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Ganapathi Raj Murugesan
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Characterization of the effects of intestinal physiology modified by exogenous enzymes and direct-fed microbial on intestinal integrity, energy metabolism, body composition and performance of laying hens and broiler chickens

by

Ganapathi Raj Murugesan

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Major: Nutritional Sciences

Program of Study Committee:
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Iowa State University
Ames, Iowa
2013

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DEDICATION

To my understanding and loving wife Sumi, who has put up with the requirements of a graduate student’s life and supported me each step of the way. To my steadfast father Murugesan Ramasamy for being the authentic inspiration, who taught me the meaning of life by his example. To my wonderful mother Annapoorani Murugesan for just being the most caring mother. And finally to my niece Anmitha who refreshed my days with her magnificent smile and taught me how to be always happily engaged for no reason.
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ABSTRACT

The objective of this dissertation research was to characterize the effects of intestinal physiology modulated by dietary supplementation of EE and DFM on intestinal integrity, energy metabolism, body composition and performance of laying hens and broiler chickens. The effects of reduced dietary metabolizable energy (ME) on performance, intestinal integrity and body composition of laying hens were evaluated in two experiments. A reduction of 90 kcal/kg of dietary ME over a period of 12 weeks did not significantly alter performance or nitrogen corrected apparent ME (AMEn), but reduced abdominal fat pad. In the follow-up experiment, no significant differences were noted in the performance of laying hens when fed a 100 kcal/kg reduced ME diet supplemented with EE or EE and DFM for 16 weeks. However, EE significantly increased AMEn and nutrient transport, whereas DFM increased intestinal epithelial barrier function, mucin expression and reduced zoonotic pathogen colonization. These data indicate that modern laying hens have little or no sensitivity to small differences in dietary ME, as they prioritize to meet the energy demands of production. The effects of DFM on performance and intestinal integrity of broiler chickens exposed to coccidial challenge or raised on built-up litter were evaluated. Ileal nutrient transport, mucin expression and intestinal epithelial barrier function were increased by DFM, although the performance was not altered. The effects of EE and DFM on dietary energy utilization in broilers were determined in two experiments. Both additives increased apparent ileal starch, amino acid and crude protein digestibility non-additively, while the combination additively increased AMEn. The effects of EE were exerted through increased activity of brush border digestive enzymes, and DFM through increased cecal butyrate synthesis. The final experiment examined the effects of EE and DFM on performance, energy utilization, and body composition
in broiler chickens. The combination of EE and DFM additively increased feed efficiency and positive energy balance of chicks. Changes in body composition indicated that the combination tends to increase lean accretion during early stages of life overcoming the immaturity of digestive tract. Overall, the combination of EE and DFM increased broiler performance additively through pathways independent of each other.
CHAPTER 1

INTRODUCTION

Energy has been an expensive component in poultry feed and will continue to drive feed costs as the global consumption of energy increases. Grains that were once reserved for animal production are being directed towards bio-fuels, driven by high crude oil prices and increasingly strict environmental legislations (USDA, 2011). The new reality of energy costs necessitates efficient energy utilization in animal production. The paradigm shift from farming for food energy production to fuel energy production is a great opportunity to increase the production efficiency of poultry using technology advancements including feed additives. Exogenous enzymes (EE) supplementation in poultry diets has been shown to increase energy and nutrient availability. This increased energy availability allows for the reduced inclusion of high cost dietary energy ingredients without negative consequences on bird performance. Numerous reports have validated the supplementation of EE to improve energy utilization and performance in chickens (Cowan et al., 1996; Fuente et al., 1998; Sakomura et al., 1999; Rutherfurd et al., 2007; Yu et al., 2007; Wang et al., 2009).

Another concern for poultry producers is the energetic cost involved in maintaining healthy flocks as sub-clinical pathogenic challenges, which are common in modern poultry production, can increase the cost of production (Klasing, 2007). Sub-clinical pathogenic challenges increase energy expenditure due to perpetual activation of acute phase immune response (Applegate et al., 2010). Productivity is depressed due to channeling of energy and nutrients to immune responses at the expense of productivity. This cost of immune activation has been estimated to be 1.3 times
that of maintenance energy requirement (Webel et al., 1998). Evaluation of methods such as using dietary additives to increase intestinal integrity can increase the energetic efficiency of the birds under sub-clinical pathogenic challenges. Supplementation of direct-fed microbial (DFM) has been used as a viable option to increase intestinal integrity (Applegate et al., 2010). A growing body of scientific research has extensively demonstrated the beneficial effects of DFM which include i) performance, ii) nutrient digestibility, iii) modulation of intestinal microflora, iv) pathogen inhibition, and v) immunomodulation and gut mucosal immunity (Mountzouris et al., 2010).

One important aspect that needs to be researched is the effects of both EE and DFM on the energy metabolism and energetic balance of poultry species. This combination is of interest because EE are known to hydrolyze non-starch polysaccharides, which are inaccessible to endogenous digestive enzymes and possibly providing prebiotic substrate for beneficial DFM in the hind gut. This combined effect may result into increased utilization of dietary energy, apart from the energy savings contributed by DFM through increased intestinal integrity. The hypothesis for this dissertation research was that supplementation of EE and DFM to laying hens and broiler chickens fed corn-soybean meal based diets will improve intestinal integrity and increase dietary energy release. The effective increase in energy availability as well as efficiency in energy utilization will be expressed in additive performance and altered body composition. The objective of the dissertation research was to characterize the effects of intestinal physiology modulated by dietary supplementation of EE and DFM on intestinal integrity, energy metabolism, body composition and performance of laying hens and broiler chickens.
REFERENCES


CHAPTER 2

LITERATURE REVIEW

A paper for submission to World’s Poultry Science Journal

Energy Metabolism

NUTRIENT DIGESTIBILITY

The objectives of poultry feeding are to satisfy the nutrient requirements of birds for specific production targets and to manage the feed ingredients in a sustainable and economic manner. From an economic and ecological perspective, the need to improve dietary energy and nutrient utilization are becoming increasingly important. Careful attention to digestibility of nutrients and exploring the means to improve the nutrient retention will result in increased energy utilization and productivity of birds. Application of feed additives such as EE and DFM has been explored to increase the nutrient digestibility and utilization in poultry. Changes in apparent ileal digestibility (AID) of crude protein (CP) and amino acids (AA) can be mediated through improved digestion and absorption of dietary AA as well as reduced loss of AA-rich endogenous proteins, resulting in increased energy utilization (Cowieson and Ravindran, 2008).

During the process of digestion there is a net movement of nutrients from the intestinal lumen into the circulation. However, there is a simultaneous movement of compounds including mucins, endogenous enzymes, bile, electrolytes, bicarbonate, sloughed cells and other nitrogenous compounds such as uric acid from gut-associated tissues into the lumen (Fuller and Reeds, 1998). Although these endogenous secretions are necessary for efficient digestion, they represent an energetic cost. For example, increased release of trypsinogen is associated with the presence of
trypsin inhibitors (Clarke and Wiseman, 2005). Presence of anti-nutritional factors such as non-starch polysaccharides (NSP) (Angkanaporn et al., 1994; Cowieson and Ravindran, 2008) and phytate (Cowieson and Ravindran, 2007) in the diet leads to excess release of mucoproteins. Endogenous secretions are primarily made up of AA, for example Thr, Ser, Asp, Glu and Gly make up more than 55% of the mucoproteins (Lien et al., 1997). Thus anti- or pro-nutrients that influence endogenous secretion and loss will have more profound effect on the component AA digestion and absorption (Lien et al., 1997; Piel et al., 2004).

DIGESTIVE ENZYME ACTIVITY

Endogenous digestive enzymes include pancreatic enzymes such as α-amylase, protease and lipase, and brush border enzymes such as maltase, sucrase-isomaltase complex and aminopeptidase. Complex polysaccharides are broken down to di- and mono-saccharides by α-amylase, while peptides are hydrolyzed to AA, di- and tri-peptides by protease, and triglycerides into fatty acids, esters and phospholipids by lipase in the lumen of intestinal tract (Lanham-New et al., 2011). Disaccharides reaching the microvillus are further degraded by disaccharidases (maltase and sucrase-isomaltase complex), while the di- and tri-peptides are degraded by aminopeptidase (Maroux et al., 1973; Galand, 1989). Disaccharidase activity is lowest in the duodenum and highest in jejunal and ileal segments. In chickens, increased activities of sucrase and maltase were observed in the upper half of villus, whereas reduced activities in the lower half of villus and in the crypt (Uni et al., 1998b). The gastrointestinal tract (GIT) modulates endogenous digestive enzyme activity in response to physiological needs, primarily with increased luminal polysaccharide and AA load rather than constantly maintaining high digestive enzyme activity (Pinheiro et al., 2004). This change is executed by increased variations in the type of epithelial
cells, primarily through increased proportion of mature enterocytes, along the crypt-villus axis to increase the release of digestive enzymes (Uni et al., 1998a; Chotinsky et al., 2001).

NUTIRENT ABSORPTION

Intestinal active nutrient transport is the process of transferring nutrients from the lumen through the apical membrane into circulation via the basolateral membrane. The epithelium tightly regulates nutrient transport by employing electrophysiological processes against electrical and concentration gradients (Ray et al., 2002). Previous research, using modified Ussing chambers, were able to determine the nutrient transport across epithelium. Typically, the epithelium pumps ions from apical to basolateral side, but the ions leak back through the tight junctions (TJ) creating a potential voltage difference across the epithelium (Ray et al., 2002). This voltage difference is zeroed out by injecting external current using modified Ussing chambers and the amount of short-circuit current (Isc) injected is the exact measure of net ion transport across the epithelium (Albin et al., 2007; Awad et al., 2007). Substrate concentrations at the apical membrane plays a primary role in regulating the absorption (Pinheiro et al., 2004). Increased glucose concentration at the apical membrane has been observed to increase the Isc by 20 to 60% in small intestine (Amat et al., 1999). When glucose is actively transported, it is coupled with Na\(^+\) in a fixed molar ratio and activates the apical Na\(^+-\)H\(^+\) exchange resulting in increased Na\(^+\)-glucose co-transport (Turner and Black, 2001). This increase in Na\(^+-\)H\(^+\) exchange also contributes to increased absorption of other nutrients, including AA and water (Amat et al., 1999; Awad et al., 2007). Furthermore, active Na\(^+\)-glucose co-transport has also been found to increase the passive transport rate of nutrients by increasing the number of small pores in villus (Fihn et al., 2000). Stressors such as pathogenic, heat or starvation play a major role in nutrient transport as increased intestinal stress decreased the
transport of nutrients in mammals (Gardiner et al., 1995; Albin et al., 2007) and laying hens (Awad et al., 2007).

FERMENTATION AND SHORT-CHAIN FATTY ACIDS

The major fermentation products of gut microbes are short-chain fatty acids (SCFA) such as acetate, propionate and butyrate, which can be used to indirectly monitor the microbial population and composition in the hind gut (Taylor, 2002). Although the growth of germ-free chicks on a nutrient adequate diet is often faster than that of conventional chicks (Muramatsu et al., 1994), the bird’s ability to extract energy from a diet is compromised in the germ-free state (Muramatsu et al., 1991). This is presumably due to the ability of GIT microbes fermenting dietary NSP, which the host is unable to digest, and producing byproducts such as SCFA that have energy value for the host. There are some primitive correlations observed between the type of carbohydrates and their fermentation products. For example, fermentation of soluble pectins produce approximately 80% acetate and only a small amount of butyrate, whereas starch and brans produce approximately 20% butyrate with relatively less acetate (Cummings and Macfarlane, 1997). Although numerous other roles are also suggested for SCFA in chickens (Cherbut, 2003), their direct relevance to host nutrition is energy contribution. In poultry, SCFA are absorbed effectively and the net efficiency of dietary energy utilization via hind gut fermentation is estimated to be 65% and 50% of that of glucose absorbed in adult cockerels and broiler chickens, respectively (Carré et al., 1995). Moreover, SCFA are believed to enhance the absorption of $\text{Na}^+$, stimulate the blood flow and regulate the nutrient absorption (Sakata, 1987).

BODY COMPOSITION
The efficiency of energy conversion of carbohydrates to lipids is approximately 80% in poultry, and fat pad is the key indicator of retained excess energy (Nir et al., 1978). In chickens, carbohydrates primarily execute their lipogenic effect through triiodothyronine (T3) due to the ineffective action of insulin in the absence of GLUT4 (Braun and Sweazea, 2008). Increased concentrations of circulating glucose enhance the effects of T3 on lipogenic enzymes by increasing the activities of malic enzyme and fatty acid synthase, independent of insulin (Mariash et al., 1980). This suggests that carbohydrates play a primary role as regulatory signals for lipogenic enzymes and that T3 amplifies and glucagon attenuates this signal (Hillgartner et al., 1995).

Glucose-6-phosphate is the glucose-signaling metabolite that is essential for the activation of carbohydrate response element binding protein, which plays a critical role in converting excess glucose into triglycerides through de novo lipogenesis (Dentin et al., 2012). Published studies have also reported that birds fed diets high in carbohydrates tend to increase energy intake (Newcombe and March, 1988) and have been shown to increase hepatic lipogenesis (Madappally et al., 1971; Hillard et al., 1980).

Feed restriction leads to reductions in circulating concentrations of T3 and insulin-like growth factor-1 in domestic fowl, which tends to change the body composition, primarily at the cost of fat storage (Cunningham and Morrison, 1977). Growing birds have higher ME requirement for maintenance due to the high-energy expenditure involved in protein synthesis. But the increase in fat deposition in mature birds reduces maintenance ME, as the metabolic ratio in fat tends to be lower than in other tissues (Blaxter, 1989). The energy utilization beyond maintenance depends on its partition into protein and lipid synthesis and their respective efficiencies (Sakomura, 2004). However, laying hens utilize energy continuously rather than storage, owing to the demands of egg production, unlike pullets and broilers (Husbands, 1972). The relationship between feed intake
and the energy demands of egg production is finely controlled such that the body weight is balanced through different stages of egg production (Macleod et al., 1979). Nevertheless, the energy requirement to produce one g of egg mass was lower for hens with low body weight compared to hens with medium or high body weight regardless of the basis of selection (Harms et al., 2000).

**Integrity of gastrointestinal tract**

The GIT consists of several anatomically and functionally distinct regions such as duodenum, jejunum, ileum, ceca, and colon. A well-functioning GIT has to allow transport of nutrients from the lumen into systemic circulation, while increasing the resistance to the translocation of pathogens. The GIT plays a significant role in saving host energy, serving as a protective barrier shielding the body from organisms and substances that do not serve as nutrients. The major barriers of the intestinal tract are the TJ and mucus layer on the epithelium.

**EPITHELIUM**

The GIT of chickens undergoes morphologic and physiologic changes immediately after hatching, increasing the surface area for digestion and absorption which is an essential step for the chicks to fully express their genetic potential for growth (Nitsan, 1995). Intestinal development is related to nutrient intake, which increases intestinal diameter and consequently intestinal weight relative to body weight (Gomide et al., 2004). The high plasticity of the intestinal mucosa is a special feature that allows for the response to exogenous agents, such as presence or absence of food and pathologic situations. Such responses consist of changes in villus height (VH), crypt depth (CD), villus density and rate of epithelial turnover (Gomide et al., 2004). Stressors in the
diet such as pathogens or mycotoxins can lead to relatively quick changes in the intestinal mucosa due to close proximity of mucosal surface and digesta, thus changes in the structure of intestinal mucosa can be used to assess potential changes due to dietary treatments (Xu et al., 2003). Crypts are considered as villus factories as they contain stem cells, hence deeper crypts indicate faster tissue turnover and higher demand for new tissue (Yason et al., 1987; Awad et al., 2009). Increased tissue turnover to permit the renewal of villi and crypts is required in response to inflammation from pathogens or their toxins (Yason et al., 1987). Reduced VH and increased CD (reduced VH:CD ratio) lead to increased endogenous secretion and reduced nutrient absorption, disease resistance and performance (Xu et al., 2003). In contrast, increased VH:CD ratio is directly correlated with increased epithelial cell turnover, although it leads to increased nutrient absorption and performance (Gomide et al., 2004).

TIGHT JUNCTIONS

Tight junctions are a specialized complex consisting of gap junctions, desmosomes, adherence junctions and zonula occludens, which join individual intestinal epithelial and endothelial cells to each other (Denker and Nigam, 1998). These TJ complexes act as major paracellular barriers that allow for the generation and maintenance of compartments with different compositions, a fundamental requirement for the physiological functioning of epithelium (Balda and Matter, 1998). They function as a “fence” separating apical and basolateral domains (Anderson and van Itallie, 1995). The trans-epithelial electrical resistance (TER) is the ability of TJ to differentially restrict free passage of water, ions, and larger solutes based on size and charge (Shen et al., 2011). However, TJ do not form absolute diffusion barriers, but are semipermeable to allow the selective passage of certain solutes (Anderson and van Itallie, 1995). By increasing
the TER, TJ maintain and increase barrier integrity (Shen et al., 2011). Increased epithelial barrier function helps to reduce the energy spent from activating and maintaining acute phase immune response and can improve productivity (Klasing and Johnstone, 1991). Leaky epithelia are a condition when ion conductance across the TJ is far greater than across apical and basolateral membranes leading to leaky TJ complexes (Albin et al., 2007).

Lipopolysaccharides (LPS) are cell wall components of gram-negative bacteria, which play a major role in establishing a disease condition through epithelial invasion. Both hematopoietic and non-hematopoietic cells are activated by LPS via CD14 directly mediated by Toll-like receptor 4 signaling (Poltorak et al., 1998). These cells in turn produce several pro- and anti-inflammatory hormones and cytokines that regulate different metabolic responses and cause behavioral changes, inflammation, sepsis and final recovery (Gardiner et al., 1995). The negative effects of LPS have been well characterized in chickens (Koh et al., 1996; Xie et al., 2000), and have been observed to reduce nutrient absorption and increase energy expenditure through increased immune challenge (Gardiner et al., 1995). Animals typically respond to wide range of stressors such as LPS by increasing intestinal permeability and conductance and hence, decreasing TER (Albin et al., 2007). Compromised barrier function by LPS results in further translocation of pathogens across the epithelia (Tomita et al., 2004). As TER is reduced due to compromised TJ, it is a good indicator of TJ permeability and epithelial barrier function (Shen et al., 2011). The TER is typically determined using modified Ussing chambers by measuring the electrical potential in ohms per cm² of the intestinal tissue (Albin et al., 2007).
Intestinal mucins are high-molecular-weight glycoproteins, secreted by goblet cells, characterized by a high content of carbohydrates and O-glycosidic bonds between N-acetylgalactosamine and either Ser or Thr in the peptide backbone (Mack et al., 1999). The overlying mucus-gel layer acts as the first line of defense against pathogens, while providing a viscous matrix to trap foreign antigens (Forstner and Forstner, 1994). An optimal protection against bacterial infection requires an intact mucus layer at the surface, dependent on both its quantitative (thickness) and qualitative (ability to fix bacteria) parameters (Smirnov et al., 2005). Binding to epithelial cells is the primary step for many enteric pathogens to affect the secretion and translocation of net fluid and electrolytes, therefore the interruption of entero-pathogenic adherence could provide therapeutic benefit to the host (Mack et al., 1999). Mucins prevent bacterial translocation by virtue of their negatively charged filamentous protruding structure (Smirnov et al., 2005). On the basis of AA sequences, mucins are categorized into three distinct families in humans: gel-forming (MUC2, MUC5AC, MUC5B and MUC6), soluble (MUC7), and membrane-bound (MUC1, MUC3, MUC4 and MUC12) (Moniaux et al., 2001). In chickens, MUC2 mucins are observed to be widely expressed in goblet cells of the small intestine and colon (Smirnov et al., 2005).

GUT MICROBIOTA

The GIT microflora is characterized as a mixture of bacteria, fungi and protozoa, with bacteria as the predominant microorganisms. Because bacterial species have different substrate preferences and growth requirements, the chemical composition of the digesta can alter the compositions of the microbial community (Apajalahti, 2005). There is also significant diversity in bacterial populations among segments of the GIT and the microbial population density tends to
increase from proximal to distal GIT (Apajalahti, 2005). Each region of the GIT develops its unique microbial profile, and this community becomes more complex as chickens age (Gong et al., 2002). Apajalahti et al. (2005) have shown that a day after hatch, bacterial densities in the ileum and ceca of broiler chicks reach $10^8$ and $10^{10}$ cells per g of digesta, respectively. The numbers of microbes reach $10^9$ per g of ileal digesta and $10^{11}$ per g of cecal digesta by d 3 post-hatch and remain relatively stable for the following 30-d.

Growing evidence suggests that the existence of commensal enteric flora can stimulate the maturity of the neonatal immune system (Umesaki and Setoyama, 2000). Germ-free animals have a large number of immune deficiencies, higher susceptibility to infection, and decreased bacterial clearance compared to conventional animals (Zachar and Savage, 1979). It has also been observed that germfree animals have relatively poor IgA production and weak systemic antibody responses compared to conventional animals, and the responses form following the introduction of gut microbes (Umesaki and Setoyama, 2000). This indicates the existence of dynamic mechanisms through which intestinal microbiota communicate among themselves as well as with the host immune system.

PATHOGENS AND DISEASE

The GIT provides mechanisms by which the body derives nutrients from its environment while attempting to safeguard the bird through protective mechanisms. The etiology of an enteric disease is complex as it may involve the combinations of virus, bacteria and other infectious agents. Moreover, physical, chemical or biological disturbances of digestion and absorption can result in enteric disease (Dekich, 1998). Coccidiosis is an intestinal specific disease caused by *Eimeria spp.* which compromises intestinal integrity by destructing the mucosa and interrupting
nutrient digestibility and absorption (Persia et al., 2006). Moreover, coccidiosis increases the susceptibility to secondary bacterial infections, resulting in reduced broiler performance (Dalloul et al., 2003). The mucosal damage caused by the onset of sub-clinical coccidiosis facilitates the establishment and multiplication of *Clostridium perfringens*, which is a part of the normal gut microbiota of poultry (Al-Sheikhly and Truscott, 1977; Al-Sheikhly and Al-Saieg, 1980). Toxins produced by *C. perfringens* are responsible for intestinal mucosal necrosis, and the characteristic lesion of necrotic enteritis (Al-Sheikhly and Truscott, 1977). Furthermore, *C. perfringens* initiates secondary bacterial infections and plays a vital role in increasing the severity of poor performance (Collier et al., 2008).

At the same time, poultry remains an important zoonotic vehicle of bacterial human pathogens, leading to foodborne diseases through the consumption of contaminated poultry products. Over the last three decades, *Campylobacter spp.* have emerged as a worldwide concern (Vandeplas et al., 2008), since they are the major causative factor of the acute bacterial enteritis known as campylobacteriosis in humans (Van Vliet and Ketley, 2001). *Campylobacter spp.* associated with food poisoning include *C. jejuni* which is predominant, and *C. coli* and *C. lari* which account for remainder of the cases (Hariharan et al., 2004). *C. jejuni* colonizes mucus overlying the epithelial cells, primarily in ceca and small intestine (Vandeplas et al., 2008), and has been observed to reduce body weight gain in broiler chicks (Morishita et al., 1997).

**Exogenous Enzymes**
NEED FOR THE USE OF EXOGENOUS ENZYMES

Starch is the major carbohydrate in corn-soybean meal (SBM)-dried distillers grains with solubles (DDGS)-based poultry diets, although NSP are also prominent. Corn contains 70% starch and sugars, 8% protein and 10% highly insoluble NSP, primarily composed of arabinose, xylose and cellulose (Knudsen, 1997; Choct, 2006). The composition of SBM is approximately 15% starch and sugars, 45% protein and 22% highly insoluble NSP, composed of arabinose, xylose, mannose, galactose, and cellulose (Knudsen, 1997; Choct, 2006). Corn derived DDGS contain lower starch (5%), but higher protein (32%) and insoluble NSP (23%), although the composition of NSP remains the same as corn (Parsippany, 2008). Insoluble NSP are relatively resistant to digestion, thus it is of relevance to consider exogenous enzyme (EE) strategies to hydrolyze NSP and maximize nutrient utilization in corn-SBM-DDGS-based diets. Supplementations of EE in wheat or barley or rye based poultry diets, which are rich in soluble NSP, have been done successfully over the last two decades with increased performance of both broilers and laying hens (Brenes et al., 1993; Pan et al., 1998; Oloffs et al., 2001; Jozefiak et al., 2007; Pirgozliev et al., 2010). Soluble NSP increase the viscosity of digesta and thus interfere in the digestion and absorption processes, which is overcome by the addition of EE. However the proportion of soluble NSP in corn-SBM-DDGS-based diets is relatively low compared to wheat or barley or rye as corn, SBM and DDGS have high concentration of insoluble NSP (Knudsen, 1997), leading to low viscosity of digesta. With the absence of this opportunity in corn-SBM-DDGS-based diets, one apparent approach is to add EE that could increase the substrate availability by addressing wider spectrum of substrates as well as complement the endogenous digestive enzymes (Cowieson et al., 2010).
As digesta passes through the small intestine, there is a progressive decline in digestive enzyme activity as they are either catabolized (Noy and Sklan, 1995), or anti-nutritional substances such as NSP and phytate bind to reduce their effectiveness (Liebert et al., 1996). Limitations in endogenous secretion of NSP-degrading enzymes have been implicated in reduced nutrient utilization in the small intestine (Angkanaporn et al., 1994). Physical access of endogenous digestive enzymes to nutrients bound in cell wall matrix rich in NSP is another factor limiting energy and nutrient digestibility (Bedford, 1996). The aleurone fraction of grain cell wall is known to contain relatively high quality protein, rich in Lys and Arg. Phytin, also abundant in grains, has been associated with the reduced solubility of aleurone associated proteins (Fulcher, 1986). Increased Lys and Arg digestibility by xylanase or phytase addition has been linked to efficient breakdown of aleurone associated proteins (Cowieson and Bedford, 2009). The beneficial effects of EE on complementing and increasing endogenous digestive enzyme activity have been extensively demonstrated (Bedford, 1996). In addition, hindgut microbes are limited in capability to ferment large NSP molecules that escape enzymatic digestion in the small intestine, resulting in a loss of dietary energy (Chocet et al., 1996). Supplemental EE can partially degrade these larger molecular NSP into smaller oligosaccharides resulting in increased synthesis of SCFA in the ceca (Chocet et al., 1996). Wang et al., (2005) observed linear increase in cecal SCFA synthesis with the addition of EE in broiler chickens.

EXOGENOUS AMYLASE, XYLANASE AND PROTEASE

Among EE combinations, amylase, xylanase and protease are commonly used to improve starch, NSP and protein digestion in non-ruminant animals fed corn-SBM based diets (Burnett, 1996). The principal exogenous amylase used in animal feed is synthesized from Bacillus
*amyloliquefaciens* (Bedford and Partridge, 2010). The primary amylose hydrolysis products by exogenous α-amylase are malto-triose (DP 3) and malto-hexaose (DP 6) compared to glucose to malto-tetraose (DP 1–4) as well as α-limit dextrins with one or two α-1-6 linkages produced by pancreatic α-amylase (Robyt, 2009). Exogenous amylase has a higher tendency to break the inner chain bonds of amylpectin and is faster in fragmenting amylpectin to lower molecular sizes in comparison to pancreatic α-amylase (Goesaert *et al.*, 2010). Exogenous amylase added to pancreatic α-amylase increases the rate of amylpectin and amylose breakdown (Bedford and Partridge, 2010). The use of exogenous amylase significantly reduced pancreatic mass without influencing the weight of other organs, indicating that exogenous amylase may lower pancreatic amylase production (Gracia *et al.*, 2003).

Xylanases in commercial preparations are derived from both bacterial and fungal sources such as *Bacillus subtilis*, *Trichoderma reesei*, etc. (Bedford and Partridge, 2010). Xylanase (endo-1, 4-β-xylanase) cleaves the arabinoxylan backbone randomly, resulting in non-substituted or branched xylo-oligosaccharides (Collins *et al.*, 2006). The xylo-oligosaccharides formed during degradation of xylans can be hydrolyzed by beneficial bacteria such as *Bifidobacterium* spp., and *Lactobacillus* spp., resulting in an increase in their population and a decrease in pathogenic bacteria such as *C. perfringens* (Thammarutwasik *et al.*, 2009). Xylanases may play a role in determining gut flora populations as a result of the identity of the dominant oligomers produced.

A number of potential modes of action have been suggested to explain the beneficial effects of proteases in poultry diets. Exogenous proteases may augment endogenous peptidase by improving protein digestibility. Additionally, they denature proteinaceous anti-nutrients such as lectins or trypsin inhibitors and hydrolyze antigenic proteins in protein sources such as SBM (Douglas *et al.*, 2000; Ghazi *et al.*, 2002). These researchers demonstrated reduced endogenous
loss and increased nutritional value of SBM for poultry with the addition of exogenous protease. Furthermore, the AA in DDGS have a low bioavailability due to Maillard reactions during drying, which may be particularly acute for Lys (Lumpkins and Batal, 2005). Thus, the nutritional value of DDGS can also be improved by the addition of exogenous protease. Protease supplementation has been employed to lower dietary protein level without a reduction in broiler performance, resulting in reduced protein waste and nitrogen excretion into the environment (Yu et al., 2007).

EFFECTS OF EXOGENOUS ENZYMES ON NUTRIENT AND ENERGY DIGESTIBILITY

Enzymatic combination of amylase, xylanase and protease has been observed to increase the AID of CP by 2.9 % (Zanella et al., 1999), while the AID of nitrogen was increased by 6.6% in another experiment (Rutherfurd et al., 2007). Supplementation of EE increased the retention of CP of broilers fed corn-SBM diets with low metabolizable energy (ME) content in starter, grower and finisher phases (Zhou et al., 2009). Xylanase was found to increase the overall AID of AA by 15% in a corn based diet while the improvement for wheat and rye based diets were 16% and 30%, respectively (Cowieson and Bedford, 2009). Addition of EE also increased the AID of AA for broiler chickens fed corn-SBM diets (Cowieson and Ravindran, 2008; Cowieson et al., 2010). In another experiment, the AID of Thr and Val were increased by 3.0 % and 2.3 % respectively with EE supplementation, however Lys, Met and Arg were not altered (Zanella et al., 1999). It has been demonstrated that a portion of the AA benefits were derived from the reduction of NSP’s anti-nutritive effects (Angkanaporn et al., 1994). Moreover, AA which are readily digested in diets not supplemented with EE have reduced responses to EE supplementation (Cowieson et al., 2010). These differences in response to EE primarily depend on the proportion of undigested fraction,
which would provide an ideal opportunity for the EE to maneuver beneficial responses (Cowieson and Bedford, 2009).

A cocktail of cell wall degrading enzymes including xylanase was found to improve ME efficiency in corn-SBM diets which was attributed to the increased AID of starch and NSP (Meng et al., 2005). Multiple peer reviewed reports have demonstrated that the addition of EE to corn-SBM diets increased AID of starch (Choct et al., 1996; Marsman et al., 1997). Supplementation of protease was found to increase the true ME and true nitrogen digestibility in chickens fed corn-SBM diets (Ghazi et al., 2002). The combination of amylase, protease and pectinase increased apparent nitrogen corrected ME (AMEn) when added to a corn-SBM diet with lower ME and protein concentrations in comparison to a control diet (Kocher et al., 2003). Apparent ME (AME) has been found to be increased with the addition of various combinations of EE containing amylase, protease, xylanase, glucanase, cellulase, mannanase, and pectinase added to corn-SBM diets (Meng et al., 2005; Cowieson and Ravindran, 2008). Supplementation of EE was found to increase the AME of corn-SBM diets with low ME levels in starter, grower, and finisher phases and the improved values of AME in lower energy diets were greater than that of higher energy diets (Zhou et al., 2009). The effectiveness of EE on nutrient digestibility has been shown to decrease with age in chickens as they have been more effective when used in birds with immature digestive tracts (Olukosi et al., 2008).

EFFECTS OF EXOGENOUS ENZYME COMBINATION ON PERFORMANCE

It is important to use synergistic EE such as amylase, xylanase and protease to increase the energy extraction potential of birds. Exogenous α-amylase increased the body weight gain by 4.9% and lowered the feed conversion ratio by 1.3% in broiler chickens fed acorn-SBM diet (Gracia et
Addition of exogenous xylanase to corn-SBM diet increased broiler weight gain by 5.6%, while reducing the feed conversion ratio by 4.6% (Cowieson et al., 2010). Protease supplementation has been demonstrated to increase the performance of broiler chickens fed a corn-SBM diet (Odetallah et al., 2005). However, various reports in literature suggest that the nutritive value of corn-SBM-based broiler diets can be enhanced by the combination of amylase, xylanase and protease. A 5.6% increase in body weight gain and 4.0% increase in feed conversion ratio of chicks fed EE containing amylase, xylanase and protease were observed by Simbaya et al., (1996). Zanella et al., (1999) observed a 1.9% increase in body weight gain and 2.2% increase in feed conversion ratio for chicks fed the same EE cocktail. Significantly increased body weight gain (5.8%) and feed efficiency (4.2%) were observed (Cowieson and Adeola, 2005), while a 7.0% increase in body weight gain and 8.1% reduction in feed conversion ratio were reported in another experiment (Cowieson and Ravindran, 2008). These findings may however not be attributed solely to the effects of one enzyme but the combination of amylase, xylanase and protease, suggesting a positive interaction among these enzymes.

**Direct-fed microbial**

Direct-fed microbial (DFM) or probiotics are defined as “live microbial feed supplements which beneficially affects the host animal by improving its intestinal microbial balance” (Fuller, 2001). The most commonly used DFM are strains of *Lactobacillus* spp., *Bifidobacteria* spp., *Bacillus* spp., *Bacteroides* spp., *Streptococcus* spp., yeasts, and various fungi including *Aspergillus* spp. (Fuller, 2001). Survival in the GIT and adherence to mucosal cells are considered to be important selection criteria for probiotic activity (Patterson and Burkholder, 2003).
MODES OF ACTION OF DIRECT-FED MICROBIAL

The beneficial modes of action of DFM include, regulation of intestinal microbial homeostasis, stabilization of gastrointestinal barrier function (Salminen et al., 1996), expression of bacteriocins, immunomodulatory effects, inhibition of pro-carcinogenic enzymes and interference with the ability of pathogens to colonize and infect the mucosa (Gill, 2003). One of the main strategies of DFM treatment relies on the benefit from the competitive nature of intestinal bacteria to exclude pathogens that negatively affect bird performance or food safety, a phenomenon called competitive exclusion (CE). The pioneering evidence of CE concept was originally designed for *Salmonella* reduction in chickens (Nurmi and Rantala, 1973). The Nurmi principle proposes that the survival of chicks can be improved by inoculating them early with an “adult” gut microbiota (Nurmi and Rantala, 1973). However, this principle has been expanded to protect against enteropathogens such as enterotoxigenic *E. coli*, *C. perfringens*, *Yersinia enterocolitica*, *Listeria monocytogenes*, and *Campylobacter spp.* (Schneitz, 2005). In addition to pathogen control, claims have been made that CE treatment enhances the performance of birds (Corrier et al., 1995). Of the latest strategies, multispecies or strain combinations derived from the intestinal contents or mucosa might provide several advantages compared with the use of single strains, simply by an additive or synergistic effect (Timmerman et al., 2004). It has been demonstrated in humans that DFM increase endogenous digestive enzyme activity and nutrient absorption (Hooper et al., 2002). Moreover, DFM play a major role in regulating hind gut fermentation and the synthesis of SCFA, which are used to meet the host energy requirements (Caballero-Franco et al., 2007).

EFFECTS OF DIRECT-FED MICROBIAL ON INTESTINAL INTEGRITY
**Intestinal morphology**

Increased VH suggests an increased surface area capable of greater absorption of available nutrients (Caspary, 1992). Broiler chicks fed *Bacillus subtilis* had increased ileal VH but no changes in CD or VH:CD were observed (Lee *et al.*, 2010a). In another experiment, supplementation of *Lactobacillus* *spp.* increased ileal VH:CD, while no change was observed in VH or CD (Awad *et al.*, 2009). The combination of *Bifidobacterium thermophilum* and *Enterococcus faecium* has been reported to increase VH and decrease CD in the jejunum (Chichlowski *et al.*, 2007). Furthermore, increased VH was found in the ileum of adult male layers with slight improvement in feed efficiency after dietary addition of *B. subtilis* (Samanya and Yamauchi, 2002) and in broilers after addition of *E. faecium* (Samli *et al.*, 2007) or *Eubacterium* *spp.* (Awad *et al.*, 2006).

**Mucin barrier**

Very strong interactions exist between the mucus layer and gut microbes, and research indicates that an action on mucus layer is involved in the beneficial effects of DFM. It has been shown in pigs that *Lactobacillus* strain *GG* inhabits the mucous layer of the small intestine and can grow and adhere to the ileal mucus (Rojas and Conway, 1996). Experimental data indicate that DFM display a trophic action on the mucosa and reduce mucus degradation (Ruseler-van Embden *et al.*, 1995). On the other hand, indigenous flora is able to degrade mucin, and the production of mucin-degrading enzymes has been suggested as a determinant of virulence for a number of entero-pathogens (Colina *et al.*, 1996). Another major action of DFM is to reinforce the intestinal mucosal barrier against deleterious agents (Mahdavi *et al.*, 2005). *Enterococcus faecium* has been found to inhibit the adhesion of enterotoxigenic *E. coli K88* to porcine small intestinal mucus (Jin
et al., 2000a). Along with native microbes, DFM are also involved in the control of amount and nature of the mucus secreted by modifying the expression of MUC genes. Supplementation of DFM has been shown to up-regulate intestinal mucin production to increase the colonization resistance against pathogens or their receptors (Caballero-Franco et al., 2007).

**Digestive enzyme activity**

It has been established that DFM alter gastrointestinal pH and microflora to favor an increased activity of intestinal enzymes and digestibility of nutrients (Dierick, 1989). Inclusion of DFM in broiler diets has been reported to increase the number of beneficial bacteria (e.g. *Bifidobacteria* spp., and *Lactobacillus* spp.,) in the GIT that potentially improved the digestion of carbohydrates, protein and minerals (Jin et al., 1998). *Lactobacilli* spp., have been reputed to modify the intestinal milieu and to produce digestive enzymes, particularly amylase, and other beneficial substances (Jin et al., 2000b). Furthermore, supplementation of *Lactobacillus* spp., to chickens for 40-d significantly increased the level of endogenous α-amylase (Jin et al., 2000b). These results are in agreement with data published in piglets, that supplementation of a mixed DFM containing multiple strains of *Lactobacillus* spp., and *Streptococcus faecium*, resulted in significantly higher carbohydrase enzyme activities in the small intestine (Collington et al., 1990). It has been observed that active amylolytic and proteolytic enzymes released by *Aspergillus oryzae* influenced the digestibility of nutrients (Schneitz, 2005). Chickens fed diets with mixed *Lactobacillus* spp., found to have reduced fecal β-glucosidase activity due to the hydrolysis of glucuronides and reduction in the generation of noxious metabolites such as toxic aglycones (Jin et al., 2000b).
Short-chain fatty acid synthesis

Supplementation of DFM has been reported to increase cecal SCFA concentrations, particularly butyrate, by establishing a dominant probiotic colony primarily in hindgut, as well as increasing the efficiency of gut microbes to ferment NSP (Caballero-Franco et al., 2007). Marounek et al. (1999) demonstrated that fermentation of low molecular weight carbohydrates could yield increased SCFA than that of large molecular carbohydrates in the broiler ceca. Besides being used as the major substrate for energy production, butyrate has been observed to be the most effective SCFA to promote proliferation and functional maturation of intestinal epithelial cells resulting in increased arterial blood flow linked to increased nutrient absorption (Cherbut, 2003).

EFFECTS OF DIRECT-FED MICROBIAL ON NUTRIENT AND ENERGY DIGESTIBILITY

Inclusion of DFM in broiler diets has been reported to increase the number of beneficial bacteria (e.g. Bifidobacteria spp., and Lactobacilli spp.,) in the GIT that potentially improved the digestion of carbohydrates, protein and minerals (Jin et al., 1998). Previous research indicates that supplementation of DFM increased the AID of AA for broiler chicks (Li et al., 2008). These beneficial DFM organisms promoted the uptake of glucose, AA and minerals across the intestinal epithelium (Wu, 1998). Moreover, DFM may affect luminal nutrient metabolism, resulting in changes in the amounts of carbohydrate and AA (free and protein-bound) in ileal digesta (Wu, 1998; Snel et al., 2002). Published data indicate that supplementation of DFM in corn-SBM diets fed to broilers increased the AMEn (Li et al., 2008; Mountzouris et al., 2010), although few studies have examined this response.
EFFECTS OF DIRECT-FED MICROBIAL ON DISEASE AND PATHOGEN COLONIZATION

Several experiments with undefined and defined probiotic cultures have been conducted to control or reduce pathogen colonization. It has been shown experimentally that DFM treatment protects chicks against *C. jejuni*, *Listeria monocytogenes*, pathogenic *E. coli*, *Yersinia enterocolitica* and *C. perfringens* (Schneitz, 2005). Supplementation of *Lactobacillus* based probiotic cultures significantly reduced *Salmonella enteritidis* recovery in challenged neonatal broiler chicks (Vicente *et al.*, 2008), while *B. cereus* var. *toyoii* reduced colonization and invasion of *S. enteritidis*, both in broiler chickens and white leghorn chickens. It has been shown that the presence *C. jejuni* was reduced in broiler chickens fed multi-species of DFM containing *L. acidophilus*, *L. casei*, *B. thermophilus*, and *E. faecium* (Willis and Reid, 2008). Feeding *L. acidophilus* and *Streptococcus faecium* has been found to reduce *Campylobacter spp.* shedding in broiler chickens (Morishita *et al.*, 1997). *Lactobacillus* culture reduced the *in vitro* colonization and shedding of *C. jejuni* by lowering the environmental pH (Fooks and Gibson, 2002). Pathogen exclusion by DFM in poultry has been well documented, including a reduction of *E. coli*, *Salmonella spp.*, *C. jejuni*, and *Eimeria acervulina* (Morishita *et al.*, 1997; Dalloul *et al.*, 2003). The symbiotic DFM enhance resistance to infection by competing with pathogens for nutrients or attachment sites, or more directly by antagonistic action against undesirable microorganisms, i.e., a barrier effect (Schneitz, 2005). Therefore, DFM could be a possible strategy to control pathogenic colonization and shedding and thus to maintain a healthy indigenous gut microbiota.

EFFECTS OF DIRECT-FED MICROBIAL ON PERFORMANCE
Supplementation of DFM has been shown to increase body weight gain and feed efficiency in broilers fed corn-SBM diets (Jin et al., 1998; Markovicva et al., 2009; Mountzouris et al., 2010). Body weight gain, carcass yield and feed efficiency were significantly increased for broiler chicks fed DFM for a 45-d period (Awad et al., 2009). Improved bird performance including increased body weight gain, feed efficiency and low mortality were reported with birds supplemented with DFM and challenged with *E. acervulina* (Dalloul et al., 2003a). Addition of DFM in corn-SBM diet fed to laying hens resulted in increased egg characteristics including shell thickness and Haugh unit (Lee et al., 2010b). Feed intake, feed conversion ratio and number of damaged eggs were reduced in another experiment when laying hens were fed DFM supplemented corn-SBM diet, although no difference in egg production or egg weight were noted (Balevi et al., 2001). Increased performance in pullets fed DFM in two different experiments was observed with increased hen-day egg production, egg weight, egg mass, and egg size and reduced feed intake and feed conversion ratio (Nahashon et al., 1994).

**Conclusions**

Supplementation of EE with corn-SBM-DDGS diets to poultry has been reported to increase the activity of endogenous digestive enzymes and digestibility of nutrients and energy. This results in increased efficiency in extracting dietary energy. Addition of DFM in poultry diets has been demonstrated to improve intestinal integrity by increasing epithelial barrier functions and reducing pathogen colonization. Increased intestinal integrity results in energy contributions by lowering the demands of activating acute immune response. Therefore the beneficial effects of EE and DFM on energy metabolism could be attributed to their action in increasing intestinal efficiency as well as integrity independently. Data described above demonstrate that specific EE
and DFM can communicate with the intestinal epithelium to modulate the physiology through complex mechanisms. These modulations may lead to increased energy savings as well as a reduction in energy spent for constant activation of acute immune response, resulting in a positive energy balance. Tracking this excess energy will be a challenge, particularly when using the combination of EE and DFM, and more research is necessary to explore and define these mechanisms as well as the resulting effects.
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CHAPTER 3

VALIDATION OF THE EFFECTS OF SMALL DIFFERENCES IN DIETARY METABOLIZABLE ENERGY AND FEED RESTRICTION IN FIRST-CYCLE LAYING HENS

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ABSTRACT

An experiment was conducted to evaluate energy utilization of laying hens fed diets containing 2 ME concentrations, using response criteria including performance, BW, abdominal fat pad and energy digestibility. The experiment was a 2 x 2 factorial with 2 feeding regimens (\textit{ad libitum} and restriction fed), and 2 dietary ME levels (2,880 kcal/kg ME (CON); and 2,790 kcal/kg ME (LME)). A total of 60 Hy-Line W36 first-cycle laying hens were fed experimental diets, resulting in 15 individually caged hens for each of the 4 treatments. Hens in the restriction fed group were fed 90 g of feed per day. The CON diet was formulated to meet or exceed the NRC (1994) recommendations with 2,880 kcal/kg, whereas LME diet was similar with the exception of a 90 kcal/kg reduction in ME. Hens were fed experimental diets for 12 wk from hen 28 to 39 wk of age. Hen day egg production, weekly feed intake and every 2 wk egg weights and egg mass were recorded, whereas hen BW was measured every 4 wk. Excreta samples were collected over

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the last 5 d of experiment to determine AMEn. Abdominal fat pads were measured individually for all hens at the end of experiment. There were no interactions between feeding regimens and dietary ME levels throughout the experiment. Feed restriction resulted in reductions ($P \leq 0.01$) in hen day egg production, BW, and abdominal fat pad, indicating reduced nutrient availability to partition toward production, maintenance, and storage functions. The reduction in energy intake between CON and LME fed birds (90 kcal/kg) did not change the energy partitioned towards production or maintenance, but reduced ($P = 0.03$) the energy stored (reduced fat pad) of LME-fed hens. These results suggest that energy is used following the pattern of production and maintenance before storage requirements and that fat pad (energy storage) may be the most sensitive indicator of dietary energy status over short-term in Hy-Line W36 laying hens.

Key words: layer, energy metabolism, feed restriction, abdominal fat pad, performance
INTRODUCTION

Dietary energy has always been an expensive component in poultry rations and as consumption of energy increases rapidly around the world (Pardue, 2010), energy costs will continue to drive grain prices. Understanding energy intake and partitioning patterns of the modern laying hen has become increasingly important to improve the efficiency of dietary energy utilization and to control feed costs. Research over time has demonstrated that laying hens can change feed intake patterns to meet energy requirement, thus feed intake and subsequent hen productivity change with dietary energy content (Hill et al., 1956; Harms et al., 1966; Leeson and Summers, 1997; Grobas et al., 1999). More recently, published results have reported certain current genetic lines of laying hens are less sensitive to dietary ME; thus feed intake may no longer be driven by the ME content of diet. Hy-Line W36 hens were found to be less sensitive to dietary ME compared to Hy-Line W98 or Hy-Line Brown over an 8-wk experimental period (Harms et al., 2000). In another similar experiment, Hy-Line W36 laying hens did not adjust their feed intake when dietary ME was increased over 10% (from 2,519 to 2,798 kcal/kg) and egg production was not affected (Harms and Russell, 2004). Hy-Line W36 hens did not increase their feed intake when the dietary ME was reduced from 2,996 to 2,783 kcal/kg during a 12 wk experimental period (Bohnsack et al., 2002).

In addition to egg production, energy can be used to maintain and grow BW and stored in adipose tissue to meet future energy needs. Changes in egg production account only for a portion of energetic balance and performance responses alone do not represent complete accounting of hen energy. Exploring the utilization pattern of dietary energy in a short-term experiment will allow for faster evaluation of feed additives or situations that affect energy metabolism than
performance variables alone. In an attempt to validate the effects of small changes in dietary ME fed to laying hens, a short term experiment was proposed with the hypothesis that small changes in dietary ME will be reflected by a modification in the combination of maintenance, productive and storage energy. The objective was to characterize the effects of small reductions in dietary ME in laying hens fed by a corn-soybean meal-dried distillers grains with solubles-based diet when fed either ad libitum or restricted feeding regimen over a 12-wk experimental period.

**MATERIALS AND METHODS**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University before the start of the experiment.

**Experimental Design**

A total of 60 Hy-Line W36 (Hy-Line International, Dallas Center, IA) hens were procured from a local commercial facility at 22 wk of age. The hens were provided a transition period of 5 wk during which time they were fed a corn-soybean meal-dried distillers grains with solubles-based standard diet before experimental diets were provided. The experiment utilized a 2 x 2 factorial arrangement of treatments, with 2 feeding levels (ad libitum and restriction fed) and 2 dietary ME levels (2,880 kcal/kg ME (CON), and 2,790 kcal/kg ME (LME)). Ad libitum hens were provided free access to feed, while the feed restricted hens were provided 90 g of feed per day individually throughout the experiment, when the standard feed intake would be 90 g/d per hen at wk 28 to 96 g/d at wk 39 as per the breed standard (Hy-Line, 2011). The CON diet was formulated to meet or exceed the NRC (1994) recommendations for all nutrients and contained
2,880 kcal/kg of ME. The LME diet was similar to the CON diet but was formulated with a 90 kcal/kg reduction in ME (Table 3.1). Titanium dioxide was added to all diets at the rate of 0.25% as an inert dietary marker for AMEn determination.

Each experimental unit (EU) was defined as an individually caged hen (1,239 cm²) resulting in 15 EU for each of the 4 treatments. The individual housing model was selected to better quantify individual bird feed intake and reduce aggressiveness among hens over competition for feed. The 15 EU were used to account for possible mortality or poor egg producers to maintain suitable replication over the 12-wk experimental period. Hens were weighed at the start of wk 28 to determine initial BW and were assigned to single-tier cages using a completely randomized design. A photoperiod of 16L:8D and temperature between 21°C to 24°C were maintained throughout the experimental period.

Data Collection

Hens were monitored twice daily and hens in the feed-restricted group were individually fed 90 g of feed daily. Any remaining feed was measured and removed at the end of each week. Unlimited access to feed was given to ad libitum fed groups, and total feed consumption was measured weekly. Feed intake was determined by measuring feed refusal (initial feeder weight with feed + feed added over the week - final feeder weight with remaining feed). Eggs were collected and recorded within an hour time period at approximately the same time every day to determine hen-day egg production (HDEP). After initial BW was determined, hens were weighed individually at 4-wk intervals. Eggs were collected and saved over a 5-d period every 2 wk to determine egg weight and egg mass. Egg mass produced per d per hen was calculated as follows:

\[
\text{mean egg mass (g)} = \left[\frac{\text{mean egg weight (g) for 5 d} \times \text{no. of eggs produced over the wk}}{7}\right]
\]
Clean excreta trays were placed under individual cages (EU) for the last 5 d of wk 12. Excreta samples were collected daily and frozen at -20°C on the same day. Upon completion of the 5-d excreta collection, the samples were thawed and pooled by EU to generate a homogenous sample for AMEn determination. At the end of wk 12, hens were euthanized via carbon dioxide asphyxiation. All hens were then dissected and abdominal fat pads (AFP) were separated and weighed.

**Chemical Analysis**

In total, 10 excreta samples were selected from the 15 available hens of each treatment for AMEn determination. Excreta of hens with similar mean HDEP during the last wk of the experiment were selected to minimize differences in nutrient requirements between productive and non-productive hens. Representative excreta samples were dried at 65°C for 3 d (Jacobs et al., 2011) and ground through a 1.0-mm screen (Brinkmann Instruments Inc., Westbury, NY). Feed samples were dried at 100°C for 24 h using a convection oven (Yamato Scientific America Inc., Santa Clara, CA) and ground through 0.5-mm screen (Brinkmann Instruments Inc., Westbury, NY). Nitrogen concentration was determined in excreta and diet samples by the micro-Kjeldahl method (AOAC, 2006) on a Kjeltech 1028 distilling unit (Foss Inc., Eden Prairie, MN), and the gross energy was determined using an adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL). The concentration of titanium dioxide was determined in excreta and feed samples as described by Leone (1973). All excreta and feed samples were analyzed in duplicate. The AMEn was calculated using the methods of Scott et al. (1982) modified to replace the chromic oxide marker with titanium dioxide.
**Statistical Analysis**

The statistical analysis was carried out as a 2 x 2 factorial in a completely randomized design to determine the main and interactive effects of dietary ME level and feeding regimen. The data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) as a two-way ANOVA with protected least square means to separate means. Student’s t-test ($\alpha = 0.05; t = 1.98698$) was used to separate significant least square means with the probability set at $P \leq 0.05$. Student’s t-test was performed only on the results with an ANOVA (overall $P$-value) $\leq 0.05$.

**RESULTS AND DISCUSSION**

There was no mortality and no hens were removed or culled during the experimental period. There were no interactions found between dietary ME levels and feeding regimen for feed intake, BW, HDEP, egg weight, egg mass, AFP or AMEn, so main effects will be reported and discussed. Restricted feeding reduced ($P \leq 0.01$) feed intake of restriction fed group as expected (Table 3.2). Overall feed intake of the restricted group was approximately 10% lower than the *ad libitum* fed group. There were no differences ($P > 0.05$) in feed intake between hens fed the 2 dietary ME levels over the 12-wk experimental period. This is in agreement with recent reported observations that Hy-Line W36 laying hens fed corn-soy based diets of low ME and standard ME did not differ in their feed intake (Scheideler et al., 2005; Jalal et al., 2007). The observations on feed intake from this experiment are in agreement with the above published observations that feed intake of Hy-Line W36 laying hens is less sensitive to dietary ME content.

The mean HDEP (Table 3.2) and egg mass (Table 3.3) of feed-restricted group were 4% and 6% lower ($P \leq 0.01$), respectively, while there was no significant difference in egg weight
(Table 3.3) in comparison with the *ad libitum* fed group. This reduced productivity of the feed restricted hens validates previously reported effects of limit feeding (Reid et al., 1978; Macleod et al., 1979). Macleod et al. (1979) reported that reducing nutrient intake by 20% as well as dietary ME by 20% had the same effect on energy metabolism in hens. There were no significant differences in any of the productivity variables (HDEP, egg weight, and egg mass) between hens fed differing dietary ME concentrations (Table 3.2 and 3.3). Feed intake:egg mass conversion ratio (Table 3.2) did not differ among the treatment groups (*P* > 0.05). This is in agreement with the reports that changes in dietary ME did not significantly affect egg production, egg weight and egg mass. Reducing dietary ME from 2,850 to 2,750 kcal/kg (Freitas et al., 2000), or from 2,798 to 2,519 kcal/kg (Harms and Russell, 2004), or from 3,097 to 2,990 kcal/kg (Jalal et al., 2006) failed to significantly alter the performance (HDEP, egg weight and egg mass) of Hy-Line W36 laying hens. No difference in egg weight or egg mass were observed between the first-cycle Hy-Line W36 laying hens fed 2,900 and 2,810 kcal/kg ME diets (Jalal et al., 2007). Although most of the reported experiments have had no performance difference in Hy-Line W36 hens with changes in dietary ME concentrations, reduced egg weight and egg mass were reported with 2,785 kcal/kg ME compared to 2,871 kcal/kg ME in Bovans White Leghorn hens (Novak et al., 2008). Mathlouthi et al. (2003) reported that reducing dietary ME from 2,753 to 2,653 kcal/kg in a wheat-barley-based diet reduced egg production and egg mass in ISA Brown laying hens.

The mean BW of hens across all treatment groups was similar at the start of experiment. After 8 wk on experimental diets, significant reduction was observed in BW of the feed-restricted hens in comparison with the *ad libitum* fed hens (*P* ≤ 0.01; Table 3.3). This is in agreement with the published data that a 20% reduction in feed intake due to feed restriction resulted in an almost 20% reduction in BW compared with hens fed *ad libitum* (Macleod et al., 1979). The hens fed
different dietary ME levels did not differ in their BW throughout the experiment \((P > 0.05)\). This is in agreement with the absence of BW difference observed in first-cycle Hy-Line W36 laying hens fed differing dietary energy concentrations (Freitas et al., 2000; Scheideler et al., 2005; Jalal et al., 2006; Jalal et al., 2007).

There were no significant differences in AMEn between the feeding regimen \((P = 0.92)\) or different dietary ME levels \((P = 0.16)\) and interactions \((P = 0.36)\) were absent. The AMEn for *Ad libitum* fed group was 3,002 kcal/kg (SEM = 26.02) with a range of 348 kcal/kg (2,771-3,119 kcal/kg), while it was 2,998 kcal/kg (SEM = 26.02) for the feed restricted group with a range of 539 kcal/kg (2,711-3,240 kcal/kg). The AMEn for CON group was 3,027 kcal/kg (SEM = 26.02) with a range of 470 kcal/kg (2,770-3,240 kcal/kg) and it was 2,973 kcal/kg (SEM = 26.02) for the LME group with a range of 408 kcal/kg (2,711-3,119 kcal/kg). Peer reviewed data on the AMEn differences in Hy-Line W36 laying hens fed differing dietary ME levels are limited in the past 15 yr (Scheideler et al., 2005; Wu et al., 2005; Jalal et al., 2006). The approximate spread of the AMEn data for 500 kcal/kg among the various treatment groups may preclude AMEn as a response criterion, particularly when evaluating relatively small differences in dietary ME.

A 51% reduction \((P \leq 0.01)\) in the AFP of feed-restricted hens (22.7 g/hen) in comparison to *ad libitum* fed hens (46.5g/hen, Table 3.3) was observed. This is in agreement with previous reports that limit feeding laying hens reduced fat deposition compared with *ad libitum* fed hens (Combs et al., 1961). The AFP of CON fed birds weighed 39.0 g/hen while the AFP of LME fed birds was decreased by 23% to 30.2 g/hen \((P = 0.03)\). It has been reported that reducing dietary energy reduced the fat content of liver as well as fat deposition in the AFP (Cunningham and Morrison, 1977). The reduced AFP weight of the LME and feed-restricted groups may be due to the insufficient dietary energy. Different dietary ME levels did not affect feed intake, HDEP or
BW, but did change the amount of energy stored in the AFP. The results of this experiment are in agreement with the previously reported observation that metabolism of laying hen favors continuous utilization of energy to meet egg production requirements rather than storage during the periods of energy insufficiency (Husbands, 1972). Limiting energy supply tends to change body composition, primarily at the cost of fat deposition in laying hens (Cunningham and Morrison, 1977). These data suggest that, over a short term experimental period, AFP may be a more sensitive response criterion. The long term effect of this reduced energy storage could ultimately result in either loss of BW or egg production, but body fat reserves seem to respond to differences in dietary ME intake before changes in BW or HDEP and are more sensitive than direct measurement of AMEn.

In conclusion, feed intake of Hy-Line W36 hens was not significantly altered with relatively small differences in dietary ME, validating observations that feed intake has little or no sensitivity to the dietary ME levels evaluated. Small differences in dietary ME levels and or feed restriction significantly reduced AFP over a 12-wk experimental period, but failed to influence AMEn after 12 wk. These results suggest that energy is utilized following a pattern of production and maintenance before storage requirements in laying hens. This outcome also underscores the importance of a comprehensive approach to laying hen energy metabolism rather than reliance on performance variables alone. Consideration of AFP, BW and HDEP was able to detect relatively small differences in dietary ME in a short time period (12 wk).

ACKNOWLEDGEMENTS
We recognize the care for the birds provided by W. Larson, J. Tjelta, W. Rogers, and R. Holbrooke of Iowa State University poultry research center, Ames, IA, and also like to thank K. Nesheim, N. Nachtrieb, M. Higgins and J. Green of Iowa State University, Ames, IA for assistance in conducting this experiment.
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Cunningham, D. C., and W. D. Morrison. 1977. Dietary energy and fat content as factors in the nutrition of developing egg strain pullets and young hens. 3. Effects on hepatic lipogenic enzyme activity and body chemical composition during the first 20 weeks of lay. Poult. Sci. 56:1783-1791.


Table 3.1 Composition of laying hen diets fed from 28 to 39 wk of age.

<table>
<thead>
<tr>
<th>Phase</th>
<th>28 to 34 wk</th>
<th>35 to 39 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>(%) unless otherwise indicated</td>
<td>CON&lt;sup&gt;1&lt;/sup&gt;</td>
<td>LME&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Ingredient composition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn</td>
<td>53.95</td>
<td>53.86</td>
</tr>
<tr>
<td>Soybean meal with 48% CP</td>
<td>24.71</td>
<td>22.17</td>
</tr>
<tr>
<td>Dried distillers grains with solubles</td>
<td>5.00</td>
<td>9.30</td>
</tr>
<tr>
<td>Meat/Bone meal</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Animal and vegetable blended fat</td>
<td>3.82</td>
<td>2.24</td>
</tr>
<tr>
<td>Salt</td>
<td>0.40</td>
<td>0.38</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>L-Lysine&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.07</td>
<td>0.16</td>
</tr>
<tr>
<td>Di-Calcium phosphate</td>
<td>1.88</td>
<td>1.70</td>
</tr>
<tr>
<td>Limestone</td>
<td>9.13</td>
<td>9.15</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Vitamin mineral premix&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Titanium dioxide</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td><strong>Chemical composition (calculated)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME (kcal/kg)</td>
<td>2,880</td>
<td>2,790</td>
</tr>
<tr>
<td>CP</td>
<td>17.90</td>
<td>17.89</td>
</tr>
<tr>
<td>Ether extract</td>
<td>6.67</td>
<td>5.46</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>2.48</td>
<td>2.70</td>
</tr>
<tr>
<td>Calcium</td>
<td>4.00</td>
<td>3.96</td>
</tr>
<tr>
<td>Non-Phytate phosphorus</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>Digestible Met + Cys</td>
<td>0.70</td>
<td>0.71</td>
</tr>
<tr>
<td>Digestible Lys</td>
<td>0.85</td>
<td>0.85</td>
</tr>
<tr>
<td>Digestible Thr</td>
<td>0.61</td>
<td>0.61</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>2.10</td>
<td>1.79</td>
</tr>
<tr>
<td><strong>Chemical composition (analyzed)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>16.75</td>
<td>16.33</td>
</tr>
<tr>
<td>Ether extract</td>
<td>4.98</td>
<td>4.12</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>2.27</td>
<td>2.39</td>
</tr>
<tr>
<td>Moisture</td>
<td>11.99</td>
<td>11.81</td>
</tr>
<tr>
<td>Crude ash</td>
<td>13.78</td>
<td>13.94</td>
</tr>
</tbody>
</table>
Control ME diet

Low ME diet

Contained 50.7% of L-lysine in the form of L-lysine sulfate, 0.1% Methionine, 0.1% Cystine, 0.3% Threonine, 0.1% Tryptophan, 0.6% Arginine, 0.3% Isoleucine, 0.5% Leucine, and 0.4% Valine.

Provided per kg of diet: Selenium-200 µg; Vitamin A-6,600 IU; Vitamin D₃-2,200 IU; Vitamin E-14.3 IU; Menadione-880 µg; Vitamin B₁₂-9.4 µg; Biotin-33 µg; Choline-358 mg; Folic acid-1.1 mg; Niacin-33 mg; Pantothenic acid-8.8 mg; Pyridoxine-880 µg; Riboflavin-4.4 mg; Thiamine-1.1 mg; Iron- 226 mg; Magnesium-100 mg; Manganese-220 mg; Zinc-220 mg; Copper-22 mg; Iodine- 675 µg.
Table 3.2 Effects of dietary ME levels, with or without feed restriction in laying hens from 28 to 39 wk of age on feed intake, hen-day egg production (HDEP) and feed:egg mass ratio (Feed:EM).\(^1\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Feed intake (g/d per hen)</th>
<th>HDEP(^2) (%)</th>
<th>Feed:EM(^3) (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary ME level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON(^4)</td>
<td>91.2</td>
<td>93.8</td>
<td>1.72</td>
</tr>
<tr>
<td>LME(^5)</td>
<td>92.5</td>
<td>94.5</td>
<td>1.73</td>
</tr>
<tr>
<td>Feeding regimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>96.8(^A)</td>
<td>95.9(^A)</td>
<td>1.75</td>
</tr>
<tr>
<td>Restricted(^6)</td>
<td>86.8(^B)</td>
<td>92.4(^B)</td>
<td>1.70</td>
</tr>
<tr>
<td>Pooled SEM</td>
<td>1.04</td>
<td>0.83</td>
<td>0.02</td>
</tr>
<tr>
<td>ANOVA P-value</td>
<td>≤ 0.01</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>Dietary ME P-value</td>
<td>0.40</td>
<td>0.52</td>
<td>0.87</td>
</tr>
<tr>
<td>Feeding regimen P-value</td>
<td>≤ 0.01</td>
<td>≤ 0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Dietary ME x Feeding P-value</td>
<td>0.58</td>
<td>0.71</td>
<td>0.07</td>
</tr>
</tbody>
</table>

\(^A,B\) Values in the same column not sharing a common superscript differ significantly, \(P \leq 0.05\).

\(^1\) \(n = 30\). Data are reported as least square means.

\(^2\) Hen day egg production.

\(^3\) Feed consumed per egg mass produced.

\(^4\) Control ME diet with 2,880 kcal/kg.

\(^5\) Low ME diet with 2,790 kcal/kg.

\(^6\) Feed offered was limited to 90 g/hen per d.
Table 3.3 Effects of dietary ME levels, with or without feed restriction in laying hens from 28 to 39 wk of age on egg weight, egg mass, body weight (BW) and abdominal fat pad.1

<table>
<thead>
<tr>
<th>Item</th>
<th>Egg weight (g/egg)</th>
<th>Egg mass (g/hen per d)</th>
<th>BW (kg/hen)</th>
<th>Fat pad2 (g/hen)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dietary ME level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON(^3)</td>
<td>57.0</td>
<td>53.6</td>
<td>1.41</td>
<td>39.0(^a)</td>
</tr>
<tr>
<td>LME(^4)</td>
<td>56.8</td>
<td>53.9</td>
<td>1.39</td>
<td>30.2(^b)</td>
</tr>
<tr>
<td><strong>Feeding regimen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad libitum</td>
<td>57.6</td>
<td>55.5(^A)</td>
<td>1.44(^A)</td>
<td>46.5(^A)</td>
</tr>
<tr>
<td>Restricted(^5)</td>
<td>56.2</td>
<td>52.0(^B)</td>
<td>1.36(^B)</td>
<td>22.7(^B)</td>
</tr>
<tr>
<td>Pooled SEM</td>
<td>0.59</td>
<td>0.77</td>
<td>0.02</td>
<td>2.74</td>
</tr>
<tr>
<td><strong>ANOVA P-value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.40</td>
<td>0.03</td>
<td>0.05</td>
<td>≤ 0.01</td>
</tr>
<tr>
<td><strong>Dietary ME P-value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.82</td>
<td>0.74</td>
<td>0.54</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Feeding regimen P-value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>≤ 0.01</td>
<td>≤ 0.01</td>
<td>≤ 0.01</td>
</tr>
<tr>
<td><strong>Dietary ME x Feeding P-value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>0.46</td>
<td>0.86</td>
<td>0.57</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Values between the groups fed differing dietary ME levels not sharing a common superscript differ significantly, \(P ≤ 0.05\).

\(^{A,B}\)Values between the feeding regimens not sharing a common superscript differ significantly, \(P ≤ 0.05\).

\(^{1}\)n = 30. Data are reported as least square means.

\(^{2}\)Abdominal fat pad weight.

\(^{3}\)Control ME diet with 2,880 kcal/kg.

\(^{4}\)Low ME diet with 2,790 kcal/kg.

\(^{5}\)Feed offered was limited to 90 g/hen per d.
CHAPTER 4

EFFECTS OF DIRECT-FED MICROBIAL ON BROILER PERFORMANCE, INTESTINAL NUTRIENT TRANSPORT AND INTEGRITY UNDER INCREASED MICROBIAL CHALLENGE

A paper for submission to British Poultry Science Journal

ABSTRACT

1. Effects of *Aspergillus oryzae* and *Bacillus subtilis* based direct-fed microbial (DFM) on performance, ileal nutrient transport and intestinal integrity of broiler chickens, raised under experimental conditions with increased microbial challenge, were investigated.

2. The first model utilized a 2 × 2 factorial arrangement, with and without DFM supplementation, and with and without challenge. Chicks were fed DFM supplemented diets from 1-28 days, while the challenge was provided by vaccinating with 10X dose of commercial coccidial vaccine on day 9.

3. The challenge reduced performance ($P \leq 0.01$), while no differences were observed between dietary treatments from 8-28 days. The challenge reduced the ileal epithelial flux for D-glucose, L-lysine, DL-methionine, and phosphorus on day 21 ($P \leq 0.05$). Epithelial flux for D-glucose, L-lysine, DL-methionine were increased by DFM ($P \leq 0.05$), while a trend was observed for L-glutamine and phosphorus ($P \leq 0.10$). Ileal trans-epithelial electrical resistance (TER) was increased in challenged broilers fed DFM although this was not observed in unchallenged birds as indicated by the significant interaction ($P \leq 0.01$).
4. In the second experiment, 2 groups of day-old broilers, housed on built-up litter (uncleaned from two previous flocks), were fed the same diets from 1-21 days.

5. Ileal mucin (MUC2) mRNA expression, and colon TER ($P \leq 0.01$) were increased, while colon endotoxin permeability ($P = 0.04$) was reduced by DFM on day 21.

6. Addition of DFM increased the intestinal integrity of broiler chickens raised under experimental conditions designed to provide an increased challenge over typical research conditions.
INTRODUCTION

Direct-fed microbial (DFM) are commensal micro-organisms used as feed additives and can have beneficial effects in reducing the colonization of pathogenic bacteria and improving the performance in chickens (Lee et al., 2010). A growing body of scientific research has extensively demonstrated the beneficial effects of DFM which include i) performance, ii) nutrient digestibility, iii) modulation of intestinal microflora, iv) pathogen inhibition, and v) immunomodulation and gut mucosal immunity (Mountzouris et al., 2010). Nevertheless, inconsistencies in validating the efficacy of DFM on broiler performance are also equally reported (Gunal et al., 2006; Liu et al., 2007; Lee et al., 2010). Reduced pathogenic load in relatively clean research environments was observed as one of the possible reason for this inconsistent performance (Edens, 2003). In commercial conditions, broiler chickens are raised under environments that contain increased opportunity for pathogenic challenge compared to environments in research setting. Unsanitary rearing environments are major contributors of sub-clinical pathogenic challenges as chickens reared in these environments had reduced growth and breast meat yield (Bregendahl et al., 2005). Sub-clinical pathogenic challenges compromise intestinal integrity by pathogenic attachment to mucosa, and increase energy expenditure towards perpetual activation of acute phase immune response and depress growth performance (Klasing and Johnstone, 1991). Thus conditions simulating commercial environment are essential to validate the effects of DFM on gut integrity and performance of broiler chickens.

Two models were utilized in an attempt to reduce overall intestinal health by increasing microbial challenge under research conditions to validate the effects of DFM. The first model used a concentrated dose of coccidial vaccine to damage intestinal structure and increase the
susceptibility of birds to secondary bacterial infections. Coccidiosis is an intestinal disease caused by *Eimeria spp.* which compromises intestinal integrity by destructing the mucosa (Collier *et al*., 2008), resulting in reduced broiler performance (Persia *et al*., 2006). Secondary bacterial infections, primarily caused by *Clostridium perfringens* associated with coccidiosis, play a vital role in increasing the severity of poor performance (Collier *et al*., 2008). Determining the effects of DFM on broiler performance, and ileal nutrient absorption were the objectives of this experiment. The second model utilized unsanitary conditions in the form of built-up litter to increase pathogenic exposure to birds. Under commercial conditions in North America, the litter is often reused over multiple flocks by spreading a layer of new litter on top, which may cause transmission of pathogens among the flocks, resulting in increased microbial challenge (Hooge *et al*., 2003). Built-up litter with high moisture increases the risk of microbial growth which would adversely affect the performance compared to birds raised on unused litter (Barker *et al*., 2010). To determine the effects of DFM on ileal and colon integrity were the objectives of this experiment. Ileal and colon trans-epithelial electrical resistance (TER), colon lipopolysaccharide (LPS) permeability and ileal mucin (MUC2) mRNA expression were determined to elucidate the effects of DFM in altering intestinal integrity. The hypothesis for both of these experiments was that dietary supplementation of DFM to broiler chickens would increase performance and intestinal integrity under research conditions with increased microbial challenge.

**MATERIALS AND METHODS**

Two experiments were conducted using broiler chicks exposed to coccidial challenge or built-up litter to increase intestinal microbial challenge at the Poultry Research and Teaching Unit of Iowa
State University. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Iowa State University before the initiation of experiments.

**Experiment 1**

*Animals and housing*

A total of 200 day-old Ross 308 (Aviagen Group, Huntsville, AL) male chicks were obtained from a commercial hatchery (Welp Hatchery, Bancroft, IA). The chicks were brooded in environmentally controlled (EC) chambers for 7 days. On day 8, chicks were individually weighed, sorted, wing banded and placed, utilizing a randomized block design, into floor pens with fresh litter in the EC chambers. The experiment was arranged as a $2 \times 2$ factorial with two dietary (with and without DFM) and two challenge (with and without coccidial challenge) treatments. Chicks were allotted to pens (experimental units) based on body weight to minimize differences in the average bodyweight among pens. Each of the 4 treatments consisted of 5 pens of 10 chicks (0.18 m$^2$ per chick) resulting in 50 chicks per treatment.

The EC chambers were chosen to allow birds to have access to the floor and litter material for possible re-infection and secondary infection, and to reduce the chance of infection to non-challenged birds. All of the 8 EC chambers were located within the same barn and the chambers were adjacent to each other. The challenged treatments were placed utilizing a randomized block design. The first 4 chambers close to the entry door housed sham inoculated treatments, and the remaining 4 chambers (close to the exit door) housed coccidial challenged birds. Dietary treatments were randomly assigned across the sham and challenge EC chambers with either 2 or 3 pens in each chamber. Access to the building was limited to the sham inoculated control side, and the exit was located at the end of challenge side. Daily chores (management and monitoring) were
done using new disposable coveralls and boot covers on the sham inoculated side before moving into infected side. Care was taken to ensure that personnel did not move from infected side back to clean side without at least 24-h of withdrawal time between visits.

**Diets**

Chicks were provided two experimental mash diets from 1-28 days; a corn-soybean meal based control diet (CON, Table 4.1) and CON diet supplemented with a DFM containing a minimum of $1.2 \times 10^6$ cfu/g *Aspergillus oryzae* and $1.2 \times 10^7$ cfu/g *Bacillus subtilis*. The DFM was added at a dose rate of 2 g/kg of diet as per the manufacturer’s recommendation. Starter diets (1-14 days) and grower diets (15-28 days) were formulated to meet or exceed the breeder recommendations (Aviagen, 2011). Feed and water were provided *ad-libitum* throughout the experimental period.

**Coccidial challenge**

Coccivac-B® (Schering-Plough Animal Health Corporation, Union, NJ), a live-modified coccidiosis vaccine containing oocysts of *Eimeria* spp. (*E. acervulina, E. maxima, E. mivati*, and *E. tenella*) was used for the coccidial challenge. On day 9, all chicks in the coccidial treatment were challenged by oral gavage with 10X vaccination dose of Coccivac-B® (1 ml of vaccine per chick, diluted at a ratio of 1,000 doses in 100 ml of non-chlorinated water). All the birds in control were sham inoculated with 1 ml tap water through oral gavage to mimic experimental handling between the two treatments.

**Sample collection**
Pen feed intake (FI) and body weight gain (BWG) for individual chick were determined on 8, 21, and 28 days. Mortalities were removed immediately upon recording the BWG. The feed efficiency (BWG:feed intake; FE) was corrected for mortality for 8-21 days and 8-28 days. On day 21, a total of 10 chicks per treatment, 2 per pen, were euthanized by carbon dioxide asphyxiation. Ileal intestinal samples of 4 cm in length were collected from mid-way between the Meckel’s diverticulum and the ileo-cecal junction. The sampling of 2 birds per pen were treated as mortality for calculation of FE although they were not recorded as mortality.

*Modified Ussing chamber method*

Freshly harvested ileal tissues were used to measure the ileal TER, and mucosal transport of D-glucose (Glucose), DL-methionine (Met), L-lysine (Lys), L-glutamine (Gln), and phosphorus (P) (Albin *et al.*, 2007). Ileal tissue samples were immediately excised from the euthanized birds and placed into chilled, aerated Krebs-Henseleit buffer (pH 7.4) for transfer to the lab for continuous analysis of nutrient transport, TER and endotoxin permeability. Once clamped in the Ussing chambers (Physiologic Instruments, San Diego, CA), the samples were continuously aerated with 95% O₂ + 5% CO₂ mixture and both the apical and basolateral sides were immersed in 8 ml of Krebs buffer. After a short-circuit current was established and stabilized (about 5 to 10 min), basal short circuit current (Isc) and resistance measurements were recorded using Acquire & Analyze® software (Physiologic Instruments, San Diego, CA). The epithelial resistance was expressed as Ω/cm².

The electrophysiological nutrient transport was determined by measuring the changes in basal charge (Δµ Isc/cm²) induced by the addition of specific nutrients (Albin *et al.*, 2007). Glucose was the first nutrient added to the mucosal chamber at a concentration of 10 mM and 10 mM
mannitol was added to the basolateral chamber as an osmotic control. The potential difference across the tissue was measured for 30 min after challenge by open-circuit conditions for every 10 sec due to the Isc being delivered by a voltage clamp apparatus. The change in maximal current was recorded, and tissue conductance was calculated from Isc with potential difference using Ohm's law (Gabler et al., 2009). The transport of Met, Lys, Gln, P (Sodium phosphate di-basic) were examined in this order, at a concentration of 7.5 mM, and the results were expressed as µA/cm².

**Experiment 2**

**General procedure**

Male, day-old Ross 308 (Aviagen Group, Huntsville, AL) chicks were obtained from a commercial hatchery (Hoover’s Hatchery, Rudd, IA). Chicks were individually weighed, sorted, wing banded and allocated into one of 2 treatments (10 chicks per treatment) in floor pens (0.15 m²/chick), based on body weight. New diets were generated using the same two dietary formulations as the first experiment (Table 4.1). The built-up litter used was not removed from two previous flocks of birds to provide an increased microbial challenge to the birds, simulating industry conditions (Barker et al., 2010). Feed and water were provided ad-libitum throughout the experimental period. On day 21, 8 chicks from each treatment were euthanized by carbon dioxide asphyxiation. Colon segments (4 cm in length) were immediately collected mid-way between the ileo-cecal junction and cloaca, to determine TER (described under Experiment 1) and permeability of LPS (endotoxins) using modified Ussing chambers. Ileal mucosal samples were collected by scraping the mucosal layer mid-way between Meckel’s diverticulum and ileo-cecal junction, to determine mucin (MUC2) messenger RNA expression.
Lipopolysaccharide permeability

The LPS permeability co-efficient for colon epithelial tissue was assessed using fluorescein isothiocyanate labeled LPS (FITC-LPS) from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO) as outlined by Tomita *et al.* (2004) using Ussing chambers. After a 20 µg/ml concentration of FITC-LPS was applied to the mucosal side of colon tissue, and basolateral FITC-LPS concentrations were determined at the basolateral side at intervals of 20 min. This sampled FITC-LPS concentrations were determined using a Synergy-4® fluorescence spectrophotometer (BioTek US, Winooski, VT) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. An apparent permeability co-efficient (Papp) was calculated for each tissue as follows:

\[
P_{\text{app}} = \frac{dQ}{dt \times A \times C_0}
\]

Where: \(dQ/dt\) is the transport rate (µg/s) and corresponds to the slope of the regression line, \(C_0\) is the initial concentration in the mucosal side of the chamber (µg/ml) and \(A\) is the area of the membrane which was 1 cm² (Tomita *et al.*, 2004). The LPS permeability co-efficient results were expressed as µg/ml.min.cm².

Ileal MUC2 mRNA expression

Ileal mucosal scrapings for total mRNA extraction were stored in Ambion® RNA later (Life Technologies, Grand Island, NY) immediately upon collection. The scrapings were then washed with Phosphate-Buffer Saline (pH 7.2), homogenized and transferred to -80°C for final storage. Total RNA was isolated using Ambion® RNAqueous kit (Life Technologies, Grand Island, NY) from the homogenized samples. Isolated RNA was treated with Ambion® DNA-free kit (Life Technologies, Grand Island, NY) to remove any DNA contamination. The final RNA
concentration was diluted to 50 ng/µl by measuring the concentration using Nanodrop1000®
spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA) at 260 nm and 280 nm.

The mRNA expression levels of MUC2 were determined by quantitative real time RT-PCR,
using QuantiTect SYBR Green® RT-PCR kit (Qiagen Inc., Valencia, CA). The RT-PCR was
carried out with primers from chicken similar to MUC2 precursor (Intestinal mucin 2; Gallus
gallus, XM-421035; forward: 5’-GCTGATTGTCACTCAGCCTT-3’; reverse: 5’-
ATCTGCTGAATCACAGGTGC-3’) (Smirnov et al., 2006) and primers from the Gallus gallus
28S ribosomal RNA gene (primer sequence forward: 5’-GGCGAAAGCCAGGAAA-3’;
reverse: 5’GACGACCGATTTCAGCTC-3’). Each RT-PCR reaction was ran in triplicate and
consisted of 50 ng of total RNA, 12.5 µl QuantiTect SYBR Green master mix, 0.25 µl QuantiTect
RT mix, forward-reverse primers and RNAse-free water for a final volume of 25 µl. The RT-PCR
reactions were performed on a MyiQ single color real-time PCR detection system (Bio-Rad
Laboratories, Philadelphia, PA) with the following program: reverse transcription at 50°C for 30
min, initial activation at 95°C for 15 min followed by 40 cycles of denaturing at 94°C for 15 sec,
annealing at 59°C for 30 sec and extension at 72°C for 30 sec followed by reading of the plate.
Gene slopes were determined with a series of ten-fold plasmid dilutions. A melting curve from
65°C to 95°C with a reading at every 1°C was also performed for each individual RT-PCR plate
(Cheeseman et al., 2007). Cycle threshold (Ct) values were calculated and adjusted relative to 28S
expression, as follows:

\[
[40 - (\text{Mean } \text{Ct}_{\text{MUC2 gene}} + [\text{Median } \text{28S} - \text{Mean } \text{Ct } \text{28S}]) \times (\text{Slope } \text{MUC2 gene} / \text{Slope } \text{28S})]
\]

Where: \( \text{Ct}_{\text{MUC2 gene}} = \) mean of the triplicate cycle threshold (Ct) values of the gene tested (MUC2);
\( \text{Ct } \text{28S} = \) mean of the triplicate Ct value of the housekeeping gene (28S); \( \text{Mean } \text{28S} = \) overall
experimental mean of \( \text{Ct } \text{28S} \); \( \text{Slope } \text{MUC2 gene} = \) slope from 10-fold test gene standard regression
Statistical analysis

The statistical analysis for Experiment 1 was carried out as a $2 \times 2$ factorial in a randomized block design. The data from Experiment 1 were analyzed by MIXED procedure (SAS, 2012) with two-way ANOVA, using the pen as an experimental unit for performance variables such as BWG, FI and FE, and individual chick as an experimental unit for ileal TER and nutrient transport assays. Data from Experiment 2 were analyzed using MIXED procedure using individual chick as an experimental unit. Student’s t-test ($\alpha = 0.05; t = 1.98698$) was used to separate significant least square means with the probability of type-I error set at $P < 0.05$ in both experiments.

RESULTS

Experiment 1

Performance

There were no significant interactions in BWG, feed intake, and FE among dietary and challenge treatments (Table 4.2). The challenge lowered feed intake ($P \leq 0.01$), BWG ($P \leq 0.01$) and FE ($P \leq 0.01$) in comparison with unchallenged birds over both 8-21 days and 8-28 days. No significant differences were observed in the performance variables between the dietary treatments. No mortalities were observed throughout the experimental period.
Electrophysiological ileal nutrient transport

No significant interactions were noted between dietary and challenge treatments for glucose, Lys, Met, Gln or P transport across the ileal mucosa (Table 4.3). Challenged birds had lower \( (P \leq 0.01) \) glucose, Lys, Met and P transport in comparison to unchallenged birds, while Gln transport did not differ. Supplementation of DFM increased \( (P \leq 0.01) \) the mucosal transport of glucose, Lys, and Met, while trends \( (P \leq 0.10) \) were observed for Gln and P transport.

Ileal integrity

Interactions between diet and challenge treatments were observed (Fig. 4.1). There were no differences in ileal TER between the unchallenged treatments. Irrespective of challenge, the CON fed birds also did not differ in ileal TER. However, supplementation of DFM increased the TER in challenged birds \( (P \leq 0.01) \).

Experiment 2

Colon integrity

Colon TER for DFM fed birds was higher \( (P \leq 0.01) \) when compared to CON fed birds (Table 4.4). The \( P \text{app} \) for LPS permeability in the colon was decreased \( (P = 0.04) \) with DFM treatment, indicating reduced epithelial LPS permeability, compared to CON fed birds (Table 4.4).

Ileal mucin mRNA expression

The mRNA expression for mucin (MUC2) in the ileal mucosa was increased \( (P \leq 0.01) \) for DFM fed birds compared to CON fed birds (Table 4.4).
DISCUSSION

The performance reduction by challenge in Experiment 1 was significant with reduction of 11.7% and 7.8% in BWG during the 8-21 and 8-28 day periods, respectively. Challenge also reduced FE by 6.9% and 12.1% during 8-21 and 8-28 days, respectively. This is in agreement with data reported by Persia et al. (2006), that coccidial challenge reduced the nutrient digestibility and performance of broiler chickens. The absence of dietary main effects in all performance variables measured indicates that DFM did not alter the performance in either sham or challenged birds. A coccidial infection was successfully established as validated by the reduced performance of the challenge group in comparison to the sham inoculated group. Hence, it is questionable whether the birds were exposed to secondary microbial infections as the facilities and litter used in this experiment were clean and disinfected before the introduction of chicks. This may have resulted in reduced opportunity for secondary bacterial infections compromising the environment from being suitable for the DFM in maneuvering its response on performance (Lee et al., 2007).

Reduced nutrient flux across the mucosa in challenged birds may be due to the destructive effects of coccidiosis on ileal mucosa (Collier et al., 2008). This is in agreement with published data that coccidial challenge decreased ileal amino acid and energy digestibility in broiler chickens (Persia et al., 2006). Increased ileal mucosal nutrient flux for glucose, Lys, and Met for DFM fed birds are supported by increased dietary nutrient digestibility with DFM supplementation (Li et al., 2008). Direct support for these data are reported by Awad et al. (2008) in that mucosal flux of glucose was increased with addition of DFM to broiler diets. Increased luminal presence of glucose has also been observed to increase the absorption of dietary amino acids and water by activating
the apical \( \text{Na}^+-\text{H}^+ \) exchange (Amat et al., 1999; Awad et al., 2007). Moreover, active \( \text{Na}^+ \)-glucose co-transport has also been found to increase the passive transport of dietary nutrients by increasing the number of small pores in villus (Fihn et al., 2000).

The barrier function of tight junctions (TJ) is the ability to differentially restrict free passage of water, ions, macromolecules, and LPS based on size and charge (Tomita et al., 2004). Previous research has demonstrated that TER is a good indicator of TJ barrier function and therefore epithelial integrity (Putaala et al., 2008). Coccidiosis can compromise epithelial integrity, especially at the sites of infection (Collier et al., 2008). In this experiment, although no differences were observed between CON fed birds irrespective of challenge, DFM was able to increase the IET in ileum, away from the expected areas of coccidial damage in duodenum and ceca. In Experiment 2, the increased colon TER is validated by the reduced colon LPS permeability by DFM. In addition to the current data, bioactive metabolites produced by DFM increased TJ integrity, protecting epithelia from the deleterious effects of pathogenic bacteria, under \textit{in vitro} conditions (Putaala et al., 2008). These combined observations suggest that DFM supplemented in the current experiments influenced TJ barrier function positively, and increased the intestinal epithelial integrity of broiler chickens.

To further assess barrier integrity, the mucin gene (MUC2) expression in the ileum was assayed. Mucins are glycoproteins secreted by goblet cells, and act as a selective barrier protecting the enterocytes, preventing bacterial translocation by virtue of their negatively charged filamentous protruding structures (Smirnov et al., 2005). The overlying mucin layer is the first line of defense barrier the pathogens encounter when trying to traverse intestinal mucosa (Smirnov et al., 2005). In chickens, MUC2 mucins are widely expressed in the goblet cells of small intestine and colon (Smirnov et al., 2005). Increased expression of MUC2 mucin in Experiment 2 is in
agreement with the observation that DFM mediate intestinal mucin up-regulation to increase the colonization resistance against pathogens or their receptors (Caballero-Franco et al., 2007).

Coccidial challenge was used to try to induce a secondary bacterial infection, but the clean and sanitary research conditions may have precluded any secondary infection. The challenge did reduce performance of broiler chickens and DFM addition did not alter the bird performance. In the absence of increased performance, DFM treatment was able to increase nutrient flux across the ileal mucosa, regardless of challenge and increase ileal TER in the challenged group. In the second experiment, DFM increased ileal mucin mRNA expression and increased colon integrity of broiler chickens raised under conditions simulated to provide commercial environment. These data indicate that dietary DFM supplementation was efficient in maintaining and increasing the intestinal integrity of broiler chickens.

ACKNOWLEDGEMENTS

We recognize the bird care provided by W. Larson, J. Tjelta, W. Rogers, and R. Holbrooke of the Poultry Research and Teaching Unit of Iowa State University, and also like to thank M. Jeffrey, K. Nesheim, M. Higgins, J. Green, J. Hanson and J. Santiago for assistance in conducting this experiment.
REFERENCES


Table 4.1 Composition of diets fed to broiler chickens in from 1 to 28 days in Experiment 1 and from 1 to 21 days in Experiment 2.

<table>
<thead>
<tr>
<th>Ingredient Composition</th>
<th>1 to 14 days</th>
<th>15 to 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>558.2</td>
<td>591.3</td>
</tr>
<tr>
<td>Soybean meal (48% CP)</td>
<td>284.1</td>
<td>214.7</td>
</tr>
<tr>
<td>Dried distiller grains with solubles</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Meat/bone meal</td>
<td>20.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>50.5</td>
<td>48.9</td>
</tr>
<tr>
<td>Salt</td>
<td>4.0</td>
<td>2.6</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>2.8</td>
<td>2.5</td>
</tr>
<tr>
<td>L-lysine(^1)</td>
<td>2.5</td>
<td>3.0</td>
</tr>
<tr>
<td>L-threonine</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Limestone</td>
<td>7.9</td>
<td>7.1</td>
</tr>
<tr>
<td>Di-calcium phosphate</td>
<td>12.7</td>
<td>6.1</td>
</tr>
<tr>
<td>Choline chloride (60%)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin and mineral premix(^2)</td>
<td>6.3</td>
<td>5.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical Composition (Calculated)</th>
<th>1 to 14 days</th>
<th>15 to 28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME (MJ/kg)</td>
<td>13.39</td>
<td>13.18</td>
</tr>
<tr>
<td>Crude protein</td>
<td>210.0</td>
<td>185.0</td>
</tr>
<tr>
<td>Ether extract</td>
<td>82.0</td>
<td>81.8</td>
</tr>
<tr>
<td>Calcium</td>
<td>9.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Non-phytate phosphorus</td>
<td>4.5</td>
<td>3.7</td>
</tr>
<tr>
<td>Digestible methionine + cysteine</td>
<td>8.6</td>
<td>7.7</td>
</tr>
<tr>
<td>Digestible lysine</td>
<td>10.9</td>
<td>9.5</td>
</tr>
<tr>
<td>Digestible threonine</td>
<td>7.2</td>
<td>6.8</td>
</tr>
</tbody>
</table>
1Contained 50.7% of L-lysine in the form of L-lysine sulfate, 0.1% Methionine, 0.1% Cystine, 0.3% Threonine, 0.1% Tryptophan, 0.6% Arginine, 0.3% Isoleucine, 0.5% Leucine, and 0.4% Valine.

2Provided per kg of premix: Selenium-40 mg; Vitamin A-1,320,000 IU; Vitamin D₃-440,000 IU; Menadione-176 mg; Vitamin B₁₂-1.9 mg; Biotin-6.6 mg; Choline-71.5 g; Folic acid-220 mg; Niacin-6.6 g; Pantothenic acid-1.8 g; Pyridoxine-176 mg; Riboflavin-880 mg; Thiamine-220 mg; Iron-45 g; Magnesium-20 g; Manganese-44 g; Zinc-44 g; Copper-4.4 g; Iodine-135 mg.
Table 4.2 Main effects of direct-fed microbial (DFM) and challenge on feed intake (FI), body weight gain (BWG) and feed efficiency (FE) of broiler chickens in Experiment 1.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Challenge</th>
<th>8-21 days</th>
<th>8-28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FI (kg/pen)</td>
<td>BWG (g/chick)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>8.53</td>
<td>581</td>
</tr>
<tr>
<td>Control + DFM</td>
<td></td>
<td>8.67</td>
<td>581</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>0.11</td>
<td>10</td>
</tr>
<tr>
<td>Sham</td>
<td></td>
<td>8.82</td>
<td>617</td>
</tr>
<tr>
<td>Challenge*</td>
<td></td>
<td>8.38</td>
<td>545</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>0.11</td>
<td>10</td>
</tr>
</tbody>
</table>

| Diet P value  | 0.36      | 0.97      | 0.25      | 0.40      | 0.78      | 0.57       |
| Challenge P value | ≤ 0.01  | ≤ 0.01    | ≤ 0.01    | 0.09      | ≤ 0.01    | ≤ 0.01     |
| Diet x Challenge P value | 0.48   | 0.91      | 0.36      | 0.75      | 0.72      | 0.92       |

*a,b Least square means within the same column without a common superscript differ significantly, \( P \leq 0.05 \)

\( n = 10 \) pens of 10 chicks each per group.

*Coccidial challenge was delivered with 10X dose of commercial vaccine at 9 days of age.
Table 4.3 Main effects of direct-fed microbial (DFM) and challenge on the ileal electrophysiological transport of D-glucose, DL-methionine (Met), L-Lysine (Lys), L-glutamine (Gln) and sodium phosphate dibasic (P), measured by modified Ussing chambers, in broiler chickens at day 21 in Experiment 1.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Challenge</th>
<th>Glucose (Δ μA/cm²)</th>
<th>Met</th>
<th>Lys</th>
<th>Gln</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.61</td>
<td>2.14</td>
</tr>
<tr>
<td>Control + DFM</td>
<td></td>
<td>2.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.99</td>
<td>3.14</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>0.11</td>
<td>0.11</td>
<td>0.15</td>
<td>0.18</td>
<td>0.42</td>
</tr>
<tr>
<td>Sham Challenge</td>
<td></td>
<td>1.52&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.52&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.22&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.57</td>
<td>1.85&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Challenge*</td>
<td></td>
<td>1.16&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.16&lt;sup&gt;B&lt;/sup&gt;</td>
<td>2.57&lt;sup&gt;B&lt;/sup&gt;</td>
<td>1.40</td>
<td>0.85&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>0.11</td>
<td>0.11</td>
<td>0.15</td>
<td>0.18</td>
<td>0.42</td>
</tr>
</tbody>
</table>

| Diet <i>P</i> value | 0.02 | 0.05 | ≤ 0.01 | 0.07 | 0.08 |
| Challenge <i>P</i> value | ≤ 0.01 | ≤ 0.01 | 0.01 | 0.14 | ≤ 0.01 |
| Diet x Challenge <i>P</i> value | 0.12 | 0.88 | 0.57 | 0.90 | 0.56 |

<sup>a,b</sup> Least square means within the same column without a common superscript differ significantly, <i>P</i> ≤ 0.05

<sup>n</sup> = 20 chicks per group.

* Coccidial challenge was delivered with 10X dose of commercial vaccine at 9 days of age.
Table 4.4 Effects of direct-fed microbial (DFM) on colon trans-epithelial electrical resistance (TER), apparent permeability co-efficient ($P_{\text{app}}$) for *Escherichia coli* lipopolysaccharides and ileal mucin (MUC2) mRNA expression at day 21 in broiler chickens raised under unsanitary condition in Experiment 2.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Colon TER (Ω/cm²)</th>
<th>Colon $P_{\text{app}}$ (µg/ml.min.cm²)</th>
<th>Ileal MUC2 (adjusted Ct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>191.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control + DFM</td>
<td>225.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
<td>8.26</td>
<td>0.35</td>
<td>1.9</td>
</tr>
</tbody>
</table>

$P$-value: ≤ 0.01 for Colon TER, ≤ 0.04 for Colon $P_{\text{app}}$, ≤ 0.01 for Ileal MUC2

<sup>a,b</sup> Least square means within the same column without a common superscript differ significantly, $P \leq 0.05$

n = 8 chicks per treatment.
**Figure 4.1** Interactions of direct-fed microbial (DFM) supplementation, with or without coccidial challenge* on ileal trans-epithelial electrical resistance, measured by modified Ussing chambers, of broiler chickens at day 21 in Experiment 1.

\[ \text{Ω/cm}^2 \]

<table>
<thead>
<tr>
<th></th>
<th>CON + Sham control</th>
<th>DFM + Sham control</th>
<th>CON + Coccidial</th>
<th>DFM + Coccidial</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>!bc</td>
<td>!c</td>
<td>!b</td>
<td>!a</td>
</tr>
</tbody>
</table>

\[ ^{a,b} \text{Least square mean columns without a common superscript differ significantly,} \; P < 0.01. \]
\[ ^n \text{= 10 chicks per treatment.} \]
\[ ^* \text{Coccidial challenge was delivered with 10X dose of commercial vaccine at 9 days of age.} \]
CHAPTER 5

EFFECTS OF EXOGENOUS ENZYMES AND DIRECT-FED MICROBIAL ON PERFORMANCE, ENERGY DIGESTIBILITY, ILEAL NUTRIENT TRANSPORT, INTESTINAL INTEGRITY AND CAMPYLOBACTER COLONIZATION IN LAYING HENS

A paper for submission to British Poultry Science Journal

ABSTRACT

1. An experiment was conducted to evaluate the effects of exogenous enzymes (EE) and direct-fed microbial (DFM) supplementation on performance, energy digestibility, nutrient transport, intestinal integrity and pathogen colonization of first-cycle (25-40 weeks) Hy-Line W36 laying hens.

2. Experimental diets were corn-soybean meal (SBM)-dried distiller’s grain with solubles (DDGS) based, with a control diet (CON) formulation to industry standards and a reduced energy (RE) diet similar to the CON but with a 0.42 MJ/kg reduction in ME. Two additional experimental diets were generated by the addition of EE only or the combination of EE and DFM to the RE diet.

3. There were no significant differences in feed intake, body weight, hen-day egg production, egg weight, egg mass, and egg characteristics, throughout the experiment. Supplementation of EE increased the nitrogen corrected apparent ME in comparison to RE fed birds over
week 38 ($P \leq 0.01$) and week 40 ($P = 0.03$), while a trend was observed in week 36 ($P = 0.09$).

4. Ileal transport of D-glucose and L-lysine was increased ($P \leq 0.01$) with EE supplementation. Supplementation of DFM with EE resulted in increased ileal mucin mRNA expression, and colon trans-epithelial electrical resistance ($P \leq 0.01$), and reduced colon endotoxin transport ($P \leq 0.01$) and *Campylobacter* spp. colonization ($P = 0.05$).

5. Supplementation of the EE and DFM combination in corn-SBM-DDGS based diets increased energy digestibility, enhanced gut integrity, and reduced zoonotic pathogen load in first-cycle laying hens.
INTRODUCTION

Dietary energy is an expensive component of poultry feed and will continue with the increased cost of dietary corn and oil (Pardue, 2010). As laying hen feed constitutes nearly 75% of the cost of egg production (Donohue and Cunningham, 2009), effective and complete utilization of dietary energy is essential for efficient and sustainable production. Another concern for poultry production is the energetic cost of sub-clinical microbial challenge, which increase energy expenditure towards activation of acute phase immune response (Klasing and Johnstone, 1991). Using dietary additives to increase the effective utilization of dietary energy and to increase intestinal integrity can increase the energetic efficiency of the birds under sub-clinical pathogenic challenges which are common in modern poultry production.

Numerous research reports have explored the efficacy of exogenous enzymes (EE) in releasing energy from corn-soybean meal-based diets fed to laying hens (Yörük et al., 2006; Gunawardana et al., 2009). Direct-fed microbial (DFM) are viable commensal micro-organisms which can reduce colonization of pathogenic bacteria by competitive exclusion (Xu et al., 2006). Moreover, supplementation of DFM to laying hen diet has been found to increase egg production, FI:egg mass, egg weight and specific gravity (Balevi et al., 2001). In addition to influencing host nutrient absorption, EE may produce additional breakdown products of non-starch polysaccharides that can be used by specific populations of intestinal microbes (Bedford and Cowieson, 2011). These breakdown products may have value in combination with DFM as additional substrates for microbial fermentation to further enhance the efficiency of energy release. However, little information has been reported concerning the benefits of EE and DFM when fed in combination. Therefore, the objective of this experiment was to determine the effects of the combination of EE
and DFM on performance, energy digestibility, nutrient transport, intestinal integrity and pathogen colonization in first-cycle laying hens.

MATERIALS AND METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University before the initiation of the experiment.

Animals and housing
A total of 288, 22 weeks old Hy-Line W36 (Hy-Line International, Dallas Center, IA) laying hens were transferred from a local commercial facility to the Poultry Research and Teaching Unit of Iowa State University. Hens were provided a transition period and standard commercial type diet until the end of week 24 to allow for adjustment to the experimental facilities before experimental diets were administered (from 25 to 40 weeks of hen age). An experimental unit (EU) was defined as 3 adjacent cages of 3 hens each (439 cm²/hen), resulting in 8 EU per dietary treatment. Hens were allocated to EU based on body weight (BW) and dietary treatments were randomly assigned to EU at the start of experiment. The photoperiod provided was 13L:11D at the start of the experiment and light was increased 0.5 h per week till 16L:8D was reached. House temperature was maintained between 21°C to 24°C throughout the experimental period.

Diets
Dietary treatments included a control (CON) formulated to meet or exceed breeder recommendations (Hy-Line, 2011) with 12.14 MJ/kg ME, a reduced energy diet (RE) with 11.72
MJ/kg ME, the RE with EE (REE), and the RE with EE and DFM (RED). All diets were corn-soybean meal-dried distiller’s grain with solubles based and differences in dietary energy were generated by the removal of fat (Table 5.1). The EE used was a cocktail containing xylanase, amylase and protease, while the DFM contained 3 different strains of *Bacillus subtilis*. Both EE and DFM were added at a dose rate of 375 mg/kg of diet and 500 mg/kg of diet, respectively, as recommended by the manufacturer. All diets were supplemented with 300 FTU phytase enzyme at a dose of 100 mg/kg of diet. Titanium dioxide was added to late phase diets (from 34 to 40 weeks) at a dose of 2.5 g/kg of diet as an inert dietary marker for digestibility assay.

Sample collection

Hens were monitored twice daily and mortalities were removed from cages, weighed and recorded as they occurred. Feed and water were provided *ad-libitum* and feed intake (FI) was determined weekly after correcting for mortality. Eggs were collected daily at 10:00 ± 1:00 h and recorded by EU as hen-day egg production (HDEP). Hens were weighed by cage at the initiation of experiment and every 4-weeks thereafter. Eggs were collected for 5-days every 4-weeks to determine egg weight, egg mass, and egg characteristics such as egg solids, shell weight, yolk weight, albumen weight and Haugh unit. Excreta samples were collected for 48-h during week 36, 38 and 40 to determine nitrogen corrected apparent ME (AMEn). Colon swabs were collected aseptically from all hens fed REE and RED diets at 40 weeks. Fifteen cm long cotton-tipped applicators (Harwood Products, Guilford, ME) were inserted approximately 7.5 cm into the cloaca, with care taken to avoid contact with the surrounding feathers and skin. Lower half of the wooden applicator with cotton end was placed into a 16- by 125-mm round-bottomed polystyrene tube (Becton-Dickinson, Franklin Lakes, NJ) containing 13.5 ml blood-free enrichment broth (BFEB), allowing for the
requisite headspace air to take up 16 to 17% of the tube’s total capacity. Tubes were transported to the laboratory, where they were immediately incubated (24 h, 42°C, ambient atmosphere).

On the last day of experiment, one hen per EU was euthanized by carbon dioxide asphyxiation. Hens were then dissected for collection of intestinal tissue samples. Ileum was defined as the segment from the point of Meckel’s diverticulum to the ileo-cecal junction. A sample of ileal cross section (0.5 mm height) was collected from mid-ileum to determine morphology. Additional 4 cm ileal mucosal tissue samples were collected from mid-ileum for nutrient transport. Approximately a 4 cm sample of ileal tissue was utilized for ileal mucosal scraping collection from mid-ileum to determine mucin mRNA expression. Colon tissue samples (4 cm) were collected from the ileo-cecal junction to the cloaca from one randomly selected hen per EU. Colon tissue and swab samples were only collected from REE and RED group hens to determine the effects of DFM supplementation on colon integrity and *Campylobacter* spp., colonization.

*Performance and energy digestibility*

Mean egg weight was determined by weighing eggs collected over a 5-day period every 4 weeks. Egg mass was calculated using egg weight and HDEP during the 5-day period. FI:egg mass ratio was calculated using FI and egg mass produced per week. Eggs (5 per EU) were used to determine percent egg solids (Gunawardana *et al.*, 2009). An additional 5 eggs per EU were used to determine egg characteristics including shell, albumen and yolk weight and Haugh units (Silversides and Villeneuve, 1994; Silversides and Budgell, 2004). All excreta and feed samples were analyzed in duplicate to determine gross energy, nitrogen, and titanium dioxide levels to calculate AMEn (Murugesan and Persia, 2013).
**Ileal morphology**

Segments of ileum were cleaned, processed, fixed in formalin, stained with hematoxylin-eosin, and 3 histological slides were prepared from 3 different cross-sections (5 µm thick) of each segment (Yi et al., 2005). Images were obtained from the slides using an Olympus IX70® inverted microscope (Olympus America, Inc., Melville, NY), processed with a Vay Tek® deconvolution imaging system (Vay Tek, Fairfield, IA). Villus height (VH) and crypt depth (CD) were measured from the images using Image-Pro Plus® software (Media Cybernetics, Silver Spring, MD) (Applegate et al., 1999; Touchette et al., 2002). Measurements of 10 complete villi for VH and associated crypts for CD were taken from each segment. Ratio of VH:CD was calculated from VH and CD measurements for each segment.

**Trans-epithelial electrical resistance**

Freshly harvested ileal and colon tissues were used to measure the ileal trans-epithelial electrical resistance (TER). Intestinal tissue samples were immediately excised from the euthanized birds and placed into chilled, aerated Krebs-Henseleit buffer (pH 7.4) for transfer to the lab for continuous analysis. Once clamped in the Ussing chambers (Physiologic Instruments, San Diego, CA), the samples were continuously aerated with 95% O₂ + 5% CO₂ mixture and both the apical and basolateral sides were immersed in 8 ml of Krebs buffer. After a short-circuit current was established and stabilized (about 5 to 10 min), basal resistance measurements were recorded using Acquire & Analyze® software (Physiologic Instruments, San Diego, CA) (Albin et al., 2007). The epithelial resistance was expressed as Ω/cm².
Electrophysiological nutrient transport

The electrophysiological nutrient transport at the ileum was determined using modified Ussing chamber by measuring the changes in basal short circuit current ($\Delta \mu\text{Isc/cm}^2$) induced by the addition of specific nutrients (Albin et al., 2007). D-Glucose (glucose) was the first nutrient added, at a concentration of 10 mM, to the mucosal chamber and 10 mM mannitol was added to the basolateral chamber as an osmotic control. The potential difference across the tissue was measured for 30 min after challenge by open-circuit conditions for every 10 sec due to the Isc being delivered by a voltage clamp apparatus. The change in maximal current was recorded, and tissue conductance was calculated from Isc with potential difference using Ohm's law (Gabler et al., 2009). The transport of DL-methionine (Met), L-lysine (Lys), L-glutamine (Gln), and phosphorus (Sodium phosphate di-basic; P) were examined in this order, at a concentration of 7.5 mM, and the results were expressed as $\Delta \mu\text{A/cm}^2$.

Lipopolysaccharide permeability

The lipopolysaccharide (LPS) permeability co-efficient for colon epithelial tissues were determined using fluorescein isothiocyanate labeled LPS (FITC-LPS) from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO) as outlined by Tomita et al. (2004) using Ussing chambers. After a 20 µg/ml FITC-LPS was applied to the mucosal side of colon tissue, and serosal FITC-LPS concentrations were determined at intervals of 20 min. This sampled FITC-LPS concentrations were determined using a Synergy-4® fluorescence spectrophotometer (BioTek US, Winooski, VT) at an excitation wavelength of 495 nm and an emission wavelength of 525 nm. The apparent permeability co-efficient ($P_{\text{app}}$) was calculated for each tissue as follows:

$$P_{\text{app}} = \frac{dQ}{dt \times A \times C_0}$$
Where: $\frac{dQ}{dt}$ is the transport rate (µg/s) and corresponds to the slope of the regression line, $C_0$ is the initial concentration in the mucosal side of the chamber (µg/ml) and $A$ is the area of the membrane which was 1 cm$^2$ (Tomita et al., 2004). The LPS permeability co-efficient results were expressed as µg/ml.min.cm$^2$.

**Ileal mucin (MUC2) mRNA expression**

Ileal mucosal scrapings for total mRNA extraction were stored in Ambion® RNA later (Life Technologies, Grand Island, NY) immediately upon collection. The scrapings were then washed with Phosphate-Buffer Saline (pH 7.2), homogenized and transferred to -80°C for final storage. Total RNA was isolated using Ambion® RNAqueous kit (Life Technologies, Grand Island, NY) from the homogenized samples. Isolated RNA was treated with Ambion® DNA-free kit (Life Technologies, Grand Island, NY) to remove any DNA contamination. The final RNA concentration was diluted to 50 ng/µl by measuring the concentration using Nanodrop1000® spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA) at 260 nm and 280 nm.

The mRNA expression levels of MUC2 were determined by quantitative real time RT-PCR, using QuantiTect SYBR Green® RT-PCR kit (Qiagen Inc., Valencia, CA). The RT-PCR was carried out with primers from chicken similar to MUC2 precursor (Intestinal mucin 2; *Gallus gallus*, XM-421035; forward: 5’-GCTGATTGTC ACTCACGCCTT-3’; reverse: 5’-ATCTGCCTGAATCACAGGTGC-3’) (Smirnov et al., 2006) and primers from the *Gallus gallus* 28S ribosomal RNA gene (primer sequence forward: 5’-GGCGAAGCCAGGAAACT-3’; reverse: 5’GACGACCGTTTGACACGTC-3’). Each RT-PCR reaction was ran in triplicate and consisted of 50 ng of total RNA, 12.5 µl QuantiTect SYBR Green master mix, 0.25 µl QuantiTect RT mix, forward-reverse primers and RNAse-free water for a final volume of 25 µl. The RT-PCR
reactions were performed on a MyiQ single color real-time PCR detection system (Bio-Rad Laboratories, Philadelphia, PA) with the following program: reverse transcription at 50°C for 30 min, initial activation at 95°C for 15 min followed by 40 cycles of denaturing at 94°C for 15 sec, annealing at 59°C for 30 sec and extension at 72°C for 30 sec followed by reading of the plate. Gene slopes were determined with a series of ten-fold plasmid dilutions. A melting curve from 65°C to 95°C with a reading at every 1°C was also performed for each individual RT-PCR plate (Cheeseman et al., 2007). Cycle threshold (Ct) values were calculated and adjusted relative to 28S expression, as follows:

\[40 - (\text{Mean Ct}_{\text{MUC2 gene}} + [\text{Median 28S} - \text{Mean Ct 28S}]) \times (\text{Slope}_{\text{MUC2 gene}} / \text{Slope 28S})]\]

Where: \(\text{Ct}_{\text{MUC2 gene}}\) = mean of the triplicate cycle threshold (Ct) values of the gene tested (MUC2); \(\text{Ct 28S}\) = mean of the triplicate Ct value of the housekeeping gene (28S); Mean 28S = overall experimental mean of Ct 28S; \(\text{Slope}_{\text{MUC2 gene}}\) = slope from 10-fold test gene standard regression equation; and \(\text{Slope 28S}\) = slope from 10-fold 28S standard regression equation (Abasht et al., 2008). All protocols were followed according to manufacturer’s instructions provided with the kits.

*Campylobacter colonization*

Colonization levels of *Campylobacter coli*, *Campylobacter jejuni* and *Campylobacter lari* in REE and RED fed hens were quantified from colon swabs as described by Wesley et al. (2005). Briefly, an aliquot (50 µl) of BFEB enriched colon swab content was streaked onto Campy-cefex agar and incubated (48 h, 42°C) micro-aerobically (5% O₂, 10% CO₂, 85% N₂) in a three-gas incubator (Forma Scientific, Marietta, Ohio). Three presumptive *Campylobacter* colonies from each Campy-cefex agar plate (flat, shiny, and mucoid, with a pink hue) were randomly picked to
ensure an even representation of colony types. The colonies were sub-cultured on brain heart infusion agar (Becton Dickinson, Sparks, MD) supplemented with 0.6% yeast extract and 10% defibrinated sheep blood and then were incubated micro-aerobically (24 h, 42°C). Presumptive colonies were identified by use of a multiplex PCR assay and the colonies that were not identified as either C. jejuni or C. coli were further subjected to a PCR for the Campylobacter genus and then a PCR for C. lari (Wesley et al., 2005). All isolates confirmed as Campylobacter spp. were stored in brain heart infusion broth (Becton Dickinson, Sparks, Md.) with 20% glycerol (-80°C).

Statistical analysis

Statistical analysis was carried out using a completely randomized design. Data were analyzed by MIXED procedure of SAS (SAS, 2012) using replicates of 3 cages of 3 hens each as an experimental unit for HDEP, FI, BW, Feed:eggmass, egg characteristics, AMEn and Campylobacter spp., colonization. Individual hen was used as an experimental unit for ileal nutrient transport, TER, mucin mRNA expression, colon TER and Papp. Student’s t-test (α = 0.05; t = 2.08596) was used to separate significant least square means with the probability of type-I error set at P ≤ 0.05. Significance was accepted at P ≤ 0.05 with trends noted at P ≤ 0.10.

RESULTS

Performance

There were no differences among dietary treatments for FI (P = 0.11), HDEP (P = 0.34), or FI:egg mass (P = 0.33, Table 5.2). Dietary treatment did not result in significant differences in BW, egg
mass, egg solids, or Haugh units (Fig. 5.1), and egg, shell, albumen, or yolk weight (Fig. 5.2). The number of mortalities for CON, RE, REE, and RED were 5, 1, 3, and 3, respectively.

**Energy digestibility**

There were no differences observed among the dietary treatments in AMEn at week 36, however, a trend was noted with EE supplemented hens (REE or RED) having higher AMEn compared to the RE fed hens ($P = 0.09$; Table 5.3). Hens fed RE diets had reduced AMEn in comparison to hens fed the CON diets and both EE supplementation as well as EE and DFM supplementation increased hen AMEn over RE fed hens at week 38 ($P \leq 0.01$) and week 40 ($P = 0.03$).

**Ileal electrophysiological nutrient transport**

The Isc for ileal mucosal transport of D-glucose and L-lysine were increased in hens fed both REE and RED diets compared to hens fed either CON or RE diets ($P \leq 0.01$; Table 5.4). There were no significant differences in either DL-methionine or L-glutamine flux among the dietary treatments.

**Ileal and colon epithelial integrity**

There were no differences ($P > 0.05$) among dietary treatments in ileal VH, CD and VH:CD. The least square means for VH (SEM = 39.5) are 569.1, 507.4, 521.6, and 544.0, CD (SEM = 10.0) are 117.0, 112.0, 103.1, and 119.0, and VH:CD (SEM = 0.4) are 5.1, 4.6, 5.2, and 4.7, for CON, RE, REE and RED respectively. Hens fed RED diet in this experiment had increased ileal mucin (MUC2) mRNA expression compared to hens fed other dietary treatments ($P \leq 0.01$; Table 5.4). Ileal TER did not differ among dietary treatments ($P = 0.60$; Table 5.4), but hens fed RED diet
had increased TER for colon epithelia compared to hens fed RE diet ($P \leq 0.01$; Table 5.5). The $P_{\text{app}}$ for LPS from the apical to basolateral side of colon epithelia was reduced in hens fed RED diet in comparison to hens fed RE diet ($P \leq 0.01$; Table 5.5).

*Campylobacter colonization in colon*

The colonization levels of *C. coli* and *C. jejuni* were decreased in hens fed RED diet in comparison with hens fed RE diet ($P = 0.05$; Table 5.5). No detectable levels of *C. lari* were observed in either REE or RED fed hens.

**DISCUSSION**

The results of HDEP are consistent with published data on Hy-Line W36 laying hens, in that short-term reduction of dietary energy (Harms et al., 2000), with or without EE supplementation did not affect performance (Scheideler et al., 2005; Jalal et al., 2007). In contrast, larger framed laying hens may have a greater ability to respond to dietary energy as the combination of xylanase and $\beta$-glucanase fed to ISA Brown hens from 45-57 weeks increased BW and improved FI:egg mass (Mathlouthi et al., 2003). A cocktail of EE fed to Lohmann hens from 30-46 weeks resulted in reduced FI and FI:egg mass (Yörük et al., 2006). The same trend of strain differences have been noted with DFM supplementation as *Bacillus subtilis* did not affect FI, HDEP, FI:egg mass, egg weight, egg mass and Haugh units in 28-39 weeks old Hy-Line W36 hens (Mahdavi et al., 2005). In contrast, supplementation of *B. subtilis* has been found to increase HDEP and decrease FI:egg mass in Brown-Nick hybrids and in Lohmann Brown layers (Kurtoglu et al., 2004; Xu et al., 2006), while reduced FI:egg mass was observed in 40-53 weeks old HiSex Brown layers (Balevi et al.,...
2001). The above results are in agreement with limited available peer-reviewed data that may indicate that Hy-Line W36 egg production rates are less sensitive to differences in dietary energy, at least over initial stages of egg production, (Murugesan and Persia, 2013).

The current AMEn data are in agreement with previous research that supplementation of EE increased energy digestibility in laying hens (Mathlouthi et al., 2003; Pirgozliev et al., 2010). However, published data in Hy-Line W36 laying hens have been mixed as EE did not change AMEn in several reports (Scheideler et al., 2005; Jalal et al., 2007; Gunawardana et al., 2009). Increased transport of glucose and Lys by EE in this experiment suggests that EE increased luminal concentration of these nutrients through increased digestion. The activity of mucosal transporters increase monotonically with increased substrate concentration, such as sugars and amino acids, at the mucosa (Diamond and Karasov, 1987). Hence the increased electrophysiological transport of glucose and Lys validate the increased dietary energy digestibility for EE fed hens.

Previous research has shown that broiler chicks fed B. subtilis had increased ileal VH but there were no changes in CD or VH:CD (Lee et al., 2010). In the present experiment, absence of changes in ileal morphology may be associated with the adult birds’ ability to regenerate epithelial cells suggesting a more robust challenge may be needed to result in differences in intestinal morphology. To further assess the effects of EE and DFM on ileal integrity, the ileal mucin gene (MUC2) expression was assayed. The overlying mucous-gel layer is the first line of defense the pathogens encounter when trying to traverse intestinal mucosa (Smirnov et al., 2005). In chickens, MUC2 mucins are observed to be widely expressed in the goblet cells of small intestine and colon (Smirnov et al., 2005). Mucin expression data from the current experiment are in agreement with published data outside laying hens by Caballero-Franco et al. (2007), that DFM may mediate
intestinal mucin up-regulation to increase the colonization resistance against pathogens or their receptors.

Previous research has demonstrated that TER is the best direct indicator of TJ barrier function and therefore epithelial integrity (Putaala et al., 2008). Increased TER for colon epithelia in the present experiment indicates enhanced TJ barrier function and increased TJ integrity at the colon in DFM supplemented chicks compared to chicks fed diets without DFM. These data are in agreement with the results from Mennigen et al. (2009) that supplementation of DFM protected the colon epithelial barrier by preventing decreased TJ protein expression in a murine model of colitis. In addition to the current data on reduced LPS permeability by DFM, bioactive metabolites produced by DFM increased TJ integrity protecting epithelia from the deleterious effects of pathogenic bacteria under in vitro conditions (Putaala et al., 2008). Reduced colon LPS permeability by DFM in the current experiment validates the increased colon TER response observed, indicating increased TJ barrier function in the colon.

Over the last three decades, Campylobacter spp. have emerged as a major cause of acute bacterial enteritis in humans (Van Vliet and Ketley, 2001). Campylobacter spp. associated with food poisoning include C. jejuni which is predominant, and C. coli and C. lari which account for most of the remainder (Hariharan et al., 2004). The reduced colonization levels of C. coli and C. jejuni are in agreement with published data that supplementation of DFM reduced shedding of Campylobacter spp. in broiler chicks (Morishita et al., 1997). Moreover, increased colon TER and Papp for LPS, indicating increased colon epithelial integrity, are in agreement with the reduced colonization of Campylobacter spp., in the present experiment.

In conclusion, reducing dietary energy did not alter BW or hen performance parameters over the 16 week experimental period, even though the combination of EE alone or in combination
with DFM increased AMEn in laying hen diets. This suggests that at least over the short-term Hy-Line W36 hens are less sensitive to productive interruptions due to dietary energy. Supplementation of EE increased ileal nutrient uptake, while DFM addition increased intestinal epithelial barrier function and integrity and reduced *Campylobacter spp.* colonization compared to hens fed control diets. It appears that EE and DFM have independent effects in layer production and can be used in combination for increased dietary energy availability as well as improved intestinal integrity.

**ACKNOWLEDGEMENTS**

We recognize the care for the birds provided by W. Larson, J. Tjelta, W. Rogers, and R. Holbrooke of the Poultry Research and Teaching Unit of Iowa State University, and also like to thank M. Jeffrey, K. Nesheim, M. Higgins, N. Nachtrieb and J. Green for assistance in conducting this experiment.
REFERENCES


Table 5.1 Composition of diets fed to first-cycle laying hens from 25 to 40 weeks of hen age.

<table>
<thead>
<tr>
<th>Ingredient Composition</th>
<th>25 to 33 weeks</th>
<th>34 to 40 weeks</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control (g/kg)</td>
<td>RE1 (g/kg)</td>
</tr>
<tr>
<td>Corn</td>
<td>487.9</td>
<td>514.0</td>
</tr>
<tr>
<td>Soybean meal (48% CP)</td>
<td>199.7</td>
<td>195.0</td>
</tr>
<tr>
<td>Dried distillers grains with solubles</td>
<td>150.0</td>
<td>150.0</td>
</tr>
<tr>
<td>Animal and vegetable fat</td>
<td>46.3</td>
<td>24.9</td>
</tr>
<tr>
<td>Salt</td>
<td>3.8</td>
<td>3.8</td>
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<tr>
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<td>1.8</td>
</tr>
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<td>Choline chloride</td>
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<td>1.0</td>
</tr>
<tr>
<td>Vitamin and mineral premix3</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Phytase4</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Titanium dioxide</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical Composition (Calculated)</th>
<th>25 to 33 weeks</th>
<th>34 to 40 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME (MJ/kg)</td>
<td>12.14</td>
<td>11.72</td>
</tr>
<tr>
<td>Crude protein</td>
<td>180.0</td>
<td>179.9</td>
</tr>
<tr>
<td>Ether extract</td>
<td>81.0</td>
<td>60.9</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>29.0</td>
<td>29.5</td>
</tr>
<tr>
<td>Calcium</td>
<td>38.9</td>
<td>38.9</td>
</tr>
<tr>
<td>Non-phytate phosphorus</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Digestible methionine + cysteine</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td>Digestible lysine</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Digestible threonine</td>
<td>6.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>
1 Contained 0.42 MJ/kg reduced ME compared to control diet.
2 Contained 50.7% of L-lysine in the form of L-lysine sulfate, 0.1% Methionine, 0.1% Cystine, 0.3% Threonine, 0.1% Tryptophan, 0.6% Arginine, 0.3% Isoleucine, 0.5% Leucine, and 0.4% Valine.
3 Provided per kg of diet: Selenium-200 µg; Vitamin A-6,600 IU; Vitamin D₃-2,200 IU; Vitamin E-14.3 IU; Menadione-880 µg; Vitamin B₁₂-9.4 µg; Biotin-33 µg; Choline-358 mg; Folic acid-1.1 mg; Niacin-33 mg; Pantothenic acid-8.8 mg; Pyridoxine-880 µg; Riboflavin-4.4 mg; Thiamine-1.1 mg; Iron-226 mg; Magnesium-100 mg; Manganese-220 mg; Zinc-220 mg; Copper-22 mg; Iodine-675 µg.
4 Contained 300 FTU phytase enzyme activity.
Table 5.2 Effects of dietary supplementation of exogenous enzymes (EE) and direct-fed microbial (DFM) from 25 to 40 weeks of hen age on hen-day egg production, feed intake and feed:egg mass ratio.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hen-day (eggs/100 birds/day)</th>
<th>Feed intake (g/day/hen)</th>
<th>Feed : Egg mass ratio (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.6</td>
<td>98.5</td>
<td>1.85</td>
</tr>
<tr>
<td>Reduced ME (RE)²</td>
<td>94.0</td>
<td>99.9</td>
<td>1.85</td>
</tr>
<tr>
<td>RE + EE (REE)</td>
<td>96.0</td>
<td>101.9</td>
<td>1.83</td>
</tr>
<tr>
<td>RE + EE + DFM (RED)</td>
<td>94.8</td>
<td>100.4</td>
<td>1.83</td>
</tr>
<tr>
<td>SEM</td>
<td>1.3</td>
<td>0.9</td>
<td>0.03</td>
</tr>
<tr>
<td><em>P</em>-value</td>
<td>0.34</td>
<td>0.11</td>
<td>0.87</td>
</tr>
</tbody>
</table>

*ab* Least square means within the same column without a common superscript differ significantly, *P* ≤ 0.05

n = 8 experimental units of 9 hens each, per treatment.

¹Contained 0.42 MJ/kg reduced ME compared to control diet.
Table 5.3 Effects of dietary supplementation of exogenous enzymes (EE) and direct-fed microbial (DFM) from 25 to 40 weeks of hen age on total tract nitrogen-corrected apparent metabolizable energy.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Week 36 (MJ/kg)</th>
<th>Week 38 (MJ/kg)</th>
<th>Week 40 (MJ/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.11</td>
<td>14.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.56&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reduced ME (RE)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>13.94</td>
<td>13.60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>RE + EE (REE)</td>
<td>14.44</td>
<td>14.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.10&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>RE + EE + DFM (RED)</td>
<td>14.40</td>
<td>14.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.46&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

SEM 0.16 0.15 0.14

<sup>P-value</sup> 0.09 ≤ 0.01 0.03

<sup>a,b</sup> Least square means within the same column without a common superscript differ significantly, <i>P ≤ 0.05</i>

n = 8 hens per treatment.

<sup>1</sup>Contained 0.42 MJ/kg reduced ME compared to control diet.
Table 5.4 | Effects of dietary supplementation of exogenous enzymes (EE) and direct-fed microbial (DFM) from 25 to 40 weeks of hen age on ileal electrophysiological transport of nutrients, trans-epithelial electrical resistance (TER) and mucin (MUC2) mRNA expression.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>D-Glucose ($\Delta \mu$A/cm²)</th>
<th>DL-Methionine ($\Delta \mu$A/cm²)</th>
<th>L-Lysine ($\Delta \mu$A/cm²)</th>
<th>L-Glutamine ($\Delta \mu$A/cm²)</th>
<th>TER (Ω/cm²)</th>
<th>MUC2 mRNA²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.78b</td>
<td>0.87</td>
<td>2.25b</td>
<td>1.42</td>
<td>1437</td>
<td>15.3b</td>
</tr>
<tr>
<td>Reduced ME (RE)</td>
<td>1.46b</td>
<td>1.89</td>
<td>2.33b</td>
<td>2.39</td>
<td>1348</td>
<td>15.1b</td>
</tr>
<tr>
<td>RE + EE (REE)</td>
<td>3.85a</td>
<td>2.14</td>
<td>4.59a</td>
<td>3.12</td>
<td>1245</td>
<td>14.6b</td>
</tr>
<tr>
<td>RE + EE + DFM (RED)</td>
<td>4.77a</td>
<td>2.09</td>
<td>4.29a</td>
<td>2.18</td>
<td>1277</td>
<td>20.1a</td>
</tr>
<tr>
<td>SEM</td>
<td>0.63</td>
<td>0.56</td>
<td>0.86</td>
<td>1.02</td>
<td>103</td>
<td>1.2</td>
</tr>
<tr>
<td>P-value</td>
<td>≤ 0.01</td>
<td>0.37</td>
<td>≤ 0.01</td>
<td>0.69</td>
<td>0.60</td>
<td>≤ 0.01</td>
</tr>
</tbody>
</table>

a,b Least square means within the same column without a common superscript differ significantly, $P \leq 0.05$

n = 8 hens per treatment.

1 Contained 0.42 MJ/kg reduced ME compared to control diet.

2 Adjusted Ct values.
Table 5.5 Effects of dietary supplementation of exogenous enzymes (EE) and direct-fed microbial (DFM) from 25 to 40 weeks of hen age on colon trans-epithelial electrical resistance (TER), apparent permeability co-efficient (Papp) for *Escherichia coli* lipopolysaccharides, and *Campylobacter spp.*, colonization.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>TER* (Ω/cm²)</th>
<th>Papp* (µg/ml.min.cm²)</th>
<th>C. coli† (× 10⁶ cfu/g)</th>
<th>C. jejuni† (× 10⁶ cfu/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RE¹ + EE (REE)</td>
<td>818b</td>
<td>2.70ᵃ</td>
<td>6.64ᵃ</td>
<td>0.53ᵃ</td>
</tr>
<tr>
<td>RE + EE + DFM (RED)</td>
<td>956ᵃ</td>
<td>1.58ᵇ</td>
<td>4.44ᵇ</td>
<td>0.29ᵇ</td>
</tr>
<tr>
<td>SEM</td>
<td>29</td>
<td>0.35</td>
<td>0.62</td>
<td>0.08</td>
</tr>
<tr>
<td>P-value</td>
<td>≤ 0.01</td>
<td>≤ 0.01</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

ᵃᵇ Least square means within the same column without a common superscript differ significantly, P ≤ 0.05

*ⁿ = 8 hens per treatment; †ⁿ = 72 hens per treatment.

¹Reduced ME: contained 0.42 MJ/kg reduced ME compared to control diet.
Figure 5.1 Effects of dietary supplementation of exogenous enzymes (EE) and direct-fed microbial (DFM) from 25 to 40 weeks of hen age on body weight and egg characteristics.

- Least square mean columns within the same graph without a common superscript differ significantly, $P < 0.05$.
- $n = 40$ hens per treatment; †$n = 8$ experimental units of 5 eggs each, per treatment.
- CON = Control; RE = Reduced Energy; REE = RE + EE; RED = RE + EE + DFM.
**Figure 5.2** Effects of dietary supplementation of exogenous enzymes (EE) and direct-fed microbial (DFM) from 25 to 40 weeks of hen age on egg composition based on total egg weight (g).

![Bar chart showing egg composition by week and treatment]

- a,b Least square mean columns without a common superscript differ significantly, $P < 0.05$
- $n = 8$ experimental units of 5 eggs each, per treatment.
- **CON** = Control; **RE** = Reduced Energy; **REE** = RE + EE; **RED** = RE + EE + DFM.
CHAPTER 6

EFFECTS OF EXOGENOUS ENZYMES AND DIRECT-FED MICROBIAL ON NUTRIENT AND ENERGY DIGESTIBILITY, ILEAL BRUSH BORDER DIGESTIVE ENZYME ACTIVITY AND CECAL SHORT-CHAIN FATTY ACID CONCENTRATION IN BROILER CHICKENS

A paper for submission to British Poultry Science Journal

ABSTRACT

1. Two experiments were conducted to determine the effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on dietary energy and nutrient utilization in broiler chickens.

2. In the first experiment, Ross 308 broiler chicks were fed diets supplemented with EE and DFM in a 2 x 2 factorial arrangement. The 4 diets (control (CON), CON + EE, CON + DFM, and CON + EE + DFM) were fed from 14-21 days of age.

3. The significant interaction ($P \leq 0.01$) between EE and DFM for starch, crude protein, and amino acid apparent ileal digestibility co-efficient indicates that both additives increased the digestibility in a non-additive manner. The lack of interaction, but significant main effects ($P \leq 0.01$) for EE or DFM for nitrogen corrected apparent ME (AMEn) indicates an additive response.

4. In a follow-up experiment, Ross 308 broiler chicks were fed the same experimental diets from 1-21 days. Ileal mucosa, cecal content and serum samples were collected on day 21, to explore the possible modes of action.
5. Activities of ileal brush border maltase, sucrase, and L-alanine aminopeptidase were increased \((P \leq 0.01)\) by EE addition. The proportion of cecal butyrate was increased \((P \leq 0.01)\) by DFM addition.

6. Increases in nutrient utilization, including starch, crude protein and amino acids involve separate but complementary mechanisms for EE and DFM, in contrast AMEn responses are separate and additive.
Supplementation of poultry diets with exogenous enzymes (EE) results in increased dietary energy and protein release in broilers by degradation of non-starch polysaccharides (NSP), resulting in increased substrate and endogenous digestive enzyme activity (Cowieson and Ravindran, 2008). The amount of insoluble NSP present in corn and soybean meal (SBM) results in an ideal opportunity to use EE, particularly with xylanase, in corn-SBM based broiler diets. Various combinations of EE containing amylase, protease, and xylanase have been found to improve the digestibility of energy (Kocher et al., 2003; Cowieson and Ravindran, 2008; Zhou et al., 2009), as well as amino acids (AA) and protein (Rutherfurd et al., 2007; Cowieson and Ravindran, 2008).

Direct-fed microbial (DFM) are commensal micro-organisms used as feed additives and have beneficial effects in reducing the colonization of pathogenic bacteria and improving the performance in chickens (Jin et al., 1998). A growing body of scientific research has extensively demonstrated the beneficial effects of DFM which include i) performance, ii) nutrient digestibility, iii) modulation of intestinal microflora, iv) pathogen inhibition, and v) immunomodulation and gut mucosal immunity (Mountzouris et al., 2010). The inclusion of a DFM in broiler diets has been reported to modify dietary nutrient uptake by increasing energy (Li et al., 2008; Mountzouris et al., 2010), and protein digestibility (Apata, 2008; Li et al., 2008). Furthermore, DFM may alter the metabolism of nutrients, particularly AA, in the lumen of the small intestine (Snel et al., 2002).

Although both EE and DFM have been demonstrated independently to increase nutrient and energy utilization, the effects of the combination of EE and DFM have not yet been researched. Hence the objective of the first experiment was to determine the effects of EE and DFM on nutrient digestibility, including the apparent ileal digestibility co-efficient (AIDC) of starch, crude protein
(CP), and AA as well as nitrogen corrected apparent ME (AMEn). The follow-up experiment explored the probable pathways through which EE and DFM affect the nutrient digestibility and utilization, including ileal brush border digestive enzyme activity - maltase, sucrase and L-alanine aminopeptidase (LAAP), and the concentration of total cecal short-chain fatty acids (SCFA) such as acetate, propionate and butyrate and their proportions. The hypothesis evaluated was that supplementation of EE and DFM to broiler chickens would increase the dietary energy and nutrient digestibility by independent modes of action resulting in additive responses.

**MATERIALS AND METHODS**

Two experiments were conducted to evaluate the effects of EE and DFM on broiler chickens, at the Poultry Research and Teaching Unit of Iowa State University. All animal procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University before the start of the experiments.

*Animals and housing*

In the first experiment, Ross 308 broiler chicks (Aviagen Inc., AL) were fed a standard starter diet, formulated to meet or exceed the breeder recommendations (Aviagen, 2011) from 1-14 days and experimental diets from 15-21 days. On day 15, chicks were individually weighed, sorted, wing banded and allocated to cages resulting in 8 cages of 6 chicks (567.7 cm²/chick) for each of the 4 treatments. Experimental diets were randomly assigned to cages in a completely randomized design. Chicks were located within an environmentally controlled room, received supplemental heat starting at 35°C and decreasing 2°C weekly. The photoperiod provided was 23-h of light and
1-h of darkness. Chicks were provided *ad libitum* access to feed and water, and monitored twice daily throughout the experimental period. Mortalities were removed, weighed and recorded as they occurred.

In the second experiment, day-old, Ross 308 chicks (Aviagen Inc., AL) were individually weighed, sorted, wing banded and allocated to floor pens. Each of the 4 dietary treatments were comprised of 10 chicks with each chick as an experimental unit. Chicks were fed experimental diets from 1-21 days. All other management practices were the similar to Experiment 1 with the exception that chicks were housed in floor pens with used litter (0.15 m²/chick).

**Diets**

Both experiments utilized the same diets organized in a 2 x 2 factorial arrangement both with and without EE and DFM supplementation. This factorial resulted in the following 4 treatments, 1. Control (CON); 2. CON + EE; 3. CON + DFM; and 4. CON + EE + DFM). The un-supplemented CON diet was corn-SBM-DDGS-based, and formulated to meet or exceed the breeder recommendations for growers (Aviagen, 2011), except for a 0.94 MJ/kg reduction in ME (Table 6.1). Since the same experimental diets were fed from 1-21 days in the second experiment, the dietary ME reduction was 0.42 MJ/kg of diet from 1-10 days, and 0.94 MJ/kg of diet from 11-21 days as per breeder recommendations (Aviagen, 2011). The EE used was a cocktail containing amylase, xylanase and protease, while the DFM contained three different *Bacillus subtilis* strains. Both EE and DFM were included at a dose of 500 mg/kg of diet as recommended by the manufacturer. Titanium dioxide (TiO₂) was added at the rate of 2.5 g/kg of diet as an indigestible dietary marker to determine the nutrient and energy digestibility in Experiment 1.
Sample collection

In the first experiment, excreta samples were collected by EU for the last 48-h of the experimental period, by placing clean trays under each cage. All birds were euthanized by carbon dioxide asphyxiation on day 21 and dissected to collect ileal digesta contents which were then pooled by EU. The section of ileum was determined from the point of Meckel’s diverticulum to ileo-cecal junction. The collected ileal digesta and excreta samples were stored at -20°C until further analysis.

In the second experiment, eight birds were randomly selected per treatment on day 21 and were euthanized by carbon dioxide asphyxiation. Blood samples were collected individually through cardiac puncture and were set at room temperature for 2-h. The blood samples were then centrifuged at 2,500 × g for 30 min at 4°C to separate serum which was stored at -80°C until further analysis. The ileal lumen was exposed and flushed with ice-cold saline to free it from food particles. The ileal mucosa was scraped using a glass slide, placed into micro-centrifuge tubes and snap frozen using liquid nitrogen. After exposing the distal ends of ceca to the point of attachment at the ileo-cecal junction, cecal contents from both ceca were squeezed into plastic vials and the vials were snap frozen in liquid nitrogen. Ileal mucosal scrapings and cecal contents were then stored at -80°C until further analysis.

Digestibility assays

Excreta and digesta samples were dried at 65°C for 3 days (AOAC, 2006a) using a convection oven (Yamato Scientific America Inc., CA) and ground through a 1.0 mm screen (Brinkmann Instruments Inc., NY). The feed samples were oven (Yamato Scientific America Inc., CA) dried at 100°C for 24-h, and ground through 0.5 mm screen (Brinkmann Instruments Inc., NY). All
samples were analyzed in duplicate. Assays for AIDC of AA were carried out on the digesta and diet samples for methionine (Met), cysteine (Cys), lysine (Lys), threonine (Thr), leucine (Leu), isoleucine (Ile), aspartate (Asp), glutamate (Glu), alanine (Ala), glycine (Gly), proline (Pro), and valine (Val). Concentrations of AA in digesta and diet samples were determined after acid hydrolysis in 6 N HCl in the presence of phenol at 110°C for 24-h (AOAC, 2006b). Total sulfur AA (TSAA) content was determined after performic acid oxidation followed by acid hydrolysis. Excreta, digesta and diet samples were assayed for nitrogen by micro-Kjeldahl method (AOAC, 2006c) on a Kjeltech 1028 distilling unit (Foss Inc., MN). From the nitrogen concentration of digesta and diet samples, the concentrations of CP were calculated using a multiplication factor of 6.25 (Scott et al., 1982). For digesta and diet samples, the concentration of starch was determined using Total Starch® kit (Megazyme International, Ireland) for both digesta and diet samples. Complete solubilization of starch was achieved by cooking samples in the presence of thermostable α-amylase followed by amyloglucosidase hydrolysis to glucose, while maltodextrins were hydrolyzed to glucose by glucoamylase. Glucose produced was measured using glucose oxidase/peroxidase reagent (AACC, 2011). Excreta, digesta and diet samples were assayed for the concentration of TiO₂ (Leone, 1973), to calculate apparent ileal AA, CP and starch digestibility (Lemme et al., 2004). Gross energy (GE) was determined using an adiabatic oxygen bomb calorimeter (Parr Instrument Co., IL) for diet and excreta samples. The AMEn was calculated by modifying the equation proposed by Scott et al. (1982), by replacing chromic oxide marker with TiO₂ (Leone, 1973).

*Ileal brush border digestive enzyme activity*
Total protein was extracted from the ileal mucosal scrapings to measure the activities of maltase (EC 3.2.1.20), sucrase (EC 3.2.1.48) and LAAP (3.4.11.2). Briefly, a buffer mixture, containing phosphate buffer saline (pH 7.2), 0.1% triton, aprotinin (2 µg/ml), leupeptin (2 µg/ml), pepstatin (1 µg/ml), sodium orthovanadate (1 mM), and PMSF (1 mM), was added at 4 mL to 0.5 g of each of the mucosal samples. The buffer added samples were then centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was transferred to 5 ml tubes. The protein concentration was determined using Pierce® BCA Protein assay kit (Thermo Scientific, IL) and expressed in g/dl. The samples were maintained in microcentrifuge tubes at -80°C until they were used for further analysis. The activity of maltase and sucrase were assayed colorimetrically at 540 nm using a spectrophotometer (Biotek Instruments Inc., VT), by measuring µ moles of glucose released per min per g of protein from maltose and sucrose, respectively (Dahlqvist, 1964). The activity of LAAP was determined colorimetrically at 384 nm using a spectrophotometer (Biotek Instruments Inc., VT), by measuring the amount of enzyme activity liberating one µ moles of L-alanine p-nitroanilide per min per g of protein from 4-Nitroaniline (Maroux et al., 1973).

Cecal short-chain fatty acid concentration

Proportions of the SCFA such as acetate, propionate, and butyrate, as well as the concentration of total SCFA were determined from the cecal contents. Approximately 0.5 g cecal contents were gently squeezed into a micro-centrifuge tube containing 1 ml of 10% meta-phosphoric acid with 0.4 µl of 4-methyl valeric acid per ml added as an internal standard. The solution was thoroughly mixed using a vortex mixer and centrifuged at 5,700 × g for 20 min at 4°C. The SCFA content of the supernatant was measured using a HP Agilent 6890 series gas chromatography (Agilent Technologies Inc., CA) fitted with a HP 5973 series mass spectrometry (Agilent Technologies
Inc., CA). The HP-FFAP columns (Agilent Technologies, CA) used were free fatty acid polyester stationary phase capillary columns of 30 m long with a 0.25 mm internal diameter, of PEG (polyethylene glycol) on Shimalite TPA 60/80. The parameters were as follows: 1 μl injection volume, 240°C injector temperature, 12.15 psi pressure, with 1.1 ml per min constant flow and helium carrier. The following running conditions in the oven program were used: 80°C initial temperature hold for 5 min, ramp 10°C per min to 240°C and 12 min hold at 240°C. Total SCFA concentration was expressed as μmol per g of wet cecal content, while the proportions of individual SCFA were expressed in %.

Statistical analysis

The statistical analysis was carried out as a 2 x 2 factorial arrangement in a completely randomized design in both the experiments. Data were analyzed by MIXED procedure of SAS (SAS, 2012) using cage as an experimental unit in the first experiment, and individual chick as an experimental unit in the second experiment. Student’s t-test ($\alpha = 0.05$; $t = 2.08596$) was used to separate significant least square means with the probability of type-I error set at $P \leq 0.05$. While significance was accepted at $P \leq 0.05$, trends were noted at $P \leq 0.10$.

RESULTS

Performance
Feed intake, body weight gain and mortality data were collected to verify typical growth responses in both the experiments, but were not statistically analyzed. The mean body weight gain (g/chick) from 15-21 days for CON, CON + EE, CON + DFM and CON + EE + DFM treatments were 344, 351, 334 and 342 respectively in Experiment 1. The mean body weight gain (g/chick) from 1-21 days for CON, CON + EE, CON + DFM and CON + EE + DFM treatments were 715, 688, 743 and 701 respectively in Experiment 2. The performance data from both experiments were typical of Ross 308 broiler chickens over this time period (Aviagen, 2011).

**Digestibility**

There were interactions ($P \leq 0.01$) in AIDC between the DFM and EE fed groups in the first experiment for starch, CP and all AA (Met, Cys, Lys, Thr, Leu, Ile, Asp, Glu, Ala, Gly, Pro, and Val) analyzed (Table 6.2). Regardless of dietary supplementation, AIDC of starch was high, ranging from 97% to 99%. When fed alone or in combination, both EE and DFM increased ($P \leq 0.01$) AIDC of starch compared to the CON diet. Supplementation of either EE or DFM increased the AIDC of CP, but the magnitude was higher with EE. The AIDC for the combination did not differ significantly from either of the additives when fed alone. Both EE and or DFM increased the AIDC significantly compared to the CON diet for all AA, except Met. Although EE and DFM did not differ between themselves in AIDC of Met, the combination reduced the AIDC which was significantly lower than EE alone. Overall, the AIDC of CP and AA were increased ($P \leq 0.01$) an average of 12% by EE, 8% by DFM and 10% by the combination. Diets supplemented with EE and / or DFM resulted in increased AMEn ($P \leq 0.01$), but there were no significant interactions (Fig. 6.1).
Ileal brush border digestive enzyme activity

Supplementation of EE increased \((P \leq 0.05)\) ileal brush border maltase, sucrase and LAAP activity in Experiment 2. Although, DFM did not alter the activities of any of the brush border digestive enzymes significantly, a trend for increased sucrase activity was observed \((P = 0.07)\). No interactions between EE and DFM were noted (Fig. 6.2).

Cecal short-chain fatty acid concentration

Overall interactions were absent among the dietary treatments. Total SCFA concentration did not differ significantly among the dietary treatments. Total SCFA concentration (µmol per g of wet cecal content) of chickens fed with and without EE were 61.1±2.0 and 57.8±2.0, whereas with and without EE were 59.8±2.0 and 59.2±2.0, respectively. The proportion of butyrate was increased \((P \leq 0.01)\) with DFM addition, although there were no significant differences observed in the proportions of acetate or propionate in Experiment 2 (Fig. 6.3).

DISCUSSION

Published data on the AIDC of AA are in agreement with current data on the effects of EE. Cowieson and Ravindran (2008) reported a 3% increase in the overall AIDC of AA, ranging from 0.44% for Met to 9.1% for Cys, by supplementing the EE cocktail containing amylase, xylanase and protease. Increases in the AIDC of starch by 2.0% and CP by 3.6% were also observed by supplementation of the same EE cocktail to broiler diets in a different experiment (Zanella *et al.*, 1999). Changes in the AIDC of dietary starch, CP and AA can be mediated through improved digestion and absorption of carbohydrates and proteins as supplementation of EE complements the
endogenous digestion (Cowieson and Ravindran, 2008). The rate of amylopectin and amylose hydrolysis was significantly increased when exogenous amylase was added to pancreatic α-amylase (Bedford and Partridge, 2010). Exogenous proteases may augment endogenous peptidases by improving protein digestibility as well as denaturing proteinaceous anti-nutrients such as lectins or trypsin inhibitors and hydrolyzing antigenic proteins in protein sources such as SBM (Douglas et al., 2000; Ghazi et al., 2002). Furthermore, EE also reduce the endogenous secretions attributed to dietary NSP activity as by hydrolyzing NSP and resulting in increased energy utilization (Cowieson and Ravindran, 2008).

The gastrointestinal tract swiftly adapts to dietary changes and the activities of endogenous digestive enzymes are modulated in response to physiological needs, primarily with increased luminal sugar and AA content, rather than constantly maintaining high enzyme activity (Pinheiro et al., 2004). Therefore, the increased activity of disaccharidases (maltase and sucrase) and LAAP at the ileal mucosa of birds fed EE might be attributed to increased substrate presence at the apical membrane due to EE enhanced hydrolysis of dietary carbohydrate and protein. Furthermore, it has been noted that the xylo-oligosaccharides released from xylanase mediated breakdown of arabinoxylan can be hydrolyzed by beneficial bacteria such as Bifidobacterium and Lactobacillus spp., in the hindgut resulting in increased synthesis of SCFA (Thammarutwasik et al., 2009). Published data demonstrated increased cecal SCFA concentrations in broiler chickens fed diets supplemented with EE (Wang et al., 2005). However, results of the current experiment were in contrary to this as supplementation of EE with corn-SBM-based diets did not alter the cecal proportions of acetate, propionate, or butyrate in broiler chickens in Experiment 2.

Supplementation of corn-SBM-DDGS-based diets with DFM increased the AIDC of starch, CP and AA in the present experiment which is in agreement with published data (Apata,
Supplementation of DFM has been reported to increase the number of beneficial bacteria (e.g. *Bifidobacteria spp.* and *Lactobacilli spp.*) in the gastrointestinal tract that can potentially improve the digestion of carbohydrates, particularly NSP, and protein (Jin et al., 1998). These bacteria are also beneficial for maintaining the intestinal integrity and function, which then promotes the absorption and transport of glucose, amino acids and other nutrients (Wu, 1998). Additionally, DFM may affect the luminal metabolism of dietary nutrients (particularly amino acids) by altering their synthesis and catabolism, therefore resulting in changes in the amounts of amino acids (free and protein-bound) in the ileal digesta (Wu, 1998). Furthermore, Dietary supplementation of DFM has been observed to increase the level of endogenous α-amylase in broiler chickens, resulting in increased carbohydrate digestion (Jin et al., 2000). The trend in increased sucrase activity by DFM was in agreement with Jin et al. (2000) that DFM increased the activity of brush border carbohydrases in broiler chickens. Present results reinforce the influence exhibited by gut microbes in modulating enterocyte activity and expressing tissue functions (Collington et al., 1990). The current data suggests that supplementation of EE and DFM, independent of each other, increased ileal nutrient digestibility, although the underlying mechanisms may be overlapping as the combination did not result in additive response.

A growing body of research demonstrates increased AMEn in broiler chickens fed EE (Kocher et al., 2003; Rutherford et al., 2007; Cowieson and Ravindran, 2008; Zhou et al., 2009), which is in agreement with current data. Primary reasons attributed are increased digestibility of starch, NSP, and AA and reduced endogenous loss (Meng and Slominski, 2005; Olukosi et al., 2008). Peer reviewed data indicate the magnitude of increase in AMEn by EE was greater for diets with low energy compared to high energy diets (Kocher et al., 2003; Zhou et al., 2009). This is in agreement with current results which were obtained by feeding diets with a reduction of 0.94
MJ/kg of ME. Although only few experiments have examined the effects of DFM on energy digestibility in broiler chickens, they are in agreement with the results of this experiment (Li et al., 2008; Mountzouris et al., 2010). The actions of DFM in increasing SCFA synthesis have been reported to increase dietary energy utilization (Caballero-Franco et al., 2007). In addition to being used as the major substrate for energy production, butyrate has been observed to be the most effective SCFA to promote the proliferation and functional maturation of intestinal epithelial cells resulting in increased arterial blood flow linked to increased nutrient absorption (Cherbut, 2003). Moreover, butyrate modifies the motility of upper gastrointestinal wall involving polypeptide YY by inducing relaxation of the proximal stomach and lower esophageal sphincter resulting in reduced emptying (Cherbut, 2003). These physiological functions of butyrate in increasing nutrient digestibility and absorption could be attributed to the increased AIDC of nutrients and AMEn by DFM addition in Experiment 1. Unlike previous reports, current data do not support increased total SCFA production as the mechanisms for DFM improvement, however the increased concentration of butyrate might be involved. Although, unlike the AIDC of starch, CP and AA, the effects of EE and DFM are additive for AMEn and could be mediated at the small intestine for EE and through modifications of hindgut fermentation for DFM.

In conclusion, EE or DFM supplementation to broiler chickens increased the AIDC for starch, CP and AA through independent but complementary mechanisms. The effects of EE were primarily exerted through up-regulation of brush border enzyme activity possibly due to increased substrate presence at the apical membrane. Supplementation of DFM increased cecal butyrate proportion which could be linked to increased energy utilization. The effects of EE and DFM are additive for AMEn and could be mediated at the small intestine for EE and through modifications of hindgut fermentation for DFM.
ACKNOWLEDGEMENTS

We recognize the care for the birds provided by the staff of the Poultry Research and Teaching Unit of Iowa State University, and also like to thank M. Jeffrey, K. Nesheim, N. Nachtrieb, J. Vande Vorde and J. Santiago for assistance in conducting the experiments.
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AOAC. (2006c) Total Nitrogen by Kjeldahl (984.13 (a,b,c,d)): Official methods of analysis. 18th ed. *Journal of AOAC International*.


Table 6.1 Composition of diets fed to broiler chickens from 15 to 21 days of age in Experiment 1 and from 1 to 21 days of age in Experiment 2.

<table>
<thead>
<tr>
<th>Ingredient Composition</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>529.4</td>
</tr>
<tr>
<td>Soybean meal (48% CP)</td>
<td>293.8</td>
</tr>
<tr>
<td>Dried distillers grains with solubles</td>
<td>120.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>10.8</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>2.2</td>
</tr>
<tr>
<td>L-lysine(^1)</td>
<td>4.4</td>
</tr>
<tr>
<td>Salt</td>
<td>4.0</td>
</tr>
<tr>
<td>Limestone</td>
<td>13.0</td>
</tr>
<tr>
<td>Di-calcium phosphate</td>
<td>12.7</td>
</tr>
<tr>
<td>Choline chloride (60%)</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin and mineral premix(^2)</td>
<td>6.3</td>
</tr>
<tr>
<td>Titanium dioxide</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical Composition (calculated)</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME (MJ/kg)</td>
<td>12.24</td>
</tr>
<tr>
<td>Crude protein</td>
<td>222.5</td>
</tr>
<tr>
<td>Ether extract</td>
<td>46.2</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>31.1</td>
</tr>
<tr>
<td>Calcium</td>
<td>9.0</td>
</tr>
<tr>
<td>Non-phytate phosphorus</td>
<td>3.8</td>
</tr>
<tr>
<td>Digestible methionine + cysteine</td>
<td>8.5</td>
</tr>
<tr>
<td>Digestible lysine</td>
<td>12.0</td>
</tr>
<tr>
<td>Digestible threonine</td>
<td>7.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical Composition (analyzed)</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>227.4</td>
</tr>
<tr>
<td>Ether extract</td>
<td>41.4</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>28.3</td>
</tr>
</tbody>
</table>
1Contained 50.7% of L-lysine in the form of L-lysine sulfate, 0.1% Methionine, 0.1% Cystine, 0.3% Threonine, 0.1% Tryptophan, 0.6% Arginine, 0.3% Isoleucine, 0.5% Leucine, and 0.4% Valine.

2Provided per kg of diet: Selenium-250 µg; Vitamin A-8,250 IU; Vitamin D₃-2,750 IU; Vitamin E-17.9 IU; Menadione- 1.1 mg; Vitamin B₁₂-12 µg; Biotin-41 µg; Choline-447 mg; Folic acid-1.4 mg; Niacin-41.3 mg; Pantothenic acid-11 mg; Pyridoxine-1.1mg; Riboflavin-5.5 mg; Thiamine-1.4 mg; Iron-282 mg; Magnesium-125 mg; Manganese-275 mg; Zinc-275 mg; Copper-27.5 mg; Iodine-844 µg.
Table 6.2 Interactions of exogenous enzymes (EE) and direct-fed microbial (DFM) on apparent ileal digestibility co-efficient of starch, crude protein (CP) and amino acids of broiler chickens at 21 days of age in Experiment 1.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Starch</th>
<th>CP</th>
<th>Methionine</th>
<th>Cysteine</th>
<th>Lysine</th>
<th>Threonine</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON1</td>
<td>0.97b</td>
<td>0.78c</td>
<td>0.88c</td>
<td>0.68b</td>
<td>0.85b</td>
<td>0.69c</td>
</tr>
<tr>
<td>CON + EE</td>
<td>0.98a</td>
<td>0.86a</td>
<td>0.95a</td>
<td>0.80a</td>
<td>0.91a</td>
<td>0.83a</td>
</tr>
<tr>
<td>CON + DFM</td>
<td>0.99a</td>
<td>0.83b</td>
<td>0.94ab</td>
<td>0.76a</td>
<td>0.89a</td>
<td>0.79ab</td>
</tr>
<tr>
<td>CON + EE + DFM</td>
<td>0.99a</td>
<td>0.84ab</td>
<td>0.93b</td>
<td>0.78a</td>
<td>0.90a</td>
<td>0.80ab</td>
</tr>
<tr>
<td>SEM</td>
<td>0.003</td>
<td>0.01</td>
<td>0.01</td>
<td>0.02</td>
<td>0.01</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>EE P-value</th>
<th>DFM P-value</th>
<th>EE x DFM P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ 0.01</td>
<td>≤ 0.01</td>
<td>≤ 0.01</td>
</tr>
<tr>
<td></td>
<td>≤ 0.01</td>
<td>0.17</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>≤ 0.01</td>
<td>0.10</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>≤ 0.01</td>
<td>0.11</td>
<td>0.05</td>
</tr>
</tbody>
</table>

a,b Least square means in the same column without a common superscript differ significantly, $P \leq 0.05$

n = 8 chickens per treatment.

1Formulated to meet breeder requirements for 14-21 d old broiler chicks except a reduction of 0.94 MJ/kg of ME.
**Figure 6.1** Main effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on nitrogen corrected apparent metabolizable energy of broiler chickens at 21 days of age in Experiment 1.

\[a,b\] Least square mean columns without a common superscript differ significantly, \(P \leq 0.01\) 
n = 16 chickens per group.
Figure 6.2 Main effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on ileal brush border digestive enzyme activity of broiler chickens at 21 days of age in Experiment 2.

Least square mean columns without a common superscript differ significantly, $P \leq 0.05$

$n = 16$ chickens per group.
**Figure 6.3** Main effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on cecal short-chain volatile fatty acid molar proportion in broiler chickens at 21 days of age in Experiment 2.

![Graph showing molar proportion of acetate, propionate, and butyrate](image)

*a,b* Least square mean columns without a common superscript differ significantly, $P \leq 0.05$

$n = 16$ chickens per group.
EFFECTS OF EXOGENOUS ENZYMES AND DIRECT-FED MICROBIAL ON
PERFORMANCE, ENERGY UTILIZATION AND BODY COMPOSITION OF
BROILER CHICKENS

A paper for submission the Journal of Nutrition

ABSTRACT

An experiment was conducted to examine the effects of exogenous enzymes (EE) and direct-fed
microbial (DFM) on performance, energy utilization, and body composition in broiler chickens.
Ross 308 chicks were fed one of the four experimental diets (control (CON), CON + EE, CON +
DFM and CON + EE + DFM) from d 1-21, and were sampled on d 21. Feed efficiency was
increased by either EE or DFM, but the magnitude of increase was higher by the combination.
Chick lean mass was increased, while fat mass was reduced by the combination on d 7 ($P = 0.01$),
but not over the remaining period of the experiment. Serum non-esterified FFA were decreased ($P$
$\leq 0.01$) in treatment chicks compared to CON chicks, while TG and cholesterol levels were
lowered ($P \leq 0.01$) by DFM. Cecal proportions of propionate and butyrate, as well as total SCFA
concentration were increased ($P \leq 0.01$) by DFM. Hepatic glucose-6-phosphate dehydrogenase
(G6PDH) activity was increased ($P \leq 0.01$) by DFM whereas no difference in GAPDH or fatty
acid synthase activity was noted. Hepatic glycogen content was increased ($P \leq 0.01$) by EE, DFM
or both. Changes in serum metabolite profile of chicks fed EE, DFM or both indicate their
efficiency in swiftly transitioning from fasting to feeding metabolism. Elevated hepatic glycogen
and G6PDH activity for DFM supplemented chicks indicate increased glucose-sparing, which may be associated with the effects of butyrate. The combination of EE and DFM appear to result in an additive response for feed efficiency suggesting independent mechanisms involved in increasing energy utilization and retention.

Keywords: enzyme, direct-fed microbial, broiler, energy metabolism
INTRODUCTION

Exogenous enzymes (EE) have been used in broiler diets to increase dietary energy release by hydrolyzing non-starch polysaccharides (NSP) resulting in increased endogenous enzyme activity through increased substrate abundance (1, 2). The increase in substrate abundance and subsequent absorption with EE supplementation have been associated with changes in the composition of gut microflora (3). Direct-fed microbial (DFM) or probiotics are live microbial feed additives which supplement the natural flora of the birds to establish a dominant colony of beneficial bacteria, primarily in the hindgut (4). Recent literature suggests that DFM can positively influence dietary energy uptake by increasing nutrient digestibility (4, 5), and SCFA synthesis (6). Polysaccharides released from NSP hydrolysis by EE can be used as substrates by DFM or the beneficial microflora (7). Hence the combination of EE and DFM may increase the efficiency of dietary energy extraction as well as energy retention in the body.

The energy contributed by increased nutrient uptake goes through anabolic process and could either be stored as fat in adipose tissue or lean tissue in skeletal muscle, depending on the extent of energy availability and heat production (8). It is well documented in chickens that excess energy is primarily retained as body fat in adipose tissue and is mobilized as non-esterified fatty acids (NEFA) to contribute energy in times of need, such as fasting (9). Excess energy released by the addition of EE was found to be retained in the adipose tissue of broiler chicks (10). In contrast, supplementation of DFM lowered abdominal fat deposition while increasing lean accretion (11). These data indicate that both EE and DFM share the ability to increase the uptake of dietary energy as well as to modify the retention pattern of energy. The changes exerted on body
composition due to the effects of EE and DFM may also affect the energy status of the bird, resulting in altered performance.

An experiment was conducted to determine the effects of EE and DFM supplementation on performance, body composition, serum metabolites concentration, cecal SCFA concentration, activities of hepatic metabolic enzymes and hepatic glycogen concentration. A 12-h fasting and 2-h re-feeding at the end of the experiment was done to determine the efficiency by which the birds can transition from fasted to feeding metabolism, indicating their energy status. The hypothesis was that supplementation of EE and DFM to broiler diets would increase the energy utilization efficiency of chicks in an additive manner, which will be reflected by changes in performance, energy status and body composition.

MATERIALS AND METHODS

Animals and housing. All animal procedures were approved by the Institutional Animal Care and Use Committee of Iowa State University before the initiation of the experiment. A total of 256, male, Ross 308 chicks (Aviagen Inc., AL) were obtained from a local commercial hatchery immediately after hatch and transferred to the Poultry Research and Teaching Unit of Iowa State University. Upon arrival, chicks were individually weighed, sorted, wing banded, and allocated to raised-wire battery cages (Petersime, IL). Dietary treatments were randomly assigned to each cage of chicks. Each of the four dietary treatments was comprised of eight cages of seven chicks. Chicks were provided ad libitum access to experimental diets and water for the duration of the experiment (d 1-21). Chicks were housed within an environmentally controlled room, and received supplemental heat starting at 35°C and decreasing 2°C every wk, with 23 h of light and one h of
darkness. Chicks were monitored twice daily throughout the experimental period, and mortality were removed, weighed and recorded as they occurred.

**Diets.** The dietary treatments (1. control (CON); 2. CON + EE; 3. CON + DFM; and 4. CON + EE + DFM) were arranged as a 2 x 2 factorial with EE and DFM supplementation as the factors. The CON diet was corn - soybean meal - dried distiller’s grain with solubles based (Table 7.1), and formulated to meet or exceed breeder recommendations (12), with the exception of 75 kcal/kg and 200 kcal/kg reductions in metabolizable energy for starter (d 1-10), and grower (d 11-21) diets, respectively. The second group was fed the same CON diet with 50 mg/kg of EE containing xylanase enzyme with a determined activity of 7757 U/g. The third group was fed the CON diet with 500 mg/kg of DFM. The DFM contained 5 different probiotic strains (*Lactobacillus reuteri, Lactobacillus salivarius, Enterococcus faecium, Bifidobacterium animalis* and *Pediococcus acidilactici*), isolated from the gut of healthy adult chickens, with a determined colony count of $2 \times 10^{11}$ cfu/kg. The fourth group was fed CON diet with the combination of EE and DFM at the above inclusion rates.

**Sample collection.** Body weight gain (BWG) by chick and feed intake (FI) by pen were determined from d 1-7, d 1-14, and d 1-21, and feed efficiency (FE) was calculated over the same periods. Mortality and chicks sampled for DXA analysis were weighed in order to correct for FE. One chick per cage was randomly selected and euthanized on d 7, 14, and 21, to determine body composition using DXA. Chicks were fasted for 12-h on the evening of d 20 before re-fed in a synchronized manner for 2-h on the morning of d 21. On d 21, blood samples were collected via cardiac puncture, from one randomly selected chick per cage, after carbon-dioxide asphyxiation.
To follow-up on the effects of energy status of the chicks and the efficiency by which they could transition from fasting to feeding state was determined by determining various serum metabolites. Liver samples were collected and packed in aluminum foil before snap freezing in liquid nitrogen. After exposing the distal ends of ceca from the point of attachment at the ileo-cecal junction, cecal contents from both ceca were squeezed into plastic vials and the vials were snap frozen in liquid nitrogen. Blood samples were set at room temperature for 2-h before being centrifuged at $2,500 \times g$ for 30 min at $4^\circ C$ to separate serum. Serum, liver and cecal samples were stored at $-80^\circ C$ until further analysis.

**Body composition.** The bone mineral content, lean, fat, and total body mass for each chick was determined on d 7, 14, and 21 using DXA (Discovery A, Hologic Inc., CO) analysis (13). The accretion rates of lean and fat mass were calculated using the initial (d 0) values as a covariate, and expressed in g/day.

**Serum metabolites concentration.** The serum samples were assayed for concentration of glucose using Autokit Glucose Enzymatic Method (Wako Diagnostics, VA), Triglyceride (TG) using Serum Triglyceride Determination Kit (Sigma-Aldrich Inc., MO), NEFA using NEFA C, ACS-ACOD Colorimetric Method (Wako Diagnostics, VA), urea nitrogen using QuantiChrom™ Colorimetric Urea Assay Kit (Sigma-Aldrich Inc., MO), uric acid using Uric Acid Fluorometric Assay Kit (Cayman Chemical Company, MI) and cholesterol using Cholesterol Fluorometric Assay Kit (Cayman Chemical Company, MI). The results were expressed in mg/dL.
**Hepatic enzyme activity.** Total protein was extracted from the liver samples to measure the activities of fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PDH) and GAPDH. Briefly, 5 mL of buffer mixture, containing phosphate buffer saline (pH 7.2), 0.1% triton, aprotinin (2 µg/mL), leupeptin (2 µg/mL), pepstatin (1 µg/mL), sodium orthovanadate (1 mM), and PMSF (1 mM), was added at 0.5 g of each of the tissue samples. The buffer added tissue samples were homogenized using Tissumiser (Fisher Scientific, IL) before being centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant was transferred to 5 mL tubes. The protein concentration was determined using Pierce® BCA Protein assay kit (Thermo Scientific, IL) and expressed in g/dL. The samples were maintained in microcentrifuge tubes at -80°C until they were used for further analysis. The assay for FAS was performed as described by Martin et al. (14), with modifications from Ingle et al. (15). Briefly, lysate was added to 100 µL of reaction buffer (50 mM potassium phosphate pH 6.8, 2.5 mM β-mercaptoethanol, 0.26 mM NADPH, 0.2 mM acetyl CoA, 0.2 mM malonyl CoA) and the absorbance was read at 340 nm, every 30 s for 7 min at 25°C, using a spectrophotometer (Biotek Instruments Inc., VT). The assay for G6PDH was performed as described by Deutsch (16). Briefly, tissue lysate was added to 100 µL of reaction buffer (125 mM Tris pH 7.5, 1 M KCl, 50 mM MgCl₂, 0.25 mM NADP⁺, 5 mM glucose-6-phosphate) and the absorbance was read at 340 nm, every 30 s for 7 min at 25°C, using a spectrophotometer (Biotek Instruments Inc., VT). The assay for GAPDH was performed as described by Gamou et al (17). Briefly, tissue lysate was added to 100 µL of reaction buffer (125 mM Triethanolamine-HCl pH 7.5, 2.5 mM EDTA, 0.5 mM NADH, 1.1 mM dihydroxyacetone phosphate, 0.125 mM β-mercaptoethanol) and the absorbance was read at 340 nm, every 30 s for 7 min at 25°C, using a spectrophotometer (Biotek Instruments Inc., VT). All biochemical reagents were purchased from Sigma-Aldrich Inc., MO. The activities of FAS, G6PDH and GAPDH were calculated from the
change in absorbance at 340 nm using the slope and expressed as nmol NAD(P)H per min per mg of protein.

\[
\text{Activity} \left( \frac{\text{units}}{\text{mL/mg}} \right) = \frac{\text{ABS}[\Delta OD_{340}] \times A (mL)}{6.22 \text{ mM}^{-1} \text{ cm}^{-1} \times B (mL) \times C (cm) \times mg \ (\text{protein} \ or \ \text{tissue})} \times 1000 \left( \frac{\text{ng}}{\mu g} \right)
\]

ABS[ΔOD₃₄₀]: Absolute change in the absorbance at 340 nm per minute
A (mL): Total reaction volume
B (mL): The volume of the enzyme solution added
C (cm): Pathlength
6.22 mM⁻¹ cm⁻¹: Molar absorption coefficient for NADH

**Hepatic glycogen concentration.** Liver tissues were homogenized 0.05 M EDTA (pH 5.9) and analyzed immediately for glycogen. Homogenate aliquots (1 mL) were digested with 30% KOH at 100°C for 20 min and glycogen was isolated by precipitation using 95% ethanol. The protein concentration of homogenates was determined using Pierce® BCA Protein assay kit (Thermo Scientific, IL) and expressed in g/dL. The glycogen was suspended in 0.1 M acetate buffer (pH 4.0), and reacted with 0.05 mL amyloglucosidase for 1-h for glucose liberation (18). The liberated glucose was assayed using commercial kit (Autokit Glucose Enzymatic Method, Wako Diagnostics, VA) and the final concentration was expressed in µg/mg of protein. The biochemical reagents were purchased from Sigma-Aldrich Inc., MO.

**Cecal short-chain fatty acid concentration.** Proportions of the SCFA such as, acetate, propionate and butyrate, as well as the total SCFA concentration were determined from cecal contents. Approximately 0.5 g of cecal contents were gently squeezed into a micro-centrifuge tube
containing 1 mL of 10% meta-phosphoric acid with 0.4 µL of 4-methyl valeric acid per mL added as an internal standard. The solution was thoroughly mixed using a vortex mixer and centrifuged at 5,700 × g for 20 min at 4°C. The SCFA content of the supernatant was measured using a HP Agilent 6890 series gas chromatography (Agilent Technologies Inc., CA) fitted with a HP 5973 series mass spectrometry (Agilent Technologies Inc., CA). The HP-FFAP columns (Agilent Technologies, CA) used were free fatty acid polyester stationary phase capillary columns of 30 m long with a 0.25 mm internal diameter, of PEG (polyethylene glycol) on Shimalite TPA 60/80. The parameters were as follows: 1 µL injection volume, 240ºC injector temperature, 12.15 psi pressure, with 1.1 mL per min constant flow and helium carrier. The following running conditions in the oven program were used: 80ºC initial temperature hold for 5 min, ramp 10ºC per min to 240ºC and 12 min hold at 240ºC. Total SCFA concentration was expressed as µmol per g of wet cecal content, while the proportions of individual SCFA were expressed in %.

**Statistical analysis.** The statistical analysis was carried out as a 2 x 2 factorial in a completely randomized design. Data were analyzed by MIXED procedure of SAS (19) using cage as an experimental unit for performance variables such as BWG, FI and FE, and individual chick as an experimental unit for the remaining variables assayed. Student’s t-test ($\alpha = 0.05; t = 2.08596$) was used to separate significant least square means with the probability of type-I error set at $P \leq 0.05$. While significance was accepted at $P \leq 0.05$, trends were noted at $P \leq 0.10$.

**RESULTS**
**Performance.** No significant main effects or interactions were observed for BWG throughout the experiment (Table 7.2). Supplementation of EE lowered FI from 1-14 d ($P = 0.02$), and 1-21 d ($P = 0.05$), whereas a trend was observed over 1-7 d ($P = 0.07$). Interactions between EE and DFM were present as either EE or DFM alone or in combination increased in FE during 1-7 d ($P = 0.04$), and 1-21 d ($P = 0.02$) in comparison to CON fed birds. The magnitude of increase in FE was higher by the combination during 1-21 d period, followed by EE and then DFM. The mortality rate was generally low and evenly distributed among the dietary treatments including 2, 4, 2 and 3 mortalities for CON, CON + EE, CON + DFM, and CON + EE + DFM groups, respectively.

**Body composition.** Significant interactions were observed in percent fat and lean mass at d 7 ($P = 0.05$; Table 7.3). Chicks supplemented with the combination of EE and DFM had lower fat mass and higher lean mass compared to chicks fed either EE alone supplemented diet or un-supplemented CON diet. Although the same effects were seen with DFM supplemented chicks, they did not differ significantly from other treatments. No significant differences were observed in fat or lean mass among the dietary treatments at either d 14 or d 21. Bone mineral content (data not shown) did not differ significantly among the dietary treatments.

**Serum metabolite concentration.** There were no significant differences in serum glucose or urea nitrogen among the dietary treatments (Fig. 7.1). Addition of DFM increased serum uric acid ($P = 0.04$), and decreased serum TG concentration ($P \leq 0.01$). Interactions were observed in serum NEFA and cholesterol as they were reduced with EE, DFM or the combination ($P \leq 0.01$).
However, the effect of DFM was significantly higher ($P \leq 0.01$) in lowering cholesterol levels compared to either EE or the combination.

**Hepatic energy metabolism.** Overall, no interactions were observed in the activities of hepatic FAS, G6PDH, or GAPDH enzymes (Fig. 7.2). There were no significant differences in the activities of FAS or GAPDH, while the activity of G6PDH was increased ($P \leq 0.01$) with DFM supplementation. Interactions were noted as EE, DFM or the combination increased liver glycogen concentration compared to CON diet (Fig. 7.2). The magnitude of increase was higher in the chicks fed DFM alone in comparison with EE, while the combination was intermediate in effect ($P \leq 0.01$).

**Cecal short-chain fatty acid concentration.** Overall interactions were absent among the dietary treatments. Supplementation of DFM increased the total SCFA concentration in cecal contents in comparison to the chickens fed diets without DFM. Total SCFA concentration (µmol per g of wet cecal content) of chickens fed with and without DFM were 66.2±1.9 and 50.7±1.9, whereas with and without EE were 60.4±1.9 and 56.5±1.9, respectively. The proportions of propionate and butyrate were significantly increased with DFM addition ($P \leq 0.01$; Fig. 7.3). There were no differences observed in the proportions of acetate among the dietary treatments. Valerate proportions (data not shown) were noted but not statistically analyzed due to low overall concentration.

**DISCUSSION**
Reduced feed intake in the current experiment by EE resulted in increased FE by 3.0%, in comparison to CON fed chicks, and in agreement with published data (1, 2). In the same manner, increased FE (1.1%) for chicks fed diets supplemented with DFM in the current experiment is in agreement with previously reported experiments (4, 20). However, the combination of EE and DFM increased FE by 6.2% compared to CON group. This increase is more than the sum of both EE and DFM when supplemented alone, indicating an additive response. This more than additive effect also suggests that both EE and DFM exert their effect through pathways independent of each other. The positive effects of EE are suggested to be due to increased digestibility of dietary nutrients (1, 2), and digestive enzyme activity (21, 22). Previous research indicates that DFM increased FE of broiler chickens through altered microbial metabolism (4), increased SCFA synthesis (6) and nutrient retention (20).

The significant interaction of EE and DFM on body composition at d 7 indicates that supplementation of EE alone did not affect body composition, while DFM increased and the combination of EE and DFM further increased lean accretion and decreased fat tissue. This is in agreement with previous research, in which supplementation of DFM alone lowered abdominal fat deposition while increasing lean accretion (11, 23). Increased DFM associated lean mass accretion, early in life, may be due to increased gut integrity by DFM which minimizes the use of glucose and amino acids by cells of the immune system (24, 25). However, these effects on body composition were lost at 14 and 21 d. One of the proposed reason could be the immaturity of digestive tract early in life enhancing the energy utilization efficiency by EE and DFM, as the effects were absent after the maturity occurred around the second week (26).
Feed deprivation or fasting causes a shift from anabolism to catabolism and a reduced metabolic rate (27), resulting in residual effects that can be detected after re-feeding (28). Reduced serum NEFA concentration for birds fed feed additives in this experiment can be attributed to reduced lipolysis (8) as feed additives may have hastened the transition from fasting to feeding metabolism. Increased serum uric acid for the DFM fed birds may indicate increased catabolism of amino acids, although the differences were small and biologically questionable provided the normal range of uric acid concentration in chickens (8, 27). Both NEFA and amino acid are catabolized for energy as well as to maintain circulating glucose levels in fasted chickens (8). The lack of difference in serum glucose concentration among treatments indicates that circulating glucose concentration is tightly regulated in chickens (9).

Because all avian TG are processed and released from the liver, reduced serum TG due to DFM addition in the current experiment suggests reduced hepatic de novo TG synthesis, and reduced TG transported to adipose tissue (11). It is in agreement with published data that addition of DFM to broiler diets significantly reduced serum TG concentration (23). Serum cholesterol concentration was strongly reduced by DFM treatment, the combination was still reduced, although somewhat mitigated in comparison to DFM alone. This is in agreement with published data that DFM down regulates hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate limiting enzyme in de novo cholesterol synthesis (23). Moreover, DFM have been demonstrated to lower serum cholesterol directly through deconjugation of bile salts, elevated bile salt hydrolase activity, and co-precipitation of cholesterol with deconjugated bile (29), while indirectly through increased propionate synthesis. Propionate has been reported as an effective inhibitor of de novo TG and cholesterol synthesis by impairing acetate incorporation, independent of the acetate
concentration (30, 31). This is in agreement with the current data as chicks fed DFM had increased cecal propionate concentration in comparison to chicks fed diets without DFM.

As the first relay station for processing dietary information, liver contains the whole biochemical machinery for both glucose and lipid storage and disposal (32). Reduced serum TG and cholesterol concentration from the current experiment suggest reduced de novo synthesis in the liver of chicks supplemented with DFM. However, the unaltered hepatic activity of FAS among the dietary treatments is in contrast to these data. The duration of re-feeding (2-h) and immediate sampling may not have allowed chicks to transition enzyme activity to post-absorptive metabolism. Increased hepatic activity of G6PDH for DFM fed chicks is associated with increased removal of glucose-6-phosphate through pentose phosphate pathway rather than glycolysis. Glucose-6-phosphate is the glucose-signaling metabolite that is essential for the activation of carbohydrate response element binding protein, which plays a critical role in converting excess glucose into TG through de novo lipogenesis (33). Hence reduced availability of glucose-6-phosphate indicates reduced de novo lipogenesis, which is in agreement with the reduced serum TG concentration data. Furthermore, increased utilization of glucose-6-phosphate in the pentose phosphate pathway provides reducing power in the form of NADPH, which enables cells to counterbalance oxidative stress and to preserve the reduced form of glutathione (34). Unchanged GAPDH activity among the dietary treatments may indicate absence of differences in glucose utilization through glycolysis.

In the present experiment, hepatic glycogen concentration was increased by EE, DFM or the combination. But the magnitude of increase was higher with DFM alone whereas the combination of EE and DFM resulted in intermediate response. The avian liver contributes significantly to circulating glucose levels and is responsive to decreased feed intake (28). A 24-h
fast resulted in reduced liver glycogen content by 77%, while re-feeding for 12-h increased the liver glycogen concentration by 380% (28). The energy need for net hepatic glycogen synthesis from exogenous glucose corresponds to ~50% of basal mitochondrial ATP turnover in rats fasted for 48-h followed by feeding for 2-h (35). However these researchers demonstrated that the total ATP content increased concomitantly with the progressive repletion of hepatic glycogen, while the mitochondrial ATP supply remained stable in the same rats (35). Hence increased hepatic glycogen concentration in chicks fed EE, DFM or the combination in the present experiment, after 12-h of fasting followed by 2-h of feeding, indicates that these chicks were efficient in energy conservation and utilization.

The hepatic metabolism of propionate contributes, after entering the TCA cycle at the level of succinyl CoA, to the synthesis of oxaloacetate, which may enter the gluconeogenic pathway (36). This hepatic glucose sparing effect of propionate may have increased the amount of glucose available for pentose phosphate pathway as well as glycogen synthesis. Butyrate of dietary origin has been observed to delay the transient increase in mitochondrial ATP turnover, contributing glucose to glycogen metabolism, hence inducing a glucose sparing effect from oxidation (35, 37). Butyrate oxidation feeds acetyl CoA into TCA cycle thus impairing pyruvate oxidation, the end-product of aerobic glycolysis, and sparing glucose from oxidation. Butyrate has also been shown to reduce glycogenolysis from the newly synthesized glycogen (37). Increased proportions of butyrate and propionate in the cecal content of chicks fed DFM are in agreement with these observations. Overall, increased hepatic glycogen content within 2-h after re-feeding, combined with swift transition from fasting to feeding metabolism by EE, DFM or the combination of EE and DFM supplemented chicks suggest a positive energy state.
CONCLUSIONS

Supplementation of EE, DFM or both increased the FE of broiler chickens from d 1-21. However, the combination of EE and DFM resulted in an additive FE response, suggesting involvement of pathways independent of each other. The combination increased the energy utilization efficiency of chicks early in life, helping to overcome the inefficiency of immature digestive tract. Chicks fed EE, DFM or both were in a positive energy state as they were able to transition swiftly from fasting to feeding metabolism. Supplementation of DFM increased cecal SCFA concentration which may have resulted in altered hepatic energy metabolism.

ACKNOWLEDGEMENTS

We recognize the care for the birds provided by the staff of the Poultry Research and Teaching Unit of Iowa State University, and also like to thank M. Jeffrey, K. Bolek, E. Bobeck, and K. Nesheim of Iowa State University for assistance in conducting this experiment.
Literature Cited


Table 7.1 Composition of diets fed to broiler chicks from 1-21 d of age.

<table>
<thead>
<tr>
<th>Ingredient Composition</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>531.0</td>
</tr>
<tr>
<td>Soybean meal (48% CP)</td>
<td>270.5</td>
</tr>
<tr>
<td>Dried distillers grains with solubles</td>
<td>120.0</td>
</tr>
<tr>
<td>Meat/bone meal</td>
<td>30.0</td>
</tr>
<tr>
<td>Soy oil</td>
<td>9.5</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>3.0</td>
</tr>
<tr>
<td>L-lysine$^1$</td>
<td>5.0</td>
</tr>
<tr>
<td>L-threonine</td>
<td>0.6</td>
</tr>
<tr>
<td>Salt</td>
<td>3.5</td>
</tr>
<tr>
<td>Limestone</td>
<td>9.0</td>
</tr>
<tr>
<td>Di-calcium phosphate</td>
<td>10.6</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin and mineral premix$^2$</td>
<td>6.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical Composition (calculated)</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolizable energy (kcal/kg)</td>
<td>2,950</td>
</tr>
<tr>
<td>Crude protein</td>
<td>227.5</td>
</tr>
<tr>
<td>Ether extract</td>
<td>47.3</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>30.9</td>
</tr>
<tr>
<td>Calcium</td>
<td>10.0</td>
</tr>
<tr>
<td>Non-phytate phosphorus</td>
<td>4.9</td>
</tr>
<tr>
<td>Digestible methionine + cysteine</td>
<td>9.2</td>
</tr>
<tr>
<td>Digestible lysine</td>
<td>12.3</td>
</tr>
<tr>
<td>Digestible threonine</td>
<td>8.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chemical Composition (analyzed)</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross energy (kcal/kg)</td>
<td>4,017</td>
</tr>
<tr>
<td>Crude protein</td>
<td>227.2</td>
</tr>
<tr>
<td>Ether extract</td>
<td>39.9</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>26.1</td>
</tr>
</tbody>
</table>
1Contained 50.7% of L-lysine in the form of L-lysine sulfate, 0.1% Methionine, 0.1% Cystine, 0.3% Threonine, 0.1% Tryptophan, 0.6% Arginine, 0.3% Isoleucine, 0.5% Leucine, and 0.4% Valine.

2Provided per kg of diet: Selenium-250 µg; Vitamin A-8,250 IU; Vitamin D3-2,750 IU; Vitamin E-17.9 IU; Menadione- 1.1 mg; Vitamin B12-12 µg; Biotin-41 µg; Choline-447 mg; Folic acid-1.4 mg; Niacin-41.3 mg; Pantothenic acid-11 mg; Pyridoxine-1.1 mg; Riboflavin-5.5 mg; Thiamine-1.4 mg; Iron-282 mg; Magnesium-125 mg; Manganese-275 mg; Zinc-275 mg; Copper-27.5 mg; Iodine-844 µg.
Table 7.2 Effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on body weight gain, feed intake and feed efficiency of broiler chicks from 1-21 d of age.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Body weight gain (g/chick)</th>
<th>Feed intake (kg/cage)</th>
<th>Feed efficiency (gain in g/feed in kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d 1-7</td>
<td>d 1-14</td>
<td>d 1-21</td>
</tr>
<tr>
<td>Control (CON)</td>
<td>100</td>
<td>353</td>
<td>672</td>
</tr>
<tr>
<td>CON + EE</td>
<td>93</td>
<td>335</td>
<td>644</td>
</tr>
<tr>
<td>CON + DFM</td>
<td>100</td>
<td>346</td>
<td>685</td>
</tr>
<tr>
<td>CON + EE + DFM</td>
<td>102</td>
<td>358</td>
<td>690</td>
</tr>
<tr>
<td>SEM</td>
<td>4</td>
<td>9</td>
<td>15</td>
</tr>
</tbody>
</table>

EE<sup>2</sup> DFM<sup>2</sup>

<table>
<thead>
<tr>
<th></th>
<th>Body weight gain (g/chick)</th>
<th>Feed intake (kg/cage)</th>
<th>Feed efficiency (gain in g/feed in kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>100</td>
<td>350</td>
<td>679</td>
</tr>
<tr>
<td>+</td>
<td>97</td>
<td>347</td>
<td>667</td>
</tr>
<tr>
<td>SEM</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>-</td>
<td>96</td>
<td>344</td>
<td>658</td>
</tr>
<tr>
<td>+</td>
<td>101</td>
<td>352</td>
<td>688</td>
</tr>
<tr>
<td>SEM</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>

EE <i>P-value</i> | 0.48 | 0.75 | 0.44 | 0.09 | 0.12 | 0.15 | 0.02 | 0.02 | ≤ 0.01 |
DFM <i>P-value</i> | 0.30 | 0.38 | 0.06 | 0.97 | 0.03 | 0.61 | 0.05 | 0.40 | ≤ 0.01 |
EE x DFM <i>P-value</i> | 0.33 | 0.10 | 0.26 | 0.07 | 0.02 | 0.05 | 0.04 | 0.58 | 0.02 |

<sup>a,b</sup> Least square means in the same column without a common superscript differ significantly, <i>P</i> ≤ 0.05

<sup>1</sup>n = 8 replicates of 7 chicks per treatment for interactions.

<sup>2</sup>n = 16 replicates of 7 chicks per group for main effects.
Table 7.3 Effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on the body composition of broiler chicks at 7, 14 and 21 d of age.

<table>
<thead>
<tr>
<th>Treatments(^1)</th>
<th>Fat mass (% of total body mass)</th>
<th>Lean mass (% of total body mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d 7</td>
<td>d 14</td>
</tr>
<tr>
<td>Control (CON)</td>
<td>18.8(^a)</td>
<td>16.6</td>
</tr>
<tr>
<td>CON + EE</td>
<td>20.0(^a)</td>
<td>18.4</td>
</tr>
<tr>
<td>CON + DFM</td>
<td>18.0(^{ab})</td>
<td>17.9</td>
</tr>
<tr>
<td>CON + EE + DFM</td>
<td>15.4(^b)</td>
<td>16.3</td>
</tr>
<tr>
<td>SEM</td>
<td>1.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>

\(^2\) n = 16 chicks per group for main effects.

<table>
<thead>
<tr>
<th>EE(^2)</th>
<th>DFM(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>18.4</td>
</tr>
<tr>
<td>+</td>
<td>17.7</td>
</tr>
<tr>
<td>SEM</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EE(^2)</th>
<th>DFM(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>19.4</td>
</tr>
<tr>
<td>+</td>
<td>16.7</td>
</tr>
<tr>
<td>SEM</td>
<td>0.7</td>
</tr>
</tbody>
</table>

EE \(P\)-value 0.44 0.98 0.50 0.39 0.30 0.81
DFM \(P\)-value \(\leq 0.01\) 0.85 0.27 0.01 0.39 0.65
EE x DFM \(P\)-value 0.05 0.42 0.38 0.05 0.51 0.57

\(^a\) Least square means in the same column without a common superscript differ significantly, \(P \leq 0.05\)
\(^b\) n = 8 chicks per treatment for interactions.
\(^1\) n = 8 chicks per treatment for interactions.
\(^2\) n = 16 chicks per group for main effects.
Supplemental Figure 7.1 Effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on serum metabolite concentration of broiler chicks at 21 d of age. A. Glucose; B. Triglycerides; C. Cholesterol; D. Uric acid; E. Urea nitrogen; F. NEFA; G. Cholesterol.
Uric acid; D. Urea nitrogen; E. Non-esterified fatty acids; F. Cholesterol. \textsuperscript{a,b} Least square mean columns in the same graph without a common superscript differ significantly, $P \leq 0.01$. n = 16 chicks per group for main effects and 8 chicks per treatment for interactions.
**Supplemental Figure 7.2** Effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on hepatic enzyme activity and glycogen content of broiler chicks at 21 d of age. A. Fatty acid synthase activity; B. Glucose-6-phosphate dehydrogenase activity; C. Glyceraldehyde-3-phosphate dehydrogenase activity; D. Glycogen concentration. a,b Least square mean columns in the same graph without a common superscript differ significantly, \( P \leq 0.01 \). n = 16 chicks per group for main effects and 8 chicks per treatment for interactions.
**Supplemental Figure 7.3** Main effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on the molar proportion of cecal short-chain volatile fatty acid in broiler chicks at 21 d of age. a,b Least square mean columns without a common superscript differ significantly, $P \leq 0.01$. $n = 16$ chicks per group.
CHAPTER 8

CONCLUSIONS

Laying hens fed diets supplemented with exogenous enzymes (EE) and direct-fed microbial (DFM) on top of a 100 kcal/kg metabolizable energy (ME) reduction over a 16 week period resulted in increased nitrogen corrected apparent ME (AMEn) and the DFM appeared to increase the consistency of the response. However, individual hens fed diet with a 90 kcal/kg reduction in ME over a 12 week period did not differ in AMEn in comparison to hens fed the standard ME diet. In broiler chickens, supplementation of either EE or DFM resulted in increased AMEn, whereas the combination of EE and DFM additively increased the AMEn. This additive response indicated the involvement of independent pathways for EE and DFM in exerting their effects.

The effects of EE were primarily exerted through increased digestion and absorption of dietary nutrients as increased ileal digestibility for starch, amino acids and crude protein were observed with the supplementation of EE. Up-regulated activity of brush border carbohydrases and aminopeptidase by EE may have led to an increase in substrate presence at the apical membrane. The activity of mucosal transporters increase monotonically with increased substrate concentration at the mucosa. This is in agreement with the increased ex vivo electrophysiological ileal mucosal D-glucose and L-lysine transport observed in laying hens fed diets supplemented with EE.

Supplementation of DFM increased ileal starch, amino acids and crude protein digestibility of broiler chickens. Furthermore, DFM increased total cecal short-chain fatty acid concentration
as well as the proportions of propionate and butyrate. However when using a different DFM, only an increase in the proportion of butyrate was observed. Differences in the strain composition between DFM may be attributed to differences in the results observed in short-chain fatty acids. Butyrate has also been observed to increase the arterial blood flow as well as reduce the gastrointestinal motility involving peptide YY resulting in increased nutrient absorption. This is in agreement with the increased ex vivo electrophysiological ileal mucosal nutrient transport for D-glucose, DL-methionine and L-lysine in broiler chickens fed DFM. Increased ileal mucin mRNA expression and ileal and colon epithelial barrier function were also observed in DFM fed broiler chickens raised under conditions simulated to commercial environment. In laying hens, supplementation of DFM for a 16 week period increased ileal mucin mRNA expression and colon epithelial barrier function while reducing the colonization of zoonotic pathogens such as Campylobacter spp. These data on increased utilization of dietary nutrient and increased gut integrity may have contributed to increased utilization of dietary energy.

Broiler chickens supplemented with EE, DFM or the combination were able to transition swiftly from fasting to feeding metabolism. Chicks fed EE, DFM or the combination had reduced serum non-esterified fatty acid concentration compared to chicks fed the un-supplemented diet after 12 hours of fasting followed by 2 hours of re-feeding. Increased hepatic glucose-6-phosphate dehydrogenase activity and glycogen concentration for chicks fed DFM supplemented diet indicated that excess glucose was diverted to pentose phosphate pathway and glycogen synthesis in the form of glucose-6-phosphate. This may be due to the increased concentration of butyrate sparing the glucose in hepatocytes for glycogen synthesis. Moreover, the hepatic glucose sparing effect of propionate may have increased the amount of glucose available for pentose phosphate pathway as well as glycogen synthesis. The avian liver contributes significantly to circulating
glucose levels through glycogenolysis and an increased hepatic glycogen store indicated a positive energy state of these chicks. Increased utilization of glucose in the pentose phosphate pathway also provides reducing power in the form of NADPH. This enables cells to counterbalance the energy expensive oxidative stress.

The effects of energy on body composition is apparent as energy retention is the net result of energy intake and expenditure. Reduction of 90 kcal/kg of dietary ME over a 12 week period significantly reduced the abdominal fat pad weight of individually fed laying hens by 23%. These data suggest that energy is preferentially utilized for production and maintenance before storage requirements in laying hens as these hens did not alter performance. Supplementation of the combination of EE and DFM to broiler chickens resulted in reduced fat deposition and increased lean tissue accretion in an additive manner after the first week of life. The enhanced energy utilization efficiency by EE and DFM could be attributed to the immaturity of the digestive tract early in life, as these changes in body composition were not observed either at the end of second or third weeks. However, chicks fed DFM supplemented diets for a period of 3 weeks had reduced serum triglyceride (TG) and cholesterol concentration in comparison to chicks fed un-supplemented diets. This indicated a reduction in the de novo TG and cholesterol synthesis as all avian TG and cholesterol are processed and released from the liver. Reduced lipogenesis may be attributed to increased propionate as it is an effective inhibitor of the incorporation of acetate into TG and cholesterol esters. Moreover, increased hepatic glucose-6-phosphate activity due to increased removal of glucose-6-phosphate, the glucose-signaling metabolite for de novo lipogenesis by activating carbohydrate response element binding protein, through pentose phosphate pathway is in agreement with reduced hepatic de novo lipogenesis.
In summary (Fig. 8.1), laying hens fed diet with 90 kcal/kg reduced metabolizable energy (ME) over a 12 week period did not significantly alter performance variables such as feed intake, hen-day egg production, egg weight and egg mass, or body weight. A reduction of 100 kcal/kg of dietary ME over a 16 week period also did not significantly alter feed intake, hen-day egg production, egg weight and egg characteristics, or body weight of laying hens. Supplementation of EE and DFM on top of diets with 100 kcal/kg reduced ME also did not change hen performance. These data indicate that modern laying hens have little to no sensitivity to small differences in dietary ME contributed by EE and DFM as these hens prioritize to meet the energy demands of production. However, supplementation of EE, DFM or the combination on top of low ME diets fed to broiler chicks resulted in increased feed efficiency in an additive manner. Moreover, the additional energy contributed by EE and DFM is directed towards maintaining a positive energy state, resulting in increased performance of the birds. Hence increased retention and efficient utilization of energy with the addition of EE and DFM can be beneficial in increasing the productivity of broiler chickens.
Figure 8.1 A comprehensive outline on the effects of exogenous enzymes (EE) and direct-fed microbial (DFM) on the intestinal integrity, energy metabolism, energy utilization and performance of chickens. *Results observed in the small intestine; †Results observed in the large intestine. The dotted lines represent observations reported in published data.
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