faecalibacterium prausnitzii* and *Roseburia sp.* produce high amounts of butyrate, whereas *Bacteroidetes* produce high levels of acetate and propionate (DenBesten et al., 2013). Bacteroidetes, Firmicutes and Actinobacteria are the groups of phyla that are actively involved in the fermentation of RS (Turroni et al., 2012; Chen et al., 2024). The SCFA are efficiently taken up from the lumen through either passive or active transport mechanisms, facilitated by the monocarboxylate transporter 1 (MCT1) or the Na-dependent monocarboxylate transporter 1 (Tan et al., 2021).

The oxidation of SCFA provides about 70% of the energy required by colonocytes while others are transferred via the basolateral membrane by MCT4 to the hepatic, portal, and peripheral blood. Acetate and propionate are transported to the liver where they serve as substrates for

peripheral adipogenesis and gluconeogenesis, respectively, whereas colonic epithelial cells use butyrate as a major source of energy. They modulate lipid, glucose, and cholesterol metabolism in various tissues (Sun et al., 2021; Chen et al., 2024). Short-chain fatty acids modulate metabolism through their regulatory effect on histone deacetylase (HDAC) and G protein-coupled receptors (GPRs), namely GPR43(FFAR2) and GPR41 (FFAR3). FFAR2 are expressed in immune cells, enteroendocrine cells and adipocytes with propionate as its most effective stimulator whereas, FFAR3 are highly expressed in adipose tissue, spleen and colon (Liu et al., 2021).

In broiler chickens, GPR43 (FFAR2) has higher responsiveness to acetate than GPR41 (FFAR3), conversely FFAR3 demonstrates little more sensitivity to propionate and butyrate than FFAR2 in primary intestinal epithelial cells. Through the reduced pH in the ceca, SCFA interacts with Toll-like receptors and the GPRs on the immune cells controlling the differentiation of T cells into T regulatory cells (Tregs). These processes not only increase the expression of genes linked with enhanced metabolic functions but also promote overall host well-being (Sun et al., 2021; Zhang et al.,2020). In addition, the SCFAs aid intestinal musculature and vasculature through increased enterocytes and colonocytes proliferation. They also increase mucus secretion and the expression of tight junction genes and thereby improve gut microbial barrier function and contribute to general gut homeostasis (Liu et al., 2021).

2.7 DIVERSITY IN FUNCTIONALITY AMONG INDIVIDUAL RESISTANT STARCH ARCHITECTURES

Starch granules derived from diverse plant sources exhibit peculiarities in their crystalline structure, granule size, surface area, amylose to amylopectin ratio, and porosity which ultimately govern their differential susceptibility to enzymatic and chemical reactions (Tan et al., 2021). The specific type of dietary RS plays a determining role in shaping the bacterial species that flourish

in the ceca of any avian species. The preference of each bacterium for substrate binding influences the ability of individual RS to modify the relative composition of the gut microbial community.

Certain RS1, 2, and 4 have been linked with increased *Firmicutes* abundance and the abundance of *Actinobacteria* with dietary inclusion of native potato starch (RS2). RS2 from high-amylose maize was associated with the abundance of *Ruminococcus bromii* (Chen et al., 2024). The abundance of butyrate-producing bacteria such as *Roseburia spp*. and *Ruminococcus bromii* have also been related to feeding retrograded starches (RS3) (Li and Hu, 2022; Jiang et al., 2020; Zeng et al., 2018). The major health benefits of RS to the intestine is modification and stabilization of intestinal microbiota, regulation of tight junction integrity, and increase in the expression of genes crucial for gut development through SCFA production.

2.8 IMPORTANCE OF FEEDING DIETARY RS IN POULTRY AND PIGS

2.8.1 INTERACTION OF RS WITH GROWTH PERFORMANCE

RS lowers the caloric density of feed and could increase feed intake in birds. Therefore, the birds could consume more to compensate for reduced energy intake and could influence the body weight positively (Oluseyifunmi et al., 2024) ; Higgins, 2014; Zhou et al., 2008;). In addition, the availability of microbial-produced SCFA as energy source could reduce glucose oxidation, mediating the effects of RS on growth performance especially in birds with ad libitum access to feed (Van Erp et al., 2020). Similarly, in rodent and human study, RS intake relative to digestible starch diets did not show any change in total energy intake and satiety scores at ad-libitum feeding and during a short-term feeding (Higgins, 2014).

However, the energy from SCFAs is considered to be less efficient than from glucose (Tan et al., 2021) and the influence of free fatty-acids receptors on the release of satiety hormones due to increased SCFA production cannot be over-emphasized but the effects of dietary RS on growth

performance responses are variable as dictated by the physiological status of animals, length of feeding, RS source, concentrations, experimental conditions, species, among other factors (Regassa and Nyachoti, 2018).

Although, studies on the effects of RS on growth performance responses in poultry are few. Liu et al. (2020) investigated the impacts of feeding corn RS diets at 4, 8 and 12 % in a 42- day Arbor acres broiler chickens were fed corn RS diets at 4, 8 and 12 % in a 42- days and observed a lower weight gain and feed intake with a decline in feed efficiency as the dietary RS level increased as against those fed control diet (corn-soybean-based diet with 20 % corn starch). The depression in growth were attributed to high crystallinity of RS which could reduce starch digestibility and leads to inadequate energy intake.

Similar sets of RS diets with the same concentrations were further studied by Zhang et al. (2020) in Arbor Acres broiler chickens but an additional normal corn–soybean control diets were used. The birds that received diets having 20% corn starch performed worse than those fed corn RS diets at 4, 8 and 12 % and normal corn–soybean control diet. On the other hand, the supplementation of day-old duckling diets with 6, 12, 18, and 24 % RPS did not affect their growth performance significantly as compared with the control diet (Qin et al., 2019).

Dietary inclusion of RS has variable effects on feed efficiency in animals despite its regulatory effects on satiety and feed intake (Regassa and Nyachoti, 2018; Tan et al., 2021). Bhandari et al. (2009) reported that feeding 7% raw potato starch (RPS) showed no significant effect on growth performance in weaned piglets. Similar study by Pluske et al. (1998) observed no significant effect of RS on average daily gain with inclusion of 280 g of RS in the diets of growing pigs.

Nofrarías et al. *(*2007) further observed almost comparable growth performance responses in groups of growing pigs fed equal amounts of RPS and corn starch. However, the

supplementation of barrow's diets with 50% high amylose maize starch (RS2), relative to control and digestible corn starch groups, negatively affected the growth performance through reduced feed intake and efficiency (Li et al., 2007).

The conflicting reports on the effects of RS on feed intake, feed efficiency, and growth performance may be due to different experimental models, age, species of animal, RS sources, the amount ingested, and duration of feeding. Apart from these factors, the interaction of RS within different diets of varying compositions of feedstuff mixture and starch components would yield a multifactorial effect influencing the functionality of individual RS (Giuberti et al.,2015).

2.8.2 IMPACTS OF FEEDING DIETARY RS ON NUTRIENT AND ENERGY UTILIZATION

Estimates suggest that SCFA from microbial fermentation in birds' ceca contributes between 5 and 15% to the daily maintenance energy (Singh and Kim. 2021) and 30% of the energy requirement can be supplied by distal GIT fermentation in pigs. Studies also showed that SCFA produced during RS fermentation could supply about 15% of the total energy required for maintenance in growing pigs (Da Silva *et al.*, 2012; Zijlstra *et al.*, 2012; Regassa and Nyachoti, 2018; Tan et al., 2021).

However, energy from SCFA is considered to have less efficiency compared to the source from glucose, and it has been reported that the dilution of energy density of feed by RS may reduce the energy intake. The effects of RS on energy metabolism may be influenced by the energy density of the control diet relative to RS diets, RS source, level of inclusion in animal diet, age, the length of feeding and whether the animals have ad-libitum or restricted access to feed (Regassa and Nyachoti, 2018; Giuberti et al., 2015; Higgin, 2014). Some rodent studies have demonstrated that when fed diets of equivalent energy density, the energy and feed intake of those consuming

resistant starch and digestible starch did not differ irrespective of the type of starch in their diets (Higgin, 2014; Da Silva et al., 2012).

Oluseyifunmi et al. (2024), in a 21-day study fed Cobb 500 broiler chickens, diets containing three sources of RS, banana starch (BS), raw potato starch (RPS), and high-amylose corn starch (HCS), each at three levels of 25, 50, or 100 g/kg observed that both RS levels and sources influenced the total tract nutrient utilization of dry matter (DM), nitrogen, and apparent metabolizable energy (AMEn). The corn-soy bean-based control diet supported superior nutrient utilization compared to RS diets, except for the 100 g/kg HCS diet, which had similar values, and a similar study showed that 35 g/kg HCS increase both AME and AMEn in broiler chickens.

However, in a 42-day study, feeding Arbor Acres broiler chickens with diets containing 40, 80, and 120 g/kg Hi-Maize 260 resistant starch resulted in decreased ($P < 0.05$) apparent metabolizable energy (AME) and apparent total tract retention of crude protein (CP), dry matter (DM), total starch (TS), ether extract (EE), and nitrogen-free extract (NFE) between days 18 to 20 and days 39 to 41. Additionally, the apparent total tract retention of TS, DM, EE and NFE declined linearly as the levels of resistant starch (RS) increased during these periods (Liu et al., 2020). Inherent individual RS structural and physiochemical properties are very crucial to their ability to influence nutrient digestibility. For instance, high crystallinity of RS can lead to lower digestibility of starch and reduced energy intake. Based on Morel et al. (2005) calculations, it was reported that one percent elevation of RS content of a diet could produce 0.64 % equivalent decrease in total ileal starch digestibility.

Starches from tubers such as potatoes may exhibit B-type crystallinity patterns that increase their degree of resistance to digestion (Bednar et al., 2001), and highly retrograded maize starch may reduce energy intake, retention and digestibility in pigs (Gerrits et al., 2012). In addition, high

phosphate content in raw potato starch, its large granule size and increased gelatinization potential could impact nutrient digestibility (Monro and Mishra, 2009). Generally, inclusion of a large portion of fermentable starches in animal diet could decrease energy efficiency and affect overall nutrient metabolism (Zijlstra et al., 2012; Yin et al., 2011).

2.8.3 THE INFLUENCE OF RS ON INTESTINAL ENVIRONMENT AND MORPHOMETRY

Among the SCFA produced by RS fermentation, butyrate plays a major role in improving gut health as it serves as the major energy source for the colonocytes. It aids the proliferation of the epithelial cells and the growth of the colonocytes, improves gut integrity, possess anti-oxidative and anti-inflammatory properties (Regassa and Nyachoti, 2018).

Different RS and the amount in diets may yield variable quantities and profiles of SCFA under distinct physiological conditions and in different species of animals. The type of SCFA produced determines the effects elicited on the enterocytes. Butyrate can further modulate the expression of mucin genes. Supplementation of tributyrin had reportedly increase mucin-2 production in the ileum of broilers chickens infected with coccidiosis (Hansen et al., 2021). Propionate production may favor the production of tight junction proteins such as ZO-1 and occludin which contribute to increased gut barrier function (Liu et al., 2021). Resistance to amylolytic enzymes and invariably the digestibility of individual resistant starches dictates the amount and the physical structure of the oligosaccharide that reaches the distal GIT and the subsequent accessibility to microbial activity. This influence the degree of penetration of bacteria, depolymerization and fermentation as well as the resulting total SCFA production and individual SCFA profile ((MacFarlane and MacFarlane, 2003; Bird et al., 2007; Haenen et al., 2013; Giuberti et al., 2015; Regassa and Nyachoti, 2018; Oluseyifunmi et al., 2024).

Dietary RPS 23 and 28% RPS elevated the relative mRNA expression mucin genes in the colon of growing and finishing pigs (Zhou et al., 2017). Feeding 12 and 18% RPS diets to day-old duckling compared to 0% RPS diet increased the mucosal thickness on day-14, ileal villi height to crypt depth ratio (VH/CD) on days 14 and 35 whereas the villi height (VH) increased at 12 % RPS on days 14 and 35, 18 % RPS on day 35 days (Qin et al., 2019). Ariza-Nieto et al. (2012) also reported greater duodenal and jejunal VH/CD in birds fed RPS compared with those on diets with no RS inclusion. Deeper jejunal crypt was reported in broiler chickens fed diets with inclusion of RPS and HCS diets and greater cecal total SCFA and acetate concentration was observed in the cecal digesta of birds fed HCS diets than other diets, along with tendency for longer jejunal villi in birds fed dietary HCS diets (Oluseyifunmi et al., 2024).

Conversely, in Zhang *et al.* (2020) study, lower duodenal CD, jejunal VH and VH/CD ratio were observed in broiler chickens that received 4% RS, 8% RS and 12% corn resistant starch diets compared to the normal corn- soybean diet group. In addition, broiler chickens fed diets containing 8% or 12% Hi-Maize 260® RS for 21 and 42 days had reduced jejunal VH and VH/CD and higher relative weights of the duodenum, jejunum and ileum compared with corn-soybean control diet (Liu et al., 2019). Weaned piglets fed diets with 160 g/kg RPS had increased VH and those that received 80 g/kg RPS diets had higher crypt depth. The weight of the colon and the amount of butyrate produced increased with increased concentration of RS (Hedemann and Knudsen, 2007). These studies indicated increased proliferation of intestinal epithelial cells due to increased production of SCFA through distal fermentation of RS-containing diets by resident micro-organisms. The SCFA in-turn support the proliferation of SCFA-producing micro-organisms.

2.8.4 INTERACTION OF RESISTANT STARCH WITH DIETARY PROTEIN AND ENVIRONMENT

The fermentation of proteins in the hindgut generates both SCFAs and branched chain fatty acids along with other toxic and proinflammatory factors that predispose to intestinal diseases. Branched-chained fatty acids originated from branched-chained amino acids (valine, leucine and isoleucine). Incorporating RS into feed containing high crude protein reduced the incidence of harmful nitrogenous metabolites in the ceca (Liu et al., 2021).

It has been well-established that RS administration shifts the focus of fermentation in the distal GIT away from proteins resulting in production of more beneficial metabolites such as SCFA and reduction in the release of nitrogenous waste into the environment. Long-term consumption of dietary RPS in pigs reduced ammonia-nitrogen, tryptamine, tyramine, phenol, cresol, indole and skatole contents in the colon (Zhou et al., 2017).

Maize and tapioca RS (the latter being RS4) reduced proteolytic fermentation in the colon of human subjects by decreasing BCFAs (isobutyrate and isovalerate) in the feces and increasing butyrate and total SCFAs emphasizing beneficial effects of RS on gut health (Deehan et al., 2020). Heo et al. (2014) reported that feeding resistant potato starch (RPS) to weaned pigs for a period 28 days increased the total SCFA concentrations in cecal digesta but reduced the molar proportion of BCFA.

In addition, Oluseyifunmi et al. (2024) observed reduced soluble caecal protein in broiler chickens fed diets containing banana starch and high amylose corn starch relative to corn-soybean based control diet. These suggest that RS may increase the fermentation of carbohydrate relative to protein in the caeca and possibly elevate the ratio of C-N of the substrates fermentable by the microbes (Sun et al., 2016).

2.8.5 MODULATION OF CECAL MICROBIAL PROFILE BY RS

The ceca house the largest community of microbes in the gastrointestinal tract (GIT) of poultry ranging from 1011 to 1012 colony forming unit (CFU)/g of luminal content, being the main site of fermentation and SCFA production. Families of bacteria found in the chicken caeca include *Lachnospiraceae, Ruminococcaceae,* and *Veillonellaceae*. These belong to the order *Clostridiales* which are non-pathogenic (Apajalahti et al., 2004). The non-pathogenic microbes produce SCFA and promotes intestinal health by modulating host immune response and maintains gut integrity (Singh and Kim, 2021).

Different SCFA production is frequently associated with different bacteria group. For example, butyrate concentration has been observed to be positively correlated with the abundance of *Faecalibacterium* and *Ruminococcaceae*, whereas propionate concentration is positively correlated with *Bacteroides* (Portincasa et al., 2022). Studies have shown that the use of prebiotics showed positive effects on some taxa in the colon. In addition, dietary fiber, among other factors plays important role in modulating gut microbial profile and diversity.

Dietary inclusion of 12 % RPS increased the relative abundance of *Firmicutes* and the butyrate-producing bacteria and 24 % RPS significantly increased the abundance of *Lactobacillus* and *Bifidobacterium in* Cherry Valley male ducks fed graded levels of dietary raw potato starch (RPS) for a period of 35 days (Qin et al., 2019). Resistant starch diets fed for a period of 42 days increased the abundance of *Anaerofilum, Bacteroides, Desulfovibrio, Parasutterella* in broiler chickens (Zhang et al., 2020b). Zhang et al. (2020c) reported that diets containing 80 and 120 g/kg corn RS significantly ($P<0.05$) decreased the abundance of cecal *Firmicutes* by 23.08 and 20.47 % and the *Bacteroidetes* increased by 24.33 and 21.92 %, respectively, compared with those on

normal corn-soybean diet. Apart from modulating cecal microbial community, RS diets may also alter metabolic pathways in the microbes (Gardiner et al., 2020).

However, further studies are necessary to be able to characterize the class of microbes influenced by different types of RS in poultry and how these relate to production performance responses. Some authors found lower species richness in the intestine of chickens with greater feeding efficiency and higher bacterial diversity within the intestinal tract of birds with lower feed conversion ratio or high feed efficiency (Carrasco et al., 2019). It will be interesting to see how species diversity is influenced by resistant starch inclusion in broiler chickens and how it translates to production efficiency.

The population of *Clostridia clusters IV* and *XIVa* were reduced in the digesta from the caeca and colon of weaned pigs fed diets containing 0-63 % dietary amylose, whereas that of *Bifidobacterium spp*. increased (Fouhse et al., 2015). Resistant starch from pea also modulated the fecal microbiota of sows during gestation, increasing the *Firmicutes:Bacteroidetes* ratio and the relative abundance of *Bifidobacterium* but these differences were not observed during lactation and maternal diets did not impact the colonic microbiota of the progeny (Leblois *et al.*, 2018). Variable effects of resistant starch diets on gut microbiota in different species of animals could be a function of different types and concentrations of RS with variable fermentability yielding different SCFA profiles and initial microbial ecology of different species and individual animal as influenced by different factors such as age and physiological status of the animals.

2.8 6 INTERACTIONS OF RS WITH IMMUNE RESPONSE AND GUT INFLAMMATION

Adverse effects of feeds, metabolites, environmental factors and enteric challenges such as coccidiosis and necrotic enteritis cause inflammations, bleeding, tissue necrosis, lesions in

gastrointestinal tract of birds, disrupt gut homeostasis, modulate the microbial ecology and eventually retard the growth performance However, SCFA produced from bacterial fermentation of RS can modulate immune response either in the absence or presence of inflammation, through their regulatory effects on cytokine production in immune and mononuclear cells (Wade et al., 2016; Yu et al., 2022).

The SCFA modulate a variety of intestinal inflammatory responses through different pathways. They regulate the mTOR and promote the differentiation of T-cell into T-reg and Th cells through inhibition of histone deacetylases (HDACs), which play a crucial role in anti-inflammatory response. Propionate and butyrate could activate FFAR2, FFAR3, or GPR109A or inhibit histone deacetylase (HDACs) to reduce nitric oxide synthase or the production of pro-inflammatory cytokines, acting as anti-inflammatory factors. They may also inhibit HDAC without necessarily going through FFAR3 and FFAR 2 (Chang et al., 2014; Sun et al., 2018; Zou et al., 2019; Liu et al., 2021). However, acetate cannot act as an inhibitor of histone deacetylase (Zheng et al., 2015).

The depression in the production of pro-inflammatory cytokines through inhibition of HDAC on immune cells and mononuclear cells by SCFA protects against gut inflammation. For example, administration of butyrate and propionate safeguarded the gut and enhanced recuperation from inflammation by suppressing the production of pro-inflammatory cytokines such as IL-1β, IL- 6, TNF-α, NF-κB via repression of HDAC on macrophages, dendritic cells and neutrophils (Chang et al., 2014; Liu et al., 2018). Butyrate being a histone deacetylase inhibitor (HDACSi) can alleviate inflammatory reaction by inhibiting NF-κB activation which downregulates the expression of pro-inflammatory cytokines such as IL-6 and TNF-α in broiler chickens (Liu et al., 2021).

In addition, butyrate may also regulate MUC-2 production through GPR109A in goblet cells, strengthening the gut's chemical barrier and aiding pathogen resistance. During coccidia infection in chickens, SCFA increase the expression of mucosal barrier genes such as claudin-1, claudin-4, and JAM-3 (Hansen et al., 2021). Additionally, butyrate activates the NLRP3 inflammasome via FFAR2, upregulating IL-18 and controlling gut inflammation by modulating Th1 and Th17differentiation while enhancing IL-10 production (Marcia et al., 2015; Liu et al., 2021).

Acetate exhibits both anti-inflammatory and pro-inflammatory properties (Liu et al., 2021; Ali et al., 2022). It acts as an anti-inflammatory factor by decreasing the production of pro-inflammatory cytokines such as IL-4 and TNFα through the activation of FFAR2 during inflammation induced by lipopolysaccharides and in allergic reaction in human and mice, respectively (Ali et al., 2022). Acetate may enhance the production of pro-inflammatory cytokines and chemokines by activating downstream signalling pathways of extracellular signal-regulated kinase 1/2 (ERK1/2) and MAPK/p38 through the activation of FFAR2 (GPR 43) and FFAR3(GPR 41) (Liu et al., 2021; Yao et al., 2020). Figure 2.1 represents the summary of how dietary RS may regulate immune response during inflammation (partly adapted from Liu *et al.,* 2021).

2.8 7 INTERACTIONS OF RESISTANT STARCH WITH IMMUNE RESPONSE AND BONE FORMATION

Gut-bone signaling pathways, along with gut microbiota, play significant roles in regulating bone health via modulation of immune status (Sjogren et al., 2012). As stated previously, the modulation of the intestinal microbiota by dietary RS increased the abundance of beneficial bacteria such as, *Lactobacillus*, *Bifidobacterium* and *Bacteroides* spp. and increased the

production of SCFA which regulate the expression of cytokines, modulating immune system and inflammatory responses (Tousen et al., 2019).

Furthermore, the mucosal immune system or the gut immunologic barrier constitute the third defense of the gut barrier. It is referred to as the gut associated lymphoid tissue and situated deep down in the epithelium or below the intestinal epithelial cells and contain various immune cells like T cells, B cells, macrophages, and dendritic cells. These cells work together to shield the system against pathogens and enhance intestinal health. The numerous immune cell populations, housed by this system manage the production of pro-inflammatory cytokines (e.g., IFN-γ, TNF-α and IL-6) and anti-inflammatory cytokines (e.g., IL-4, IL-10 and TGF-β) (Liu *et al.,* 2021).

During intestinal infections, there is an increase in the differentiation of naive CD4+ cells into Th1, Th2, Treg, and Th17 and these T-cells increase the expression of a variety of pro and anti-inflammatory cytokines of the innate immune system and antigen- presenting cells like macrophages and dendritic cells (Fathima et al., 2022; Sharma *et al.,* 2023). The nuclear factor kappa-B ligand (RANKL), a regulatory factor in bone remodeling, is highly expressed by some of these pro-inflammatory cytokines such as TNF-α and IL-17 (Takayanagi *et al.,* 2000), and its interaction with its receptor RANK increases recruitments of cell into osteoclastic pathways increasing bone resorption (Ono et al., 2020).

Eimeria-challenged chickens had characteristic elevated osteoclasts population with a decline in bone mineral density and volume. However, an increase in the recruitment of antigen-presenting cells and CD4+, along with anti-inflammatory cytokines do not suffice in mediating bone resorption during intestinal infection due to elevated production of pro-inflammatory cytokines that favor osteoclastic pathways (Sharma *et al.,* 2023). Feeding of functional fibers such as resistant starch has been shown to reduce RANKL expression, suppress the production of pro-

inflammatory cytokines, and elevate the expression of anti-inflammatory cytokines, favoring bone repair and formation (Tousen et al., 2019). The interaction of RS with immune system and bone formation is summarized in Figure 2.2.

Meat -type ducks with *E. coli*-induced bone loss that were administered SCFAs through drinking water and fed dietary 12 % RPS had reduced expression of TNF-α in the serum and decreased relative mRNA of TNF-α, IL-1β, nuclear factor-κB (NF-κB), and Malt1 in the bone marrow as against the control birds. Dietary RPS at 12% inclusion also reduced the relative mRNA expression of *RANKL*, decreased the *RANKL*/*OPG* ratio and at the same time increased the bone ash and strength as well as reduced osteoclast number in the tibia of birds infected with *E. coli* (Zhang *et al.,* 2022). Feeding dietary 34% tapioca starch reduced NF-κB expression and TGF-β in the proximal colon of growing pig (Haenen *et al.,* 2013). Qin et al. (2019) also observed a reduction in the expression of pro-inflammatory cytokines (IL-1β, IL-6, TNFα) in the blood plasma of meat-type ducks fed diets with inclusion of 12% RPS. Furthermore, ducks fed 7.4% inclusion of dietary RPS for a period of 35 days had reduced plasma TNF-α, IL-1β and endotoxin (Qin et al., 2020).

The downward expression of pro-inflammatory cytokines by SCFA reduced the expression of RANKL and inhibit its interaction with the receptor RANK, increasing osteoprotegerin (OPG) from osteoblast. In addition, the inhibition of RANKL can reduce excessive bone loss or resorption during infection by suppressing osteoclastogenesis (Ono et al., 2020). The downward expression of *RANKL* reduced the *RANK*/*OPG* ratio. OPG can further inhibit the binding of RANKL to its active receptor RANK, which promotes the formation of osteoblasts, which are specialized bone-forming cells capable of repairing the existing bones (Sharma *et al.,* 2023). They secrete a bone matrix that facilitate the transformation of proteins into new bone tissue, filling in gaps and

strengthening the bone structure. Therefore, bone health could be enhanced through the regulation of cytokines via SCFA produced from RS fermentation (Zhang et al., 2022).

An alternative way by which RS could improve bone health could be through the effect of SCFA on proliferation and enlargement of intestinal epithelial cells which increases the surface area for absorption of minerals and other nutrients (Han et al., 2023; Nofrarias *et al.,* 2007). The decreased luminal pH by SCFAs also facilitates the rapid conversion of minerals into more soluble ions elevating mineral absorption (Han *et al.,* 2023; Metzler-Zebeli *et al.,* 2019). Zhang *et al,* (2022) reported an increase in the serum P concentration of meat-type duck fed 12% dietary RPS and given drinking water containing SCFA. This was related to the elevation of transcription of *NaPi- IIb*, a regulator of P transport in the intestine.

2.9 CONCLUSION

Resistant starches are functional fibers that are not digestible in the proximal intestine but are fermentable in the distal segment to produce microbial metabolites such as short-chain fatty acids (SCFA), which are beneficial for gut health. Dietary RS modulates energy metabolism, immune response, gut health and bone health in broiler chickens. They may increase the fermentation of carbohydrate relative to protein in the caeca and possibly elevate the ratio of C-N of the substrates fermentable by resident microbes. However, different RS and concentrations in diets may yield variable quantity and profiles of SCFA under distinct physiological conditions and in different species of animals. Individual SCFA is linked with different bacteria group and taxa, playing significant roles in modulating gut microbial profile and possess regulatory effects on cytokine production. The suppression of pro-inflammatory cytokines such as NF-κB, TNF- α may enhance improved bone health in poultry, but this needs further investigation through in vivo and

invitro studies to identify and establish probable pathways. Some pathways were suggested but these require further investigation.

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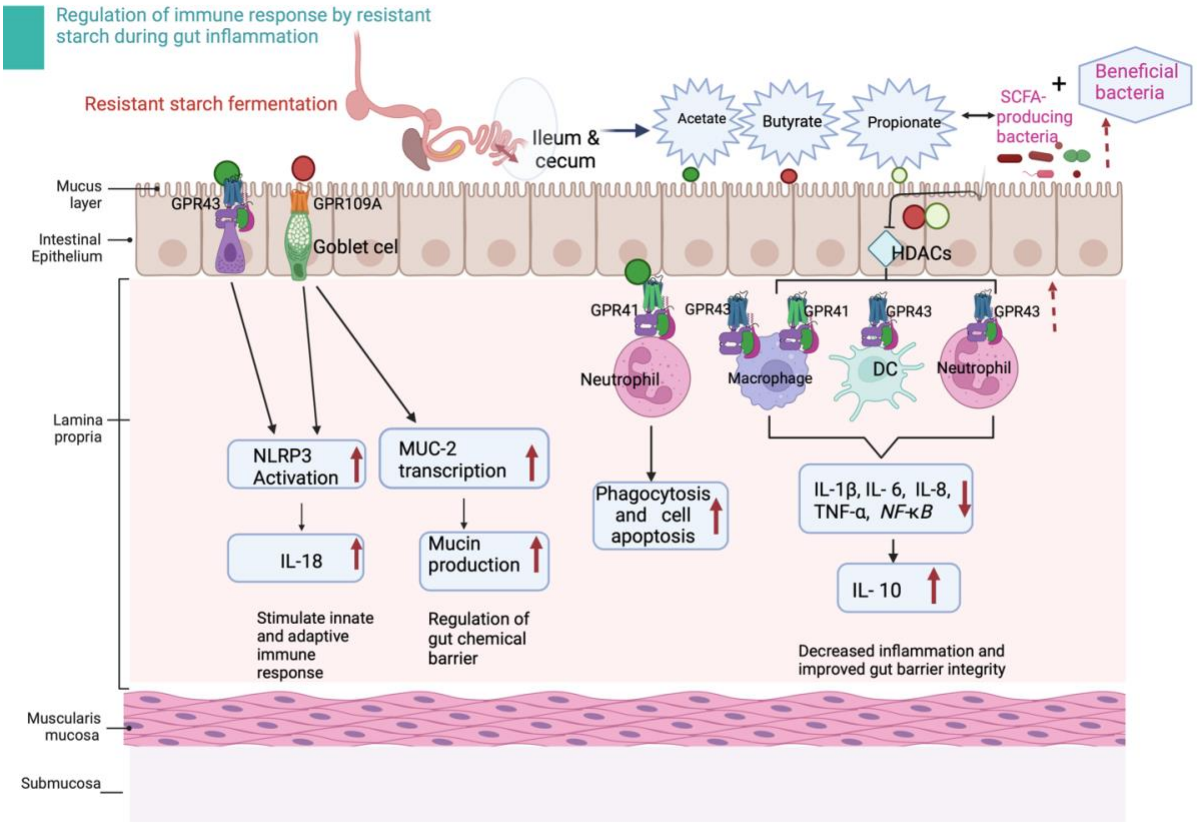

Figure 2.1: Summary of regulatory role of dietary RS on immune response during inflammation. Short-chain fatty acids (SCFA), including propionate and butyrate, modulate intestinal inflammatory responses by regulating mTOR, promoting T-cell differentiation, and inhibiting histone deacetylases (HDACs). This inhibition reduces pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and NF-κB, thus alleviating inflammation. Butyrate also supports mucosal barrier function and regulates the NLRP3 inflammasome, enhancing gut immunity. Acetate exhibits both anti- and pro-inflammatory roles, dependent on specific receptor activation

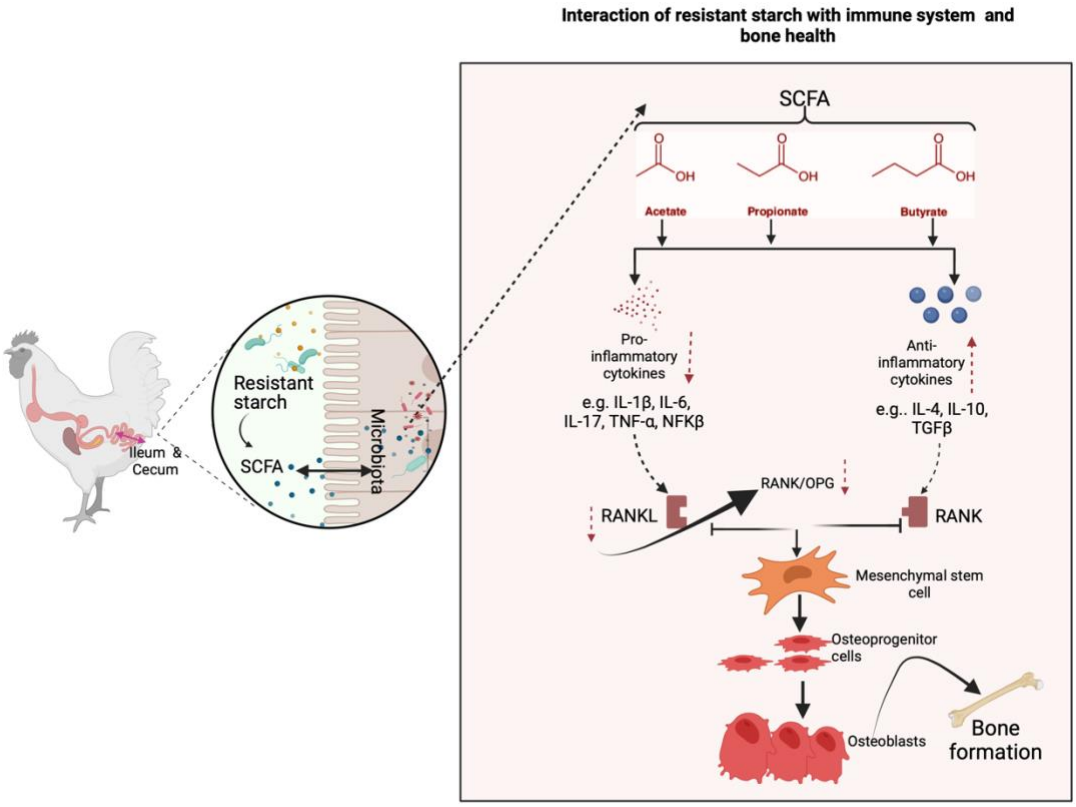

Figure 2.2: The interaction of RS with immune system and bone formation. Gut-bone signaling pathways, influenced by gut microbiota, modulate bone health via immune regulation. Dietary resistant starch (RS) promotes beneficial bacteria and short-chain fatty acid (SCFA) production, reducing pro-inflammatory cytokines (e.g., TNF-α, IL-6) and increasing anti-inflammatory cytokines (e.g., IL-10, TGF-β), which suppress RANKL expression. This reduces osteoclastic activity, lowering bone resorption and enhancing bone formation and repair. SCFA-mediated downregulation of RANKL/OPG ratio supports bone health during infection by inhibiting osteoclastogenesis.

CHAPTER 3

3. THE INTERACTIVITY OF SOURCES AND DIETARY LEVELS OF RESISTANT STARCHES – IMPACT ON GROWTH PERFORMANCE, STARCH, AND NUTRIENT DIGESTIBILITY, DIGESTA OLIGOSACCHARIDE PROFILE, CAECAL MICROBIAL METABOLITES, AND INDICATORS OF GUT HEALTH IN BROILER CHICKENS[2]

[2] Oluseyifunmi, I. W.; Lourenco, J.; Olukosi, O. A., Poultry Science, 2024, 103, 12, 104337. Reprinted here with permission of the publisher.

3.1 ABSTRACT

In a 21-day study, 480 Cobb 500 (off-sex) male broiler chicks were used to investigate the effects of feeding different sources and levels of resistant starches (RS) on growth performance, nutrient and energy utilization, and intestinal health in broiler chickens. The birds were allocated to 10 dietary treatments in a 3×3+1 factorial arrangement. The factors were 3 RS-sources (RSS): banana starch (BS), raw potato starch (RPS), and high-amylose corn starch (HCS); each at 3 levels (RSL) 25, 50 or 100 g/kg plus a corn-soybean meal control diet. Birds and feed were weighed on d 0, 8, and 21. On d 21, samples of jejunal tissue and digesta were collected for chemical analysis. Data were analyzed using the mixed model procedure of JMP with factor levels nested with the control. In the 0-21 phase, the birds fed the RPS diets had higher ($P = 0.011$) FI than those fed HCS or control diets and FCR was greater ($P = 0.030$) in birds that received BS diets than in other diets. RSS × RSL was significant ($P < 0.05$) for total tract nutrient retention, AME, and AMEn on d 21. The starch digestibility was higher ($P < 0.001$) in birds that received the control diet than in RS diets, and decreased RS levels increased, except for HCS. The apparent metabolizable energy (AME) and nitrogen-corrected AME (AMEn) were higher ($P < 0.001$) in birds fed 100 g/kg HCS diet, with both decreasing with increasing levels of BS and RPS, except for HCS. Relative ileal oligosaccharides profile showed significant ($P < 0.05$) RSS × RSL with a higher relative abundance of Hex(3) ($P = 0.01$) and Pent(3) ($P = 0.001$) in HCS diets. In conclusion, RS may influence gut health and growth performance in broiler chickens through modulation of cecal SCFA and nutrient digestion, but these depend largely on the botanical origin and concentrations of individual RS.

3.2 INTRODUCTION

Consumer concern coupled with the ban of antibiotic feed additives in some parts of the world has necessitated the search for feed components that could improve growth performance and maintain gut health in poultry (Vondruskova et al., 2010). Exogenous enzymes, probiotics, prebiotics, amino acids among others have been in use for modulating intestinal health but functional carbohydrates are another promising nutritional approach for improving intestinal health (Dobranowski and Stintzi, 2021). Resistant starches (RS**)** belong to the group of functional dietary fibers that are capable of modulating gut health, exhibiting prebiotic effects in the hindgut. As gut health is characterized by efficient digestion and nutrient absorption, the absence of gastrointestinal disorders, a balanced and stable intestinal microbiome, a robust immune system, and in all, an overall state of well-being (Bischoff, 2011).

Resistant starches are starches that escape digestion in the proximal intestinal tract and undergo fermentation in the ceca, producing beneficial metabolites such as short-chain fatty acids (SCFA) which are critical for gut health, enhanced immunity, and better nutrient utilization (Landon et al., 2012). There are five classes of RS based on sources and factors affecting their resistance to digestion (Champ, 2004). These include RS type 1 (RS1) which are starches that are encapsulated within plant cell walls and are inaccessible to amylolytic enzymes, present in unprocessed whole grains (Tan et al., 2021). RS type 2 (RS2) are naturally occurring RS granules and good examples are raw potato and green banana starches; RS type 3 (RS3) are starches that are retrograded and occurs when starchy foods are cooked and then cooled; RS type 4 (RS4) are chemically modified starches whereas the RS type 5 (RS5) are mainly starches that form complexes with lipids (Tan et al., 2021).

Resistant starches increase the proportion of fermentable carbohydrates relative to protein in the caeca, and the fermentable starches are possible prebiotics in the distal part of the digestive tract. The increased SCFA production lowers the caeca pH and creates a hostile environment for the harmful microbes preventing their proliferation, and this, in turn, dictates the rate and profile of SCFA production (Roy et al., 2006; Regassa and Nyachoti, 2018). The SCFA produced in the ceca include propionate, acetate and butyrate (Macfarlane and Macfarlane, 2003). Although acetate and propionate have beneficial impacts on health, butyrate seems most important for gut health and is reported as the SCFA most increased by RS consumption (Fuentes-Zaragoza et al., 2010; DeMartino and Cockburn, 2020). Dietary inclusion of 120 g/kg raw potato starch (**RPS**) for 35 days increased cecal acetate, propionate, and butyrate profile in Cherry Valley meat ducks (Qin et al., 2019). In a broiler study by Zhang et al. (2020), dietary levels of 40, 80, and 120 g/kg corn RS increased cecal concentrations of acetic and butyric acid. Fermentation of diets rich in RS and high amylose starch has also been reported to facilitate increased intraluminal SCFA concentrations and lowered digesta and fecal pH in pigs. (Tan et al., 2021).

Short-chain fatty acids support the healthy functioning of the lower gastrointestinal tract by influencing the metabolism of enterocytes and colonocytes, thereby affecting the intestinal musculature and vasculature. In mice and humans, RS consumption has been associated with various health benefits such as prebiotic, hypocholesterolemic, and hypoglycemic effects, improved gut barrier function, and reduced risk of colon cancer and inflammation (Bojarczuk et al., 2022). However, resistant starches belonging to the same group may behave differently based on the type of crystallinity exhibited and different botanical origins (Giuberti et al., 2015).The crystallinity pattern influences the degree of resistance to pancreatic amylase, which ultimately determines the digestibility of the starches and the oligosaccharide profile in the distal digestive

tract. These dictate the degree of bacteria penetration into the starch granules and the rate of depolymerization as well as availability for bacterial fermentation (Macfarlane and Macfarlane, 2003; Martinez-Puig et al., 2003).

In spite of the information available in the literature for effect of RS in different species, there is a dearth of information on how the site and extent of starch digestion, sources, and levels of resistant starches can interact to influence the growth performance and intestinal environment in broiler chickens. The aim of the current study was, therefore, to investigate how sources and levels of RS influence the growth performance and nutrient utilization in broiler chickens in the starter and grower phases. Possible effects on starch digestibility in the small intestine, gut morphology and morphometry, ileal oligosaccharides profile, cecal protein and SCFA profile, and jejunal expression of nutrient transporters, and gut integrity genes were used in accounting for the effect of the treatments on growth performance, nutrient and energy utilization by the birds.

3.3 MATERIALS AND METHODS

Birds and housing

The Institutional Animal Care and Use Committee of the University of Georgia approved the experimental procedures (IACUC number: A2021-06-006).

Animal housing, diets and experimental design

The birds used in the experiment were raised in metabolism cages in a controlled environment following recommended lighting and temperature regimes for Cobb 500 broiler chickens. A total of 480 male broiler chicks (byproducts of the female line) were allocated to 10 treatments in a 3×3+1 nested factorial arrangement. There were eight replicates per treatment and six birds per replicate. The diets included a corn-soybean meal control diet and the factors, three

RS sources (RSS): banana starch (BS), raw potato starch (RPS), and high-amylose corn starch (HCS); each at 3 inclusion levels (RSL) 25, 50 or 100 g/kg.

The analyzed chemical composition of the resistant starches is presented in Table 3.1. Feed and water were provided ad libitum throughout the experiment. The diets were presented as mash in both starter and grower phases and formulated to meet the nutrient and energy recommendations for Cobb 500 broiler chickens (Cobb 500 Broilers, 2022). All the diets were isocaloric and isonitrogenous. The diet compositions are presented in Tables 3.2 and 3.3 for the starter and grower phases, respectively.

Growth performance measurements

Birds and feed were weighed on days 0, 8, and 21 to evaluate growth performance response. Body weight gain (WG), feed intake (FI), and FCR were corrected for mortality.

Nutrient digestibility, ileal oligosaccharides, cecal short-chain fatty acids, and cecal protein measurements

On day 21, birds were euthanized by CO_2 asphyxiation, and jejunal and ileal digesta, caecal content, and excreta were collected for digestibility measurement, the determination of the ileal oligosaccharide profile, the cecal SCFA profile and protein content, and total tract N retention and metabolizable energy. Digesta from the jejunum (end of pancreatic loop to Meckel's diverticulum), ileum (from Meckel's diverticulum up to 2cm proximal to ileocecal junction), and excreta were also analyzed for starch digestibility to study site-specific starch disappearance based on RSS and RSL.

Digesta pH measurements

Digesta samples (one bird per pen) were collected from the duodenum, jejunum, ileum, and caeca for pH determination. The digesta was diluted at the ratio of 1 to 9, with 9ml as distilled water and 1g as digesta, vortexed and stirred with a magnetic stirrer for proper homogenization. The pH was then measured using a sterile glass pH electrode (Thermo Scientific, Beverly, MA, USA).

Digestive tract morphometry and histomorphology

The digestive tract was excised from one randomly selected bird per cage and sectioned into the crop, proventriculus, gizzard, duodenum (pancreatic loop), jejunum (end of the pancreatic loop to Meckel's diverticulum), ileum (from Meckel's diverticulum to the ileocecal junction) and ceca. The adjoining tissues were carefully removed, and the sections were weighed with the digesta. The weights and lengths of each of the GIT (gastrointestinal tract) sections were expressed relative to the body weight.

Two-centimeter sections of mid-jejunum were collected from another two randomly selected birds per cage and fixed in 10% neutral buffered formalin for morphological assessment. Subsequently, the samples were dehydrated with ethanol, cleared with sub-x/xylene, embedded in paraffin, and sectioned into 5 μm. The sections were then stained with hematoxylin-eosin stain for morphology measurement. Images of the stained samples were captured at 4 × magnification using BZ microscope (BZ-X800; Keyence Inc., Itasca, IL) and analyzed using BZ-X800 Analyzer. Villus height (the tip of the villus to the villus-crypt junction) and the corresponding crypt depth (villus-crypt junction to the base of the crypt) for six villi and crypts per section were determined. The villus height-to-crypt depth ratio was subsequently calculated.

Chemical analyses

Diets, excreta, ileal, and jejunal digesta were oven-dried and ground (0.5mm sieve) prior to chemical analyses. Except stated otherwise, all analyses followed the AOAC (2006; 2012) procedure. The dry matter (Method 934.0) was determined by drying the samples at 100°C for a period of 24 hours. The N content of the samples was measured with the aid of a combustion nitrogen analyzer (LECO, St. Joseph, MI) using 15 mg of sample. The gross energy was measured using an isoperibol bomb calorimeter (Model 6200, Parr Instruments, Moline, IL), with benzoic acid serving as the calibration standard. Total starch was analyzed using the Megazyme assay kir (K-AMYL). These RSS used in the current experiment belong to the RS2 category and are native RS. The RS assay was done by Eurofins Food Testing, Netherlands, using the AOAC method 2011.25. The titanium dioxide contents of the samples were evaluated as outlined by Short et al. (1996).

Caecal short-chain fatty acid profile

One gram of cecal digesta was diluted with 3-ml deionized water, mixed, and centrifuged (10,000 × g) for 10 minutes. The supernatant (1 ml) was mixed with 0.2 ml 25 % (w/v) metaphosphoric acid solution and frozen overnight. The thawed samples were then centrifuged, and 0.75 ml of the supernatant was mixed with 0.15 ml of internal standard to have 0.9 ml solution. 1.8 ml ethyl acetate was then added and tubes were vortexed for 15 seconds. The solution was allowed to settle for at least 5 minutes, and about 1.2 ml of the top layer was transferred into a glass vial, and the SCFA content was measured using gas chromatography (Shimadzu GC-2010 Plus; Shimadzu Corporation, Japan). Results were compared to known standards, and concentrations of SCFA were expressed in m*M* (Lourenco et al., 2020).

Caecal protein analysis

The soluble protein concentration of the cecal content was determined using a BCA protein assay kit, according to the manufacturer's instructions (EMD Millipore Corp, MA, USA).

Relative ileal oligosaccharide profile analysis

Matrix-assisted laser desorption ionization mass spectrometry was used for the evaluation of the ileal digesta oligosaccharide profile, as outlined by Lin and Olukosi (2021). Briefly, the ileal digesta from three randomly selected birds were collected, stored at -20°C prior to freeze-drying. About 30 mg of the digesta sample was dispersed in 7 ml of 80% ethanol, chilled at 4 °C for 1 hour, and centrifuged at 4 °C for 20 minutes at 1200 × g. The supernatant was dried by a stream of nitrogen, weighed, and then diluted with 1 ml of water. Approximately 100 µl of the diluted sample was then freeze-dried in a small glass vial overnight. The dried sample was permethylated using dimethyl sulfoxide and dichloromethane, washed with water, dried, dissolved in methanol, and crystallized with ʊ-dihydroxybenzoic acid. The relative abundance of each oligosaccharide was then determined using the relative intensity from matrix-assisted laser desorption ionization mass spectrometry (MALDI- MS) detection (Applied Biosystems, MDS Analytical Technologies). The abundance of each sugar was calculated relative to the total intensity of the sugars evaluated in percentage.

Quantitative real-time PCR analysis

Total RNA extraction was done using QiAzol lysis reagent (QIAGEN, Hilden, Germany (Invitrogen, Carlsbad, CA, USA). The RNAs extracted were cleaned using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as outlined by the manufacturer. The concentrations were measured with NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and adjusted to 200 ng/mL with nuclease-free water. 10 µl of the RNAs were reverse transcribed to cDNA in a

20 µl reaction volume using a high-capacity cDNA reverse transcription kit (Thermo Fischer Scientific, Waltham). The cDNA was diluted to 20 ng/µl following concentration measurement and the RT-qPCR was done using StepOnePlus (Applied Biosystems, Carlsbad, CA, USA) with reaction master mix iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). The housekeeping gene used was beta-actin and the fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The primer sequence and their functions are presented in Table 3.4.

Calculations

All calculations are presented on a dry matter basis. The total tract nutrient retention and apparent ileal digestibility of starch and energy, and DM were calculated using the index method by the following equations:

$$Digestibility\ (\%) = 100 \times [1 - ((Ci/Co) \times (No/Ni))]$$

$$DM\ Digestibility\ (\%) = 100 \times [1 - (Ci/Co)]$$

Where Ci and Co are the % concentrations of titanium dioxide in the diet and excreta/digesta, respectively; Ni and No are the % nutrient contents in the diet and excreta/digesta, respectively. Nitrogen-corrected apparent metabolizable energy (AMEn) and apparent metabolizable energy (AME) in kcal/kg were calculated as follows:

$$AME = GEi - [(Ci/Co) \times (GEo)]$$

$$AMEn = AME - [8.22 \times (NR/DMI)]$$

where GE_i and GE_o are gross energy contents (kcal/kg) of the diets and excreta, respectively. The NR is the retained nitrogen (g) and DMI is the dry matter intake (g) and calculated as follows:

$$NR\ (g) = (NI - NO)$$

$$DMI\ (g) = Feed\ intake, g \times Feed\ DM(coefficient)$$

Where NI and NO are nitrogen intake (g) and output (g), respectively.

3.4 STATISTICAL ANALYSIS

The statistical model for the experiment was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + [\beta(\gamma)]_{jk}\,[\beta(\delta)]_{jl} + [\beta(\gamma\delta)]_{jkl} + \varepsilon_{ijkl}$$

where $Yijkl$ is the response variable, μ is the overall mean, α_i is the random effect of *i*th block, β_j is the fixed effect of *j*th treatment group, $\beta(\gamma)_{jk}$ represent the fixed effect of *k*th resistant starch source within *j*th treatment group, $\beta(\delta)_{jl}$ represents the fixed effect of *l*th resistant starch level within *j*th treatment group. $\beta(\gamma\delta)_{jkl}$ *is the* interaction effect of *k*th resistant starch source and *l*th resistant starch level within *j*th treatment group, and the ε_{ijkl} is the residual error. Data were analyzed using the mixed model procedure of JMP Pro 13.2.0 (SAS Institute Inc., Cary, NC) in a 3 × 3 + 1 factorial arrangement. The factors were 3 resistant starch sources, each at 3 inclusion levels nested with the control diet. Means with significant differences were separated using Tukey's HSD, and the significant P-value was set at $P \leq 0.05$. Main effects are discussed when there are no significant interactions, whereas the simple effects are described in cases of significant interactions.

3.5 RESULTS

Growth performance

In the starter phase, RSS × RSL was significant ($P < 0.05$) for FCR (Table 3.5). The FCR of birds receiving BS or RPS was not influenced by inclusion levels, whereas birds receiving 50 g/kg HCS had higher FCR than other treatments except RPS at 100 g /kg. There were significant main effects of RSL and RSS ($P < 0.05$) for the WG and FI, respectively. Birds fed 100 g/kg RS had higher ($P = 0.034$) WG than 50 g/kg. The feed intake was greater ($P = 0.05$) in the birds that

received BS and RPS diets and similar across all the diets. During the grower phase, there was a significant ($P < 0.05$) RSS × RSL for FI. Birds fed the 100 g/kg RPS diet had higher FI ($P = 0.042$) than 25 g /kg BS and the control diet. The main effects of RSS and RSL were significant ($P < 0.05$) for FCR. Birds that received BS diets had increased FCR ($P = 0.018$) than other treatments, whereas the birds that received 100 g/kg RS had greater ($P = 0.045$) FCR than others. In the overall phase, the main effects of RSS were significant for FI and FCR ($P < 0.05$). Birds fed the RPS diets had higher ($P = 0.011$) FI than those that received HCS or control diets. On the other hand, FCR was greater ($P = 0.030$) for birds that received BS diets than for all other diets. There were no significant effects on the final body weight.

Nutrient digestibility, total tract retention, and metabolizable energy

There were significant ($P < 0.05$) RSS × RSL for all the responses on d 21 (Table 3.6). For jejunal DM, digestibility was greater ($P = 0.01$) in the control diet than all levels of BS, 100 g/kg RPS and 25 g/kg HCS. Jejunal starch digestibility was greater ($P < 0.001$) in the control diet than all levels of BS and 50 and 100 g/kg RPS. Jejunal starch digestibility decreased ($P < 0.05$) with increasing RSL for all RS except for HCS.

For apparent ileal nutrient digestibility, the DM digestibility was greater ($P < 0.001$) in birds fed the control diet than in all levels of BS, 100 g/kg RPS, and 25 g/kg HCS. Nitrogen digestibility was greater ($P = 0.01$) in the control diet, all levels of RPS, and 50 and 100 g/kg HCS than in 50 g/kg BS diets. The ileal starch digestibility was greater ($P < 0.001$) in the control diet than in all RS diets. The ileal starch digestibility decreased as the RSL increased for all RS except for HCS.

For total tract nutrient utilization, N retention was greater ($P < 0.001$) in birds fed the control diet, 100 g/kg RPS, and 100 g/kg HCS diets than all levels of BS and 25 g/kg HCS. The N retention increased with increasing RPS and HCS levels but decreased with increasing levels of BS. Starch

retention was greater ($P < 0.001$) in the control diet than in all RS diets. The AME and AMEn were greater ($P < 0.001$) in birds fed 100 g/kg HCS diet than in other RS diets, and both decreased with increasing RSL for all RS except HCS.

Relative Ileal Oligosaccharides Profile

There were ($P < 0.05$) RSS × RSL for the relative abundance of Hex (3) and Pent (3) (Table 3.7). The relative abundance of Hex (3) was greater ($P = 0.01$) in birds fed 50 g/kg BS, 100 g/kg RPS, 25 and 100 g/kg HCS and the control diets. Birds fed 50g/kg HCS had greater ($P = 0.001$) relative abundance of Pent (3) than 50 and 100 g/kg BS, 25 and 100 g/kg RPS and HCS. Pent (3) had greater relative abundance ($P = 0.001$) in birds fed 50 g/kg of RPS and HCS than other inclusion levels except BS with higher relative abundance of Pent (3) at 25 g/kg. There was a significant RSL main effect ($P = 0.01$) on Hex (5) relative abundance, with birds fed 50 g/kg RS diets having lower relative abundance than the control and 100 g/kg.

Cecal short-chain fatty acids profile

The main effects of RSL were significant ($P < 0.05$) for all the cecal SCFA analyzed and there was a significant main effect of RSS and RSL for total SCFA (Table 3.8). The individual and total SCFA concentrations ($P < 0.05$) were increased by 25 g/kg and 100 g/kg RS diets. Isobutyrate and isovalerate concentrations were greater ($P < 0.05$) at 25 g/kg than at 50 g/kg RSL. The birds that received HCS diets had elevated ($P = 0.029$) cecal total SCFA than those fed RPS diets.

Intestinal digesta pH and cecal protein

There were no significant treatment effects on the duodenal, jejunal, ileal, and cecal digesta pH in this experiment (Table 3.9). However, the main effects of RSS and RSL were significant (P

< 0.05) for cecal protein. Dietary BS and HCS reduced (P = 0.001) the cecal protein and in addition, 25 g/kg RS diets reduced (P = 0.03) the cecal protein more than the other RS levels.

Digestive organs index

The main effect of RSL was significant (P <0.05) for relative duodenal weight and cecal weights whereas the RSS × RSL was only significant for relative ileal weight (Table 3.10). The relative weight of duodenum generally increased (P < 0.037) with increasing RSL. Birds fed the 100 g/kg BS diet had higher (P = 0.03) relative ileal length than those fed 25 g/kg RPS, and this increased with increasing BS level. The relative cecal weight was greater (P = 0.01) in birds fed 100 g/kg RS than 25 and 50 g/kg RS.

Jejunal Histomorphology

There were no significant RSS × RSL or main effects for jejunal histomorphology except for the significant RSS effect on crypt depth (Table 3.11). Birds that received RPS and HCS diets had deeper (P < 0.01) jejunal crypts than those that received the BS diets.

Jejunal mRNA expression of glucose and peptide transporters and epithelial integrity genes and caecal expression of fatty acid transporters

The jejunal GLUT-2 mRNA expression was greater (P = 0.02) in birds that received RPS or BS than those fed HCS diet (Table 3.12). There was significant RSS × RSL for jejunal PEPT1 relative expression. Generally, relative expression of PEPT1 increased (P < 0.05) with increasing BS level but decreased with increasing level of RPS or HCS. The relative mRNA expression of jejunal MUC-2 was lower (P < 0.05) for birds that received BS and HCS than the control diet. The main effects of RSL were significant (P < 0.05) for caecal FFAR2 and MCT4. Birds fed 25 g/kg RS had greater (P = 0.003) mRNA expression of FFAR2 than those fed the 100 g/kg RS diet. On

the other hand, birds fed 50 g/kg RS or the control diet had greater (P = 0.05) mRNA expression of MCT4 than those fed 100 g/kg. The RSS × RSL was significant (P < 0.05) for cecal MCT1. Birds fed the control diet had higher (P = 0.001) mRNA expression of MCT1. Among RS diets, birds fed 25 and 100 g/kg BS had greater MCT1 expression than 50 g/kg BS, and those fed 50 g/kg HCS had greater relative expression MCT1 than those fed 100 g/kg.

3.6 DISCUSSION

Fermentation of resistant starch (RS) can enhance gut health through the production of microbial metabolites such as short-chain fatty acids (SCFAs). However, the growth performance of the host organism may be compromised depending on various factors such as the source, concentrations, and duration of RS feeding, as energy from SCFAs is less efficiently utilized than that from glucose. Some studies demonstrated that the weight gain, feed intake and feed efficiency may decrease (Li et al., 2007; Regmi et al., 2011) or not influenced (Nofrarías et al., 2007; Doti et al., 2014) by RS consumption. Hence, the current study was aimed at investigating the influence of different sources and levels of RS on the growth performance and nutrient utilization in broiler chickens in the starter and grower phases. We, therefore, hypothesized that the source and level of RS influence nutrient utilization with consequences on caecal SCFA production, ileal oligosaccharide profile, intestinal health and ultimately the growth performance of broiler chickens.

Growth performance

The observed increase in WG in the starter phase of the current experiment at highest concentration of RS (100 g/kg) was accompanied by higher FI observed at this level and could probably be due to lower caloric density of RS which might have fueled the increased feed intake associated with high level of RS inclusion to compensate for reduced energy intake (Zhou et al.,

2008; Higgins, 2014). Furthermore, the results obtained in the grower phase with different sources and levels of RS having varying FI and FCR (reduced FCR with RPS and HCS diets), and increased FI as RPS level increased) could be because of the differences in crystallinity and degree of resistance to pancreatic amylase which ultimately influenced their digestion and subsequent fermentability in the distal GIT. Although, despite the crystallinity pattern shared by HCS and RPS, the former is believed to be more resistant due to its higher amylose content and the additional resistance conferred by its potential to form RS3. On the other hand, BS has a different crystallinity pattern and so is considered to be less resistant (Jane et al., 1999; Giuberti et al., 2015). Some banana cultivars exhibit crystallinity similar to RPS and HCS thus showing cultivar-dependence in crystallinity patterns (Wang et al., 2017).

The results obtained on feed intake correspond with the reports of Martinez-Puig et al. (2003) and Fang et al. (2014) that the inclusion of 25% RPS in growing pigs diet elevated the FI when compared with corn starch. It is also in line with increased BW and FI in 1-14 days old ducks fed RPS supplementation in normal non-phytate phosphorous diets (Xu et al., 2021). This was attributed to the varying ability of RS to stimulate intestinal movement, increase absorption and utilization of nutrients in the intestines. Also, availability of microbial-produced SCFA as energy source could reduce glucose oxidation, mediating the effects of RS on growth performance as the birds in this study had ad libitum access to feed (Van Erp et al., 2020). The increased concentration of total SCFA (including butyrate, propionate and acetate) in this study at 25 and 100 g/kg RS diets further corroborate the increased WG in birds fed 100 g/kg RS diets during the starter phase and 25 g/kg RS that seems optimum for growth performance in the grower phase.

Nutrient Digestibility, Total Tract Retention and Metabolizable Energy

Our observations on apparent jejunal, ileal nutrient digestibility, AMEn and total tract nutrient retention partially agree with the previous finding whereby increased concentrations of RS lowered the total tract nutrient utilization as well as apparent ileal digestibility of nutrients (Gerrits et al., 2012; Cervantes-Pahm et al., 2014; Giuberti et al., 2015). The inclusion of RS in broiler diets reduced starch digestibility as expected, although 25 g/kg RPS and all levels of HCS had comparable jejunal starch digestibility to the control diet. Morel et al. (2005) postulated that a 1% dietary increase in RS may reduce the total ileal starch by 0.64 %.

The observed decrease in jejunal and ileal starch digestibility as the RSL increased for all RS except for HCS may be associated with different physiochemical properties peculiar to RS of different botanical origins resulting in changes in the rate of nutrient absorption and varying endogenous secretions (Giuberti et al., 2015) but also due to the observation that virtually all the starch in HCS was resistant. Further calculations (supplementary figure) indicating a smaller marginal increase (2.23 %) in starch digestibility between jejunum and ileum for the birds that received the HCS diets than other RS sources suggested that most of the starch digestibility in the birds fed the HCS diet occurred in the jejunum. This may not necessarily indicate a degree of resistance of the RS in HCS but a consequence of HCS having nearly 100% resistant starch by analysis compared to BS and RPS.

Greater N retention that increased with increasing RPS and HCS levels was supported by the lower FCR observed in the birds fed these RSS. In addition, the increase in AME and AMEn with increasing levels of HCS could be due to greater total cecal SCFA production and higher jejunal starch digestibility of HCS. This was corroborated by the general view that different RS or even RS of the same type at the same or varying levels of inclusion may elicit variable metabolic

responses with evident implications on fermentation kinetics, performance parameters, energy utilization, and nutrient digestibility due to different crystalline polymorphism and physiochemical properties of the starches (Deng et al., 2010; Bach Knudsen, 2011; Haenen et al., 2013; Giuberti et al., 2015).

Relative ileal oligosaccharide and short-chain fatty acid profiles

Starch digestibility, as influenced by the level and source of RS fed, could determine the type of oligosaccharides that reached the distal GIT (Wiseman, 2006). The profile of distal oligosaccharide, in turn, dictates the different degree of penetration of bacteria into these starch granules, which is related to granule dimension and surface area. This is crucial for certain bacterial strains because substrates need to be broken down by bacterial hydrolytic enzymes before fermentation. The rate of this depolymerization affects how readily RS and other non-digestible carbohydrates are available for bacterial fermentation (Macfarlane and Macfarlane, 2003; Martinez-Puig et al., 2003). These might be the likely reason why different RSS and RSL, influenced the relative abundance of ileal oligosaccharide profile differentially in this study.

Lin and Olukosi (2021) previously reported that dietary fiber modulated the digesta oligosaccharide concentrations with higher Pent(3), Pent(4), Pent(5) and greater SCFA in protease-only supplemented high-fiber diets than in protease-only supplemented low-fiber diets. In the current study, the greater abundance of Hex(3) (moderate mass and chain length) in the HCS diet than in other RS diets, along with a greater abundance of Pent (3) (smaller mass and short chain length) in the birds fed HCS was accompanied with higher total SCFA in birds receiving this diet. This could be a function of its higher starch digestibility than other RS sources which could have significant impact on the structure of the oligosaccharide that entered the ceca. The structure of these oligosaccharides, in turn, might have increased accessibility to fermentative bacteria

evidenced by greater production of SCFA in birds fed HCS diets (Tan et al., 2021). These indicated that not only the amount of RS entering the large intestine that matters (Haenen et al., 2013) but its composition and physical structure are crucial and may affect total SCFA production and individual SCFA profile (Martin et al., 1998; Jonathan et al., 2012).

The SCFA contributes to the normal functioning of the gut by acting on the intestinal musculature and vasculatures through their impacts on metabolism of enterocytes and colonocytes (Elia and Cummings, 2007; Hamer et al., 2008; Regassa and Nyachoti, 2018). The SCFA profile in response to RS feeding in the current experiment is similar to previous studies that reported an increase in SCFA production in pigs and birds fed RS diets (Haenen et al., 2013; Qin et al., 2019; Trachsel et al., 2019). Higher production of SCFA in birds fed HCS diets could be attributed to its characteristic ability to enhance substrate flow to the hindgut thereby facilitating increased fermentative production of SCFA (Bird et al., 2007; Regmi et al., 2011; Fouhse et al., 2015; Tan et al., 2021). Our finding on HCS also aligns with that of Den Besten et al. (2013) who reported an increase in cecal and fecal concentrations of butyrate and total SCFA concentration in pigs fed 63% dietary high amylose starch.

Digesta pH

Despite the increase in the cecal SCFA production observed in this study, the duodenal, jejunal, ileal, and cecal pH were not significantly affected. Although, Fouhse et al. (2015) and Tan et al. (2021) reported reduced pH through production of SCFA in the hindgut of pigs fed dietary HCS diets and a reduction in the pH of both ileal and cecal digesta were found in pigs fed daily with 0.5% or 1.0% dietary raw potato starch over a 28-day period (Heo et al., 2014) , whereas the ileal digesta pH was not significantly different in ducks fed varied levels of RPS diets for a period of 14 and 35 days (Qin et al., 2019). The disparity between these results could be due to differences

in experimental conditions, RS sources, concentrations, specie of animal and feeding length used in different studies.

Caecal protein

Functional fibers are capable of increasing carbohydrate fermentation relative to protein in the hindgut thereby minimizing the disproportionate production of branched-chain volatile fatty acids, ammonia and hydrogen sulfide which may have toxic potential on colonic cells (Magee et al., 2000; Mu et al., 2016). The decrease in cecal protein observed in RS diets is a pointer to increase in carbohydrate fermentation relative to protein in the ceca and corresponds with reduced cecal protein and N- levels reported in broiler chickens fed prebiotics and xylo-oligosaccharide (Lin et al., 2023). This further corroborates the prebiotic effects of RS in distal GIT. Another possible reason for decreased cecal protein is that, the lower total tract and apparent ileal starch digestibility in birds receiving dietary RS may have increased carbon availability in the ceca, raising the carbon-nitrogen ratio of the substrates that were available for microbial fermentation (Sun et al., 2016).

Histomorphology of the jejunum and digestive organ index

The knowledge of digestive organ histomorphology helps explain the impact of RS on nutrient utilization as longer villi is indicative of increased surface area and absorptive capacity and deeper crypt may indicate greater cellular turnover and higher organ index may be relevant for energy expenditure of the digesta tract (Montagne et al., 2003). The deeper jejunal crypt depth observed with RPS and HCS diets may be due to digesta viscosity effect of these RS diet (Guo et al., 2022), the viscosity effect being part of the mechanism behind the reduction of cholesterol level by RS through retardation of the interactions between sugars and fatty acids (Mudgil, 2017). Additionally, the tendency for longer jejunal villi in birds that received HCS diets than other diets indicated increased proliferation of jejunal epithelial cells evidenced by significant greater

production of cecal total SCFA and acetate as well as numerically higher propionate and butyrate in birds fed HCS diets than other RS diets and the control.

Generally, high inclusion of RS increased the relative weight of both proximal (duodenum) and distal GIT (caecum) which correspond with the results obtained for SCFA. The increased relative organ weights and lengths by dietary RS in the current study indicated fermentative production of SCFA which promoted the proliferation of mucosal epithelium and the growth of enterocytes, increasing the surface area for nutrient absorption. The results on organ index is in line with previous studies that reported an increase in ileal length in ducks fed 60 to 180 g/kg RPS diets (Qin et al., 2019) and increased in the length of the small intestine in rats fed dietary inclusion of 30 % HAM (Toden et al., 2007). It also agrees with the enlargement of ceca reported in rat fed dietary RS (Faulks, 2003).

Gene expression in the jejunum and caeca

Resistant starch (RS) influences the metabolism of the host by modifying the metabolites present in systemic circulation and the expression of intestinal genes that play crucial roles in gut health and nutrient metabolism (Regassa and Nyachoti, 2018). The transport of glucose (the final product of starch digestion) into the bloodstream is facilitated by GLUT-2 (Wright and Loo, 2000). The upward relative mRNA expression of GLUT-2 in the jejunum of broiler chickens fed diets containing RPS and BS implies the adjustment of their capacity for increased intestinal glucose uptake (Sajilata et al., 2006; Gilbert et al., 2008). Higher expression of jejunal PepT1 in the birds fed diets containing 50, 100 g/kg BS and 25, 50 g/kg RPS and 25 g/kg HCS suggests that dietary RS may increase luminal peptide uptake as supported by decreased cecal protein in birds fed dietary RS in the current study.

Dietary RS may increase mucin secretion and improve the gut barrier functions (Qin et al., 2019). Higher mRNA expression of MUC-2 in birds fed RPS and control diets partially aligns with the reported increase in the expression of colonic mucin genes in growing and finishing pigs fed dietary 23 and 28% RPS in comparison with a corn starch diet (Zhou et al., 2017). The jejunal mRNA expression of occludin was not significantly affected by RS inclusion in our study, although it was numerically higher in birds fed certain sources and level of RS (BS, RPS and 50 g/kg RS) than in the control diets. However, the positive effect of RS on intestinal mucin secretion and tight junction genes have only been reported in the distal GIT (ileum and ceca) (Zhou et al., 2017; Qin et al., 2023), being the major site of RS fermentation.

The activation of free fatty acid receptors 2 and 3 (FFAR2 and FFAR3, respectively) by SCFA in the gastrointestinal tract stimulate the release of satiety-stimulating hormones that regulate feed intake (Hirasawa et al., 2005). The expression of FFAR3 was comparable between the dietary treatments used in this study. The observed depression in cecal mRNA expression of FFAR2 in birds fed 100 g/kg RS, irrespective of source, corresponded with the results obtained for the feed intake. There was a numerical increase in feed intake with increasing RSL. This suggests that the reduction in the expression of FFAR2 could have inhibited the release of satiety of hormones, thereby preventing the reduction in feed intake at that concentration (Hirasawa et al., 2005; Kaji et al., 2011). Haenen et al. (2013) also observed a comparable expression of FFAR2 and FFAR3 in the pigs fed RS or digestible starch diets regardless of higher caecal and colonic SCFA production in RS-fed pigs. The depression in expression of FFAR2, irrespective of higher production of cecal SCFA in the current study could probably be the consequence of the similar results obtained for both SCFA production and FFAR2 in birds fed high RS diet and the control

diets, suggesting that the SCFA produced might not be sufficient to elicit the activation of the receptors and subsequently the satiety hormones.

The uptake of the SCFA from the lumen is facilitated by monocarboxylate transporter 1 (MCT1) and across the basolateral membrane by monocarboxylate transporter 4 (MCT4) (Den Besten et al., 2013). The results showed that the birds fed moderate RS level (50 g/kg RS) and the control diet had greater and comparable mRNA expression of MCT4 whereas, the MCT1 was more expressed in control diets than all RS diets, although 50 g/kg HCS, 25 and 100 g/kg BS diet favored higher expression of MCT1 than other RS diets. A previous study reported higher expression of MCT1 in the caecum of pigs fed RS-diet relative to digestible starch diets (Haenen et al., 2013). The little disparity in these results could be the effects of different animal model, length of feeding, concentrations and sources of RS.

3.7 CONCLUSION

The Type-2 RS used in this study differentially modulated the nutrient digestion and energy utilization in broiler chickens based on their botanical origins and dietary concentrations with consequences on the composition and structure of oligosaccharides entering the distal gastrointestinal tract. These have possible consequences on the extent of cecal fermentation and, subsequently, the total SCFA production as well as on individual SCFA profiles, each eliciting variable effects on GIT morphometrics and growth performance responses. Future studies should investigate the impact of prolonged feeding of RS on broiler chickens. In addition, phase-specific effects of individual RS at various concentrations should be evaluated as these would provide a clearer understanding of the influence of individual RS on growth performance and gastrointestinal health metrics.

We acknowledge the limitation of not having the same level of analyzed RS in all the diets using the different RSS. An attempt to achieve the same analyzed RS level in all diets will necessitate including some RSS at impractically high levels. Nevertheless, the effect of increasing the levels of each RSS can be interpreted within its context in as much as increasing the inclusion levels of each RSS was accompanied by a corresponding increase of its analyzed level in the respective diets.

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Table 3.1. Analyzed chemical profile of resistant starches used in the experiment (g/kg, as fed).

Resistant starch source	DM	Total starch	Resistant starch	N	GE, kcal/kg
Banana starch	899	729	373	4.6	3,833
Raw potato starch	894	806	651	0.7	3,442
High-amylose corn starch	898	368	350	1.9	3,744

DM-Dry matter, RS- Resistant starch, GE- Gross energy

Table 3.2. Ingredients and chemical composition (g/kg) of the starter phase (d 0 to 8) diets.

Items	Control	Banana starch			Raw potato starch			Hi-amylose corn starch		
Corn	629	598	566	495	598	560	485	593	551	469
Banana starch		25	50	100						
Raw potato starch					25	50	100			
Hi-amylose corn starch								25	50	100
Soybean meal	325	328	330	340	328	335	348	329	336	350
Soybean oil	10	12	17	28	12	18	30	16	26	44
Dicalcium phosphate	19	19	19	19	19	19	19	19	19	19
Limestone	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6
Sodium bicarbonate	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin/TM premix	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
DL- Methionine	1.5	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
L- Lysine. HCl	1.1	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Threonine	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Salt	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Calculated nutrients, g/kg										
Protein, g/kg	212	211	210	211	211	211	211	211	211	212
ME, kcal/kg	2989	2974	2974	2975	2974	2975	2978	2975	2978	2974
Available P, g/kg	4.6	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Ca, g/kg	9.0	9.0	9.0	9.0	9.0	9.0	9.1	9.0	9.0	9.1
Digestible amino acids, g/kg										
Lys	12.2	12.3	12.3	12.4	12.3	12.5	12.6	12.4	12.5	12.7
Met	4.8	4.9	4.8	4.8	4.9	4.9	4.8	4.9	4.8	4.8
Thr	8.3	8.2	8.2	8.2	8.2	8.3	8.3	8.2	8.2	8.3
TSAA	8.3	8.3	8.2	8.1	8.3	8.3	8.2	8.3	8.3	8.2
Analyzed nutrient composition, g/kg										
Protein	196	166	186	191	198	171	190	196	186	192
GE, kcal/kg	3984	3919	4197	4504	4657	4927	5246	3982	4039	4068
Total starch	380	416	399	374	382	379	385	388	376	342

The vitamin and mineral premix provided (per kg of diet): Vitamin A, 5,484 IU; vitamin D3, 2,643 IU; vitamin E, 11 IU; menadione sodium bisulfate, 4.38 mg; riboflavin, 5.49 mg, d-pantothenic acid, 11 mg; niacin, 44.1 mg, choline chloride, 771 mg; vitamin B12, 13.2 µg; biotin, 55.2 µg; thiamine mononitrate, 2.2 mg; folic acid, 990 µg; pyridoxine hydrochloride, 3.3 mg, I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 µg.

Table 3.3. Ingredients and chemical composition (g/kg) of the grower phase (d8 – 21) diets.

Items	Control	Banana starch			Raw potato starch			Hi-amylose corn starch		
Corn	662	627	591	517	627	590	514	623	582	498
Banana starch		25	50	100						
Raw potato starch					25	50	100			
Hi-amylose corn starch								25	50	100
Soybean meal	287	292	297	310	292	298	312	292	299	314
Soybean oil	10.0	15.0	21.0	32.0	15.0	21.0	32.5	18.5	28.0	47.0
Dicalcium phosphate	18.2	18.3	18.4	18.4	18.4	18.5	18.8	18.4	18.5	18.8
Limestone	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.7	7.2
Sodium bicarbonate	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin/trace minerals premix	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
DL- Methionine	1.5	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6
L- Lysine.HCl	1.2	1.3	1.3	1.3	1.3	1.3	1.2	1.3	1.3	1.2
L-Threonine	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Titanium dioxide	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Salt	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Calculated nutrients, g/kg										
Protein	196	196	197	198	196	196	196	196	196	197
ME, kcal/kg	3010	3008	3010	3008	3007	3009	3007	3007	3007	3008
Available P, g/kg	4.35	4.36	4.37	4.35	4.37	4.37	4.39	4.36	4.36	4.38
Ca	8.7	8.8	8.8	8.8	8.8	8.8	8.9	8.8	8.8	8.8
Digestible amino acids, g/kg										
Lys	11.2	11.3	11.4	11.6	11.3	11.4	11.6	11.3	11.4	11.6
Met	4.6	4.7	4.7	4.6	4.7	4.7	4.6	4.7	4.7	4.6
Thr	7.5	7.5	7.5	7.6	7.5	7.5	7.6	7.5	7.5	7.6
TSAA	7.9	7.9	7.9	7.8	7.9	7.9	7.8	7.9	7.9	7.8
Analyzed nutrients, g/kg										
Protein	180	176	179	174	182	180	188	181	181	194
Gross energy, kcal/kg	4335	4321	4385	4411	4361	4434	4382	4384	4409	4480
Total starch	439	452	425	416	459	441	428	418	428	423
Resistant starch	8.0	10.1	12.2	16.4	24.3	40.5	73.0	13.7	19.4	30.8

The vitamin and mineral premix provided (per kg of diet): Vitamin A, 5,484 IU; vitamin D3, 2,643 IU; vitamin E, 11 IU; menadione sodium bisulfate, 4.38 mg; riboflavin, 5.49 mg, d-pantothenic acid, 11 mg; niacin, 44.1 mg, choline chloride, 771 mg; vitamin B12, 13.2 µg; biotin, 55.2 µg; thiamine mononitrate, 2.2 mg; folic acid, 990 µg; pyridoxine hydrochloride, 3.3 mg, I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 µg.

Table 3.4. List of primers and their functions.

Symbol	Gene name	Function	Forward primer	Reverse primer
βeta-actin	β-actin	Housekeeping gene	ACCGGACTGTTACCAACACC	GACTGCTGCTGACACCTTCA
MUC2	Mucin 2	Mucin secretion	ATGCGATGTTAACACAGGACTC	GTGGAGCACAGCAGACTTTG
OCLN	Occludin	Tight junction	CTGCTCTGCCTCATCTGCTTCTTC	CCATCCGCCACGTTCTTCACC
GLUT- 2	Glucose transporter- 2	Glucose transporter	TCATTGTAGCTGAGCTGTT	CGAAGACAACGAACACATAC
PepT1	Peptide transporter- 1	Peptide transporter	CCCCTGAGGAGGATCACTGTT	CAAAAGACCAGCAGCAACGA
FFAR2	Free-fatty acid receptor – 2	Free-fatty acid receptor	AACGCCAACCTCAACAACTC	TGGGAGAAGTCATCGTAGCA
FFAR3	Free-fatty acid receptor – 3	Free-fatty acid receptor	GAAGGTGGTTTGGGAGTGAA	CAGAGGATTTGAGGCTGGAG
MCT4	Monocarboxylate transporter 4	SCFA transporters	GCTGGTCTCAAGTGGGTTAG	CCACCGTAATCGACAGACATAG
MCT1	Monocarboxylate transporter 1	SCFA transporters	AGCAGCATCCTGGTGAACAAG	AGGCACCCACCCACGAT

Table 3.5. Growth performance response of the broiler chickens receiving dietary resistant starches from different sources fed at different levels up to day 21.

RSL, g/kg	RSS	Starter phase (d 0 – 8)			Grower (d 8 – 21)			Overall (d 0 – 21)			
		WG, g	FI, g	FCR	WG, g	FI, g	FCR	WG	FI, g	FCR	FBW, g
Control (0)		114^{AB}	159^{ab}	1.40^{b}	717	975^{b}	1.50^{bB}	831	1134^{b}	1.47^{b}	874
Main effects means of resistant starch source (RSS)											
	BS	117	170^{a}	1.46	633	1030	1.67^{a}	750	1199^{ab}	1.63^{a}	793
	RPS	113	167^{a}	1.50	730	1066	1.50^{b}	843	1233^{a}	1.49^{b}	886
	HCS	106	158^{ab}	1.52	666	996	1.51^{b}	772	1154^{b}	1.50^{b}	816
Main effects means of resistant starch level (RSL)											
25		113^{AB}	163	1.46	692	1009	1.51^{B}	805	1172	1.49	849
50		105^{B}	162	1.57	686	1037	1.52^{B}	791	1199	1.52	835
100		118^{A}	170	1.46	651	1046	1.66^{A}	769	1216	1.61	812
Pooled SEM		4.1	4.2	0.030	35	18	0.050	34	21	0.046	34
Simple effect means											
25	BS	115	165	1.44^{b}	645	1005^{b}	1.58	760	1170	1.55	804
50	BS	114	170	1.50^{b}	681	1072^{ab}	1.58	795	1242	1.57	838
100	BS	122	174	1.44^{b}	572	1012^{ab}	1.86	694	1186	1.77	738
25	RPS	112	164	1.50^{b}	782	1028^{ab}	1.40	894	1192	1.40	937
50	RPS	112	165	1.49^{b}	680	1033^{ab}	1.53	792	1199	1.52	837
100	RPS	114	173	1.53^{ab}	728	1136^{a}	1.57	842	1309	1.56	885
25	HCS	111	160	1.45^{b}	650	995^{b}	1.54	762	1154	1.52	806
50	HCS	89	151	1.72^{a}	697	1005^{b}	1.44	786	1156	1.47	830
100	HCS	118	163	1.41^{b}	652	989^{b}	1.54	770	1152	1.52	814
Pooled SEM		5.9	6.1	0.040	52	28	0.080	51	31	0.068	501
Probabilities											
RSS		0.092	0.050	0.158	0.071	0.012	0.018	0.074	0.011	0.030	0.074
RSL		0.034	0.218	0.002	0.568	0.253	0.045	0.678	0.227	0.069	0.676
RSS × RSL		0.155	0.829	0.001	0.463	0.042	0.342	0.512	0.075	0.403	0.514

RSS- Resistant starch source, RSL- Resistant starch level, BS- Banana starch, RPS- Raw potato starch, HCS - High-amylose corn starch, WG- body weight gain, FI- Feed Intake, FCR- feed conversion ratio, FBW: Final Body Weight. n = 8 replicate cages per treatment.

$^{AB, ab}$: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$). Small letter superscripts compare the RSS group nested with control, or the simple effect means nested with control (in cases of significant interaction). Block letter superscripts compare RSL nested with control.

Table 3.6. Apparent jejunal and ileal nutrient digestibility, total tract nutrient retention, and dietary metabolizable energy on d 21 for the broiler chickens receiving dietary resistant starches from different sources fed at different levels.

RSL,	RSS	Jejunum		Ileum				Total tract		
g/kg		DM	Starch	DM	N	Starch	N	Starch	AME, kcal/kg	AMEn, kcal/kg
Control (0)		62.0^{a}	96.0^{a}	77.8^{a}	81.3^{a}	98.57^{a}	67.6^{a}	96.4^{a}	3428^{ab}	3352^{ab}
Main effects means of resistant starch source (RSS)										
	BS	44.1	88.7	66.2	72.0	91.25	53.2	87.6	3044	2935
	RPS	53.5	88.6	72.6	79.2	91.1	63.2	88.5	3249	3160
	HCS	54.0	93.2	72.8	77.73	95.4	61.5	91.7	3293	3199
Main effects means of resistant starch level (RSL)										
25		49.6	92.7	71.0	75.2	95.4	58.4	92.1	3180	3082
50		50.0	90.6	70.2	75.4	92.8	59.3	90.1	3193	3096
100		51.6	87.3	70.5	70.5	89.5	60.3	85.6	3212	3117
Pooled SEM		1.52	0.524	0.76	1.33	0.38	1.20	0.33	20.0	22.0
Simple effect means										
25	BS	43.9^{d}	91.9^{bcd}	68.7^{cde}	73.6^{ab}	95.3^{b}	57.6^{bc}	92.5^{b}	3138de	3040de
50	BS	43.8^{d}	89.6^{cd}	63.8^{e}	67.3^{b}	91.1^{c}	53.8^{cd}	88.2^{d}	3006^{ef}	2897^{ef}
100	BS	44.6^{d}	84.6^{e}	66.1^{de}	75.0^{ab}	87.4^{d}	48.3^{d}	82.2^{e}	2987^{f}	2869^{f}
25	RPS	57.2^{abc}	92.9^{abc}	73.9^{ab}	79.2^{a}	95.2^{b}	61.4^{abc}	92.1^{bc}	3244^{cd}	3152^{cd}
50	RPS	52.4^{abcd}	88.2^{d}	73.3^{abc}	78.9^{a}	91.7^{c}	62.4^{ab}	90.2^{cd}	3321^{bc}	3232^{bc}
100	RPS	51.0^{bcd}	84.7^{e}	70.3^{bcd}	79.4^{a}	86.3^{d}	65.8^{a}	83.2^{e}	3181^{d}	3097^{cd}
25	HCS	47.8^{cd}	93.2^{ab}	70.3^{bcd}	72.7^{ab}	95.8^{b}	56.3^{bcd}	91.7^{bc}	3158^{d}	3054^{d}
50	HCS	55.2^{abc}	93.9^{ab}	73.4^{abc}	80.0^{a}	95.7^{b}	61.6^{abc}	92.0^{bc}	3252^{cd}	3160^{cd}
100	HCS	59.2^{ab}	92.5^{abc}	74.9^{ab}	80.5^{a}	94.8^{b}	66.7^{a}	91.3^{bc}	3469^{a}	3384^{a}
Pooled SEM		2.24	0.77	1.11	1.95	0.55	1.76	0.48	29.4	32.3
Probabilities										
RSS		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
RSL		0.555	< 0.001	0.680	0.097	< 0.001	0.433	< 0.001	0.403	0.424
RSS × RSL		0.005	< 0.001	< 0.001	0.012	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

RSS- Resistant starch source, RSL- Resistant starch level, BS- Banana starch, RPS- Raw potato starch, HCS - High-amylose corn starch, DM – dry matter, N – nitrogen.

n = 8 replicate cages per treatment.

$^{AB, ab}$: Means in a column, within a group, but with different superscripts differ significantly ($P \leq 0.05$). Small letter superscripts compare the RSS group nested with control, or the simple effect means nested with control (in cases of significant interaction). Block letter superscript compares RSL nested with control.

Table 3.7. Relative ileal oligosaccharide profile (%) on d 21 for the broiler chickens receiving dietary resistant starches from different sources fed at different levels.

RSL (g/kg)	RSS	Hex(3)	Hex(4)	Hex(5)	Hex(6)	Pent(3)	Pent(4)	Pent(5)	Pent(6)
Control		35.9a	42.9	13.6^{A}	0.99	1.53^{b}	0.00	2.73	0.00
Main effects means of resistant starch source (RSS)									
	BS	30.4	30.8	6.28	0.59	3.63	0.02	4.19	0.00
	RPS	29.5	28.4	7.83	1.28	2.56	0.10	4.10	0.33
	HCS	32.7	36.1	7.97	0.60	4.38	0.00	5.26	0.15
Main effects means of resistant starch level (RSL)									
25		29.6	32.3	8.24^{AB}	1.04	2.89	0.12	3.76	0.07
50		26.9	28.0	4.60^{B}	0.67	5.35	0.00	4.93	0.35
100		36.1	35.1	9.25^{A}	0.76	2.34	0.00	4.85	0.06
Pooled SEM		3.45	3.98	1.751	0.550	0.897	0.081	1.588	0.170
Simple effect means									
25	BS	20.3^{c}	26.1	5.23	0.02	5.42^{ab}	0.05	3.88	0.00
50	BS	36.9^{a}	38.4	4.08	0.58	1.81^{b}	0.00	3.17	0.00
100	BS	33.9^{ab}	28.0	9.54	1.18	3.67^{b}	0.00	5.51	0.00
25	RPS	30.1^{b}	29.6	8.53	2.45	1.54^{b}	0.31	3.50	0.21
50	RPS	20.1^{c}	22.2	4.63	0.74	4.88^{ab}	0.00	4.71	0.61
100	RPS	38.2^{a}	33.5	10.3	0.63	1.26^{b}	0.00	4.09	0.18
25	HCS	38.3^{a}	41.2	11.0	0.65	1.71^{b}	0.00	3.92	0.00
50	HCS	23.7^{c}	23.4	5.07	0.68	9.37^{a}	0.00	6.92	0.45
100	HCS	36.1^{a}	43.7	7.87	0.47	2.08^{b}	0.00	4.96	0.00
Pooled SEM		4.93	5.69	2.501	0.786	1.282	0.115	2.269	0.243
Probabilities									
RSS		0.724	0.317	0.809	0.777	0.468	0.378	0.926	0.302
RSL		0.099	0.079	0.011	0.359	0.001	0.411	0.645	0.123
RSS × RSL		0.010	0.112	0.212	0.150	0.001	0.543	0.454	0.618

Hex – hexoses with the indicated number of monomers, Pent – pentoses with the indicated number of monomers.
n = 8 replicate cages per treatment.

$^{AB, ab}$: Means in a column, within a group, but with different superscripts differ significantly ($P \leq 0.05$). Small letter superscripts compare the RSS group nested with control, or the simple effect means nested with control (in cases of significant interaction). Block letter superscript compares RSL nested with control.

Table 3.8. Caecal short-chain fatty acids profile (mM) on d 21 for the broiler chickens receiving dietary resistant starches from different sources fed at different levels.

RSL, g/kg	RSS	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Total SCFA
Control (0)		87.0^{AB}	5.45^{AB}	0.48^{AB}	20.9^{AB}	0.55^{AB}	1.20^{AB}	116^{abAB}
Main effects means of resistant starch source (RSS)								
	BS	89.4	5.62	0.48	20.0	0.58	1.18	117^{ab}
	RPS	83.4	5.17	0.48	19.9	0.53	1.11	111^{b}
	HCS	94.2	5.87	0.51	22.2	0.54	1.30	125^{a}
Main effects means of resistant starch level (RSL)								
25		88.4^{AB}	6.13^{A}	0.56^{A}	21.7^{A}	0.62^{A}	1.27^{A}	119^{AB}
50		81.5^{B}	4.57^{B}	0.38^{B}	18.5^{B}	0.45^{B}	1.04^{B}	107^{B}
100		97.2^{A}	5.97^{A}	0.53^{AB}	21.9^{A}	0.58^{AB}	1.28^{A}	127^{A}
Pooled SEM		4.02	0.404	0.049	1.13	0.053	0.082	4.4
Simple effect means								
25	BS	89.8	6.79	0.51	21.6	0.58	1.25	121
50	BS	78.7	4.7	0.48	16.5	0.56	1.06	102
100	BS	99.8	5.38	0.47	21.9	0.60	1.23	129
25	RPS	85.2	5.64	0.55	20.1	0.58	1.20	113
50	RPS	78.1	3.87	0.32	19.5	0.41	0.95	103
100	RPS	86.9	5.99	0.58	20.0	0.61	1.18	115
25	HCS	90.0	5.95	0.64	23.3	0.70	1.37	122
50	HCS	87.8	5.15	0.35	19.4	0.40	1.10	114
100	HCS	105.0	6.52	0.54	23.8	0.53	1.45	138
Pooled SEM		5.88	0.59	0.072	1.65	0.078	0.120	6.3
Probabilities								
RSS		0.087	0.340	0.857	0.165	0.782	0.143	0.029
RSL		0.007	0.003	0.008	0.024	0.032	0.022	0.001
RSS × RSL		0.742	0.400	0.285	0.558	0.386	0.960	0.667

RSS- Resistant starch source, RSL- Resistant starch level, BS- Banana starch, RPS- Raw potato starch, HCS - High-amylose corn starch.

n = 8 replicate cages per treatment.

$^{AB, ab}$: Means in a column, within a group, but with different superscripts differ significantly ($P \leq 0.05$). Small letter superscripts compare the RSS group nested with control, or the simple effect means nested with control (in cases of significant interaction). Block letter superscript compares RSL nested with control.

Table 3.9. Digesta pH along the digestive tract and cecal soluble protein on d 21 for the broiler chickens receiving dietary resistant starches from different sources fed at different levels.

RSL (g/kg)	RSS	Duodenum pH	Jejunum pH	Ileum pH	Cecal pH	Cecal protein (µg/g DM)
Control (0)		6.31	6.34	6.67	6.70	214^{aA}
Main effects means of resistant starch source (RSS)						
	BS	6.31	6.32	6.62	6.55	169^{b}
	RPS	6.35	6.34	6.67	6.51	193^{a}
	HCS	6.34	6.30	6.78	6.60	158^{b}
Main effects means of resistant starch level (RSL)						
25		6.35	6.36	6.59	6.54	158^{B}
50		6.34	6.33	6.81	6.47	181^{AB}
100		6.29	6.28	6.73	6.54	180^{AB}
Pooled SEM		0.027	0.023	0.142	0.123	7.4
Simple effect means						
25	BS	6.33	6.36	6.41	6.44	138
50	BS	6.35	6.32	6.79	6.69	193
100	BS	6.26	6.29	6.66	6.53	176
25	RPS	6.38	6.36	6.61	6.76	189
50	RPS	6.35	6.33	6.70	6.27	197
100	RPS	6.32	6.34	6.69	6.49	193
25	HCS	6.39	6.35	6.55	6.77	148
50	HCS	6.33	6.33	6.95	6.45	153
100	HCS	6.29	6.22	6.85	6.60	172
Pooled SEM		0.040	0.034	0.208	0.180	10.6
Probabilities						
RSS		0.440	0.822	0.942	0.341	0.001
RSL		0.127	0.097	0.203	0.211	0.027
RSS × RSL		0.823	0.274	0.521	0.155	0.073

RSS- Resistant starch source, RSL- Resistant starch level, BS- Banana starch, RPS- Raw potato starch, HCS - High-amylose corn starch.

n = 8 replicate cages per treatment.

$^{AB, ab}$: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$). Small letter superscripts compare the RSS group nested with control, or the simple effect means nested with control (in cases of significant interaction). Block letter superscript compares RSL nested with control.

Table 3.10. Digestive organ index on d 21 for the broiler chickens receiving dietary resistant starches from different sources fed at different levels.

RSL, g/kg	RSS	Crop	Proventriculus	Gizzard	Duodenum		Jejunum		Ileum		Ceca
		W	W	W	W	L	W	L	W	L	W
Control (0)		3.14	5.07	20.8	28.6^{AB}	9.71	67.1	18.8	67.5^{ab}	14.6	4.21^{AB}
Main effects of resistant starch source (RSS)											
	BS	3.06	5.49	23.6	29.8	10.6	65.2	18.9	72	15.0	4.31
	RPS	3.02	5.33	24.1	28.6	10.5	65.8	19.0	68.1	15.7	4.33
	HCS	3.26	5.56	23.7	29.3	11.0	66.0	18.5	71.7	14.8	4.20
Main effects of resistant starch level (RSL)											
25		3.01	5.50	22.7	27.9^{AB}	9.87	64.0	18.1	67.8	14.6	4.03^{B}
50		3.11	5.28	24.3	29.1^{AB}	11.2	67.7	20.2	70.5	15.8	4.14^{B}
100		3.22	5.6	24.5	30.7^{A}	11	65.3	18.2	73.5	15.1	4.70^{A}
Pooled SEM		0.135	0.248	0.76	1.021	0.405	2.77	0.76	3.24	0.67	0.19
Simple effect means											
	BS	2.95	5.34	22.6	27.5	10.1	63.5	18.7	64.8^{ab}	14.7	3.74
	BS	3.29	5.44	23.2	27.9	10.2	65.6	19.1	68.9^{ab}	15.4	3.95
	BS	2.93	5.68	25.1	34	11.5	66.6	18.9	82.4^{a}	15.1	5.23
	RPS	2.89	5.63	21.9	27.1	9.41	61	18.1	60.0^{b}	14.1	4.1
	RPS	3.13	5.34	26	31.2	11.7	76	21.4	79.5^{ab}	16.9	4.39
	RPS	3.03	5.68	24.5	27.4	10.4	60.4	17.5	64.9^{ab}	16.1	4.48
	HCS	3.18	5.54	23.5	29.1	10.1	67.7	17.4	78.7^{ab}	15	4.24
	HCS	2.91	5.70	23.6	28.1	11.8	61.6	20	63.0^{ab}	15.1	4.09
	HCS	3.7	5.45	23.9	30.7	11	68.9	18.1	73.4^{ab}	14.2	4.28
Pooled SEM		0.198	0.364	1.111	1.498	0.590	4.058	1.125	4.760	0.976	0.277
Probabilities											
RSS		0.077	0.735	0.642	0.149	0.133	0.599	0.586	0.125	0.936	0.877
RSL		0.277	0.504	0.110	0.037	0.068	0.93	0.429	0.202	0.776	0.008
RSS × RSL		0.089	0.928	0.850	0.167	0.431	0.487	0.804	0.027	0.635	0.095

RSS- Resistant starch source, RSL- Resistant starch level, BS- Banana starch, RPS- Raw potato starch, HCS - High-amylose corn starch.

W – weight (g/kg body weight), L – length cm/kg body weight).

n = 8 replicate cages per treatment.

$^{AB, ab}$: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$). Small letter superscripts compare the RSS group nested with control, or the simple effect means nested with control (in cases of significant interaction). Block letter superscript compares RSL nested with control.

Table 3.11. Jejunal histomorphology on d 21 for the broiler chickens receiving dietary resistant starches from different sources fed at different levels.

RSL, g/kg	RSS	VH, µm	CD, µm	VW, µm	VH/CD
Control (0)		1361	206^{ab}	220	6.72
Main effects means of resistant starch source (RSS)					
	BS	1510	219^{b}	229	6.94
	RPS	1581	255^{a}	243	6.40
	HCS	1702	256^{a}	244	6.82
Main effects means of resistant starch level (RSL)					
25		1514	241	245	6.43
50		1600	242	249	6.75
100		1679	248	223	6.98
Pooled SEM		72	11.5	11.3	0.355
Simple effect means					
25	BS	1413	211	232	6.71
50	BS	1579	233	232	6.83
100	BS	1537	212	224	7.29
25	RPS	1461	245	252	6.14
50	RPS	1491	236	241	6.49
100	RPS	1793	285	236	6.57
25	HCS	1669	267	251	6.44
50	HCS	1729	255	275	6.93
100	HCS	1707	245	208	7.08
Pooled SEM		104	16.8	16.5	0.517
Probabilities					
RSS		0.076	0.013	0.474	0.358
RSL		0.157	0.861	0.116	0.434
RSS × RSL		0.325	0.173	0.356	0.996

RSS- Resistant starch source, RSL- Resistant starch level, BS- Banana starch, RPS- Raw potato starch, HCS - High-amylose corn starch. VH- Villi height. CD- Crypt depth, VW- Villi width.

n = 8 replicate cages per treatment.

$^{AB, ab}$: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$). Small letter superscripts compare the RSS group nested with control, or the simple effect means nested with control (in cases of significant interaction). Block letter superscript compares RSL nested with control.

Table 3.12. Relative mRNA expression of jejunal nutrient transporters and epithelial integrity and cecal fatty acid transporters on d 21 for the broiler chickens receiving dietary resistant starches from different sources fed at different levels.

		Jejunum				Caeca			
RSL, g/kg	RSS	GLUT 2	PEPT1	MUC 2	Occludin	FFAR2	FFAR3	MCT4	MCT1
Control (0)		1.00^{ab}	1.00^{b}	1.00^{a}	1.00	1.00^{AB}	1.00	1.00^{A}	1.00^{a}
Main effects means of resistant starch source (RSS)									
	BS	1.24^{a}	1.18	0.67^{b}	1.16	1.36	0.78	0.85	0.52
	RPS	1.12^{a}	1.13	0.89^{ab}	1.09	1.05	0.72	0.89	0.36
	HCS	0.65^{b}	1.01	0.68^{b}	0.98	1.47	0.9	1.21	0.51
Main effects means of resistant starch level (RSL)									
25		0.86	1.08	0.77	1.04	1.71^{A}	0.81	0.94^{AB}	0.51
50		1.07	1.16	0.81	1.19	1.27^{AB}	0.91	1.18^{A}	0.46
100		1.09	1.08	0.66	0.99	0.91^{B}	0.67	0.82^{B}	0.42
Pooled SEM		0.149	0.129	0.081	0.099	0.214	0.126	0.160	0.070
Simple effect means									
25	BS	0.84	0.80^{bc}	0.52	1.24	1.28	1.11	0.86	0.63^{abc}
50	BS	1.46	1.32^{a}	0.83	1.45	1.77	0.66	0.77	0.25^{c}
100	BS	1.43	1.42^{a}	0.65	0.8	1.04	0.57	0.91	0.68^{ab}
25	RPS	1.08	1.32^{a}	1.14	0.98	1.45	0.42	0.78	0.41^{bc}
50	RPS	1.07	1.20^{ab}	0.89	1.2	0.69	0.99	0.97	0.36^{bc}
100	RPS	1.22	0.88^{b}	0.64	1.09	1.01	0.75	0.93	0.32^{c}
25	HCS	0.66	1.14^{ab}	0.66	0.91	2.41	0.91	1.18	0.50^{bc}
50	HCS	0.68	0.96^{b}	0.71	0.93	1.34	1.08	1.81	0.76^{ab}
100	HCS	0.62	0.94^{b}	0.67	1.08	0.67	0.71	0.63	0.26^{c}
Pooled SEM		0.218	0.189	0.118	0.145	0.332	0.195	0.248	0.109
Probabilities									
RSS		0.023	0.485	0.031	0.503	0.189	0.492	0.204	0.119
RSL		0.766	0.730	0.206	0.275	0.003	0.421	0.050	0.349
RSS ×´ RSL		0.319	0.042	0.103	0.128	0.079	0.083	0.079	0.001

RSS- Resistant starch source, RSL- Resistant starch level, BS- Banana starch, RPS- Raw potato starch, HCS - High-amylose corn starch.

n = 8 replicate cages per treatment.

$^{AB, ab}$: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$). Small letter superscripts compare the RSS group nested with control, or the simple effect means nested with control (in cases of significant interaction). Block letter superscript compares RSL nested with control.

CHAPTER 4

4. DIETARY INCLUSION OF RAW POTATO OR HIGH-AMYLOSE-CORN RESISTANT STARCHES FED FOR DIFFERENT DURATIONS – IMPACT ON PHENOTYPIC RESPONSES AND INDICATORS OF GUT HEALTH OF BROILER CHICKENS[3]

[3]Oluseyifunmi, I. W., & Olukosi, O. A. Submitted to *Animal Nutrition*, October 1, 2024.

4.1 ABSTRACT

This 21-d study evaluated the effects of feeding durations of diets containing graded levels of raw potato starch (RPS) or high amylose corn starch (HCS) on growth performance, nutrient and energy utilization, and gut biochemical metrics in broiler chickens. A total of 300 male broiler chicks at day old were randomly allocated to ten treatments. in a 3×3+1 factorial arrangement. The factors were 3 RS levels (RSL): 25 and 50 g/kg of RPS and 35 g/kg of HCS, and three feeding durations (RSD): 21, 14, or 7 d, plus a corn-soybean meal control diet. Each treatment had 6 replicates and 5 birds per replicate cage. The birds and feeds were weighed on d 0, 7, 14, and 21. On d 21, ileal digesta, excreta, jejunal tissues, and caecal contents were collected for digestibility, histomorphology, and mRNA expression of nutrient transporters, pH, and SCFA profile analysis, respectively. Data were analyzed as 3×3+1 nested factorial. The main effect of RSL was significant ($P < 0.05$) for feed conversion ratio (FCR), apparent ileal digestible energy (AIDE), nitrogen retention, metabolizable energy (ME), and nitrogen-corrected ME (AMEn). FCR was greater ($P < 0.043$) in birds fed 25 g/kg RPS than 35 g/kg HCS diets. The AIDE ($P = 0.001$), ME, and AMEn ($P = 0.002$) were greater in birds that received 35 g/kg HCS than those fed RPS diets except AIDE at 50 g/kg RPS and control diets. The RSD×RSL was significant ($P < 0.05$) for relative mRNA expression of jejunal glucagon-like peptide 2 (*GLP-2*) and the main effects of RSD for *MUC-2* (mucin-2). The birds fed 25 g/kg RPS for 21 and 14 d had greater ($P = 0.005$) expression of *GLP-2* than those fed the same diet for 7 d, and *MUC-2* was more ($P = 0.006$) expressed in birds fed RS diets for 21 and 14 d than 7 d, except the control diet. Overall, RSL played a key role in modulating growth performance, microbial metabolite production and nutrient uptake, while gene expression was more associated with synergistic effects of RSD and RSL with implications on nutrient absorption and overall gut homeostasis.

4.2 INTRODUCTION

Resistant starches have been classified as part of complex carbohydrate fractions and considered as dietary fibers Delcour and Hoseney (2010). Therefore, resistant starches (RS), as functional fiber, could be promising for improving gut health in poultry due to the production of microbial metabolites such as short-chain fatty acids (SCFA) when fermented in the caeca (Tan et al., 2021). The metabolites in the digesta can influence the intestinal health by inducing shifts in microbial community composition or by altering the trajectory of the digestion process (Choct et al., 1999; Dittoe et al., 2022). The SCFA produced from RS fermentation act by modulating the metabolites in systemic circulation, nutrient digestion and the microbial community in the gut as a result of reduced luminal pH, and hence, improve the overall intestinal health exhibiting prebiotic effects in the distal gastrointestinal tract (GIT) (Champ, 2004).

In a previous experiment we reported that different sources and dietary levels of resistant starches (RS) differentially modulated the growth performance, nutrient digestion and utilization as well as intestinal health in broiler chicken (Oluseyifunmi et al., 2024). In addition to concentrations and sources of RS, the length of feeding can also influence the effect of RS on growth and gut health metrics (Haenen et al., 2013; Qin et al., 2019). Nofrarías et al. (2007) and Qin et al. (2019) observed an improvement in colonic mucosal integrity and enhanced villi structure, in pigs and ducks, respectively, when fed diets containing raw potato starch, which depended largely on the starch level and duration. In addition, a duration-dependent negative impact on the nutrient retention, gut morphology and feed efficiency were reported in broiler chickens fed diets containing high concentration of dietary Hi- maize resistant starch (Liu et al., 2020b).

However, only very limited studies had investigated the effects of RS feeding duration in poultry especially in broiler chickens, and the existing results showed high variability due to different RS sources and levels, necessitating further investigation. Furthermore, in our previous experiment, increasing RS levels showed variable effects on growth performance, so it becomes pertinent to further investigate if this could be feeding-duration dependent. This study, therefore, evaluated the effects of duration of feeding (or adaptation lengths) of raw potato starch (RPS) and high amylose corn starch (HCS) on growth performance, nutrient utilization, intestinal morphology, cecal SCFA profile, and mRNA expression of nutrient transporters and gut integrity genes in broiler chickens.

4.3 MATERIALS AND METHODS

Animal ethics statement

The animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Georgia (IACUC number: A2021-06-006).

Birds, diets, housing and experimental design

A total of 300 male broiler chicks (byproducts of the female line) at zero-day old were randomly allocated to ten treatments in a 3×3 +1 factorial arrangement. The factors were 3 RS levels (RSL), namely 25 or 50 g/kg of raw potato starch (RPS) and 35 g/kg of high amylose corn starch (HCS), and three durations (RSD): namely 21, 14, or 7 d, plus a corn-soybean meal control diet. The feeding durations indicate how long the birds received the RS diets. The birds received the control diet until feeding of the RS diet was initiated in respective treatments. Birds receiving RS diets for 21, 14, or 7 d received the corn-soybean meal control diets for 0, 7, or 14 d, respectively (Table 4.1). Each treatment had 6 replicates and 5 birds per replicate cage. The birds were housed in metabolism cages in a controlled environment, adhering to the recommended

lighting and temperature regimes for Cobb 500 broiler chickens. Feed and water were supplied ad libitum for the duration of the experiment. The diets were designed to meet the nutrient and energy requirements for Cobb 500 broiler chickens (Cobb-Vantress, 2022) and were isocaloric and isonitrogenous. The starter diets were presented in mash form, and the grower diets as pellets. The feedstuff and chemical compositions of the starter and grower diets are shown in Tables 4.2 and 4.3, respectively.

Growth performance measurements

The birds and feeds were weighed on d 0, 7, 14, and 21. The feed intake (FI), body weight gain (BWG), final body weight gain (FBW), and feed conversion ratio (FCR) were determined from d 0 to 21 and corrected for mortality.

Nutrient digestibility, caecal short-chain fatty acids, and caecal pH measurements

Birds were humanely euthanized using CO_2 asphyxiation on d 21, and the ileal digesta (from Meckel's diverticulum to 2cm proximal to ileocecal junction), excreta, and caecal contents were collected for evaluation of nutrient digestibility, total tract N retention, metabolizable energy, cecal short-chain fatty acids (SCFA) profile, and pH determination.

Caecal digesta short-chain fatty acid profile

The caecal content was diluted at the ratio of 1 to 3 with deionized water and centrifuged for 10 minutes at 10,000 × g. 1 mL of the supernatant was combined with 0.2 mL of 25 % metaphosphoric acid solution and stored at -20^{o} C overnight. After thawing and re-centrifugation, 0.75 mL of the supernatant was mixed with 0.15 mL of an internal standard and 1.8 mL ethyl acetate, then vortexed for 15 s. After settling for 5 min, 1.2 mL of the top layer was pipetted into

a glass vial, and the SCFA content was measured using gas chromatography (Shimadzu GC-2010 Plus; Shimadzu Corporation, Japan (Lourenco et al., 2020).

Caecal digesta pH measurements

The caecal digesta samples pooled from three randomly selected birds per pen were used for pH determination. 1 g of digesta was mixed with 9 mL of distilled water and vortexed. The pH was subsequently determined using a sterile glass pH electrode while the mixture was stirred with a magnetic stirrer for thorough homogenization (Thermo Scientific, Beverly, MA, USA).

Jejunal histomorphology

On d 21, approximately 2cm mid-jejunum tissue samples were collected from 2 randomly selected birds per pen and fixed in 10% buffered formalin. The formalin-fixed jejunum tissues were dehydrated in graded concentrations of ethanol, 50%, 70%, and 96% ethanol for 15 min each, and 100 % ethanol for 30 min, cleared with xylene, and embedded in paraffin wax. This was sectioned into 5 μm and stained with haematoxylin-eosin and the images were captured at 4 × magnification with the aid of BZ microscope (BZ-X800; Keyence Inc., Itasca, IL) and analyzed using BZ-X800 Analyzer. The villus height (the tip of the villus to the crypt junction) and the corresponding crypt depth (depth of the invagination between adjacent villi) were measured for six villi and crypts per section, and the villus height-to-crypt depth ratio was subsequently calculated.

Chemical analyses

The oven-dried samples of diets, ileal digesta, and excreta were ground (0.5 mm) and used for dry matter (DM), nitrogen (N), titanium dioxide, and gross energy determination (GE). A portion of the samples was dried at 100°C for 24 h to evaluate the DM (AOAC, 2006). The N

content of the samples was measured by the combustion method (AOAC, 2006) with a N analyzer (LECO, St. Joseph, MI), and the gross energy with the aid of isoperibol bomb calorimeter calibrated with benzoic acid (Model 6200, Parr Instruments, Moline, IL). The titanium dioxide content of the samples was measured according to the method of Short et al. (1996). The total starch analysis was done using the Megazyme assay kit (K-AMYL). The RS analysis was conducted by Eurofins Food Testing, Netherlands, based on AOAC method 2011.25 (AOAC, 2012)

Quantitative real-time PCR analysis

On d 21, samples of mid-jejunal tissue obtained from one randomly selected bird per pen were snap-frozen in liquid nitrogen and stored at -80 ^{o}C prior to RNA extraction. The RNA was extracted with QiAzol lysis reagent (QIAGEN, Hilden, Germany (Invitrogen, Carlsbad, CA, USA), purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). High-capacity cDNA reverse transcription kit (Thermo Fischer Scientific, Waltham) was used for reverse transcription of RNA to cDNA in a 20 µl reaction volume, and the RT-qPCR performed with StepOnePlus (Applied Biosystems, Carlsbad, CA, USA) and Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Beta-actin was used as the housekeeping gene, and the fold change was determined based on $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The primer sequence is shown in Table 4.4.

Calculations

The index method was used to calculate the apparent ileal digestibility and the total tract nutrient retention of starch, energy, and dry matter. The calculations were conducted on a dry matter basis. The following equations outline the calculations:

$$\text{Digestibility (\%)} = 100 \times [1 - ((\text{Ci/Co}) \times (\text{No/Ni}))]$$

$$\text{DM Digestibility (\%)} = 100 \times [1 - (\text{Ci/Co})]$$

Where Ci and Co are the % titanium dioxide concentrations in the diet and excreta/digesta, respectively; Ni and No are the % nutrient contents in the diet and excreta/digesta, respectively. Nitrogen-corrected apparent metabolizable energy (AMEn) and apparent metabolizable energy (AME) in kcal/kg were calculated by the following equation:

$$\text{AME} = \text{GEi} - [(\text{Ci/Co}) \times (\text{GEo})]$$

$$\text{AMEn} = \text{AME} - [8.22 \times (\text{NR/DMI})]$$

where GE_i and GE_o represent the diets and excreta gross energy contents (kcal/kg), respectively. The DMI (dry matter intake) and NR (retained nitrogen) were calculated as shown below:

$$\text{NR (g)} = (\text{NI} - \text{NO})$$

$$\text{DMI (g)} = \text{Feed intake, g} \times \text{Feed DM(coefficient)}$$

Where NI and NO represent the N intake (g) and output (g), respectively.

4.4 STATISTICAL ANALYSIS

The statistical model for the experiment was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + [\beta(\gamma)]_{jk}\,[\beta(\delta)]_{jl} + [\beta(\gamma\delta)]_{jkl} + \varepsilon_{ijkl}$$

where $Yijkl$ is the response variable, μ is the overall mean, α_i is the random effect of *i*th block, β_j is the fixed effect of *j*th treatment group, $\beta(\gamma)_{jk}$ represent the fixed effect of *k*th resistant starch level within *j*th treatment group, $\beta(\delta)_{jl}$ represents the fixed effect of *l*th resistant starch feeding duration within *j*th treatment group. $\beta(\gamma\delta)_{jkl}$ *is the* interaction effect of *k*th resistant starch level and *l*th resistant starch feeding duration within *j*th treatment group, and the ε_{ijkl} is the residual error. Data obtained were subjected to statistical analysis using the mixed model procedure of JMP Pro 13.2.0 (SAS Institute Inc., Cary, NC) in a 3 × 3 + 1 factorial arrangement. The factors included

three levels of resistant starch, each tested across three feeding durations and nested with the control diet. Tukey's HSD was used to separate means with significant difference and the significant P-value was established at $P \leq 0.05$. The main effects were reported when no significant interactions occur, and simple effects discussed in cases where interactions were significant.

4.5 RESULTS

Growth performance

The growth performance results showed no significant ($P > 0.05$) RSD×RSL and main effects of RSD on WG, FI, and BWG at d 21 (Table 4.5). However, there was a significant main effect of RSL ($P < 0.05$) on FCR. The FCR was lower ($P < 0.043$) in birds receiving 35 g/kg HCS than in those receiving 25 g/kg RPS.

Nutrient digestibility, total tract retention and metabolizable energy

There were no significant ($P > 0.05$) RSD×RSL and main effects of RSD for ileal nutrient digestibility, metabolizable energy (ME), nitrogen-corrected apparent metabolizable energy (AMEn), and total tract nutrient retention, except for significant ($P < 0.05$) main effects of RSL on apparent ileal digestible energy (AIDE), total tract N retention, ME and AMEn (Table 4.6). The birds receiving 35 g/kg HCS had greater ($P = 0.001$) AIDE than those on 25 g/kg RPS. Nitrogen retention was greater ($P = 0.047$) in the birds fed the control diet than those fed 50 g/kg RPS. The birds fed 35 g/kg HCS had higher ($P = 0.002$) ME and AMEn than those fed the RPS diets.

Caecal digesta short-chain fatty acids profile and pH

The caecal SCFA concentrations were not significantly ($P > 0.05$) affected by the treatments (Table 4.7). However, there was a numerical increase in concentration of acetate, propionate, butyrate and total SCFA in birds fed RS diets for 21 d, as well as numerically greater

production of acetate, butyrate and total SCFA in birds that received 35g/kg HCS diets. In addition, the birds that received the RS diets for 21 d showed a tendency for a lower (P = 0.055) caecal content pH than those that received the same diets for 0, 7, or 14 d.

Jejunal histomorphology

The RSD×RSL and the main effects of RSD and RSL were not significant for villi height, villi width, or villi height: crypt depth ratio (Table 4.8). However, there was a significant (P < 0.05) RSL main effect for the crypt depth; the crypt of birds receiving 25 g/kg RPS was deeper than for those fed 50 g/kg RPS.

Relative mRNA expression of jejunal gut integrity gene, satiety hormones, and nutrient transporters

The RSD×RSL was significant for relative mRNA expression of jejunal *GLP-2* (P = 0.005), *GLP-1* (P = 0.001) and *GLUT-2* (P = 0.033) (Table 4.9). The birds fed 25 g/kg RPS for 21 and 14 d had greater (P = 0.005) relative mRNA expression of *GLP-2* than those fed 25 g/kg RPS for 7 d. *GLP-2* also had the highest expression in birds that consumed 35g/kg HCS diet for 7 d among other durations but comparable across 7, 14, and 21 d. The relative mRNA expression of *GLP-1* was greater (P = 0.001) in birds that received the control diet, 25 and 50 g/kg RPS, and 35 g/kg HCS for 21 d, 25 g/kg RPS for 14 d than in other diets. There was a greater (P = 0.033) mRNA expression of *GLUT*-2 in birds fed the control diet, 25 and 50 g/kg RPS for 21 d, 25 g/kg RPS for 14 and 35 g/kg HCS for 7 d.

The main effects of RSD were significant (P < 0.05) for *MUC-2* (mucin-2), *PYY* (peptide YY), *y+LAT1* (AA transporter) (Table 4.9). *MUC-2* (P = 0.006) and *PYY* (P = 0.001) were more expressed in birds fed RS for 21 and 14 d than those fed for 7 d, except the control diet whereas, *y+LAT1* had greater (P < 0.001) expression in birds that received RS diet for 14 d than other

diets. The main effects of RSL were significant ($P < 0.05$) for *PYY* and *y+LAT1*. *PYY* had higher ($P = 0.002$) expression in birds fed 25 g/kg RPS and 35 g/kg HCS than 50 g/kg RPS with the exemption of control diet. The mRNA expression of *y+LAT1* was greater ($P = 0.001$) in birds that received 25 g/kg RPS than those fed 35 g/kg HCS but comparable across the experimental diets.

4.6 DISCUSSION

The potential of RS in improving gut health with little or no detrimental effect on growth performance has been demonstrated in both humans and animals (Li et al., 2007; Regmi et al., 2011). Continuous SCFA supply, especially in animal fed ad libitum, may reduce glucose oxidation and leading to no-detrimental effect or improved growth performance. This varies among RS sources and levels necessitating the need to determine the optimum source and levels as well as safe feeding duration in various species of animals (Van Erp et al., 2020; Oluseyifunmi et al., 2024). On the other hand, the feed milling process can compromise the efficiency of RS in improving gut health by altering the crystalline structure of RS depending on the stability to the shearing process and pelleting temperature (Devi et al., 2009; Alsaffar, 2011). This study investigated how different durations of the feeding of raw potato starch (RPS) and high amylose corn starch (HCS) could influence their effects on the growth performance, nutrient utilization, intestinal morphology, and cecal SCFA profile in broiler chickens during the grower phase. It was hypothesized that the varied impacts of dietary RS levels on the production of caecal SCFA, nutrient utilization, intestinal morphology, modulation of mRNA expression of jejunal nutrient transporters, and genes of gut health importance depend on the duration of feeding RS in broiler chickens.

Growth performance

The results obtained on growth performance in this study showed no detrimental effect of RS on growth performance metrics which could be due to relatively short duration of feeding RS diets and the lower FCR observed in the birds fed 35 g/kg HCS diet relative to 25 g/kg RPS diet might be the result of its greater resistance to pancreatic amylase due to its higher amylose content and its potential to form RS3, enabling HCS to maintain most of its granular integrity during processing (Morell et al., 2004; Giuberti et al., 2015). Generally, the non-significant effects of RS levels and feeding duration on growth performance in this study are in line with the previous studies that reported no significant effects of graded levels of RPS on growth performance in ducks fed for varied durations (Qin et al., 2019) and in growing pigs fed RS diets (Pluske et al., 1998). However, some studies reported detrimental effects of RS on growth in animals attributing this to low caloric density of RS and variability in crystalline configuration of different RS (Li et al., 2007; Regmi et al., 2011; Higgins, 2014; Liu et al., 2020b), as Liu et al. (2020b) suggested that high crystallinity of resistant starch (RS) may reduce starch digestibility and limit energy intake, leading to depressed growth in broiler chickens. Additional probable reasons for variable effects of RS on growth performance, aside source of RS and age might be difference in presentation of diets, either as mash or pellets influencing the viability of the crystalline structure, high concentration of RS in diets, specie difference among other factors (Devi et al., 2009; Alsaffar, 2011; Giuberti et al., 2015; Tan et al., 2021).

Nutrient digestibility, total tract retention and metabolizable energy

Greater AIDE, ME and AMEn in birds fed 35 g/kg HCS than all RPS and control diets indicated better energy utilization in HCS diets than other RS diets irrespective of the feeding duration. This was further corroborated by greater nitrogen retention in birds fed control diets and

25 g/kg RPS and 35 g/kg HCS. The greater DM and starch retention in the birds that received control diet was not a surprise as RS diet has been proven to reduce starch digestibility (Morel et al., 2005; Oluseyifunmi et al., 2024). These results along with our previous findings indicated that HCS may be a preferrable RS for broiler chickens, among those tested in our experiments. Other studies observed lower total tract nutrient utilization and apparent ileal digestibility of nutrients with higher dietary levels of RS (Cervantes-Pahm et al., 2014; Giuberti et al., 2015) but a previous study by Oluseyifunmi et al. (2024) observed an increase in N retention with increasing RPS and HCS levels and higher AME and AMEn in birds fed 100 g/kg HCS diet than in banana starch and raw potato starch in broiler chickens. Liu et al. (2020b) observed a linear decrease in the apparent retention of DM and TS with increasing RS levels from d 18 to 20 and d 39 to 41 in Arbor acre broiler chickens fed dietary 40, 80 and 100 g/kg Hi- maize resistant starch. Higher energy digestibility and availability in birds fed 35 g/kg HCS explains the lower FCR observed at this level of inclusion, although the FI and WG were not significantly different across all the diets. Sources of RS play a fundamental role in determining the crystalline organization of RS which is pertinent to its amylolytic hydrolysis (Alsaffar, 2011) and the effects of RS on nutrient digestibility and growth performance in broiler chicken may be more dependent on the amount ingested rather than on the feeding duration of the RS used in this study.

Caecal digesta short-chain fatty acid profile and pH

Short-chain fatty acids are the major beneficial end-products of the distal gastrointestinal tract microbial fermentation of dietary fibers which constitute the fundamental mode of action of RS in improving gut health in animals. The result obtained on caecal SCFA concentrations in this study showed no significant ($P > 0.05$) effects of the treatments, However, acetate, butyrate and total SCFA were numerically greater in birds fed 35 g/kg HCS and those fed RS diets for 21 d.

Previous findings showed significant increase in SCFA due to increased RS fermentation in the caeca of birds fed dietary RS (Qin et al., 2020; Zhang et al., 2020; Oluseyifunmi et al., 2024). The probable explanation for the difference in the results could be the possible alteration of the crystalline structure of RS by feed milling processes involved in pelleting the diets (Alsaffar, 2011), as the diets fed in the previous study were presented as mash through starter to grower phase, whereas in the current study, the diets were in mash form in the starter phase and as pellets in the grower phase. Possibility of alteration in RS crystalline structure cannot be ruled out during feed processing which could alter their physiochemical properties and fermentability (Svihus et al., 2005; Alsaffar, 2011). Lower luminal and digesta pH are associated with mechanism of action of SCFA in modulating intestinal microbiota which could favor the proliferation of beneficial bacteria relative to pathogenic ones (Regassa and Nyachoti, 2018). The tendency for lower caecal content pH in birds fed RS diets for a longer period (21 d) is an evidence of RS fermentation and increased SCFA production in the birds fed RS diets for a longer duration (Tan et al., 2021).

Jejunal histomorphology

The development and aborptive capacity of the intestine is measured by the villi height to crypt depth ratio (Marchewka et al., 2021). The result observed for jejunal histomorphology showed that neither the duration of feeding the RS diets nor dietary levels of RS in the diets influenced the villi height, width, or villi height: crypt depth ratio except for deeper crypt in birds fed 25 g/kg RPS diet. This observation is consistent with our previous findings (Oluseyifunmi et al., 2024).

In contrast, Qin et al. (2019) reported longer villi height, higher villi height: crypt depth ratio, and lower crypt depth in the ileum of ducks fed 120 and 180 g/kg RPS diets relative to 0% RPS diet for 14 and 35-d period and Liu et al. (2020b) observed a decrease in the jejunal villi

height and villi height: crypt depth ratio of broiler chickens fed diets containing 110.2 and 141.6 g/kg, 114.8 and 147.8 g/kg Hi-maize resistant starch, in the starter and grower phases, respectively, each for 21 and 42 d. However, the difference in the RS inclusion level, intestinal site and specie are the probable explanation for disparity in these results, especially as the major site of fermentation of RS lies in the distal part of the intestine.

Relative mRNA expression of jejunal gut integrity genes, satiety hormones and nutrient transporters

Resistant starches especially, high-amylose corn starch have been linked with increased fermentation, and elevated expression of *GLP-1* (glucagon-like peptide-1) and peptide YY *(PYY)* genes in the distal GIT, and improved gut health (Zhou et al., 2008; Keenan et al., 2012). GLP-1 and *PYY*, are satiety hormones associated with feed intake regulation (Keenan et al., 2012). Glucagon-like peptide-2 (*GLP-2*) is produced along with *GLP-1*, and it is associated with increased crypt cell proliferation and nutrient absorption in pigs and humans (Hellmich and Evers, 2006).

The increased mRNA expression of *GLP-2* by longer feeding of 25 g/kg RPS for a period of 14 and 21 d corresponded with the deeper crypt observed in birds fed at this level and a pointer to the ability of *GLP-2* in increasing crypt proliferation. This was reflected by the greater FCR in birds fed 25 g/kg RPS but showed no negative implication on the growth performance, probably because the levels of expression of *GLP-1* and *PYY* (Satiety hormones) in RS-fed birds were below the concentration capable of decreasing feed intake as they were similar to the expression observed in the birds that received the control diet.

MUC-2 lubricates the intestinal mucosa, functions in protecting the gut and maintaining intestinal homeostasis. It constitutes a part of the first line of defense against microbial invasion

(Cornick et al., 2015; Liu et al., 2020a). In the current study, the relative mRNA expression of *MUC-2* was elevated in birds that received RS for 14 and 21 d but comparable to control. Similarly, feeding 180 g/kg RPS diet in meat-type ducks for 14 d elevated *MUC-2* mRNA expression in the ileum (Qin et al., 2019) and Trachsel et al. (2018) reported a greater caecal *MUC-2* mRNA expression in the pigs fed dietary inclusion of 50 g/kg RPS. Our result demonstrated the duration-dependent effects of dietary RS on jejunal *MUC-2* expression in broiler chickens. Feeding RS diets for 7 d was not sufficiently long enough time to elicit a response except when fed for a longer period, as observed in the birds that received RS diets for 14 or 21 d.

GLUT-2 is a facilitative glucose transporter that allows large bidirectional fluxes of glucose in and out the cell (Leturque et al., 2009). The variability of mRNA expression of *GLUT-2* at different levels and durations of feeding RS indicated the interdependence of both factors in modulation of luminal glucose uptake (Sajilata et al., 2006). Furthermore, the major role of *y+LAT1* (Na-dependent cationic amino acid exchanger) is to transport amino acids across the intestinal epithelium (Wang et al., 2017; Lin et al., 2022). The greater expression of *y+LAT1* in birds fed RS diet for a period of 14 d and at 25 g/kg RPS level than 35 g/kg HCS, though comparable across diets, further demonstrated the importance of both factors in nitrogen uptake during RS consumption in broiler chickens (Lin et al., 2023; Oluseyifunmi et al., 2024).This is in line with the greater nitrogen retention observed in birds fed lower concentrations of RS or zero RS (25 g/kg RPS, 50 g/kg HCS and the control diets, respectively) which further explained the reason why the increase in crypt proliferation (evidenced by increase in crypt depth at 25 g/kg RPS inclusion) had no negative effect on the weight gain and final body weight. The improved nitrogen intake and utilization contributed to the growth of these animals which might have compensated for the endogenous nitrogen loss associated with increased enterocyte turnover rate, as deeper

crypts is suggestive of a higher enterocyte turnover rate, (Montagne et al., 2003; Wijtten et al., 2012)

4.7 CONCLUSION

The study assessed the impacts of feeding graded levels (RSL) of raw potato starch (RPS) and high amylose corn starch (HCS) on growth performance, nutrient utilization, intestinal morphology and cecal SCFA profile in broiler chickens as it relates to feeding duration or duration of adaption to RS diets. Results showed no significant effects of RSD on growth performance or nutrient digestibility. The RSL influenced the FCR, AIDE, ME, and AMEn, with 35 g/kg HCS showing better efficiency than 25 g/kg RPS diets and greater AIDE, ME and AMEn in birds fed 35 g/kg HCS diets. The mRNA expression of genes was influenced by both RSD and RSL, with notable increases in expression of certain markers of gut integrity and nutrient transporters such as *GLP-2*, *MUC*-2, and *y+LAT1*. However, it would be pertinent for future studies to investigate the effects of the feed milling process on the crystalline integrity of RS, resistance to pancreatic amylase, and the efficiency of microbial fermentation of RS when animals are fed pelletized diets.

4.8 REFERENCES

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Table 4.1. The treatment arrangement used in the experiment.

Feeding duration (RSD), d	Age (d) of birds when the experimental diet was introduced	Number of d the bird received the experimental diets[1]
0 (Control)	-	0
21	0	21
14	7	14
7	14	7

[1] Three experimental diets were fed: corn-soybean meal diets with 25 or 50 g/kg raw potato starch or 35 g/kg high-amylose corn starch. The birds in the control treatment received the corn-soybean meal without any additional source of resistant starch throughout the experiment. The birds in the other treatments (apart from the control) received the corn-soybean meal control up to the day experimental diets were fed according to the treatment schedule.

Table 4.2. Feedstuff and chemical compositions (g/kg, as fed) of the starter phase (d 0 to 7) diets.

Items	Control	Raw potato starch		Hi-amylose corn starch
Corn	633	606	573	582
Raw potato starch	0	25	50	0
Hi-amylose corn starch	0	0	0	35
Soybean meal	323	328	335	330
Soybean oil	9	12	18	19
Dicalcium phosphate	17.5	17.5	17.5	17.5
Limestone	5.9	5.9	5.87	5.87
Sodium bicarbonate	2	2	2	2
Vitamin and trace mineral premix[1]	2.5	2.5	2.5	2.5
DL- Methionine	1.5	1.6	1.6	1.6
L- Lysine. HCl	1.7	1.7	1.55	1.7
Threonine	0.7	0.6	0.6	0.7
Phytase (Q blue)	0.1	0.1	0.1	0.1
Salt	2.8	2.4	2.4	2.4
Total	1000	1000	1000	1000
Calculated nutrients, g/kg dry matter				
Protein, g/kg	212	212	212	211
ME, MJ/kg	12.5	12.5	12.5	12.5
P, g/kg	6.85	6.80	6.75	6.74
Ca, g/kg	7.96	7.97	7.97	7.96
Available P, g/kg	4.29	4.28	4.27	4.26
Digestible amino acids, g/kg				
Lys	12.60	12.68	12.68	12.67
Met	4.80	4.89	4.88	4.86
Thr	8.64	8.56	8.59	8.62
TSAA	8.27	8.34	8.32	8.28
Analyzed nutrient composition, g/kg dry matter				
Protein	213	208	212	198
GE, MJ/kg	18.6	18.6	18.7	18.9
Total starch	463	440	452	435

[1]The vitamin and mineral premix provided (per kg of diet): Vitamin A, 5,484 IU; vitamin D3, 2,643 IU; vitamin E, 11 IU; menadione sodium bisulfate, 4.38 mg; riboflavin, 5.49 mg, d-pantothenic acid, 11 mg; niacin, 44.1 mg, choline chloride, 771 mg; vitamin B12, 13.2 µg; biotin, 55.2 µg; thiamine mononitrate, 2.2 mg; folic acid, 990 µg; pyridoxine hydrochloride, 3.3 mg, I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 µg.

Table 4.3. Ingredients and chemical composition (g/kg, as fed) of the grower phase (d 7 to 21) diets.

Items	Control	Raw potato starch		Hi-amylose corn starch
Corn	663	637	606	611
Raw potato starch	0	25	50	0
Hi-amylose corn starch	0	0	0	35
Soybean meal	295	300	305	302
Soybean oil	8	10	15	19
Dicalcium phosphate	10.5	10.6	10.7	10.7
Limestone	9.2	9.15	9.1	9.1
Sodium bicarbonate	2	2	2	2
Vitamin and trace mineral premix[1]	2.5	2.5	2.5	2.5
DL- Methionine	1.6	1.6	1.7	1.7
L- Lysine. HCl	1.8	1.7	1.6	1.7
Threonine	0.5	0.5	0.5	0.5
Phytase (Q blue)	0.1	0.1	0.1	0.1
Titanium dioxide (TiO2)	3	3	3	3
Salt	2.8	2.4	2.4	2.4
Total	1000	1000	1000	1000
Calculated nutrients, g/kg dry matter				
Protein, g/kg	201	201	201	200
ME, kcal/kg	3020	3007	3009	3009
P, g/kg	5.53	5.51	5.47	5.47
Ca, g/kg	7.37	7.39	7.40	7.40
Available P, g/kg	3.03	3.03	3.04	3.04
Digestible amino acids, g/kg				
Lys	11.92	11.92	11.91	11.91
Met	4.77	4.76	4.84	4.82
Thr	8.00	8.02	8.02	7.98
TSAA	8.09	8.06	8.12	8.10
Analyzed nutrient composition, g/kg dry matter				
Protein	228	226	214	211
GE, MJ/kg	12.64	12.58	12.59	12.59
Total starch	513	519	474	465
Resistant starch	6	8	12	13

[1]The vitamin and mineral premix provided (per kg of diet): Vitamin A, 5,484 IU; vitamin D3, 2,643 IU; vitamin E, 11 IU; menadione sodium bisulfate, 4.38 mg; riboflavin, 5.49 mg, d-pantothenic acid, 11 mg; niacin, 44.1 mg, choline chloride, 771 mg; vitamin B12, 13.2 µg; biotin, 55.2 µg; thiamine mononitrate, 2.2 mg; folic acid, 990 µg; pyridoxine hydrochloride, 3.3 mg, I, 1.11 mg; Mn, 66.06 mg; Cu, 4.44 mg; Fe, 44.1 mg; Zn, 44.1 mg; Se, 300 µg.

Table 4.4. List of primers and their sequences.

Symbol	Gene name	Function	Forward primer	Reverse primer
β-actin	βeta-actin	Housekeeping gene	ACCGGACTGTTACCAACACC	GACTGCTGCTGACACCTTCA
MUC-2	Mucin-2	Mucin secretion	ATGCGATGTTAACACAGGACTC	GTGGAGCACAGCAGACTTTG
GLP-2	Glucagon-like peptide-2	Cell proliferation	CGTGCCACAGCCATTCTTA	AGCGGCTCTGCAAATGATTA
PYY	Peptide tyrosine tyrosine	Satiety regulation	CGCCATTACATCAACCTGGT	GAGTCATCGTCGATGTCGGA
GLP-1	Glucagon-like peptide-1	Satiety regulation	GATAGTTCAAGGCAGCTGGC	CTTGAGAGCTGATCCGGGAA
GLUT-2	Glucose transporter- 2	Glucose transporter	TCATTGTAGCTGAGCTGTT	CGAAGACAACGAACACATAC
PepT1	Peptide transporter-1	Peptide transporter	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA
y+LAT1	y+L amino acid transporter 1	Cationic amino acid transporter	CAGAAAACCTCAGAGCTCCCTTT	TGAGTACAGAGCCAGCGCAAT

Table 4.5. Growth performance response (d 0 to 21) of broiler chickens fed dietary resistant starches with different feeding durations.

RSL, g/kg	RSD, d	WG, g	FI, g	FCR	FBW, g
Control (0)		1071	1441	1.35^{AB}	1112
Main effects means of resistant starch feeding duration (RSD)					
	21	1035	1386	1.34	1074
	14	1029	1379	1.34	1070
	7	1015	1362	1.34	1056
Main effects means of resistant starch level (RSL)					
25 RPS		1030	1396	1.36^{A}	1070
50 RPS		1027	1375	1.34^{AB}	1067
35 HCS		1023	1356	1.33^{B}	1064
Pooled SEM		14.5	17.0	0.009	14.6
Simple effect means					
25 RPS	21	1052	1411	1.34	1112
50 RPS	21	1010	1367	1.35	1090
35 HCS	21	1041	1380	1.33	1051
25 RPS	14	1018	1384	1.36	1082
50 RPS	14	1033	1384	1.34	1058
35 HCS	14	1036	1370	1.32	1074
25 RPS	7	1020	1393	1.37	1078
50 RPS	7	1036	1376	1.33	1060
35 HCS	7	990	1319	1.33	1077
Pooled SEM		21.3	24.9	0.013	1031
Probabilities					
RSD		0.529	0.486	0.968	0.557
RSL		0.916	0.159	0.043	0.942
RSD × RSL		0.325	0.574	0.417	0.340

RPS- Raw potato starch, HCS - High-amylose corn starch, WG - Weight gain, FI - Feed intake,

FCR- Feed conversion ratio, FBW- Final body weight

n = 6 replicate cages for the simple effects and the control, and 18 cages for the main effects of feeding duration and resistant starch level.

$^{AB, ab}$: Means in a column, within a group, but with different superscripts differ significantly ($P \leq 0.05$). Block letter superscript compares RSL nested with control.

Table 4.6. Apparent ileal nutrient digestibility (%), total tract nutrient retention (%), and ileal digestible and metabolizable energy for broiler chickens fed dietary resistant starches with different feeding durations.

		Ileal				Total tract				
RSL, g/kg	RSD, d	DM	N	AIDE, MJ/kg	Starch	DM	N	ME, MJ/kg	AMEn, MJ/kg	Starch
Control (0)		74.7	83.6	13.88^{AB}	96.8	71.6	64.0^{A}	13.39^{AB}	12.94^{AB}	96.8
Main effects means of resistant starch feeding duration (RSD)										
	21	70.4	79.6	13.88	95.8	68.7	58.5	13.53	13.03	95.9
	14	70.6	80.1	13.85	95.6	69.2	60	13.65	13.18	96.1
	7	70.2	80.1	13.82	95.5	67.6	58.3	13.38	12.88	95.9
Main effects means of resistant starch level (RSL)										
25 RPS		70.2	80.3	13.58^{B}	95.8	68.7	58.8^{AB}	3183^{B}	12.81^{B}	96.2
50 RPS		70.0	79.5	13.79^{AB}	95.4	67.1	56.9^{B}	3182^{B}	12.81^{B}	95.5
35 HCS		71.1	80.0	14.20^{A}	95.7	69.7	61.1^{AB}	3329^{A}	13.48^{A}	96.2
Pooled SEM		0.691	0.746	0.121	0.199	0.875	1.39	0.152	0.167	0.221
Simple effect means										
25 RPS	21	69.7	79.3	13.50	96.4	68.6	57.6	13.31	12.79	96.4
50 RPS	21	70.1	79.1	13.87	95.4	67.4	56.5	13.28	12.77	95.1
35 HCS	21	71.4	80.4	14.28	95.6	70.1	61.4	13.99	13.54	96.3
25 RPS	14	71.0	80.7	13.71	95.9	68.5	58.9	13.29	12.78	96.1
50 RPS	14	70.6	80.3	13.84	95.2	69.0	60.2	13.66	13.19	95.9
35 HCS	14	70.4	79.3	14.00	95.8	70.0	60.9	14.02	13.56	96.3
25 RPS	7	70.0	80.8	13.56	95.2	68.9	59.9	13.35	12.85	96.3
50 RPS	7	69.2	79.1	13.65	95.7	64.9	54.0	13.01	12.46	95.6
35 HCS	7	71.4	80.3	14.2	95.5	68.9	61.1	13.78	13.33	95.9
Pooled SEM		1.012	1.092	0.180	0.29	1.28	2.03	0.223	0.245	0.383
Probabilities										
RSD		0.874	0.830	0.894	0.403	0.319	0.55	0.324	0.344	0.82
RSL		0.403	0.686	0.001	0.251	0.056	0.047	0.002	0.002	0.055
RSD □ RSL		0.705	0.693	0.599	0.068	0.517	0.411	0.608	0.587	0.56

RPS- Raw potato starch, HCS - High-amylose corn starch, DM - dry matter, N - Nitrogen, AIDE - Apparent ileal digestible energy, ME - Metabolizable energy, AMEn- Nitrogen- corrected apparent metabolizable energy.

n = 6 replicate cages for the simple effects and the control, and 18 cages for the main effects of feeding duration and resistant starch level.

^{AB}: Means in a column, within a group, but with different superscripts differ significantly ($P \leq 0.05$). Block letter superscript compares RSL nested with control.

Table 4.7. Quantitative caecal short-chain fatty acids (SCFA) profile (mM) and caecal content pH of broiler chickens fed dietary resistant starches with different feeding durations.

RSL, g/kg	RSD, d	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Total SCFA	Caecal pH
Control (0)		70.2	4.62	0.64	14.3	0.62	0.95	91.3	7.24
Main effects means of resistant starch feeding duration (RSD)									
	21	77.3	4.36	0.52	17.7	0.50	0.95	101.4	6.92
	14	72.7	4.35	0.51	15.6	0.47	0.99	94.6	7.18
	7	73.4	3.86	0.50	16.3	0.49	0.96	95.5	7.17
Main effects means of resistant starch level (RSL)									
25 RPS		75.1	4.21	0.55	15.8	0.53	0.94	97.1	7.09
50 RPS		71.3	4.21	0.50	16.0	0.48	0.92	93.4	7.06
35 HCS		77.0	4.14	0.47	17.8	0.46	1.03	101.0	7.12
Pooled SEM		2.991	0.348	0.047	1.057	0.054	0.051	3.802	0.096
Simple effect means									
25 RPS	21	79.7	4.46	0.52	16.6	0.48	0.93	102.7	6.96
50 RPS	21	72.1	3.82	0.49	18.2	0.48	0.86	95.9	6.77
35 HCS	21	80.3	4.80	0.55	18.4	0.54	1.07	105.6	7.05
25 RPS	14	76.3	4.39	0.54	15.1	0.47	0.93	97.7	7.14
50 RPS	14	67.8	4.61	0.52	15.1	0.51	1.02	89.6	7.21
35 HCS	14	74.0	4.04	0.46	16.7	0.43	1.01	96.6	7.18
25 RPS	7	69.3	3.78	0.61	15.7	0.63	0.96	91.0	7.17
50 RPS	7	74.1	4.22	0.50	14.7	0.46	0.90	94.9	7.21
35 HCS	7	76.8	3.58	0.40	18.5	0.39	1.02	100.6	7.12
Pooled SEM		4.401	0.512	0.069	1.556	0.080	0.075	5.595	0.141
Probabilities									
RSD		0.377	0.416	0.969	0.250	0.885	0.827	0.278	0.055
RSL		0.288	0.982	0.331	0.235	0.525	0.180	0.278	0.894
RSD ´× RSL		0.564	0.515	0.537	0.845	0.361	0.617	0.803	0.702

RPS- Raw potato starch, HCS - High-amylose corn starch.

n = 6 replicate cages for the simple effects and the control, and 18 cages for the main effects of feeding duration and resistant starch level.

Table 4.8. Jejunal histomorphology for broiler chickens fed dietary resistant starches with different feeding durations.

RSL, g/kg	RSD, d	VH, µm	CD, µm	VW, µm	VH:CD
Control (0)		1028	204^{B}	185	5.22
Main effects means of resistant starch feeding duration (RSD)					
	21	1042	215	169	5.10
	14	1274	236	167	5.42
	7	1205	235	173	5.25
Main effects means of resistant starch level (RSL)					
25 RPS		1236	251^{A}	165	5.01
50 RPS		1187	207^{B}	165	5.82
35 HCS		1098	228^{AB}	179	4.94
Pooled SEM		127	14.75	5.74	0.474
Simple effect means					
25 RPS	21	1156	248	168	4.88
50 RPS	21	996	169	159	6.03
35 HCS	21	975	227	181	4.38
25 RPS	14	1415	261	157	5.43
50 RPS	14	1311	230	171	5.56
35 HCS	14	1096	219	171	5.27
25 RPS	7	1138	245	169	4.71
50 RPS	7	1254	221	165	5.88
35 HCS	7	1224	238	184	5.16
Pooled SEM		180.6	21.0	8.2	0.675
Probabilities					
RSD		0.241	0.421	0.717	0.700
RSL		0.566	0.035	0.066	0.215
RSD × RSL		0.810	0.495	0.568	0.848

RPS- Raw potato starch, HCS - High-amylose corn starch. VH- Villi height. CD- Crypt depth, VW- Villi width.

n = 6 replicate cages for the simple effects and the control, and 18 cages for the main effects of feeding duration and resistant starch level.

^{AB}: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$). Block letter superscript compares RSL nested with control.

Table 4.9. Relative mRNA expression of jejunal gut integrity gene, satiety hormones, and nutrient transporters in broiler chickens fed dietary resistant starches with different feeding durations.

RSL, g/kg	RSD, d	MUC-2	GLP-2	GLP-1	PYY	GLUT-2	PEPT1	y+LAT1
Control (0)		1.00^{ab}	1.00^{abc}	1.00^{a}	1.00^{ab,AB}	1.00a	1.00	1.00^{bc,AB}
Main effects means of resistant starch feeding duration (RSD)								
	21	1.04^{a}	1.20	0.91	1.27^{a}	0.74	1.41	1.06^{b}
	14	1.09^{a}	1.34	0.50	1.29^{a}	0.70	1.10	1.63^{a}
	7	0.68^{b}	1.02	0.18	0.72^{b}	0.71	1.63	0.50^{c}
Main effects means of resistant starch level (RSL)								
25 RPS		0.94	1.24	0.61	1.22^{A}	0.78	1.27	1.33^{A}
50 RPS		0.80	1.06	0.42	0.74^{B}	0.65	1.43	1.06^{AB}
35 HCS		1.07	1.19	0.56	1.32^{A}	0.72	1.44	0.80^{B}
Pooled SEM		0.107	0.083	0.088	0.131	0.074	0.074	0.113
Simple effect means								
25 RPS	21	1.04	1.40^{ab}	0.88^{ab}	1.23	0.85^{abc}	1.00	1.55
50 RPS	21	0.95	1.04^{abc}	0.80^{ab}	0.94	0.81^{abc}	1.63	0.97
35 HCS	21	1.12	0.95^{abc}	1.05^{a}	1.65	0.56^{c}	1.60	0.66
25 RPS	14	1.09	1.53^{a}	0.89^{ab}	1.70	0.83^{abc}	1.37	1.89
50 RPS	14	1.11	1.24^{abc}	0.44^{bc}	0.89	0.57^{c}	0.92	1.74
35 HCS	14	1.07	1.25^{abc}	0.16^{c}	1.27	0.69^{abc}	1.00	1.25
25 RPS	7	0.70	0.79^{c}	0.06^{c}	0.73	0.65^{bc}	1.43	0.55
50 RPS	7	0.32	0.89^{bc}	0.02^{c}	0.39	0.57^{c}	1.73	0.47
35 HCS	7	1.02	1.37^{abc}	0.46^{bc}	1.03	0.91^{ab}	1.73	0.50
Pooled SEM		0.157	0.118	0.128	0.187	0.103	0.103	0.167
Probabilities								
RSD		0.006	0.010	<0.001	0.001	0.838	0.086	<0.001
RSL		0.121	0.175	0.172	0.002	0.321	0.721	0.001
RSD × RSL		0.248	0.005	0.001	0.229	0.033	0.340	0.101

RPS- Raw potato starch, HCS - High-amylose corn starch, *MUC-2* - Mucin 2, *GLP-2* - Glucagon-like peptide 2, *GLP-1* - Glucagon-like peptide 1, *PYY* - Peptide tyrosine tyrosine, *GLUT-2* - Glucose transporter- 2, *PEPT1* - Peptide transporter-1, *y+LAT1* - y+L amino acid transporter 1.

n = 6 replicate cages for the simple effects and the control, and 18 cages for the main effects of feeding duration and resistant starch level.

$^{AB, ab}$: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$). Small letter superscript compares the RSD group nested with control, or the simple effect means nested with control (in cases of significant interaction). Block letter superscript compares RSL nested with control.

CHAPTER 5

5. THE OUTCOME OF DIETARY ADDITION OF RESISTANT STARCHES ON GROWTH PERFORMANCE, APPARENT ILEAL AMINO ACID DIGESTIBILITY, CAECAL SHORT-CHAIN FATTY ACIDS PROFILE, JEJUNAL MORPHOLOGY, AND GUT INTEGRITY METRICS IN BROILER CHICKENS CHALLENGED WITH A MIXED *EIMERIA* INFECTION[4]

[4] Oluseyifunmi, I. W.; Lourenco, J.; Olukosi, O. A. To be submitted to *PLOS ONE*.

5.1 ABSTRACT

A mixed *Eimeria* spp. challenge model was used to investigate the possible ameliorative effect of dietary addition of resistant starches (RS), a functional fiber, on the growth performance, amino acid digestibility, cecal short-chained fatty acid profile (SCFA), jejunal histomorphology and gut integrity indices in broiler chickens during *Eimeria* infection. A total of 1056 day-old Cobb 500 broiler chickens were allocated into 8 treatments in a 4 x 2 factorial arrangement, which include corn-soybean-based diets with inclusions of 0, 25, and 50 g/kg raw potato starch (RPS) and 50 g/kg high-amylose corn starch (HCS) with or without *Eimeria* challenge. On day 13, the challenged groups were orally gavaged with a solution containing *E. maxima*, *E. acervulina* and *E. tenella* oocysts. Feed intake and body weights were measured on d9, d13, d16, d19, d28, and d35 to evaluate the growth performance response at different phases of *Eimeria* infection. There were no significant *Eimeria* × *diets* interactions for the growth responses. During the starter phase (d0-9), the weight gain and final body weight (WG, FBW) were greater $P < 0.05$) in birds fed 25 g/kg RPS diet than in control group but comparable to other RS diets. Weight gain, FBW and gain:feed were higher ($P < 0.05$) in the 50 g/kg HCS group than others in the early grower phase (d9-13) and in the prepatent stage (d13-16). The main effects of *Eimeria* were significant ($P < 0.05$) during the acute, recovery, and compensatory growth phases. The *Eimeria* infection depressed the WG, FI, G: F, and FBW ($P <0.05$) in the acute phase (d16-19); WG, FI, and FBW ($P < 0.05$) in the recovery phase(d19-28) and WG, G: F and FBW in the compensatory growth phase (d28-35). The *Eimeria* × diets and main effects of diets were not significant ($P > 0.05$) for the cecal SCFAs determined at d20 and d35. *Eimeria* challenge reduced butyrate ($P = 0.001$) and tended to reduce total SCFAs ($P = 0.08$) but increased ($P < 0.05$) the branched-chain fatty acids (BCFA) and propionate ($P = 0.01$) on d20. Butyrate and total SCFAs concentrations were depressed in the challenged group at d35

(P < 0.05 The main effects of *Eimeria* challenge were significant for jejunal histomorphology. The challenge group had lower villi height, villi height: crypt depth (P< 0.05), and deeper crypts (P=0.001). The intestinal permeability increased (P < 0.05) in the challenged group. Results showed no significant (P > 0.05) diet × infection interaction or main effects of diet for dry matter digestibility (DMD) or AID of indispensable amino acids, except for Trp. Birds fed 50 g/kg HCS had higher (P = 0.018) AID of Trp than those on the control diet. The AID of Ile, Lys, Met, Phe, and Val were reduced (P < 0.05) and that of Trp was greater (P < 0.001) in challenged birds. Birds fed 25 g/kg RPS and 50 g/kg HCS had greater (P = 0.005) AID of Cys than the control, whereas AID of Ala decreased (P = 0.002) in challenged birds. This study showed that RS supplementation may reduce the depression of growth performance during the *Eimeria* prepatent phase and improve growth performance in the absence of infection during early life of broiler chickens but had no phenotypic effects on gut integrity and growth during the acute to compensatory growth phases.

5.2 INTRODUCTION

Coccidiosis is the most prevalent enteric disease of major economic importance that affects the broiler industry worldwide, with annual expenditures of over $3 billion on prevention and $90 million in the US. *Eimeria,* a genus of *apicomplexan* parasites, cause coccidiosis in chickens, disrupting the host's homeostasis and decreasing nutrient absorption and utilization. Seven species of Eimeria are widely recognized to infect chickens: *E. acervulina, E. mitis, E. necatrix, E. tenella, E. praecox, E. maxima, and E. brunetti* (Shirley, 1986). Each affecting different anatomical sites of the intestine. Nutrition impacts all aspects of broiler chickens' responses to *Eimeria* exposure, including susceptibility, protection, pathogenesis, recovery, and compensatory growth.

Several nutritional strategies have been explored in alleviating the effects of this protozoan disease that predisposes chicken to further bacterial infection which include the use of various vitamins, enzymes, prebiotics, combinations of amino acid feed formulations, organic acids, and others (Gómez-Osorio et al., 2021). Functional carbohydrates are another promising nutritional approach for improving intestinal health in poultry and may be beneficial during enteric challenge as reported in weaned pigs and ducks by altering microbial composition, improving short-chain fatty acid production and immune status (Dobranowski and Stintzi, 2021, Zhang et al., 2022, Yi et al., 2023). Some gut bacteria have been shown to stimulate anti-coccidial antibodies and enhance immune response. The existence of *Lactobacillus* at various locations in the gastrointestinal tract enhances nutrition utilization, impede the colonization of the intestines, and reduce the intestinal lesions resulting from invasion by *E. tenella* (Madlala et al., 2021).

Starches are generally highly digestible, but certain starch molecules escape digestion in the proximal intestinal tract and exert positive effects on a distal part by undergoing fermentation

in the ceca to produce beneficial metabolites such as short-chain fatty acids (SCFAs) and beneficial digesta oligosaccharide profile. These types of starches are categorized as resistant starches (Landon et al., 2012). Resistant starches have similar physiological functions as soluble fermentable fiber and were considered a part of the total dietary fiber assay (Haralampu, 2000). They increase the proportion of fermentable carbohydrates relative to protein in the caeca, and the fermentable starches are possible prebiotics in the distal part of the digestive tract. The increased production of SCFA by RS fermentation lowers the cecal luminal pH and change the digesta pH; creates an acidic environment that is not conducive for proliferation of pathogenic microbes.

The production of short chain fatty acids favor the proliferation of beneficial bacteria, such as B*ifidobacteria* and *Lactobacilli* which in turn stimulate SCFA synthesis. Firmicutes bacteria such as *Faecalibacterium prausnitzii, Roseburia sp*. produce high amounts of butyrate, whereas Bacteroidetes produce high levels of acetate and propionate (Liu et al., 2021, Tan et al., 2021). Butyrate serves as a major source of energy to cecal epithelial cells and increases the proliferation of the cells and modulate the expression of mucin genes and tight junction proteins, thereby improving intestinal barrier function. The SCFA modulate immune response through regulation of cytokines production by inhibition of histone deacetylases (HDACs) and activation of G protein-coupled receptors (GPRs) on immune cells. Butyrate being a histone deacetylase inhibitor can inhibit NF-kB activation which can downregulate the expression of pro-inflammatory cytokines in broilers whereas, acetate seems to be more involved in the production of pro-inflammatory cytokines and chemokines by the activation of GPR41 or GPR43 (Liu et al., 2021). Cytokines are natural proteins essential for stimulation and regulation of immunity against infectious diseases. They also hinder the secretion of siderophores, restricting iron availability for parasite invasion (Madlala et al., 2021).

However, most publications are on pigs, mice and human with just a few in ducks. In addition, no study has investigated the influence or possible ameliorative effects of resistant starches on intestinal integrity and gut health in broilers during Eimeria challenge. This study evaluated the influence of optimal nutrition of resistant starch (RS) on intestinal integrity, immune response, growth performance, and broiler resistance to *Eimeria* challenge.

5.3 MATERIALS AND METHODS

Birds and housing

The University of Georgia Institutional Animal Care and Use Committee approved the experimental procedures (IACUC number: A2021-06-006).

Animal housing, diets and experimental design

The birds were raised in a building containing 48-floor pens (white pine shavings litter, 4.5 x 0.8 m dimension in a controlled environment following recommended lighting and temperature regimes for Cobb 500 broiler chickens, with 22 birds per pen from day old to 35 days of age. Three diets plus corn-soybean meal control diet which consist of 25 and 50 g/kg inclusion of raw potato starch (RPS) and 50 g/kg high amylose corn starch (HCS)were fed, with or without Eimeria challenge making a total of 8 treatments arranged as a 4×2 factorial. A total of 1056 Cobb 500 male broiler chicks at day old received the experimental diets from day 0 to 35. Each treatment had 6 replicates and 22 birds per replicate. The analyzed chemical composition of the resistant starches was presented in the first experiment (Oluseyifunmi et al., 2024). Feed and water were provided ad libitum throughout the experiment. The diets were presented as mash in the starter and as pellets in the grower and finisher phases and formulated to meet the nutrient and energy recommendations for Cobb 500 broiler chickens (Cobb-Vantress, 2022). All the diets were

isocaloric and isonitrogenous. The diet compositions are presented in Tables 5.1, 5.2 and 5.3 for the starter, grower and finisher phases, respectively.

Birds and feeds were weighed on days 0, 9, 13, 16, 20, 28, and 35. On d 13, the challenge group were orally gavaged with a solution containing 12,500 sporulated oocysts of *E. maxima*, 12,500 sporulated oocysts of *E. tenella*, and 62,500 sporulated oocysts of *E. acervulina* suspended in 1 ml of distilled water and the non-challenged groups were gavaged with 1 ml of distilled water. An intestinal permeability assay using FITC-dextran was done using one bird per pen on d 19. Ileal digesta and samples of jejunal tissues were collected on d 20 and analyzed for amino acid digestibility and mRNA expression (related to intestinal integrity and inflammation, selected glucose, amino acids and peptide transporters) and for histomorphology, respectively. Caecal tonsils were also collected for analysis of mRNA expression of pro and anti-inflammatory cytokines. Cecal contents were collected on days 20 and 35 for SCFAs analysis and whole ceca were used for microbiota profiling by 16S RNA sequencing.

Growth performance measurements

Birds and feeds were weighed on days 0, 9, 13, 16, 20, 28, and 35 to evaluate the growth performance response at various phases of *Eimeri*a challenge. Body weight gain (WG), feed intake (FI), and FCR were corrected for mortality.

Amino acid digestibility, cecal short-chain fatty acids, and cecal protein measurements

On day 20, three randomly selected birds per pen were euthanized by CO_2 asphyxiation and the ileal digesta and cecal content were collected for digestibility measurement, the cecal SCFA profile and soluble protein analysis, respectively. The digesta from the distal half of the ileum (from mid ileum up to 2cm proximal to ileocecal junction) was used for ileal amino acid digestibility.

Jejunal histomorphology

Two birds per pen were randomly selected and euthanized and about 2 cm segments of mid-jejunal tissues of each bird were collected after carefully removing the adhering tissues and the digesta flushed out using phosphate buffered saline. These tissues were subsequently fixed in 10% neutral buffered formalin and dehydrated in ethanol, cleared with xylene, embedded in paraffin wax and sectioned into 5 μm. The sections were then stained with haematoxylin-eosin (H&E) stains and the images were captured with the aid of BZ microscope (BZ-X800; Keyence Inc., Itasca, IL) at 4 × magnification and analyzed using BZ-X800 Analyzer. The villus height (the tip of the villus to the crypt junction) and the crypt depth (depth of the invagination between adjacent villi) were measured for six villi and the corresponding crypts per section, and the villus height-to-crypt depth ratio was determined.

Caecal short-chain fatty acids profile

A portion of caecal digesta (1g) was diluted with 3 ml of deionized water, thoroughly mixed, and centrifuged at 10,000 × g for 10 minutes. The resulting supernatant (1 ml) was combined with 0.2 ml of 25% (w/v) metaphosphoric acid solution and allowed to freeze overnight. Subsequently, the thawed samples were centrifuged and about 0.75 ml of the supernatant was mixed with 0.15 ml of an internal standard to create a 0.9 ml solution. About 1.8 ml of ethyl acetate was then added, and the mixture was vortexed for 15 seconds and allowed to settle for 5 minutes, after which approximately 1.2 ml of the top layer was transferred into a glass vial. Gas chromatography (Shimadzu GC-2010 Plus; Shimadzu Corporation, Japan) was then used to measure the SCFA content of the solutions relative to a known standard (Lourenco et al., 2020).

Quantitative real-time PCR analysis

Mid sections of jejunal tissues and caecal tonsils were collected from 1 randomly selected bird per pen and snap-frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. The extraction was done using QiAzol lysis reagent (QIAGEN, Hilden, Germany) and the RNA samples were purified and normalized accordingly using the procedures described by Oluseyifunmi et al. (2024). About 10 µl volume of the RNAs were reverse transcribed to cDNA with the aid of a high-capacity cDNA reverse transcription kit (Thermo Fischer Scientific, Waltham) and the RT-PCR carried out in duplicate using StepOnePlus (Applied Biosystems, Carlsbad, CA, USA) with reaction master mix iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). The fold change was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and the housekeeping gene used were beta-actin and glyceraldehyde-3-phosphate dehydrogenase. The primer sequence and their functions are presented in Table 5.4.

Chemical analyses

Oven-dried and ground diets and ileal digesta (0.5mm sieve) samples were used for chemical analyses following AOAC (2006; 2012). Samples were dried at 100°C for 24 hours for dry matter determination (Method 934.0) and combustion nitrogen analyzer (LECO, St. Joseph, MI) was used to evaluate the N content of the samples. The titanium dioxide analysis was done using the procedures described by Short et al. (1996). Amino acids (AAs) were analyzed following AOAC Method 982.30E (a, b, c). Samples were hydrolyzed with 6N HCl containing phenol at 110°C for 24 hours, and AAs were measured using an ion exchange analyzer with ninhydrin post-column derivatization. Chromatograms were detected at 570 and 440 nm and processed using Agilent Open Lab software. Cys and Met were analyzed as cysteic acid and methionine sulfone by oxidation with performic acid–phenol at 0 °C for 16 hours prior to hydrolysis. Trptophan was

measured via alkaline hydrolysis with barium hydroxide at 110°C for 20 hours, separated by reverse-phase HPLC, and detected using fluorescence to ensure specificity. Megazyme assay kit (K-AMYL) was used for total starch determination and the resistant starch assay was carried out by Eurofins Food Testing, Netherlands, using the AOAC method 2011.25.

Calculations

All calculations are presented on a dry matter basis. On day 20, ileal digesta were collected from 3 randomly selected birds per pen and pooled for birds within a pen. The digesta were collected from the distal half of the ileum by flushing with distilled water into plastic containers and stored frozen at −20 °C for later processing for ileal amino acid digestibility. The apparent ileal digestibility AA in the assay diets were calculated according to the following equation:

$$AID\ (\%) = 1 - (ID \times AI / AD \times II) \times 100$$

where ID = titanium concentration in the assay diet (% DM), AI = AA content in ileal digesta (% DM), AD = AA content in the assay diet (% DM), and II = titanium concentration in ileal digesta (% DM).

5.4 STATISTICAL ANALYSIS

The statistical model for the experiment was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_{j(i)} + \gamma_{k(i)} + (\beta\gamma)_{jk(i)} + \varepsilon_{ijkl}$$

where Yijkl is the response variable, μ is the overall mean, α_i is the random effect of *i*th block, β_j represent the fixed effect of jth diet within ith treatment group, γ represents the fixed effect of kth infection status within *i*th treatment group. $(\beta\gamma)_{jk}$ *is the* interaction effect of jth diet and kth infection status within ith treatment group, and the ε_{ijkl} is the residual error. Data were analyzed

using the mixed model procedure of JMP Pro 13.2.0 (SAS Institute Inc., Cary, NC) in a 4 × 2 factorial arrangements. The factors include three RS diets plus corn-soybean meal control diet with or without *Eimeria* challenge. Means with significant differences were separated using Tukey's HSD, and the significant P-value was set at $P \leq 0.05$. Main effects are discussed when there are no significant interactions, whereas the simple effects are described in cases of significant interactions.

5.5 RESULTS

Growth performance

During the starter phase (Table 5.5), the birds that received 25 g/kg RPS had greater WG ($P = 0.047$) and FBW ($P = 0.047$) than those that received the control diet but comparable among resistant starch diets whereas, the FI and FCR were not significantly affected by the dietary treatments. At d 4 pre-infection, the WG and FBW were higher in birds fed 50 g/kg HCS than the control diet and the FCR was lower in 50 g/kg HCS group than those that received the control and the RPS diets.

The diet × infection was not significant ($P > 0.05$) during the prepatent (0–3 dpi) (Table 5.6). The main effects of diets were significant ($P < 0.05$) for WG, FCR and FBW and the main effects of infection significant ($P < 0.05$) for WG, FI and FBW. The WG ($P = 0.001$) and FBW ($P = 0.003$) were higher in birds fed 50 g/kg HCS than the control diet and the FCR ($P = 0.015$) lower in same group than the control but comparable in birds that received resistant diets. The infected birds had greater WG ($P = 0.003$), FI ($P = 0.011$) and FBW (< 0.001).

During the acute (4-6 dpi) phase of infection, there were no significant ($P > 0.05$) diet × infection nor main effects of diet on the growth performance responses (Table 6). However, the main effects

of infection were significant ($P < 0.05$). The infection reduced ($P < 0.05$) the WG, FI and FBW and increased the FCR.

Neither the diet × infection nor main effects of diets were significant during the recovery (7 -15 dpi) and compensatory (16 - 22 dpi) phases but the main effects of infection were significant ($P < 0.05$) for WG, FI and FBW during the recovery phase and WG, FCR and FBW during the compensatory phase (Table 5.7). The *Eimeria* challenge reduced the WG ($P = 0.023$), FI and FBW ($P < 0.001$ during the recovery phase and also decreased the WG ($P = 0.003$, and FBW ($P = 0.001$) as well as increased the FCR ($P = 0.002$) during the compensatory phase.

Apparent ileal amino acid digestibility

Indispensable amino acid digestibility

The results showed no significant ($P > 0.05$) diet × infection interaction or main effects of diet for dry matter digestibility (DMD) or AID of indispensable amino acids, except for Trp (Table 5.8). Birds fed 50 g/kg HCS had higher ($P = 0.018$) AID of Trp than those on the control diet. The main effects of infection were significant ($P < 0.05$) for the AID of Ile, Lys, Met, Phe and Trp. The AID of Ile, Lys, Met, Phe, and Val were reduced ($P < 0.05$) and the AID of Trp was greater ($P < 0.001$) in challenged birds.

Dispensable amino acid digestibility

There was no significant ($P > 0.05$) diet × infection for AID of all the dispensable amino acids assessed (Table 5.9). However, the main effect of diets was significant ($P < 0.05$) for Cys ($P < 0.05$**)** and showed a tendency ($P < 0.10$) for Asp, Gly, Glutamate and Serine. The birds that received 25 g/kg RPS and 50 g/kg HCS diets had greater ($P = 0.005$) AID of Cys than those on control diet. The AID of Asp ($P = 0.090$), Gly ($P = 0.073$, Glutamate ($P = 0.069$), Serine ($P = 0.061$) tended to increase in birds fed 25 g/kg RPS and 50 g/kg HCS diets.

Additionally, the main effect of infection or *Eimeria* was significant ($P < 0.05$) for AID of Ala and showed a tendency for AID of Cys. The *Eimeria* challenge decreased ($P = 0.002$) the AID of Ala and tended to decrease ($P = 0.077$) the AID of Cys.

Gastrointestinal tract permeability

The gut permeability on d 19 (6 dpi) was not significantly ($P > 0.05$) affected by the diet × infection or main effects of diets. The main effect of *Eimeria*-challenge or infection was significant ($P < 0.05$) for serum FITC-d concentration (Figure 5.1). The *Eimeria*-challenged birds had significantly higher ($P < 0.001$) serum levels of FITC-d than uninfected group.

Caecal short-chain fatty acids profile

There was no significant diet × infection nor main effects of diets ($P > 0.05$) for caecal short-chain fatty acids profile on d 20 but the main effects of infection or *Eimeria* challenge were significant ($P < 0.05$) for propionate, isobutyrate, butyrate, isovalerate and total branched-chain fatty acids (BCFA) (Table 5.10).

The caecal concentration of propionate ($P= 0.008$), isobutyrate ($P < 0.001$), isovalerate ($P < 0.001$) and total branched-chain fatty acids (BCFA) ($P < 0.001$) in mM were greater in *Eimeria*-challenged birds, whereas the caecal butyrate concentration decreased ($P = 0.001$) and there was a tendency for reduced acetate ($P = 0.098$) and total short-chain fatty acids (SCFA) ($P = 0.058$) concentrations in *Eimeria*-challenged birds.

The diet × infection and the main effects of diets were not significant ($P > 0.05$) for d 35 caecal short-chain fatty acids profile except for the main effects of infection or *Eimeria* challenge that were significant ($P < 0.05$) for caecal butyrate, valerate and total SCFA concentrations (Table 5.11). The caecal butyrate ($P = 0.018$), valerate ($P = 0.015$), SCFA ($P = 0.042$) concentrations declined in the infected or *Eimeria* -challenged birds relative to the uninfected ones.

Jejunal histomorphology

The histomorphology of the jejunum was not affected ($P > 0.05$) by the interaction of diets and *Eimeria* challenge nor by main effects of diets. However, the main effects of infection were significant ($P < 0.05$) for villi height (VH), crypt depth (CD) and villi height to crypt depth ratio (VH:CD) (Table 5.12). Eimeria challenge reduced the VH ($P < 0.001$), VH:CD ($P < 0.001$), and increased the CD ($P = 0.001$).

Relative mRNA expression of jejunal nutrient transporters, tight junction proteins and selected cytokines in the caecal tonsil.

Relative mRNA expression of jejunal tight junction proteins and nutrient transporter

The relative mRNA expressions of jejunal tight junction proteins were not significantly affected by interaction of the diet × infection and main effects of diets except for main effects of infection on JAM-2 and a tendency ($P< 0.10$) for occludin (Table 5.13). The Eimeria challenge depressed ($P = 0.001$) the expression of JAM-2 and tended ($P = 0.069$) to reduce the expression of occludin. PepT1 was more expressed ($P= 0.054$) in uninfected birds fed 25 g/kg RPS than in infected birds fed 25 and 50 g/kg RPS. Among the infected birds, the control and 50 g/kg HCS had higher PepT1 expression and greater expression in uninfected birds fed 25 g/kg RPS and 50 g/kg HCS.

Relative mRNA expression of selected cytokines and annexin A1 (AnxA1)

The main effects of diets were significant ($P< 0.05$) for TNFα and IL-10 and the main effects of infection for IL β. The diet × infection was ($P< 0.05$) significant for annexin A1 (AnxA1) (5.13). The birds fed 50 g/kg RPS and HCS had higher ($P = 0.014$) expression of TNFα than those that received the control diet. IL-10 was highly expressed ($P = 0.035$) in birds fed the control and

25 g/kg RPS diets. The *Eimeria* challenged or infected birds had decreased expression (P = 0.003) of IL β.

The relative mRNA expression of annexin A1 (AnxA1) was greater (P = 0.001) in uninfected birds fed 50 g.kg RPS and HCS than in the infected birds fed RS diets and in the uninfected control diet.

5.6 DISCUSSION

Coccidiosis is one of the major enteric and parasitic disease in poultry which impairs the intestinal morphology causing lesions and inflammation of the intestinal mucosa, thereby eliciting numerous metabolic and physiological changes and compromised the immune function (Blake et al., 2020, Lee et al., 2023). However, the severity of the impacts of the infection on growth performance responses (weight gain, feed intake and feed efficiency) depend largely on various factors which include the different phases of infection, the species of *Eimeria*, pathogenicity and number of oocysts ingested, the environmental conditions (presence of other stressors and the state of the litter), sex, genetic line as well as the age and diet of the animals (Lee and Rochell, 2022, de Freitas et al., 2023, Lee et al., 2023).

Over the years, the modulation of dietary compositions and formulations constitutes parts of the major control measures for coccidiosis (Gómez-Osorio et al., 2021). Recently the use of functional fibers such as resistant starches for improving gut health is gaining more attention and could be promising for alleviating the adverse effects of coccidiosis on the gut health due to the production of beneficial metabolites such as SCFA during distal fermentation, its prebiotic effect in the hind gut and modulation of intestinal microbial ecology (Thompson et al., 2022). This study aimed to harness the possible prebiotic function of resistant starch in broiler chicken during enteric challenge.

Growth performance

The results obtained in this study indicated that 25 g/kg RPS seem to be optimum during the starter phase and 50 g/kg HCS during the early grower phase. This showed a phase-specific dependent impact of resistant starch (RS) on growth performance, suggesting that different RS source and concentration might be beneficial at different phases of development in broiler chicken. These results align with the observations reported in our previous study (Oluseyifunmi et al., 2024).

Although, Liu et al. (2020) previously reported a lower weight gain and feed intake with a decline in feed efficiency as the dietary RS level increased in chickens fed diets containing 40, 80 and 120 g/kg corn RS relative to the control diet (corn-soybean-based diet with 20 % corn starch). Zhang et al. (2020) further compared the same set of diets with normal corn–soybean diet in broiler chickens and found lower feed intake and weight gain in the birds that received RS diets and the diet containing 20 % corn starch.

On the contrary, feeding of day-old duckling diets with diets containing 60, 120, 180 and 240 g/kg RPS for a period of 14 and 35 days had no significant adverse effects on the growth performance as compared with normal corn–soybean diet (Qin et al., 2019). The disparity in results could be attributed to different RS sources, concentrations and experimental models in these studies. The increase in WG observed in the current study could be corroborated by the increase in nitrogen retention associated with these RS sources (RPS and HCS) and higher AME and AMEn reported in birds fed diets containing HCS in our previous experiment (Oluseyifunmi et al., 2024). The higher feed intake and weight gain observed at the early stage of infection (prepatent phase) might be because the infection is yet to peak at this time point and the pathological consequence of coccidiosis might still be less severe during the incubation stage (de Freitas et al., 2023). The

increase in nitrogen retention and apparent metabolizable energy associated with HCS in addition to continuous energy supply through production of SCFA by distal fermentation of RS (Oluseyifunmi et al., 2024) might have also played a role in alleviating the effects of coccidiosis at this phase of infection. The SCFA produced could boost the immune response during this phase and at the same time modulate the intestinal microbiota to favor the proliferation of microbes that could play significant immunomodulatory roles during inflammation, thereby reducing the adverse consequence on growth indices.

Coccidiosis had detrimental effects on the WG FI and FCR through acute phase to compensatory growth phase with no significant ameliorative impacts of RS observed during these periods. However, the birds fed 50 g/kg HCS had numerically greater WG and FBW with reduced FCR than other dietary treatments during the recovery phase. This can be explained by repartitioning of nutrients towards intestinal repair, immune responses and disruption of digestion/absorptive capacity which have been reported to account for about 28% reduction in body weight and 73% decrease in body weight as consequences of reduced FI during coccidiosis (Lillehoj and Trout, 1996, Lee and Rochell, 2022).

Apparent ileal amino acid digestibility

The AID value indicates the net disappearance of amino acids from the digestive tract (Kim et al., 2022). *Eimeria* infection could impair the digestive and absorptive capacity of the gastrointestinal tract and interferes with apparent ileal digestibility of amino acids and growth performance (Rochell et al., 2016a). In this study, *Eimeria* infection reduced the AID of selected indispensable amino acids which included methionine and lysine, the most limiting amino acids for broiler performance (Alagawany et al., 2021). The decrease in AID of indispensable AA observed in infected birds was not unexpected as the requirements for these AA might increase

during infection. These amino acids are important for combating oxidative stress and crucial for immune functions (Jespersen et al., 2024).

However, the increase in AID of Trp by 50 g/kg HCS and greater AID of Cys in the birds that received 25 g/kg RPS and 50 g/kg HCS diets suggested the modulation of amino acid metabolism by dietary inclusion of resistant starch as previously reported by Qin et al. (2023), although the mechanism behind this is yet to be understood. Furthermore, the increase in AID of Trp during infection partially corresponded with previous report that *E. acervulina* reduced the AID of all amino acids except for Gly and Trp, along with lower AID of Trp under mixed *Eimeria* challenge condition (Bortoluzzi et al., 2020; Rochell et al., 2016b). This is unexpected because Trp is involved in acute protein synthesis during inflammation and birds may increase their requirement for it during *Eimeria* challenge (Kim et al., 2022), although Trp may play important role in modulating gut microbiota as it serves as substrate for enzymes within the gut microbiota leading to its breakdown into various metabolites which can regulate the host's immune response (Gao et al ., 2020).

Additionally, the AID of Ala (dispensable amino acid) was reduced by mixed *Eimeria* challenge such that the challenged birds had lower ileal digestibility compared to the uninfected group, irrespective of the dietary resistant starch fed. The digestibility of Ala has been previously reported to be one of the most impacted amino acids during mixed *Eimeria* challenge model (Kim et al., 2022). The effects of dietary fiber on nitrogen and amino acid digestibility are dependent on several factors including the nature of the fiber, digestibility of dietary carbohydrate and protein. Native fibers may play a role in the sloughing of intestinal cells and have the potential to adsorb amino acids, peptides, or proteins (Eggum, 1992).

Gastrointestinal tract permeability

The intestinal permeability is measured in poultry studies using Fluorescein isothiocyanate-dextran (FITC-d) as an indicator (Liu et al., 2021). The elevated FITC-d levels in the serum of these birds on d 19 (6 dpi) points to the severity of the *Eimeria* infection at the acute phase and it is indicative of elevated leakage of the gastrointestinal tract.

Caecal short-chain fatty acids profile

Production of SCFA by microbial fermentation of RS modulated caecal microbiota with some RS possessing selective impact on the microbial composition which have implications on gut health of the host (Bird et al., 2007). In the current study, the caecal propionate concentration was increased on d- 20 (7 dpi), which is the start of the recovery phase. In poultry, *Alistipes* have been identified as producers of propionate in the chicken cecal microbiota and its abundance in cecal microbiota has been reported to increase significantly after *E. tenella* infection and might be involved in the host immune response to chicken coccidia infection, although this need to be further investigated (Yu et al., 2023). The decrease in butyrate concentration and an increase in isovalerate, isobutyrate and branched chain fatty acids (BCFA) on d- 20 (7 dpi) by *Eimeria* challenge in the current study might be consequential to sloughing of intestinal epithelial during coccidiosis which may increase the amount of substrate available for protein fermentation (Lin and Olukosi, 2021). Dietary RS used in this study did not have any significant impact on *Eimeria* infection nor on SCFA profile and concentration at the start of the recovery d- 20 (7 dpi) and during compensatory phases 35 (15-22 dpi).

Jejunal histomorphology

Villi height to crypt depth ratio is an indicator of intestinal absorptive capacity and health (Marchewka et al., 2021). The dietary RS used in the current study did not increase the absoprtive

surface area of the jejunum, as RS microbial fermentation occurs in the distal gut. A previous study observed increased VH, VH:CD and decreased CD in the caecum of ducks fed the RPS-containing diets as against those fed 0 % RPS diet (Qin et al., 2020). However, the increase in jejunal crypt depth observed in *Eimeria*-challenge bird in this study is suggestive of increased proliferative activity of crypt cells and are higher during inflammations. The reduced VH and VH:CD are indicative of reduced absorptive surface area during enteric infection (Belote et al., 2023).

Relative mRNA expression of jejunal nutrient transporters, tight junction proteins and selected cytokines in the caecal tonsil

The major beneficial effects of RS on gut health are modulation of intestinal microbial ecology, maintenance of gut homeostasis and elevation of expression of genes that are associated with gut development and health (Roy et al., 2006, Regassa and Nyachoti, 2018).

Tight junction proteins are crucial for maintaining the functionality and integrity of epithelial cell barriers, such as those in chicken intestines, by forming tight seals, controlling molecule and ion movement, and preventing pathogen entry (Graham et al., 2023). The depression in the expression of JAM-2 and occludin by *Eimeria* challenge are partly in line with the previous report (Scharl et al., 2009), whereas certain study reported no expression of tight junction protein in birds challenged with mixed *Eimeria* species (Graham et al., 2023). These might be influenced by various factors which include the number of oocysts, the integrity of ribonucleic acid (RNA), *Eimeria* species mixed and pathogenicity of the oocysts among others (Lee and Rochell, 2022, de Freitas et al., 2023, Lee et al., 2023).

PEPT1 is a protein situated in the brush-border membranes that facilitates the transport of di- and tripeptides (Verri et al., 2010). Greater expression of PepT1 during infection in birds fed 50 g/kg HCS than other infected birds that received other RS diets demonstrated the potential of HCS in facilitating the

luminal uptake of peptides during enteric challenge in broiler chickens. Additionally, a much higher expression of PepT1 in birds fed dietary RS in the absence of *Eimeria* challenge is congruential to the previous report (Oluseyifunmi et al., 2024).

Tumour Necrosis Factor alpha (TNF α), is a pro-inflammatory cytokine primarily produced by macrophages/monocytes during acute inflammation, leading to necrosis or apoptosis. Its expression fluctuates with infection phases. (Zhang et al., 1995, Idriss and Naismith, 2000, Zhang and An, 2007)

Conversely, IL-10 is an anti-inflammatory cytokine that downregulates the Th1 response by inhibiting pro-inflammatory cytokine secretion, essential during parasitic infections (Gazzinelli et al., 1996, Lee et al., 2022). The elevation of TNFα in birds fed 50 g/kg RPS and HCS than in birds fed the control diet and lower expression of 1L-10 in birds that received 50 g/kg HCS relative to the control and 25 g/kg RPS diets indicated RS source and level dependent effects of resistant starch on expression of various anti and pro-inflammatory cytokines.

IL-1β is a pro-inflammatory cytokine that induces chemokine production, promoting the recruitment of inflammatory cells to the site of inflammation (Hong et al., 2006). Suppressed relative mRNA expression of IL-1β in *Eimeria*-challenged broiler might be associated with the collection of sample tissue at the beginning of recovery phase as chickens infected with *E. maxima* and *E. tenella* previously increased the expression of IL-1β in the duodenum, jejunum, and cecum of chickens (Hong et al., 2006, Lee et al., 2022).

Annexin A1 (AnxA1) is an anti-inflammatory endogenous pro-resolving protein that modulates inflammation by triggering signaling pathways that promote the resolution of the inflammatory response, pathogen clearance, and the restoration of tissue homeostasis, which makes the expression of Annexin A1 pertinent during Eimeria challenge. It can further stimulate

the reprogramming of macrophage toward a resolving phenotype which could restore tissue homeostasis (Resende et al., 2023).

Feeding broiler chickens with diets containing 25 g/kg RPS and 50 g/kg HCS during *Eimeria* challenge spiked the relative mRNA expression of AnxA1. However, birds fed 50 g/kg RPS and HSC in the absence of infection had much more expression of AnxA1. AnxA1 pathway presents a potential alternative pathway through which RS may regulate immune response, facilitating monocyte recruitment and aiding phagocytosis of dead neutrophils by macrophages, accompanied by anti-inflammatory signals. In overall, the type and the botanical origin of RS as well as the physiological condition of the animals influence RS fermentability and the resulting SCFA, which inform the inflammatory responses elicited (Liu et al., 2021).

5.7 CONCLUSION

This study evaluated the influence of optimal nutrition of resistant starch (RS) on intestinal integrity, immune response, growth performance, and broiler resistance to *Eimeria* challenge. Dietary inclusion of 25 g/kg RSPS and 50 g/kg HCS improved the growth performance during the starter phase and 4-pre-infection, respectively and the latter equally promoted the growth performance in the prepatent phase of infection. However, *Eimeria* challenge negatively impacted the growth performance through acute to compensatory phase. Feeding broiler chickens with dietary resistant starches improved the apparent ileal digestibility (AID) of Trp and Cys but *Eimeria* challenge showed detrimental effects on the AID of all indispensable AA evaluated irrespective of the RS diet fed except for Trp. The elevated caecal BCFA and propionate concentrations at d- 20 (7 dpi) provides evidence of a shift in gut microbial ecology. In addition, the differential expression of immune regulatory proteins presents an additional pathway through which RS may regulate immune response. Future studies should fully elucidate the effects of RS

on immune system as this would clearly establish the mode of action of RS during inflammation or enteric challenge in broiler chickens.

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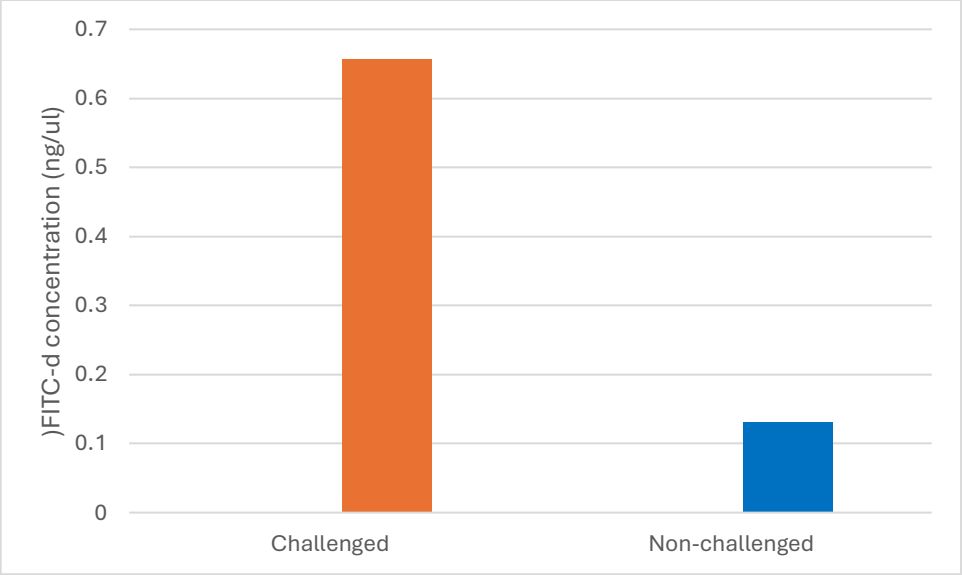

Figure 5.1: The intestinal permeability on day 19 (6 dpi). The *Eimeria*-challenged birds had significantly higher ($P < 0.001$) serum levels of FITC-d. This suggests increased gut leakage caused by Eimeria infection.

Table 5.1. Ingredients and chemical analysis (g/kg) of the starter phase (d 0 to 10) diets.

	Starter diet (d 0 - 10)			
			Potato starch	Hi-Maize starch
Ingredients, g/kg	Control	25	50	50
Corn	636	599	561	555
Potato starch		25	50	
Hi-Maize starch				50
Soybean meal	329	335	343	342
Soybean oil	1.5	7	12.8	19.8
Dicalcium phosphate	18	18	18	18
Limestone	5.9	5.9	5.87	5.87
Sodium bicarbonate	2.0	2.0	2.0	2.0
Vitamin premix[a]	0.9	0.9	0.9	0.9
Trace minerals premix[b]	0.68	0.68	0.68	0.68
Methionine	1.5	1.52	1.5	1.56
Lysine	1.52	1.3	1.23	1.33
Threonine	0.6	0.6	0.5	0.6
Titanium dioxide (TiO2)	0	0	0	0
Salt NaCl	2.3	2.3	2.3	2.3
Choline	0.7	0.7	0.7	0.7
Phytase (Q blue)	0.1	0.1	0.1	0.1
Total	1000	1000	1000	1000
Calculated Nutrients & Energy				
Protein, g/kg	215	214	215	215
ME, kcal/kg	2938	2938	2938	2938
Ca, g/kg	7.9	8.0	8.0	8.0
P, g/kg	6.9	6.8	6.8	6.8
Available P, g/kg	4.3	4.3	4.3	4.3
Starch	412	414	415	411
Digestible amino acids, g/kg				
Arg	14	14	14	14
His	5.7	5.7	5.7	5.7
Ile	9.1	9.1	9.2	9.2
Leu	19	18	18	18
Lys	13	13	13	13
Met	4.8	4.8	4.8	4.8
Tyr	7.8	7.8	7.8	7.8
Thr	8.6	8.6	8.6	8.6
Met + Cys	8.4	8.3	8.3	8.3

[a.] Vitamin Premix: Supplemented per kg of diet: thiamin mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B12 (cobalamin), 12.0 g; pyridoxine HCl, 4.7 mg; D-biotin, 0.11 mg; folic acid, 5.5 mg; menadione sodium bisulfite complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 27.5 g; transretinyl acetaote, 1,892 g; α-tocopheryl acetate, 11 mg; ethoxyquin, 125 mg.

[b.] Mineral Premix: Supplemented as per kg of diet: manganese ($MnSO_4.H_2O$), 60 mg; iron ($FeSO_4.7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4.5H_2O$), 5 mg; iodine (ethylene diaminedihydroiodide), 0.15 mg; selenium ($NaSeO_3$), 0.3 mg.

Table 5.2. Ingredients and chemical analysis (g/kg) of the grower phase (d 10 to 28) diets.

	Grower diet (d 10 - 28)			
		Potato starch		Hi-Maize starch
Ingredients, g/kg	Control	25	50	50
Corn	680	642	605	598
Potato starch		25	50	
Hi-Maize starch				50
Soybean meal	290	297	304	303
Soybean oil	1.2	6.9	12.56	19.59
Dicalcium phosphate	7.5	7.5	7.4	7.5
Limestone	8.77	8.74	8.77	8.72
Sodium bicarbonate	1.5	1.5	1.5	1.5
Vitamin premix[a]	0.9	0.9	0.9	0.9
Trace minerals premix[b]	0.68	0.68	0.68	0.68
Methionine	1.58	1.6	1.61	1.59
Lysine	1.59	1.44	1.29	1.35
Threonine	0.4	0.4	0.4	0.4
Titanium dioxide (TiO2)	3	3	3	3
Salt NaCl	2.5	2.5	2.5	2.5
Choline	0.7	0.7	0.7	0.7
Phytase (Q blue)	0.1	0.1	0.1	0.1
Total	1000	1000	1000	1000
Calculated Nutrients & Energy				
Protein, g/kg	200	200	200	200
ME, kcal/kg	2988	2988	2988	2988
Ca, g/kg	6.3	6.3	6.3	6.4
P, g/kg	5.0	5.0	4.9	4.9
Available P, g/kg	2.5	2.5	2.5	2.5
Starch	439	440	441	437
Digestible amino acids, g/kg				
Arg	13	13	13	13
His	5.3	5.3	5.3	5.3
Ile	8.4	8.4	8.5	8.4
Leu	18	17	17	17
Lys	12	12	12	12
Met	4.7	4.7	4.7	4.7
Tyr	7.2	7.2	7.2	7.2
Val	9.4	9.4	9.4	9.4
TSAA	8.1	8.0	8.0	8.0
Analyzed nutrient composition, g/kg				
Protein, g/kg	219	211	213	218
GE, kcal/kg	4450	4412	4478	4503
Total starch	454	424	414	407
Resistant starch	12	28	45	24

[a.] Vitamin Premix: Supplemented per kg of diet: thiamin mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B12 (cobalamin), 12.0 g; pyridoxine HCl, 4.7 mg; D-biotin, 0.11 mg; folic acid, 5.5 mg; menadione sodium bisulfite complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 27.5 g; transretinyl acetate, 1,892 g; *α*-tocopheryl acetate, 11 mg; ethoxyquin, 125 mg.

[b.] Mineral Premix: Supplemented as per kg of diet: manganese ($MnSO_4.H_2O$), 60 mg; iron ($FeSO_4.7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4.5H_2O$), 5 mg; iodine (ethylene diaminedihydroiodide), 0.15 mg; selenium ($NaSeO_3$), 0.3 mg.

Table 5.3. Ingredients and chemical analysis (g/kg) of the grower phase (d 28 to 35) diets.

	Finisher diet (d 28 - 35)			
		Potato starch		Hi-Maize starch
Ingredients, g/kg	Control	25	50	50
Corn	707	670	632	626
Potato starch		25	50	
Hi-Maize starch				50
Soybean meal	266	273	280	279
Soybean oil	3	8.9	15	22
Dicalcium phosphate	5.66	5.81	6	6.2
Limestone	8.6	8.5	8.3	8.2
Sodium bicarbonate	2.0	2.0	2.0	2.0
Vitamin premix[a]	0.9	0.9	0.9	0.9
Trace minerals premix[b]	0.68	0.68	0.68	0.68
Methionine	1.37	1.41	1.38	1.4
Lysine	1.1	0.9	0.85	0.9
Salt NaCl	2.4	2.4	2.4	2.4
Choline	0.7	0.7	0.7	0.7
Phytase (Q blue)	0.1	0.1	0.1	0.1
Total	1000	1000	1000	1000
Calculated Nutrients & Energy				
Protein, g/kg	190	190	190	190
ME, kcal/kg	3050	3050	3050	3050
Ca, g/kg	5.8	5.7	5.7	5.8
P, g/kg	4.6	4.6	4.6	4.6
Available P, g/kg	2.2	2.2	2.2	2.2
Starch	455	457	458	454
Digestible amino acids, g/kg				
Arg	12	12	12	12
His	5.1	5.1	5.1	5.1
Ile	7.9	8.0	8.0	8.0
Leu	17	17	17	17
Lys	11	11	11	11
Met	4.4	4.4	4.4	4.4
Tyr	6.9	6.9	6.9	6.8
Thr	7.1	7.1	7.1	7.1
TSAA	7.6	7.6	7.5	7.5

[a.] Vitamin Premix: Supplemented per kg of diet: thiamin mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B12 (cobalamin), 12.0 g; pyridoxine HCl, 4.7 mg; D-biotin, 0.11 mg; folic acid, 5.5 mg; menadione sodium bisulfite complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 27.5 g; transretinyl acetate, 1,892 g; *α*-tocopheryl acetate, 11 mg; ethoxyquin, 125 mg.

[b.] Mineral Premix: Supplemented as per kg of diet: manganese ($MnSO_4.H_2O$), 60 mg; iron ($FeSO_4.7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4.5H_2O$), 5 mg; iodine (ethylene diaminedihydroiodide), 0.15 mg; selenium ($NaSeO_3$), 0.3 mg.

Table 5.4. List of primers and their functions.

Symbol	Gene name	Function	Forward primer	Reverse primer
β-actin	βeta-actin	Housekeeping gene	ACCGGACTGTTACCAACACC	GACTGCTGCTGACACCTTCA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping gene	TGTGACTTCAATGGTGACAGC	GCTATATCCAAACTCATTGTCATACC.
PepT1	Peptide transporter- 1	Peptide transporter	CCCCTGAGGAGGATCACTGTT	CAAAAGACCAGCAGCAAC. GA
y+LAT1	y+L amino acid transporter 1	Cationic amino acid transporter	CAGAAAACCTCAGAGCTCCCTTT	TGAGTACAGAGCCAGCGCAAT
OCLN	Occludin	Tight junction	CTGCTCTGCCTCATCTGCTTCTTC	CCATCCGCCACGTTCTTCACC
CLDN-1	Claudin-1	Tight junction	GGTGAAGAAGATGCGGATGG	CTGGTGTTAACGGGTGTGA
ZO-1	Zona occluden-1	Tight junction	GCCAACTGATGCTGAACCAA	GGGAGAGACAGGACAGGACT
ZO-2	Zona occluden-2	Tight junction	TCAGCAACAGCAAGGTGAAG	GCACCCATGGCAGTAAGGTA
JAM-2	Junctional adhesion molecule 2	Tight junction	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA
AnxA1	Annexin 1	Anti-inflammatory regulator	CTGCCTGACTGCCCTTGTGA	GTTTGTGTCGTGTTCCACTCCC.
IL-1β	interleukin-1-beta	Pro-inflammatory function and cellular defense	CGAGGAGCAGGGACTTTGC	GAAGGTGACGGGCTCAAAAA
TNF-α	Tumor necrosis factor alpha	Pro-inflammatory function	CCCATCCCTGGTCCGTAAC	ATACGAAGTAAAGGCCGTCCC
IL-10	Interleukin-10	Anti-inflammatory function and regulator of cell homeostasis.	CGCTGTCACCGCTTCTTCA	CGTCTCCTTGATCTGCTTGATG

Table 5.5. Growth performance of broiler chickens fed diets with graded levels of resistant starches in the starter phase (d 0-9) and d 4 - pre-infection.

	Starter phase (d 0-9)				d 4 - pre-infection			
Treatments	WG, g	FI, g	FCR	FBW, g	WG, g	FI, g	FCR	FBW, g
Control	160[b]	207	1.29	204[b]	159[b]	220	1.39[a]	361[b]
25g/kg RPS	175[a]	220	1.26	218[a]	170[ab]	239	1.41[a]	387[ab]
50g/kg RPS	171[ab]	216	1.27	215[ab]	167[ab]	233	1.40[a]	381[ab]
50g/kg HCS	173[ab]	214	1.24	217[ab]	181[a]	235	1.30[b]	397[a]
SEM	3.77	3.73	0.018	3.76	5.37	6.48	0.020	8.26
P-value	0.047	0.104	0.226	0.048	0.040	0.233	0.001	0.023

WG- body weight gain, FI- Feed Intake, FCR- feed conversion ratio, FBW: Final Body Weight.
n = 12 replicate pens per treatment with 22 birds per pen
[ab]: Means in a column with different superscripts differ significantly ($P \leq 0.05$).

Table 5.6. Growth performance of broiler chickens fed diets with graded levels of resistant starches during the prepatent (0 - 3 dpi) and acute (4 - 6 dpi) phases when challenged or unchallenged with mixed *Eimeria* spp. oocysts.

			Prepatent phase (d 13-16)				Acute phase (d 16-19)			
Items		Treatments	WG, g	FI, g	FCR	FBW, g	WG, g	FI, g	FCR	FBW, g
Diet × Infection						Simple effect means				
	Infected	Control (0)	176	260	1.48	543	89	234	2.62	621
		25 g/kg RPS	188	262	1.40	599	87	229	2.63	673
		50 g/kg RPS	186	260	1.40	581	75	216	2.88	656
		50 g/kg HCS	197	264	1.34	608	57	212	3.72	660
	Uninfected	Control (0)	169	242	1.44	523	222	323	1.45	746
		25 g/kg RPS	176	241	1.37	539	222	328	1.48	760
		50 g/kg RPS	174	250	1.44	541	223	322	1.44	765
		50 g/kg HCS	187	257	1.38	571	239	321	1.34	810
						Main effect means				
Diets		Control (0)	172[b]	251	1.46[a]	533[b]	155	279	2.04	693
		25 g/kg RPS	182[ab]	252	1.39[ab]	569[ab]	154	278	2.03	723
		50 g/kg RPS	180[ab]	255	1.42[ab]	561[ab]	149	269	2.16	711
		50 g/kg HCS	192[a]	261	1.36[b]	589[a]	148	267	2.53	735
						Main effect means				
Infection status		Infected	187	262	1.40	583	77	223	2.96	660
		Uninfected	176	248	1.41	544	226	323	1.43	770
						Pooled SEM				
		Diets	3.26	5.19	0.022	9.77	10.98	9.83	0.439	16.67
		Infection	2.30	3.67	0.016	6.91	7.77	6.96	0.311	12.50
		Diet × Infection	4.61	7.35	0.031	13.81	15.30	14.81	0.620	24.99
						Probabilities				
		Diets	0.001	0.528	0.015	0.003	0.911	0.692	0.384	0.454
		Infection	0.003	0.011	0.823	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
		Diet × Infection	0.946	0.744	0.583	0.569	0.302	0.850	0.279	0.363

Dpi- days post-inoculation

WG- body weight gain, FI- Feed Intake, FCR- feed conversion ratio, FBW: Final Body Weight.

n = 6 replicate pens per treatment for simple effects, n = 12 replicate pens per treatment for main effects of diets, n = 24 replicate pens per treatment for main effects of infection status, with 22 birds per pen.

[ab]: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$).

Table 5.7. Growth performance of broiler chickens fed diets with graded levels of resistant starches during the recovery (7 - 15 dpi) and compensatory (16 - 22 dpi) phases when challenged or unchallenged with mixed *Eimeria* spp. oocysts.

			Recovery phase (d 19-28)				Compensatory phase (d 28-35)			
Items		Treatments	WG, g	FI, g	FCR	FBW, g	WG, g	FI, g	FCR	FBW, g
Diet × Infection						Simple effect means				
	Infected	Control (0)	772	1180	1.55	1393	834	1403	1.71	2262
		25 g/kg RPS	807	1202	1.49	1479	872	1424	1.64	2392
		50 g/kg RPS	815	1238	1.55	1471	882	1464	1.68	2391
		50 g/kg HCS	814	1173	1.44	1465	828	1422	1.72	2331
	Uninfected	Control (0)	845	1340	1.59	1591	952	1449	1.52	2584
		25 g/kg RPS	863	1342	1.56	1623	956	1478	1.55	2618
		50 g/kg RPS	865	1369	1.62	1632	983	1491	1.53	2652
		50 g/kg HCS	906	1352	1.54	1715	882	1428	1.63	2629
						Main effect means				
Diets		Control (0)	808	1260	1.57	1492	893	1426	1.61	2423
		25 g/kg RPS	835	1272	1.52	1551	914	1451	1.60	2505
		50 g/kg RPS	840	1303	1.58	1552	933	1478	1.60	2521
		50 g/kg HCS	860	1262	1.49	1590	855	1425	1.68	2480
						Main effect means				
Infection status		Infected	802	1198	1.51	1452	854	1429	1.69	2344
		Uninfected	870	1351	1.58	1640	943	1462	1.56	2621
						Pooled SEM				
		Diets	28.82	21.98	0.063	32.77	27.72	23.20	0.039	44.76
		Infection	20.38	15.54	0.045	23.17	19.60	16.40	0.027	31.65
		Diet × Infection	40.76	31.08	0.089	46.34	39.20	32.80	0.055	63.30
						Probabilities				
		Diets	0.653	0.490	0.723	0.224	0.242	0.340	0.488	0.437
		Infection	0.023	< 0.001	0.295	< 0.001	0.003	0.163	0.002	< 0.001
		Diet × Infection	0.955	0.869	0.987	0.675	0.863	0.894	0.783	0.881

Dpi- days post-inoculation

WG- body weight gain, FI- Feed Intake, FCR- feed conversion ratio, FBW: Final Body Weight.

n = 6 replicate pens per treatment for simple effects, n = 12 replicate pens per treatment for main effects of diets, n = 24 replicate pens per treatment for main effects of infection status, with 22 birds per pen.

Table 5.8. Apparent ileal digestibility of indispensable amino acid in broiler chickens fed diets with graded levels of resistant starches at d 20 (during recovery phase) when challenged or unchallenged with mixed *Eimeria* spp. oocysts.

Items	Treatments	DMD	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Trp
Diet × Infection						Simple effect means						
Infected	Control (0)	66.3	83.5	75.5	72.5	74.6	75.6	81.7	74.6	65.4	70.3	82.6
	25g/kg RPS	67.4	85.5	77.3	74.8	76.3	77.8	82.7	76.6	67.7	72.5	84.2
	50g/kg RPS	64.2	84.4	76.1	73.4	75.4	76.0	80.9	75.6	64.9	71.0	84.0
	50g/kg HCS	63.1	83.4	74.8	72.8	74.4	76.7	81.6	75.1	66.3	70.3	84.8
Uninfected	Control (0)	65.5	82.3	75.3	72.9	73.1	77.0	82.5	74.8	65.4	71.0	78.3
	25g/kg RPS	67.1	84.9	77.8	75.9	76.2	79.2	84.0	77.7	67.3	73.8	79.2
	50g/kg RPS	66.7	84.3	77.4	75.3	75.6	78.5	83.4	77.0	66.5	73.2	78.1
	50g/kg HCS	68.2	86.4	80.2	78.3	78.6	82.7	87.7	79.8	71.4	76.6	83.2
						Main effect means						
Diets	Control (0)	65.9	84.9	75.4	72.7	73.9	76.3	82.1	74.7	65.4	70.7	80.4[b]
	25 g/kg RPS	67.3	86.3	77.6	75.3	76.3	78.5	83.3	77.1	67.5	73.2	81.7[ab]
	50 g/kg RPS	65.4	86.4	76.8	74.3	75.5	77.2	82.1	76.3	65.7	72.1	81.0[ab]
	50 g/kg HCS	65.7	86.8	77.5	75.6	76.5	79.7	84.7	77.4	68.9	73.5	84.0[a]
Infection status						Main effect means						
	Infected	65.3	84.2	75.9	73.4	75.2	76.5	81.7	75.5	66.1	71.1	83.9
	Uninfected	66.9	84.5	77.7	75.6	75.9	79.4	84.4	77.3	67.6	73.7	79.7
						Pooled SEM						
	Diets	0.979	0.676	0.842	0.947	0.922	1.003	0.567	0.849	1.214	1.022	0.793
	Infection	0.692	0.478	0.595	0.670	0.652	0.709	0.401	0.600	0.858	0.723	0.561
	Diet × Infection	1.385	0.956	1.191	1.339	1.304	1.42	0.802	1.2006	1.72	1.445	1.122
						Probabilities						
	Diets	0.546	0.110	0.247	0.146	0.195	0.101	0.101	0.1199	0.164	0.226	0.018
	Infection	0.104	0.696	0.046	0.025	0.454	0.008	0.002	0.034	0.207	0.016	< 0.001
	Diet × Infection	0.131	0.156	0.105	0.246	0.180	0.339	0.115	0.287	0.380	0.231	0.276

DMD- Dry matter digestibility.

n = 6 replicate pens per treatment for simple effects, n = 12 replicate pens per treatment for main effects of diets, n = 24 replicate pens per treatment for main effects of infection status, with 22 birds per pen.

[ab]: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$).

Table 5.9. Apparent ileal digestibility of dispensable amino acid in broiler chickens fed diets with graded levels of resistant starches on d 20 (during recovery phase) when challenged or unchallenged with mixed *Eimeria* spp. oocysts.

Items		Treatments	Ala	Asp	Cys	Gly	Glu	Pro	Ser	Tyr
Diet × Infection						Simple effect means				
	Infected	Control (0)	70.5	73.6	59.5	67.8	81.3	73.8	73.6	75.4
		25g/kg RPS	72.5	76.0	66.9	70.9	83.1	76.1	76.2	77.4
		50g/kg RPS	70.6	74.6	61.2	68.8	82.1	74.4	74.1	76.6
		50g/kg HCS	69.8	74.7	61.2	68.9	81.5	73.6	74.8	75.8
	Uninfected	Control (0)	71.7	73.4	54.5	66.4	79.8	71.4	71.9	75.0
		25g/kg RPS	74.7	76.5	61.8	70.1	82.7	75.0	74.6	77.4
		50g/kg RPS	73.7	75.5	57.7	68.7	82.0	73.7	73.4	77.1
		50g/kg HCS	77.2	78.0	64.3	73.0	84.3	76.4	77.4	79.0
						Main effect means				
Diets		Control (0)	71.1	73.5	57.01[b]	67.1	80.6	72.6	72.7	75.2
		25 g/kg RPS	73.6	76.3	64.37[a]	70.5	82.9	75.5	75.4	77.4
		50 g/kg RPS	72.1	75.1	59.45[ab]	68.8	82.0	74.0	73.8	76.8
		50 g/kg HCS	73.5	76.3	62.76[a]	70.9	82.9	75.0	76.1	77.4
Infection status						Main effect means				
		Infected	70.8	74.7	62.2	69.1	82.0	74.5	74.7	76.3
		Uninfected	74.3	75.9	59.6	69.5	82.2	74.1	74.3	77.1
						Pooled SEM				
		Diets	1.027	0.872	1.461	1.095	0.682	0.895	0.931	0.967
		Infection	0.726	0.616	1.033	0.774	0.482	0.633	0.658	0.684
		Diet × Infection	1.452	1.233	2.065	1.548	0.964	1.266	1.316	1.368
						Probabilities				
		Diets	0.263	0.090	0.005	0.073	0.069	0.128	0.061	0.348
		Infection	0.002	0.205	0.077	0.701	0.780	0.708	0.740	0.393
		Diet × Infection	0.177	0.531	0.175	0.289	0.169	0.206	0.334	0.582

n = 6 replicate pens per treatment for simple effects, n = 12 replicate pens per treatment for main effects of diets, n = 24 replicate pens per treatment for main effects of infection status, with 22 birds per pen.

[ab]: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$).

Table 5.10. Caecal short-chain fatty acids profile (mM) on d 20 in broiler chickens fed diets with graded levels of resistant starches when challenged or unchallenged with mixed *Eimeria* spp. oocysts.

Items	Treatments	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	SCFA	BCFA
Diet × Infection					Simple effect means				
Infected	Control (0)	62.8	11.8	1.2	17.6	1.8	1.7	93.9	3.0
	25 g/kg RPS	81.8	9.9	1.2	18.5	1.6	1.6	111.8	2.8
	50 g/kg RPS	68.9	9.3	1.3	17.7	1.8	1.6	97.6	3.2
	50 g/kg HCS	56.9	8.3	1.4	14.3	2.0	1.5	81.1	3.3
Uninfected	Control (0)	81.0	7.0	0.8	26.1	0.9	1.6	115.7	1.7
	25 g/kg RPS	81.3	8.2	0.9	26.6	1.1	1.7	117.9	2.0
	50 g/kg RPS	78.1	6.6	0.8	26.7	0.9	1.6	113.1	1.7
	50 g/kg HCS	75.6	5.8	0.6	27.6	0.7	1.3	110.3	1.3
					Main effect means				
Diets	Control (0)	71.9	9.4	1.0	21.9	1.4	1.6	104.8	2.4
	25 g/kg RPS	81.6	9.0	1.0	22.6	1.3	1.7	114.8	2.4
	50 g/kg RPS	73.5	8.0	1.1	22.2	1.4	1.6	105.4	2.4
	50 g/kg HCS	66.2	7.1	1.0	20.9	1.3	1.4	95.7	2.3
					Main effect means				
Infection status	Infected	67.6	9.8	1.3	17.0	1.8	1.6	96.1	3.1
	Uninfected	79.0	6.9	0.8	26.8	0.9	1.5	114.2	1.7
					Pooled SEM				
	Diets	6.74	1.05	0.107	2.58	0.162	0.158	9.27	0.262
	Infection	4.76	0.745	0.076	1.83	0.115	0.112	6.56	0.186
	Diet × Infection	9.52	1.49	0.152	3.65	0.229	0.224	13.11	0.371
					Probabilities				
	Diets	0.449	0.395	0.952	0.973	0.997	0.772	0.540	0.991
	Infection	0.098	0.008	< 0.001	0.001	< 0.001	0.615	0.058	< 0.001
	Diet × Infection	0.714	0.755	0.462	0.883	0.440	0.925	0.835	0.430

n = 6 replicate pens per treatment for simple effects, n = 12 replicate pens per treatment for main effects of diets, n = 24 replicate pens per treatment for main effects of infection status, with 22 birds per pen.

Table 5.11. Caecal short-chain fatty acids profile (mM) on d 35 in broiler chickens fed diets with graded levels of resistant starches when challenged or unchallenged with mixed *Eimeria* spp. oocysts.

Items	Treatments	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	SCFA	BCFA
Diet × Infection					Simple effect means				
Infected	Control (0)	54.3	5.22	0.592	8.14	0.517	0.646	68.3	1.11
	25 g/kg RPS	74.6	6.60	0.805	14.06	0.726	1.03	96.3	1.53
	50 g/kg RPS	56.0	4.56	0.881	12.31	0.874	0.974	73.9	1.76
	50 g/kg HCS	63.0	6.14	0.844	11.80	0.796	0.926	81.9	1.64
Uninfected	Control (0)	71.1	6.11	0.745	16.12	0.700	1.15	94.5	1.45
	25 g/kg RPS	66.2	7.20	0.808	14.13	0.732	1.05	88.6	1.54
	50 g/kg RPS	76.1	6.86	0.817	16.44	0.781	1.20	100.6	1.60
	50 g/kg HCS	66.1	6.54	0.756	14.72	0.678	1.03	88.4	1.43
					Main effect means				
Diets	Control (0)	62.7	5.67	0.669	12.13	0.609	0.897	81.4	1.28
	25 g/kg RPS	70.4	6.90	0.807	14.10	0.729	1.04	92.4	1.54
	50 g/kg RPS	66.1	5.71	0.849	14.38	0.828	1.09	87.3	1.68
	50 g/kg HCS	64.6	6.34	0.800	13.26	0.737	0.979	85.2	1.54
					Main effect means				
Infection status	Infected	62.0	5.63	0.781	11.58	0.728	0.894	80.1	1.51
	Uninfected	69.9	6.67	0.782	15.35	0.723	1.11	93.0	1.50
					Pooled SEM				
	Diets	4.64	0.663	0.083	1.53	0.082	0.084	8.14	0.164
	Infection	3.28	0.468	0.059	1.08	0.058	0.059	4.34	0.116
	Diet × Infection	6.56	0.937	0.117	2.17	0.116	0.118	8.68	0.231
					Probabilities				
	Diets	0.683	0.512	0.457	0.730	0.328	0.423	0.641	0.384
	Infection	0.096	0.124	0.988	0.018	0.936	0.015	0.042	0.978
	Diet × Infection	0.129	0.741	0.728	0.343	0.570	0.214	0.155	0.650

n = 6 replicate pens per treatment for simple effects, n = 12 replicate pens per treatment for main effects of diets, n = 24 replicate pens per treatment for main effects of infection status, with 22 birds per pen.

Table 5.12. Jejunal histomorphology on d 20 in broiler chickens fed diets with graded levels of resistant starches when challenged or unchallenged with mixed *Eimeria* spp. oocysts.

Items		Treatments	VH (µm)	CD (µm)	VW (µm)	VH/CD
Diet × Infection				Simple effect means		
	Infected	Control (0)	745	252	260	3.07
		25 g/kg RPS	779	267	225	3.16
		50 g/kg RPS	789	281	247	2.87
		50 g/kg HCS	770	285	263	2.73
	Uninfected	Control (0)	1215	217	242	5.76
		25 g/kg RPS	1379	203	236	6.82
		50 g/kg RPS	1304	241	249	5.55
		50 g/kg HCS	1509	218	255	6.92
				Main effect means		
Diets		Control (0)	980	234	251	4.41
		25 g/kg RPS	1079	235	230	4.99
		50 g/kg RPS	1046	261	248	4.21
		50 g/kg HCS	1140	251	259	4.83
				Main effect means		
Infection status		Infected	771	271	249	2.96
		Uninfected	1352	220	246	6.26
				Pooled SEM		
		Diets	61.36	14.62	11.24	0.304
		Infection	43.49	10.34	7.95	0.215
		Diet × Infection	84.71	20.67	15.89	0.430
				Probabilities		
		Diets	0.330	0.493	0.361	0.257
		Infection	< 0.001	0.001	0.791	< 0.001
		Diet × Infection	0.444	0.821	0.807	0.227

VH- Villi height. CD- Crypt depth, VW- Villi width.

n = 6 replicate pens per treatment for simple effects, n = 12 replicate pens per treatment for main effects of diets, n = 24 replicate pens per treatment for main effects of infection status, with 22 birds per pen.

Table 5.13. Relative mRNA expression of jejunal nutrient transporters, tight junction proteins and selected caecal tonsil cytokines on d 20 in broiler chickens fed diets with graded levels of resistant starches when challenged or unchallenged with mixed *Eimeria* spp. oocysts.

Items	Treatments	ZO-1	ZO-2	JAM-2	Claudin	Occludin	Pept1	LAT	TNFα	1L-10	IL-1β	AnxA1
Diet × Infection							Simple effect means					
Infected	Control (0)	1.34	0.39	0.45	0.92	1.34	0.92^{ab}	0.68	0.80	0.72	0.80	0.91^{bc}
	25 g/kg RPS	0.63	0.78	0.50	1.29	0.63	0.36^{bc}	0.60	1.13	1.02	0.85	1.03^{bc}
	50 g/kg RPS	0.35	0.98	0.14	0.21	0.35	0.57^{b}	1.42	1.53	0.76	0.88	0.72^{c}
	50 g/kg HCS	1.17	0.89	0.48	1.48	1.17	0.79^{ab}	0.27	1.94	0.32	1.07	0.94^{bc}
Uninfected	Control (0)	1.00	1.00	1.00	1.00	1.00	1.00^{ab}	1.00	1.00	1.00	1.00	1.00^{bc}
	25 g/kg RPS	1.60	0.33	0.96	0.80	1.60	1.57^{a}	0.87	1.72	0.68	1.35	1.99^{ab}
	50 g/kg RPS	1.54	0.63	1.14	1.35	1.54	0.84^{ab}	1.35	1.87	0.73	1.59	2.98^{a}
	50 g/kg HCS	1.83	0.50	1.44	1.02	1.83	1.14^{ab}	0.91	1.64	0.63	1.68	2.62^{a}
							Main effect means					
Diets	Control (0)	1.17	0.70	0.73	0.96	1.17	0.96	0.84	0.90^{b}	0.86^{a}	0.90	0.96
	25 g/kg RPS	1.11	0.56	0.73	1.04	1.11	0.97	0.74	1.43^{ab}	0.85^{a}	1.10	1.51
	50 g/kg RPS	0.94	0.80	0.64	0.78	0.94	0.71	1.38	1.70^{a}	0.75^{ab}	1.24	1.85
	50 g/kg HCS	1.50	0.69	0.96	1.25	1.50	0.96	0.59	1.79^{a}	0.47^{b}	1.37	1.78
Infection status							Main effect means					
	Infected	0.87	0.76	0.39	0.97	0.87	0.76	0.74	1.35	0.71	0.90	0.90
	Uninfected	1.49	0.62	1.14	1.04	1.49	0.99	1.03	1.56	0.76	1.40	2.15
							Pooled SEM					
	Diets	0.234	0.183	0.194	0.357	0.342	0.192	0.385	0.194	0.100	0.151	0.178
	Infection	0.167	0.130	0.137	0.252	0.243	0.135	0.277	0.137	0.071	0.108	0.126
	Diet × Infection	0.331	0.259	0.277	0.505	0.482	0.271	0.539	0.274	0.141	0.213	0.251
							Probabilities					
	Diets	0.647	0.832	0.735	0.814	0.698	0.309	0.808	0.014	0.035	0.149	0.006
	Infection	0.329	0.348	0.001	0.869	0.069	0.059	0.800	0.293	0.583	0.003	< 0.001
	Diet × Infection	0.845	0.150	0.668	0.295	0.410	0.054	0.905	0.417	0.082	0.711	0.001

n = 6 replicate pens per treatment for simple effects, n = 12 replicate pens per treatment for main effects of diets, n = 24 replicate pens per treatment for main effects of infection status, with 22 birds per pen.

^{abc}: Means in a column, within a group, with different superscripts differ significantly ($P \leq 0.05$). n = 6 replicate cages per treatment

CHAPTER 6

GENERAL CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

These studies provided valuable insights on the optimum RS for broiler chickens by exploring factors such as source, length of feeding and dietary quantity of RS for improved growth performance and gut homeostasis**.** The influence of optimal nutrition of resistant starch (RS) on intestinal integrity, immune response and growth performance during *Eimeria* challenge were also assessed. Short chain fatty acids generated from microbial fermentation of RS have potentials to modulate nutrient digestion, microbial ecology and immune response as reported in human, rodents, pigs and ducks.

Our central hypothesis was that resistant starch modulates nutrient digestion, as well as generate microbial metabolites in the hindgut inducing a shift in microbial community and thus contribute to modulation of intestinal health. The Type-2 RS used in this study differentially modulated the nutrient digestion and energy utilization in broiler chickens based on their botanical origins and dietary concentrations with consequences on the composition and structure of oligosaccharides entering the distal gastrointestinal tract. These have possible consequences on the extent of cecal fermentation and, subsequently, the total SCFA production as well as on individual SCFA profiles, each eliciting variable effects on GIT morphometrics and growth performance responses.

In broilers fed RS diets for 7, 14 and 21 days; and fed diets presented as mash during starter phase and pellets in the grower phase, the feeding length of RS had no significant effects on the growth performance or nutrient digestibility but influenced the FCR, AIDE, ME, and AMEn, with

35 g/kg HCS showing better efficiency than 25 g/kg RPS diets and greater AIDE, ME and AMEn in birds fed 35 g/kg HCS diets. The mRNA expression of genes was influenced by both feeding length and levels of dietary inclusion of RS, with differential expression of nutrient transporters such as *GLP-2 and* selected markers of gut integrity.

It was hypothesized that the RS sources and levels used differentially modulated broiler resistance to *Eimeria* challenge. The inclusion of 25 g/kg RPS and 50 g/kg HCS in broiler chickens' diets improved the growth performance during the starter phase and during 4d-pre-infection or prepatent phase of infection, respectively. *Eimeria* infection adversely affected the growth performance in the acute, recovery and compensatory phases. The AID of almost all the indispensable amino acids evaluated were depressed by *Eimeria* challenge as the demand for these amino acids are expected to be higher during enteric challenge due to increase in proliferation of crypts cell and additional requirements for immune response among other needs. However, the birds fed RS diets had greater AID of Trp and Cys, although the mechanism responsible for improved AID of these amino acids is yet to be fully elucidated.

Furthermore, the caecal BCFA was increased by infection, the propionate concentration was also elevated at d- 20 (7 dpi) which is an indication of increased microbial fermentation in the caeca and possible modulation of gut microbiota. In addition, the differential expression of immune regulatory proteins presented an additional pathway through which RS may regulate immune response. The summary of these experiments is presented in Figure 6.1. However, the microbiota result is underway and would shed more lights on the potential of these RS sources to induce a shift in the caecal microbial community in favor of the proliferation of beneficial bacteria that could possibly boost immune response during *Eimeria* challenge in broiler chickens.

These studies provided valuable insights on the optimal nutritional approaches to use with the selected type-2 RS sources to achieve beneficial digesta oligosaccharides profiles and established the possible prebiotic roles of these metabolic products and metabolites with their possible influence on growth, disease resistance, and tolerance responses. Due to increasing interest in gut health, this project provided relevant information to the industry in understanding the possible role of RS on intestinal health. However, the limitation of not having the same level of analyzed RS in all the diets using different RS sources is acknowledged. An attempt to achieve the same analyzed RS level in all diets will necessitate including some RS sources at impractically high levels. Nevertheless, the effect of increasing the levels of each RS source can be interpreted within its context in as much as increasing the inclusion levels of each RS source was accompanied by a corresponding increase of its analyzed level in the respective diets.

6.2 RECOMMENDATIONS

Future studies should investigate the impact of prolonged feeding of RS on broiler chickens and plausible effects of the feed milling process on the crystalline integrity of RS, resistance to pancreatic amylase, and the efficiency of microbial fermentation of RS when animals are fed pelletized diets. Additional studies should be conducted to fully elucidate the effects of RS on protein and amino acids metabolism as well as their detailed role in immune function. These would clearly establish the mode of action of RS during inflammation or enteric challenge in broiler chickens. Studies focusing on how to harness SCFA from microbial fermentation of RS for improved bone health during inflammatory responses in chickens should be extensively evaluated.

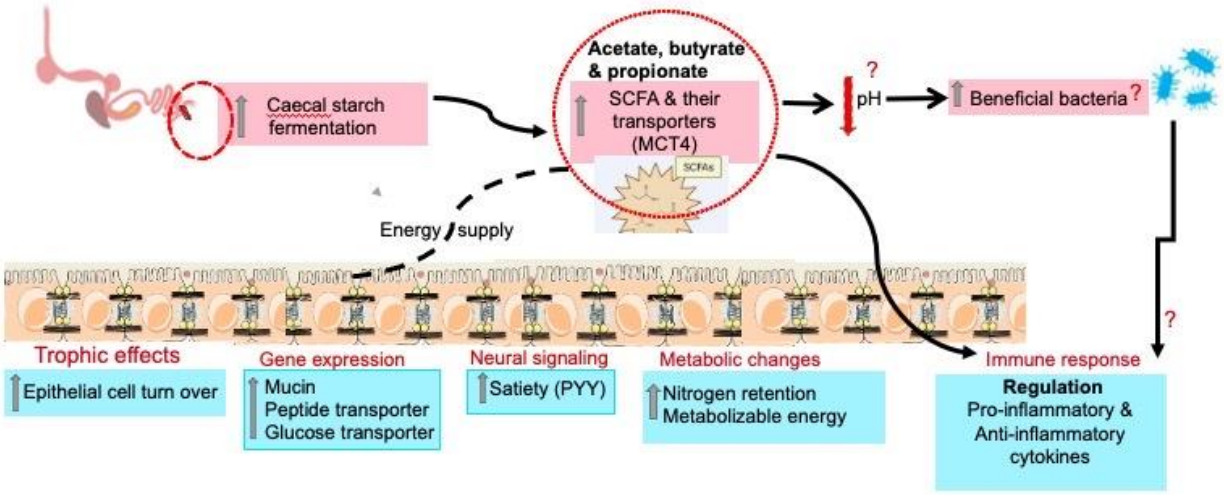

Figure 6.1: The summary of the experiments. Dietary RS sources and levels differentially modulated SCFA production without necessarily reducing the intestinal digesta pH and increased the epithelial cell turn over but leads to higher jejunal crypt proliferation. Mucin, peptide tyrosine tyrosine (PYY), peptide and glucose transporters were selectively expressed based on RS sources and levels. Dietary RS also differentially increased nitrogen retention, metabolizable energy and modulated immune response in broilers chickens. The microbiota analysis will clarify the potential of these RS sources to shift the cecal microbial community to promote beneficial bacteria that may enhance immune response during *Eimeria* challenge in broilers