PERFORMANCE AND MOLECULAR PARAMETERS OF BROILER CHICKENS FED DIET WITH OR WITHOUT EXOGENOUS SOURCE OF METHIONINE AND RAISED IN ORGANIC OR CONVENTIONAL PRODUCTION ENVIRONMENT

by

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(Under Direction of Samuel E. Aggrey)

ABSTRACT

According to the National Organic Program, synthetic amino acids are not permitted in organic production although low levels of synthetic methionine (Met) is permitted. The current national organic standard board (NOSB) recommended level (0.1% Met) pose issues and concerns for some organic producers citing lack of commercially available natural sources of methionine, and consideration pertaining to poultry health and welfare. Here, we examined the effects of complete D,L-Methionine (DLM) replacement by Brazil nut meal in organically raised broilers. It was concluded that the use of Brazil nut meal is a viable substitute for DLM in organically raised broilers, exhibiting no differences in growth and carcass yield compared to broilers fed a conventional diet and better feed conversion compared to broilers fed a NOSB organic diet. mRNA expression of genes in methionine metabolism suggests that an organic diet incorporating Brazil nut meal as a methionine source was comparable to a conventional diet containing DLM.

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CHAPTER 1

INTRODUCTION

Methionine is an essential amino acid in poultry and is important for metabolic function. It plays a vital role in transmethylation, which in turn affects many other pathways; including DNA methylation and RNA synthesis. Additionally, methionine is key in the synthesis of other sulfur-containing amino acids, such as cysteine and homocysteine. It is also a precursor to carnitine and glutathione, both of which fend off oxidative stress (Jacob, 2013; Jankowski et al., 2014).

Conventional broiler feed is corn and soybean meal based (Aguirre et al., 2016). However, corn has low lysine, cysteine, and methionine content. Additionally, soybean meal is low in methionine and cysteine. Therefore, methionine is the first limiting amino acid for this combination of feed and must be supplemented to meet bird requirements (Yodseranee and Bunchasak, 2012).

Poultry is typically given synthetic methionine in the form of dry D,L-Methionine, which consists of a 1:1 ratio of D- and L- isomer. (Burley, 2012). However, on October 1, 2012, the National Organic Standards Board (NOSB) ruled that in order to be classified as organic, no more than 2 pounds of D,L-Methionine per ton are to be used to feed organic broilers (USDA, 2012). Further, the European Union has banned all synthetic amino acids in organic poultry diets (Burley et al., 2016). It has been shown that the inclusion of only 0.1% D,L-Methionine leaves a conventional corn and soybean meal based diet deficient in methionine, requiring an increase in crude protein composition in order to meet methionine needs. Such high protein content, however, raises ammonia secretion which is harmful to both bird health and the environment (Burley et al., 2013).

Previous work has indicated non-synthetic sources of methionine such as high methionine corn, sunflower meal, and Brazil nut powder are capable of replacing D,L-Methionine in poultry diets and still meet the required amount of methionine for the birds used (Burley, 2012; Burley, 2013; Fanatico, 2010; Rao et al., 2005). Additionally, these studies found broilers fed a diet with naturally sourced methionine either performed similarly or outperformed broilers fed D,L-Methionine.

The focus of this study will be on the growth, performance, and gene expression of broilers fed an organic source of methionine. Additionally, the effect of production environment on these factors will be analyzed. Such organic diet studies in the past have been performed in either conventional floor pens or battery cages. It is believed, however, the environment in which broilers are raised will also affect growth, performance, and gene expression.

The objectives of this study are to 1) formulate a diet which meets broiler methionine requirements through the use of a non-synthetic, organic source of methionine, 2) observe the effects of the newly formulated diet in relation to an NOSB diet and a conventional diet on growth, performance, and gene expression, and 3) observe the effects of production environment on growth, performance, and gene expression.

CHAPTER 2

LITERATURE REVIEW

Amino acids

Amino acids play a vital role in protein metabolism. Proteins are formed using the 20 canonical amino acids during the process of translation, where mRNA encodes for a sequence of these amino acids, creating a protein. Along with being the fundamental building block of proteins, amino acids regulate protein synthesis and degradation. Amino acids promote protein synthesis as well as inhibit proteolysis, the process of breaking down proteins (Tesseraud et al., 2006; Tesseraud et al., 2011).

Sulfur-containing amino acids

There are four sulfur-containing amino acids, two of which are incorporated in proteins. Methionine and cysteine are both found in proteins, whereas taurine and homocysteine are both free amino acids (Figure 2.1) (Atmaca, 2004; Brosnan and Brosnan, 2006).

Methionine is a hydrophobic amino acid, mainly found in the hydrophobic core of globular proteins and in the hydrophobic layer of membrane proteins (Gomez-Tamayo et al., 2016). Cysteine is most known for its ability to form disulfide bonds, either within a protein or between two protein chains. The bond is formed without the use of an enzyme; however enzymes such as disulfide isomerase can move around a mismatched disulfide bond to help with protein folding (Brosnan and Brosnan, 2006).

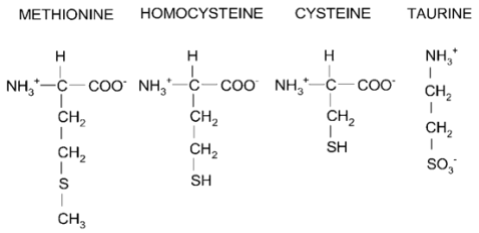

Figure 2.1. Structures of the four sulfur-containing amino acids. (Brosnan and Brosnan, 2006)

Methionine metabolism

Methionine is essential to the formation of the homocysteine, cysteine, and taurine. The formation of homocysteine is done through the transmethylation pathway; a path in which compounds are methylated. Upon the formation of homocysteine, cysteine and taurine are able to be produced via the transsulfuration pathway. Additionally, homocysteine has the ability to be methylated to form methionine through the remethylation pathway. These three pathways encompass methionine, homocysteine, cysteine, and taurine metabolism.

Transmethylation pathway

Methionine is first converted to S-Adenosylmethionine (SAM) in the transmethylation pathway. SAM is comprised of methionine linked to the 5' carbon of adenosine. The reaction is catalyzed by methionine adenosyltransferase (MAT) and requires ATP. All three phosphates are cleaved from ATP and adenosine is linked to the sulfur in methionine. The three phosphates leave as a single inorganic phosphate and an inorganic pyrophosphate, the latter being catabolized yielding energy (Atmaca, 2004).

A key reaction in transmethylation is the conversion of SAM to S-Adenosylhomocysteine (SAH), catalyzed by a methyltransferase. There are a variety of methyltransferases that transfer the methyl group of SAM onto nitrogen, oxygen, and occasionally carbon. These methyltransferases utilize an SN2 reaction where a base deprotonates either the nitrogen, oxygen,

or carbon on methyl acceptor. The reaction forces the electrons from the acceptor to attack the methyl group of SAM. The methyl group on SAM is a hot electrophile because of the positive charge on the adjacent sulfur, a charge which sulfur does not like. The leaving group for this reaction is SAH, and the product is the newly methylated compound. A common methyl acceptor in this reaction is DNA, which often has the fifth carbon of a cytosine ring methylated. DNA methylation is vital to many cellular processes including embryonic development, X-chromosome inactivation, genomic imprinting, gene suppression, and chromosome stability (James et al., 2008; Robertson, 2015).

Once the methyl group of SAM has been removed and SAH is formed, SAH hydrolase (SAHH) hydrates SAH, releasing adenosine and producing homocysteine. This hydrolysis reaction serves as a regulator for methylation reactions. SAH inhibits many methyltransferases that utilize SAM as a methyl donor. Thus, the SAM/SAH ratio indicates transmethylation and the use of SAM as a methyl donor. A decrease in the ratio points to reduced methylation capacity. Under normal conditions, SAH is in equilibrium with homocysteine, however the hydrolysis of SAH is rapid, maintaining a flux towards hydrolysis. There are then two paths for homocysteine; it can be remethylated to methionine or enter the transsulfuration pathway (Lu, 2009; Mato and Lu, 2007; Selhub and Miller, 1992; Zhang et al., 2016).

Transsulfuration pathway

Homocysteine can go through transsulfuration resulting in the production of cysteine. This is done by a couple of enzymes, the first being cystathionine-β-synthase (CBS) catalyzing a dehydration reaction. CBS is stimulated by SAM, thereby taking homocysteine through transsulfuration during times of high SAM levels. CBS produces cystathionine and water from homocysteine and serine, an alcohol containing amino acid. This step prepares for the formation

of cysteine, which is produced in the following reaction catalyzed by cystathionine γ-lyase (CGL). The ammonia group is released from the former homocysteine, forming α-ketobutyrate, and the thiol group is linked to the former serine, which is now cysteine. α-ketobutyrate and cysteine are split through hydrolysis catalyzed by CGL. Regulation of this pathway is thought to be centered around CBS because of its sensitivity to oxidative properties. Antioxidants suppress the pathway where peroxidases increase the flux (Aitken et al., 2011; Finkelstein, 1998; Mato and Lu, 2007; Selhub and Miller, 1992).

Cysteine now has two fates; it can either enter glutathione synthesis or be converted to taurine. If it is converted to taurine, cysteine dioxygenase (COO) adds O_2 to produce cysteine sulfinic acid. Sulfinoalanine-decarboxylase (CSO) oxidizes cysteine sulfinic acid by removing CO_2 and producing hypotaurine which then reduces NAD^+ to NADH by hypotaurine dehydrogenase and the addition of water, forming taurine (James et al., 2008; Lu, 2009).

Cysteine can also be combined with glutamate, forming γ-glutamylcysteine by the activity of glutamate cysteine ligase (GCL). Glutathione is formed using γ-glutamylcysteine by glutathione synthase (GS). Glutathione, an antioxidant, is then able to be oxidized and subsequently reduced in a cycle, protecting cells from oxidative stress. Glutathione is additionally able to be recycled to form cysteine in times of need (Lu, 2009).

Remethylation pathway

If the fate of homocysteine is not transsulfuration, it can be remethylated to methionine through the enzyme methionine synthase (MS). This reaction takes homocysteine and adds a methyl group donated from N^5-methyltetrahydrofolate (5-methyl-THF). The methyl group on 5-methyl-THF is first transferred to vitamin B_{12} which synthesizes methylcobalamin, which then transfers the methyl group to homocysteine, creating methionine (Finkelstein, 1998).

The important methyl donor in remethylation is 5-methyl-THF, which is produced and recycled in the folate cycle. This cycle starts with the intake of folate. Folate is reduced to dihydrofolate through dihydrofolate synthase (DHFR), which oxidizes NADPH in the process. DHFR further reduces dihydrofolate to tetrahydrofolate (THF) and oxidizes NADPH to $NADP^+$. THF is methylated by serine hydroxymethyltransferase (SHMT), which consumes serine and releases glycine, ultimately producing 5,10-methylene-THF. Methylenetetrahydrofolate reductase (MTHFR) oxidizes NADPH and reduces 5,10-methylene-THF, freeing the methyl group to be transferred to homocysteine and producing 5-methyl-THF (Finkelstein, 1998).

Another important remethylation pathway in the methionine cycle involves the incorporation of choline. Choline is an essential nutrient that plays many roles in the cell, but the one played in the methionine cycle is the reduction of homocysteine. Choline's structure is similar to homocysteine, however the thiol group in homocysteine is an alcohol group in choline. Through the action of choline oxidase, betaine is formed from choline. Betaine will be the methyl donor and hands off its methyl group to homocysteine through the action of betaine-homocysteine methyltransferase (BHMT). Betaine is a polar compound where the carboxyl oxygen has a negative charge and the nitrogen with three methyl groups has a positive charge. When a methyl group is transferred, the negatively charged oxygen picks up the hydrogen from homocysteine's thiol group, enabling the methyl group to join and form methionine. This transfer creates dimethylglycine (DMG) which is released. DMG is oxidized and a methyl group is released producing sarcosine. Sarcosine can be converted to glycine by a reversible reaction with the involvement of SAM and SAH and the transfer of a methyl group (Fu et al., 2016; Zeisel and da Costa, 2009).

Importance of the sulfur-containing amino acids in poultry

Methionine is an essential amino acid in poultry and is important for metabolic function. It plays a vital role in transmethylation, which in turn effects many other pathways; including DNA methylation and RNA synthesis. Additionally, methionine is key in the synthesis of other sulfur-containing amino acids, such as cysteine and homocysteine. It is also a precursor to carnitine and glutathione, both of which fend off oxidative stress (Jacob, 2013; Jankowski et al., 2014).

Both methionine and cysteine have important roles in poultry immune health. Methionine is an essential building block in immune cells and tissue and is included in the nonspecific mechanisms in skin and mucosa. Cysteine has been shown to increase mononuclear cell proliferation in the spleen and also enhances T cell proliferation (Wu et al., 2012; Wu et al., 2013).

Immune system associations

Humoral and cellular immunity is greatly affected by methionine, due to its role in clonal proliferation of lymphocytes, effector molecule synthesis, and immunoglobulin affinity in the bursa. The bursa is the primary lymphoid organ in poultry and plays an important role in differentiation of B lymphocytes. The integrity of the bursa is related to humoral immune function, providing a good reference when judging bird health. During times of methionine deficiency, the weight of the bursa is reduced and causes reduced lymphocyte proliferation (Wu et al., 2013). The thymus is also a vital organ for poultry immunity. When a bird is deficient in methionine, the thymus has been shown to have lower T-cell numbers, decreasing T-cell migration to the bloodstream. This decrease in T-cells negatively impacts bird health, due to the importance of T-cells during inflammatory and antigen response (Wu et al., 2012). Additional studies have shown an increase in leukocytes numbers, lymphocyte and heterophils percentage, and antibody response

to infection in poultry fed higher levels of methionine (Mirzaaghatabar et al., 2011; Tsiagbe et al., 1987).

Antioxidants

The term reactive oxygen species (ROS) refers to free radicals containing oxygen, such as: hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2), superoxide ($O^{\bullet 2-}$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$) (Colovic et al., 2018; Salami et al., 2015). An excess of free radicals can cause damage to biological molecules and create undue molecular stress (Surai et al., 2017). To combat these damaging ROS, antioxidants are used which interrupt oxidation (Halliwell and Gutteridge, 1999). Antioxidants do this by donating an electron to the freed radical, neutralizing it. Some antioxidant compounds and amino acids that incorporate sulfur in their structure are methionine, cysteine, taurine, glutathione (GSH), and N-acetylcysteine (NAC) (Atmaca, 2004).

Cysteine is the precursor for both GSH and taurine. It is also the rate-limiting amino acid for GSH synthesis. In this way, cysteine is crucial for antioxidant function and protection against oxidative stress (Atmaca, 2004; Bin et al., 2017; Colovic et al., 2018).

GSH is cysteine-based and is arguably the most vital antioxidant. GSH is found throughout the cytosol of a cell and present in some organelles. ROS easily oxidize GSH, forming glutathione disulfide (GSSG), which can be reduced back to GSH by glutathione reductase (Atmaca, 2004; Bin et al., 2017; Colovic et al., 2018; Lu, 2009).

NAC is a cysteine derivative and an intermediate in the formation of GSH. The sulfhydryl groups present in NAC give the compound antioxidant properties. NAC prevents oxidative harm by scavenging for free radicals. Further, NAC can be hydrolyzed to form cysteine, making it an important compound in GSH synthesis (Atmaca, 2004; Colovic et al., 2018).

Taurine is an additional antioxidant that scavenges for free radicals. It is a product formed in the transsulfuration pathway. The free amino acid is present in high concentrations in the muscle, kidney, liver, and brain. Taurine directly scavenges for ROS, increases GSH oxidation activity, and prevents disturbance in membrane permeability by ROS, ultimately reducing oxidative stress (Colovic et al., 2018).

Methionine is an endogenous antioxidant susceptible to oxidation and is often vulnerable when exposed on the surface of a protein. Methionine's potential oxidation protects other vital amino acids from oxidation. Even when oxidized, methionine retains its biological function. The oxidized form of methionine can be restored by methionine sulfoxide reductase, which reduces oxidized methionine back to methionine (Atmaca, 2004; Colovic et al., 2018; Zhang et al., 2013).

Complications of methionine deficiency in poultry

When accessing the effects of methionine deficiency on performance and growth, studies have shown decreased weight and poor performance in methionine deprived poultry (Deng et al., 2007; Rodenburg et al., 2008; Rubin et al., 2007). Additional studies have shown methionine's role in protein synthesis as well as poultry immune health. Poultry fed a diet deficient in methionine had reduced bursa weight in addition to low proliferation of B-cells and T-cells (Jankowski et al., 2014; Mirzaaghatabar et al., 2011; Tsiagbe et al., 1987; Wu et al., 2012; Wu et al., 2013). Results seen in these previous studies suggest methionine deficiency negatively impacts performance and health of poultry.

Amino acid requirements

Requirements for amino acids have increased over the decades (Farkhoy et al., 2012). The genetic improvement of the broiler means a faster rate of growth and decreased time to maturity. This faster growth results in a greater need for amino acids to complete metabolic processes and

protein development. Current production broilers require a higher amount of amino acids during the first stage of development (starter phase), with the requirement decreasing with age. Broilers such as Ross 308 require 1.28 and 0.95 percent lysine and cysteine + methionine (TSAA), respectively, during the starter phase and reduce to 1.06 and 0.83 percent lysine and TSAA, respectively, during the final phase of growth (Avigen, 2014).

Sources of amino acids

Conventional broiler feed is corn and soybean meal based (Aguirre et al., 2016). However, corn has low lysine, cysteine, and methionine content. Additionally, soybean meal is low in methionine and cysteine. Therefore, methionine is the first limiting amino acid for this combination of feed and must be supplemented to meet bird requirements (Yodseranee and Bunchasak, 2012).

Methionine has two conformations; D- or L- isomers (Jacob, 2013). The D-form is not biologically active; however, poultry can convert the D-isomer to the L-isomer (Yodseranee and Bunchasak, 2012). Poultry is typically given synthetic methionine in the form of dry D,L-methionine, which consists of a 1:1 ratio of D- and L- isomer. (Burley, 2012). The conversion of D-Methionine to its L-isomer is so efficient that no differences have been seen between birds fed L-Methionine and D,L-Methionine in terms of weight gain and feed conversion (Dilger and Baker, 2007). Methionine can be further supplemented through the use of 2-hydroxy-4-(methylthio) butanoic acid (HMB). Similarly, HMB is converted to the biologically active L-Methionine through a pathway utilizing multiple enzymes (Dibner and Knight, 1984; Martín-Venegas et al., 2006).

Complications facing organic poultry production

On October 1, 2012, the National Organic Standards Board (NOSB) ruled that in order to be classified as organic, no more than 2 pounds of D,L-Methionine per ton are to be used to feed organic broilers (3 pounds per ton for turkeys and all other poultry) (USDA, 2012). Further, the European Union has banned all synthetic amino acids in organic poultry diets (Burley et al., 2016). Table 2.1 illustrates the challenges of formulating a diet when synthetic methionine is limited or absent. The column labeled 5 lb/ton illustrates conventional amounts of D,L-Methionine. The column labeled 2 lb/ton illustrates an amount of D,L-Methionine tolerated by NOSB guidelines. The column labeled 0 lb/ton illustrates a diet containing no D,L-Methionine. Only at conventional levels do crude protein level and methionine content meet requirements. The remaining two diets have increased soybean meal, which results in excessive crude protein levels in order to meet methionine requirements. An increase in crude protein in poultry diets lead to excessive ammonia secretion thereby negatively impacting the environment (Burley et al., 2013).

Table 2.1. Nutrient composition of broiler starter diet with varying levels of D,L-Methionine (Burley et al., 2016)

	Broiler Starter Diet		
Ingredient (%)	5 lb/ton	2 lb/ton	0 lb/ton
Corn	41.39	38.86	15.29
Soybean meal	41.69	56.26	78.32
Wheat middlings	10.0	-	-
Soybean oil	2.93	1.03	2.79
limestone	1.38	0.50	1.30
Di-calcium phosphate	1.55	1.51	1.41
Salt	0.50	0.50	0.50
Vit-TM premix	0.40	0.40	0.40
D,L-Methionine	0.166	0.10	-
Calculated Composition			
M.E. (kcal/kg)	3025	3025	3025
Crude protein	23.68	28.32	36.05
Ether extract	7.77	6.67	9.69
Lys	1.31	1.64	2.19
Met	0.51	0.51	0.51
TSAA	0.90	0.96	1.08
Trp	0.30	0.37	0.49
Ile	1.00	1.23	1.61
Thr	0.90	1.10	1.42
Val	1.11	1.34	1.73
Calcium	1.00	1.00	1.00
Available phosphorus	0.45	0.45	0.45

Organic sources of Methionine

As a result of the importance of methionine and the restriction of synthetic methionine in organic poultry, producers are in need of an organic source of methionine for organic poultry. Table 2.2 identifies various feed ingredients with high amounts of methionine. Brazil nut meal, egg-based products, and fish meal are some ingredients with the highest amounts of methionine. Past studies have indicated Brazil nut meal in combination with wheat middlings provide adequate amounts of methionine for broilers (Burley, 2012). Fish meal has also been studied for its effect on broiler performance and yield, but the negative impacts due to ammonia excretion and meat quality have proved fish meal to be flawed (Jacob, 2013). Additional ingredients shown to have high methionine content have ultimately been ruled out because of negative impacts on health and performance, or because the ingredient is not practical or available for industrial use.

Table 2.2. Total methionine content of various feed ingredients (Burley et al., 2015)

Feed Ingredient	Methionine (%)
Brazil nut meal	3.35
Egg white	3.06
Casein	2.65
Egg membrane	2.90
Egg blend	2.44
Fish meal, Herring	2.16
Fish meal, Sardine	1.94
Fish meal, Menhaden	1.90
Potato protein	1.67
Sunflower seed meal	1.60
Corn gluten meal	1.49
Whole egg	1.48
Corn gluten meal (60% CP)	1.45
Sesame meal	1.40
Earthworm meal	1.23
Sesame meal (expeller)	1.22
Corn gluten meal (41% CP)	1.20
Sunflower seed meal (expeller)	1.04
Soy protein isolate	1.01
Poultry bi-product meal	1.01
Fish solubles	1.00
Lumbang meal	1.00
Yeast	1.00

Previous work

To address the problem facing organic poultry, Dematteˆ Filho et al. (2016) observed the effects of methionine supplementation via naturally obtained methionine. Treatment diets included a diet containing 99% D,L-Methionine and diets containing 100, 110, and 120 percent of natural methionine acquired from soybean. The group's results show most growth over the first 21 days in the treatment fed D,L-Methionine. Feed intake was highest in this treatment over that period, and lowest in the treatment fed 120% of natural methionine. At day 42, the treatment fed D,L-Methionine had the heaviest body weight while the treatments fed naturally sourced methionine all had similarly low body weights. The observed results were contributed to the possibility of differing amino acid absorption according to source and molecular availability, suggesting the alternative source of methionine used may have lower methionine availability than D,L-

Methionine. This theory is supported by numerous studies which observe differences in absorption between D,L-Methionine and D,L-Methionine hydroxyl. In conclusion of Dematthê Filho, et al. (2016), broilers fed methionine sourced from soybean showed a lower body weight and poorer feed conversion over 42 days than broilers fed D,L-Methionine (Dematthê Filho et al., 2016).

In another study, Jacob et al. (2008) examined the use of non-genetically modified high methionine (HM) corn as a methionine source for poultry diets. HM corn contains 53% more crude protein than traditional corn, with methionine content of 0.32%. In the study, two diets were formulated, one with conventional corn and synthetic methionine and one with HM corn to meet methionine requirements. Jacob et al. (2008) reported no differences in body weight, feed intake, or feed conversion. The group concluded the inclusion of HM corn in organic poultry diets is suitable for poultry (Jacob et al., 2008). However, the use of HM corn is less than ideal. The protein content is high in HM corn, making formulation an issue. Additionally, the yield of HM corn and the high moisture content make it unattractive to growers (Fanatico, 2010).

Sunflower seed meal (SSM) is high in methionine (0.63%), which lends itself to incorporation as an organic source of methionine. It was found that SSM could completely replace soybean meal in broiler diets. Broilers fed a starter diet void of synthetic methionine with SSM as a primary methionine source had improved body weight and performance compared to broilers fed a conventional starter diet containing synthetic methionine (Burley, 2013; Rao et al., 2005).

Investigation of Brazil nut powder on broiler performance was done by Burley (2012). Brazil nuts contain 3.35% methionine and 51.08% crude protein and could completely replace D,L-Methionine in broiler diets. Ross 308 broilers were raised for 21 days in battery cages and fed different diets containing various feed ingredients. The control diet was a conventional diet containing DL-Methionine, while the treatment containing Brazil nut powder contained no

synthetic methionine, but still met methionine requirements for the broilers used. The study found that inclusion of Brazil nut powder in a broiler diet during days 1 to 21 yielded similar growth and carcass yield as broilers fed a conventional diet (Burley, 2012).

Gene expression

To observe effects of diet on methionine metabolism, gene expression of vital enzymes used along transmethylation, remethylation, and transsulfuration pathways can be analyzed. Observation of gene expression is paramount in the understanding of molecular activity (Aggrey et al., 2018).

MAT

Research has shown there are at least three forms of Methionine adenosyltransferase (MAT) that perform the same reaction in mammals. Separate MATs appeared to have different kinetic characteristics along with dissimilar chromatographic behavior. MATI and MATIII are more prominent in liver tissue while MATII is present in all tissue (Table 2.3). The gene MAT1A encodes for the catalytic subunit for both MATI and MATIII. The MAT2A gene encodes for MATII catalytic subunit, α_2. Additionally, MAT2B encodes for the regulatory subunit for MATII (Table 2.4). The β subunit regulates MATII by lowering the inhibition constant (K_i) for SAM while lowering the Michaelis constant (K_m) for methionine (Halim et al., 1999; Lu and Mato, 2005; Ramani et al., 2011).

During times of methionine deficiency, the MAT1A gene in the liver is downregulated, conserving methionine suppressing the producing SAM. This leads to oxidative stress due to reduced SAM levels. Upregulation of the MAT1A gene points to increased SAM levels (Ghoshal et al., 2006; Niculescu and Zeisel, 2002).

Table 2.3. Methionine adenosyltransferase isoforms[1]

MAT Isoforms	Tissue	Inhibitor	Stimuli	Reference
MAT I	Liver	SAM (weak)		(Halim et al., 1999; Lu and Mato, 2005; Ramani et al., 2011)
MAT II	All tissues	SAM (strong)		(Halim et al., 1999; Lu and Mato, 2005; Ramani et al., 2011)
MAT III	Liver		SAM	(Halim et al., 1999; Lu and Mato, 2005; Ramani et al., 2011)

[1]SAM, S-Adenosylmethionine; MAT, Methionine adenosyltransferase

Table 2.4. Brief overview of three genes that encode for subunits on MATI, MATII, and MATIII[1]

Genes	Tissue	Encodes for	Conditions for high expression	Reference
MAT1A	Expressed mostly in the liver	α_1 catalytic subunit; MATIII (dimer) or MATI (tetramer)	High SAM levels	(Halim et al., 1999; Lu and Mato, 2005; Ramani et al., 2011)
MAT2A	Expressed in the fetal liver; gradually replaced by MAT1A	α_2 catalytic subunit; MATII	Low SAM levels	(Halim et al., 1999; Lu and Mato, 2005; Ramani et al., 2011)
MAT2B	All tissues	β regulatory subunit; MATII	Low SAM levels	(Halim et al., 1999; Lu and Mato, 2005; Ramani et al., 2011)

[1]SAM, S-Adenosylmethionine; MAT1A, Methionine adenosyltransferase 1, alpha; MAT2A, Methionine adenosyltransferase 2, alpha; MAT2B, Methionine adenosyltransferase 2, beta

GNMT

The gene GNMT encodes for the enzyme glycine N-methyltransferase (GNMT), which methylates glycine, forming sarcosine. SAM donates a methyl group for this reaction, making SAM levels important for GNMT activity. GNMT is inhibited by 5-methyl-THF (Table 2.5), a product of the reaction catalyzed by MTHFR. MTHFR is a key enzyme for remethylation, a process which occurs when there is a need for methionine, thus a need for SAM. Therefore, the need for SAM inhibits GNMT activity, which consumes SAM during transmethylation. Inversely, high SAM levels inhibit MTHFR activity, lowering 5-methyl-THF levels and relieving GNMT inhibition. Gene expression of GNMT is highest in times of high SAM levels, pointing to high methionine levels (Table 2.6) (Luka et al., 2009; Rowling et al., 2002).

The product of glycine methylation, sarcosine, has no known metabolic function. Sarcosine dehydrogenase converts sarcosine back to glycine while also producing methylene-THF. It is therefore hypothesized that the role of GNMT lies in its ability to regulate the SAM/SAH ratio (Luka et al., 2009).

Table 2.5. Brief overview of the enzyme Glycine N-methyltranferase[1]

Enzyme	Tissue	Inhibitor	Stimuli	Reference
Glycine N-methyltransferase	Largest amount in the liver and pancreas	5-methyl-THF	SAM (indirectly)	(Rowling et al., 2002)

[1]SAM, S-Adenosylmethionine

Table 2.6. Brief overview of the Gene GNMT[1]

Gene	Tissue	Encodes for	Conditions for high expression	Reference
GNMT	Largest amount in the liver and pancreas	Glycine N-methyltransferase	High SAM levels	(Luka et al., 2009)

[1]SAM, S-Adenosylmethionine

AHCY

Adenosylhomocysteinase (AHCY) is inhibited by the two products it produces, homocysteine and adenine. Additionally, AHCY is inhibited by cysteine, a product of transsulfuration (Table 2.7). The gene AHCY encodes for the enzyme adenosylhomocysteinase and is upregulated in tissues with low homocysteine levels (Table 2.8) (Chen et al., 2010; Ueland, 1982).

The concentration of SAH varies much more than SAM from tissue to tissue (Finkelstein, 1998). This supports the idea that homocysteine levels are not constant across tissues, and homocysteine metabolism varies. It has been that shown homocysteine levels in mice are highest in the liver and lowest in the spleen and brain (Chen et al., 2010). In a previous study examining gene expression of methionine deficient broilers, it was discovered that the AHCY gene expression levels differed between the duodenum and muscle tissue, suggesting homocysteine levels may differ from tissue to tissue (Aggrey et al., 2018). Skeletal muscles store the largest amount of amino acids, which can be sourced to meet amino acid requirements in other, more deficient, tissues (Wolfe, 2006). This could explain potential differences in gene expression across different tissues.

Table 2.7. Brief overview of the enzyme Adenosylhomocysteinase

Enzyme	Tissue	Inhibitor	Stimuli	Reference
Adenosylhomocysteinase	Most issues	Adenine, cysteine, homocysteine		(Chen et al., 2010; Ueland, 1982)

Table 2.8. Brief overview of the gene AHCY

Gene	Tissue	Encodes for	Conditions for high expression	Reference
AHCY	Most tissues	Adenosylhomocysteinase	Low homocysteine levels	(Chen et al., 2010)

BHMT

Expression of the BHMT gene is important when looking at remethylation of homocysteine. The enzyme betaine-homocysteine methyltransferase (BHMT) catalyzes the methylation of homocysteine through the use of betaine as a methyl donor. BHMT is inhibited by SAM (Table 2.9), and expression of the BHMT gene is highest during times of low methionine levels and high choline and betaine levels (Table 2.10) (Ji et al., 2007; Jurkowska et al., 2016; Slow and Garrow, 2006).

Downregulation of the BHMT gene points to reduced remethylation of homocysteine. However, there is another path of remethylation that utilizes MTR and MTHFR, so expression of all three genes must be examined before forming a conclusion (Aggrey et al., 2018).

Table 2.9. Brief overview of the enzyme betaine-homocysteine methyltransferase[1]

Enzyme	Tissue	Inhibitor	Stimuli	Reference
Betaine-homocysteine methyltransferase	Liver and other tissues	Taurine, SAM		(Ji et al., 2007; Jurkowska et al., 2016; Slow and Garrow, 2006)

[1]SAM, S-Adenosylmethionine.

Table 2.10. Brief overview of the gene BHMT

Gene	Tissue	Encodes for	Conditions for high expression	Reference
BHMT	Liver and other tissues	Betaine-homocysteine methyltransferase	Low methionine levels with excess choline or betaine	(Ji et al., 2007; Slow and Garrow, 2006)

MTRR

MTRR encodes for the enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTHFR). When methionine levels are high, SAM levels are high. High levels of SAM

inhibit MTHFR (Table 2.11), decreasing 5-methyl-THF levels, lowering inhibition of GNMT, thus allowing SAM to be converted to SAH. Low levels of SAM relieve MTHFR inhibition, allowing for remethylation (Selhub and Miller, 1992). Studies have shown mice deficient in MTHFR have elevated homocysteine levels, suggesting a need for the enzyme for proper remethylation of homocysteine (Mato and Lu, 2007). During times of low SAH levels, the MTRR gene is highly expressed (Table 2.12) (Chen et al., 2010).

Table 2.11. Brief overview of the enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase reductase[1]

Enzyme	Tissue	Inhibitor	Stimuli	Reference
MTHFR	Liver and other tissues	SAM		(Gaull et al., 1973; Swanson et al., 2001)

[1]SAM, S-Adenosylmethionine; MTHFR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase

Table 2.12. Brief overview of the gene MTRR[1]

Gene	Tissue	Encodes for	Conditions for high expression	Reference
MTRR	Most Tissues; high in pituitary glans	MTHFR	Low SAH levels	(Chen et al., 2010)

[1]SAH, S-Adenosylhomocysteine; MTHFR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase

MTR

The MTR gene encodes for the enzyme responsible for homocysteine methylation, methionine synthase (MS); using 5-methyl-THF as a methyl donor, resulting in the products THF and methionine. MS is inhibited by many factors, SAM analogs being most relevant currently (Table 2.13) (Zhang et al., 2012). During times of methionine deficiency, this remethylation pathway may be utilized, upregulating gene expression of MTR (Table 2.14) (Aggrey et al., 2018; Chen et al., 2010; Li et al., 1996; Ma et al., 1999).

Table 2.13. Brief overview of the enzyme methionine synthase[1]

Enzyme	Tissue	Inhibitor	Stimuli	Reference
Methionine synthase	Most tissues	SAM; Oxidative stress		(Swanson et al., 2001; Zhang et al., 2012)

[1]SAM, S-Adenosylmethionine

Table 2.14. Brief overview of the gene MTR[1]

Gene	Tissue	Encodes for	Conditions for high expression	Reference
MTR	Most tissues	Methionine synthase	Low SAH levels	(Chen et al., 2010; Li et al., 1996; Ma et al., 1999)

[1]SAH, S-Adenosylhomocysteine

CBS

Cystathionine-β-synthase (CBS) takes homocysteine down the transsulfuration pathway by converting homocysteine to cystathionine. SAM is an activator of CBS (Table 2.15), thus when SAM levels are high, the CBS gene is upregulated (Table 2.16) (Aggrey et al., 2018). CBS is also affected by oxidative stress. Free radicals induce overexpression of the CBS gene while also inhibiting MS. This stimulation of CBS allows for the production of antioxidants which alleviates oxidative stress (Del Vesco et al., 2015). Mice deficient in CBS have shown elevated homocysteine levels (Mato and Lu, 2007), suggesting the pathway is key in homocysteine metabolism.

Table 2.15. Brief overview of the enzyme Cystathionine-β-synthase[1]

Enzyme	Tissue	Inhibitor	Stimuli	Reference
Cystathionine-β-synthase	Most tissues		SAM; Oxidative stress	(Del Vesco et al., 2015; Janosik et al., 2001; Swanson et al., 2001)

[1]SAM, S-Adenosylmethionine.

Table 2.16. Brief overview of the gene CBS

Gene	Tissue	Encodes for	Conditions for high expression	Reference
CBS	High in liver and muscle	Cystathionine-β-synthase	Conditions of oxidative stress	(Chen et al., 2010; Del Vesco et al., 2015)

Reference:

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CHAPTER 3

PERFORMANCE OF BROILER CHICKENS FED A DIET WITH OR WITHOUT EXOGENOUS METHIONINE AND RAISED IN A CONVENTIONAL OR ORGANIC PRODUCTION ENVIRONMENT[1]

[1] Foutz, J.C., M.C. Milfort, A.L. Fuller, W.K. Kim, R. Rekaya, and S.E. Aggrey. To be submitted to *Poultry Science.*

Abstract

Synthetic amino acids are not permitted in organic feeds by the National Organic Standard Board (NOSB) in the United States. However, low levels of methionine (Met) are permitted, and the NOSB recommends 0.1% synthetic Met for broilers. This poses concerns pertaining to health and welfare of broilers. This research examined the effects of complete replacement of DL-Methionine (DLM) by Brazil nut powder in a 100% organic diet. 800 Ross308 chicks were hatched and randomly divided into 4 treatment groups with 4 replicates of 50 chicks and raised for 56 days. Treatments included birds fed a conventional diet and raised organically (Conv_Org), NOSB diet raised organically (NOSB_Org), a 100% organic diet and raised organically (Org_Org), and a conventional diet raised conventionally (Conv_Conv). Body weight at 56 days were 3,960, 3,995, 3,981 and 3,676 g for Conv_Org, NOSB_Org, Org_Org and Conv_Conv, respectively. Body weights of the Conv_Conv group were lower ($P<0.05$) than all the other treatments. The cumulative FCR (0-8 weeks) of Org_Org treatment group was lower ($P<0.05$) than the NOSB diet. The birds on the NOSB diet had increased feed intake perhaps to compensate for the deficiency in Met. The NOSB diet may not sufficiently meet the requirement of broiler chickens even when raised with access to pasture. There were no differences in carcass yield and composition in any treatment. These results show that the use of Brazil nut powder is a viable substitute for DLM in organically raised broilers, with no difference in growth and carcass yield and better FCR compared to current NOSB diet recommendation.

Introduction

Amino acids (AA) are vital in poultry diets. Essential AA, which cannot be synthesized in adequate amounts in birds, are provided to poultry through the diet (Jankowski et al., 2014). Methionine (Met) is the first limiting AA in a typical corn and soybean meal (SBM) diet, and plays an important role in poultry metabolism (Dematthê Filho et al., 2016; Tesseraud et al., 2011). Met, a sulfur-containing AA, is involved in over one hundred methylation reactions including the production of metabolites (Fu et al., 2016).

Though Met is central to poultry metabolism, conventional corn and SBM do not provide adequate Met for poultry (Jacob, 2013), and as a result, poultry diets are supplemented with synthetic Met (Jankowski et al., 2014). This is acceptable for conventionally raised poultry, but organically raised poultry are limited in the use of synthetic ingredients in the diet by the National Organic Standards Board (NOSB). Synthetic Met is restricted to 2 pounds per ton of feed for organic broilers feed (USDA, 2012). To provide Met to organic poultry, producers have turned to alternative organic sources of Met. Various studies (Burley et al., 2015; Jacob, 2013) have identified multiple plant and animal-based sources of Met, including Brazil nut meal, fish meal, sesame hulls, and corn gluten meal. Brazil nut meal contains about 3.35 % Met, compared to fish meal which contains about 2% Met but often leads to an undesirable fishy taste. Organic sesame hulls and corn gluten meal also contain high amounts of Met. Neither are commercially available in the United States.

For this study, Brazil nut powder was selected as a source of Met for poultry diets because of high Met content and digestibility (Milfort et al., 2017). This study directly compares a diet containing Brazil nut powder to both an NOSB and conventional diet. Conventional and organic environmental effects on growth and performance were also examined.

Materials and methods

A total of 800 Ross308 broilers were divided into 4 treatment groups of 4 replicates each with 50 birds. Birds were raised for 8 weeks. From d 1 to 14, the birds received a mash starter diet. Grower (d 15 to 28) and finisher (d 28 to 56) diets were pelleted. The conventional poultry diet (Conv_Org) was formulated using non-organic ingredients. The NOSB_Org diet was formulated using all organic ingredients and adhered to USDA National Organic Program criteria; and contained only 0.1% DL-Methionine (DLM). The Org_Org diet was formulated using Brazil nut powder as a source of Met and organic ingredients. The Conv_Conv treatment group received the same feed as the Conv_Org treatment. The starter, grower and finisher diets of all treatments are presented in Table 3.1, 3.2 and 3.3, respectively.

At hatch, 50 chicks were placed in 1.22 m by 1.83 m conventional pens with wood shavings. The birds were brooded for 14 days. Lighting and temperature management practices were in accordance with Ross 308 standards (Avigen, 2014). At 14 days of age, Conv_Org, NOSB_Org, and Org_Org treatments were moved to an organic setting that adhered to organic production guidelines. The pens at the organic setting measured 8.23 m by 2.44 m, with pasture area measuring 13.41 m by 2.44 m (1.06 m^2 per bird). The birds were given 24-hour access to pasture, along with *ab libitum* water and feed via nipple drinkers and feeders. No artificial light was used in the organic environment. Conv_Conv was left in a conventional environment with no access to pasture and pens measuring 7.32 m by 1.83 m (0.27 m^2 per bird). Conv_Conv birds were provided light for 16 h a day and no light for 8 h and *ab libitum* water and feed via identical nipple drinkers and feeders. Temperature at the conventional environment was kept at 26.5˚C; temperatures in the organic environment varied due to daily weather variation. Weekly body weights (BW) were taken from hatch till d 56. Feed intake per pen was also measured weekly over

the span of the experiment. On d 56, 3 males and 3 females from each replicate were randomly selected and processed for carcass composition which comprised of carcass-, *Pectoralis* (*P.*) *major*-, *P. minor*-, drum-, thigh- and abdominal fat- yield.

The following model was implemented using PROC GLM in SAS 9.4 (SAS, 2017)

$$Y_{ij} = \mu + T_j + e_{ij}$$

Where Y_{ij} is the measurement for any given treatment for bird i in treatment j, μ is the overall mean, T_j is the effect of treatment j, and e_{ij} is the random error term. The Tukey test evaluated statistical significance between treatments at $P<0.05$.

Results

The weekly BW of birds are presented in Table 3.4. At the end of the starter phase, birds fed conventional diets were heavier ($P<0.05$) than those fed the Brazil nut or NOSB diet. However, at the end of the grower phase, there was no difference between the Conv_Org, NOSB_Org and Conv_Conv treatments. The birds on the Org_Org treatment were significantly lighter ($P<0.05$) than all other treatments. At the end of the finisher phase, all the birds raised in the organic environment were significantly heavier ($P<0.05$) than those raised in the conventional environment. At d 56, there was no difference in BW among the Conv_Org, NOSB_Org and Org_Org treatments. The body weight gain (BWG), feed conversion (FCR) and feed intake (FI) for the treatments at the three phases of growth are presented in Table 3.5. At the end of the starter phase, the Conv_Conv and Conv_Org treatments had higher BWG ($P<0.05$) and lower FCR ($P<0.05$) than the NOSB and Org_Org treatments. The NOSB_Org treatment had higher BWG ($P<0.05$) and better FCR ($P<0.05$) than the Org_Org birds. At the end of the grower phase, the NOSB birds gained more weight ($P<0.05$) than the Org_Org treatment birds, but the Conv_Org birds had significantly better FCR ($P<0.05$) than all the other treatments. At the end of the finisher

phase, all the birds grown organically had significantly higher ($P<0.05$) BWG than their counterparts raised in the conventional production environment. The Org_Org treatment had better FCR ($P<0.05$) than the NOSB and Conv_Conv treatment birds. There was no difference in FCR between the Org_Org and Conv_Org at the end of the finisher phase. When the data was analyzed cumulatively from hatch till d 56, there were no differences in BWG among the treatments raised under organic environment, however, all the organically raised treatments had higher BWG ($P<0.05$) than their Conv_Conv counterparts. The Org_Org treatments birds had better FCR ($P<0.05$) than the NOSB_Org birds. The carcass composition yields among all treatments are provided in Table 3.6. There were no differences ($P>0.05$) in carcass-, *P. major*-, *P. minor*-, abdominal fat-, drum- and thigh-yields among all the treatments.

Discussion

This study focuses on the effects of organically sourced Met and its comparison to synthetic Met supplementation in poultry diets. Overall, broiler chickens fed a diet that included Brazil nut powder and raised organically showed a more desirable cumulative FCR and lower feed intake than chickens fed a standard NOSB diet containing synthetic Met and raised organically. Additionally, there was no difference in final BW or cumulative FCR between Org_Org and Conv_Org, demonstrating Brazil nut powder as a viable inclusion for organic broiler diets.

Burley (2012) fed a diet that contained Brazil nut protein powder (BNPP) and wheat middlings to broilers raised in battery cages. The starter and grower diets contained 5.5 and 4.26% BNPP, respectively, and met the Met requirement for the Ross308 broiler chickens used. The study concluded that over the duration of the trial, broiler chickens fed BNPP had comparable growth, feed intake and FCR with their counterparts fed a conventional balanced diet.

In the current study, broiler chicken diets that included Brazil nut powder were able to meet the Met requirement. At the starter phase, all the birds were raised in conventional production environment, therefore, the Conv_Conv and Conv_Org treatment birds were expected to grow faster than the NOSB_Org birds, since the conventional diet met the Met requirement of the birds and the NOSB diets contained less than required Met. The chickens fed the NOSB diet in the starter phase consumed more feed than their counterparts possibly to compensate for the deficient Met diet. The Org_Org treatment had comparable feed intake with the Conv_Conv group in the starter phase, yet the birds had significantly lower growth. It is possible that there are unknown nutritional elements in Brazil nut powder that have a negative effect on chicks.

At the end of the starter phase, 3 treatments were transferred to an organic environment where they had access to pasture. At the end of the grower phase (d 28), the Conv_Org, NOSB_Org and Conv_Conv treatments had similar BW, but the BW for the Org_Org treatment group was significantly lower. Pasturing may have played a role in the growth of the chickens on the NOSB diet. Even though the absolute BW of the Org_Org treatment group was lower than all other treatments, their gain during the grower phase was similar to that of the Conv_Conv and the Conv_Org groups, but remained lower than the NOSB_Org group. Chickens in the NOSB_Org treatment still had a significantly increased feed intake in the grower phase compared to all other groups. At the end of the grower phase, the Conv_Org group had a better FCR than all the other groups. Pasture is a reservoir of nutrients from the vegetation present, and Ponte et al. (2008b) shows that pasture provides high amounts of protein to foraging broilers. Broiler chickens with access to pasture show higher growth than their counterparts with no access to pasture. Additionally, broiler chickens have been shown to consume high amounts of pasture when fed a

conventional corn-SBM diet (Ponte et al., 2008a; Ponte et al., 2008b), thereby suggesting a positive relationship between access to pasture and performance.

At the end of the finisher phase, all the birds raised in the organic production environment showed no difference in either BW or BWG. Chickens raised in the conventional environment had BW and gain that were significantly different from pasture reared birds. At the finisher phase, the chickens on the NOSB diet still had a slightly increased feed intake compared to their other counterparts raised in the organic environment, but this increase was not significant. Conv_Conv chickens had lower feed intake than the NOSB chickens.

In the current study, it appears that birds that had access to pasture showed an increased growth when compared to their counterparts in the conventional environment. This is in accordance with the observation of Ponte et al. (2008a, b). However, Fanatico et al. (2009) and Moyle et al. (2014) reported no differences in BWG, feed intake and FCR of fast-growing broilers provided access or no access to pasture. Recently, Ipek and Sozcu (2017) reported that slow-growing broiler chickens with access to pasture had lower final BW and FCR (d 42-84) than their counterparts raised in conventional environment. We did not monitor the activity of birds or the amount of time they spent outdoors on pasture, however, the relationship between access to pasture and different broiler chicken strains (slow-, medium- and fast-growth) requires further investigation. Cumulatively, the chickens on the Org_Org treatment had significantly lower feed intake and FCR than their counterpart on the NOSB diet.

It has been reported that Brazil nut has high levels of selenium compared to other feed ingredients (NIH, 2016). We did not analyze the diet for selenium. However, there are studies that suggest dietary selenium levels could affect performance. Choct et al. (2004) reported that male broilers receiving 0.1 mg/kg of selenium consumed more feed than their counterparts that received

0.25 mg/kg of selenium. In that study, there were no differences in BW, but those on the 0.25 mg/kg of selenium had an improved FCR compared to those on the 0.1 mg/kg. Besides high amounts of sulfur amino acids in Brazil nut (Milfort et al., 2017), high levels of selenium may reflect the cumulative FCR of the chickens in the Org_Org treatment.

The dietary treatment and production environment did not affect the carcass yield and carcass composition. Fanatico et al. (2009) and Moyle et al. (2014) also reported similar results in fast growing broilers with or without access to pasture. However, in slow growing broiler chickens Ipek and Sozcu (2017) reported that chickens with access to pasture had lower breast- and abdominal fat-yields compared to their counterparts raised conventionally with no access to pasture. The current study focused on the utility of Brazil nut as a substitute for synthetic Met in organic broiler chicken feed. Future studies need to include cost optimization of the diet and the use of a wider array of organic ingredients.

Conclusion

As a proof of principle, the current study has demonstrated that it is possible to formulate an organic broiler chicken diet without synthetic Met supplementation. The tolerance of the chickens to an organic diet with Brazil nut powder may be an issue for young birds and needs to be investigated further. It is also apparent that, even in organic production environment, the NOSB diet is deficient in Met and may not meet the needs of broiler chickens. Also, fast-growing broilers perform better in organic production environment with access to pasture than their counterparts without access to pasture. Carcass yield is similar in fast-growing chickens with or without access to pasture. Future studies should monitor activity and time spend on pasture in order to evaluate the relationship between foraging behavior, activity, and performance.

Tables and Figures

Table 3.1. Nutrient composition for starter diet

	Treatments[1]			
Ingredient	Conv_Org	NOSB_Org	Org_Org	Conv_Conv
Corn (%)	54.07	38.00	61.20	54.07
Soybean Meal (%)	38.11	28.70	22.93	38.11
Soybean Oil (%)	3.00	3.50	0.00	3.00
Sunflower Meal (%)	0.00	22.05	0.00	0.00
Brazil Nuts (%)	0.00	0.00	12.00	0.00
Limestone (%)	0.68	0.00	0.00	0.68
Defluor. Phos. (%)	1.80	2.00	2.18	1.80
Sand (%)	0.00	4.27	0.00	0.00
Common Salt (%)	0.30	0.25	0.30	0.30
Vitamin Premix (%)	0.50	0.50	0.50	0.50
Mineral Premix (%)	0.08	0.08	0.08	0.08
DL-Methionine (%)	0.29	0.10	0.00	0.29
L-Lysine HCl (%)	0.17	0.35	0.52	0.17
Threonine (%)	0.99	0.20	0.29	0.99
	Composition[2]			
M.E. (Kcal/g)	3.06	3.05	3.06	3.06
Protein (%)	23.33	23.35	23.33	23.33
LYS (%)	1.28	1.27	1.28	1.28
MET (%)	0.62	0.43	0.63	0.62
CYS (%)	0.30	0.27	0.32	0.30

[1] Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically

[2] LYS, Lysine; MET, Methionine; CYS, Cysteine.

Table 3.2. Nutrient composition for grower diet

	Treatments[1]			
Ingredient	Conv_Org	NOSB_Org	Org_Org	Conv_Conv
Corn (%)	58.22	42.75	64.73	58.22
Soybean Meal (%)	34.20	25.00	20.75	34.20
Soybean Oil (%)	3.00	3.50	0.25	3.00
Sunflower Meal (%)	0.00	22.05	0.00	0.00
Brazil Nuts (%)	0.00	0.00	10.75	0.00
Limestone (%)	0.68	0.00	0.00	0.68
Defluor. Phos. (%)	1.60	1.80	1.95	1.60
Sand (%)	0.00	3.27	0.00	0.00
Common Salt (%)	0.30	0.30	0.25	0.30
Vitamin Premix (%)	0.50	0.50	0.50	0.50
Mineral Premix (%)	0.08	0.08	0.08	0.08
DL-Methionine (%)	0.25	0.10	0.00	0.25
L-Lysine HCl (%)	0.17	0.35	0.45	0.17
Threonine (%)	0.99	0.30	0.29	0.99
	Composition[2]			
M.E. (Kcal/g)	3.10	3.10	3.10	3.10
Protein (%)	21.75	21.69	21.64	21.75
LYS (%)	1.18	1.18	1.16	1.18
MET (%)	0.56	0.41	0.58	0.56
CYS (%)	0.29	0.25	0.30	0.29

[1] Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically

[2] LYS, Lysine; MET, Methionine; CYS, Cysteine.

Table 3.3. Nutrient composition for finisher diet

	Treatments[1]			
Ingredient	Conv_Org	NOSB_Org	Org_Org	Conv_Conv
Corn (%)	61.88	42.00	66.00	61.88
Soybean Meal (%)	29.90	21.25	18.00	29.90
Soybean Oil (%)	3.85	5.25	2.00	3.85
Sunflower Meal (%)	0.00	25.00	0.00	0.00
Brazil Nuts (%)	0.00	0.00	10.50	0.00
Limestone (%)	0.65	0.00	0.00	0.65
Defluor. Phos. (%)	1.45	1.60	1.95	1.45
Sand (%)	0.00	3.27	0.00	0.00
Common Salt (%)	0.27	0.30	0.25	0.27
Vitamin Premix (%)	0.50	0.50	0.50	0.50
Mineral Premix (%)	0.08	0.08	0.08	0.08
DL-Methionine (%)	0.25	0.10	0.00	0.25
L-Lysine HCl (%)	0.17	0.35	0.43	0.17
Threonine (%)	1.00	0.30	0.29	1.00
	Composition[2]			
M.E. (Kcal/g)	3.20	3.20	3.20	3.20
Protein (%)	19.97	20.22	20.07	19.97
LYS (%)	1.07	1.09	1.07	1.07
MET (%)	0.54	0.40	0.56	0.54
CYS (%)	0.27	0.24	0.28	0.27

[1] Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically

[2] LYS, Lysine; MET, Methionine; CYS, Cysteine.

Table 3.4. Weekly body weights of chickens raised under organic or conventional environment and fed organic, NOSB or conventional diet

Age	Treatments[1]				SEM	Pr>F
	Conv_Org (N=163)	NOSB_Org (N=166)	Org_Org (N=158)	Conv_Conv (N=176)		
0	42.53^{ab}	43.18^{a}	42.23^{b}	42.18^{b}	0.09	0.0300
7	156.09^{ab}	156.19^{ab}	151.27^{b}	161.05^{a}	0.61	<0.0001
14	430.86^{ab}	420.87^{b}	386.98^{c}	435.71^{a}	1.89	<0.0001
21	823.39^{b}	816.47^{b}	755.13^{c}	900.28^{a}	3.86	<0.0001
28	1447.34^{a}	1446.28^{a}	1364.73^{b}	1419.16^{a}	6.10	<0.0001
35	2214.94^{a}	2220.52^{a}	2117.69^{b}	2087.11^{b}	9.39	<0.0001
42	2742.69^{a}	2759.33^{a}	2670.87^{ab}	2623.44^{b}	11.58	<0.0001
49	3333.71^{a}	3361.26^{a}	3252.61^{ab}	3196.14^{b}	14.31	<0.0001
56	3960.08^{a}	3996.36^{a}	3984.58^{a}	3677.55^{b}	18.02	<0.0001

[1] Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically.

[a-c] Means within the same row with no common superscript differ significantly ($P \leq 0.05$).

Table 3.5. Mean BWG, feed intake, and FCR of treatments for all phases[1]

	Treatments[2]				SEM	Pr>F
	Conv_Org (N=163)	NOSB_Org (N=166)	Org_Org (N=158)	Conv_Conv (N=176)		
Starter Phase (0-14 d)						
BWG (g)	388.35^{ab}	378.03^{b}	345.08^{c}	393.34^{a}	7.10	<0.0001
FI (g)	465.86^{b}	510.26^{a}	465.57^{b}	467.88^{b}	6.70	<0.0001
FCR	1.20^{b}	1.35^{a}	1.34^{a}	1.19^{b}	0.02	<0.0001
Grower Phase (15-28 d)						
BWG (g)	1,016.66^{ab}	1,025.19^{a}	976.49^{b}	983.78^{b}	12.72	0.0028
FI (g)	1,461.72^{b}	1,600.84^{a}	1,541.96^{b}	1,503.66^{b}	26.85	<0.0001
FCR	1.44^{b}	1.56^{a}	1.58^{a}	1.52^{a}	0.02	<0.0001
Finisher Phase (29-56 d)						
BWG (g)	2,546.06^{a}	2,549.39^{a}	2,618.10^{a}	2,257.45^{b}	67.27	<0.0001
FI (g)	5,038.18^{ab}	5,285.01^{a}	4,924.95^{ab}	4,671.23^{b}	94.47	<0.0001
FCR	1.98^{ab}	2.07^{a}	1.88^{b}	2.07^{a}	0.04	<0.0001
Cumulative (0-56 d)						
BWG (g)	3,3951.06^{a}	3,952.62^{a}	3,939.67^{a}	3,634.56^{b}	66.03	<0.0001
FI (g)	6,965.75^{b}	7,396.10^{a}	6,932.49^{b}	6,642.77^{b}	91.85	<0.0001
FCR	1.76^{b}	1.87^{a}	1.76^{b}	1.82^{ab}	0.02	<0.0001

[1]Body weight gain, BWG; Feed intake, FI; Feed conversion ratio, FRC.

[2] Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically.

[a-c] Means within the same row with no common superscript differ significantly ($P \leq 0.05$).

Table 3.6. Mean carcass composition (%) of treatments

Composition (%)	Treatments[1]				SEM
	Conv_Org (N=24)	NOSB_Org (N=24)	Org_Org (N=24)	Conv_Conv (N=24)	
Carcass	74.68	74.16	74.11	74.85	0.45
Fat Pad	2.23	2.35	2.14	2.19	0.17
P. major	25.00	25.52	25.93	27.01	0.58
P. minor	6.26	5.24	4.93	5.58	0.48
Wings	10.11	10.67	10.09	10.54	0.17
Drums	13.30	13.57	13.92	13.02	0.20
Thighs	17.57	17.97	17.49	17.28	0.30

[1] Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically.

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CHAPTER 4

GENE EXPRESSION OF TRANSMETHYLATION, REMETHYLATION, AND TRANSSULFURATION PATHWAYS OF BROILERS FED A DIET WITH OR WITHOUT EXOGENOUS METHIONINE RAISED IN EITHER ORGANIC OR CONVENTIONAL PRODUCTION ENVIRONMENT[1]

[1] Foutz, J.C., M.C. Milfort, A.L. Fuller, W.K. Kim, R. Rekaya, and S.E. Aggrey. To be submitted to *Journal of Animal Physiology and Animal Nutrition*.

Abstract

Methionine (Met) is required for hundreds of metabolic pathways within poultry. Met metabolism includes the transmethylation and transsulfuration pathways which in turn yield cysteine (Cys), homocysteine (Hcy), and taurine, along with many other products. The restriction of only 0.1% D,L-Methionine (DL-Met), as ruled by the National Organic Standards Board (NOSB), in organic poultry diets leads to a Met deficiency, which can be detrimental to the bird's health. This study examines gene expression of vital enzymes in Met metabolism to observe differences between different feed type and production environments. A total of 800 Ross 308 chicks were hatched and randomly divided into four treatment groups with four replicates of 50 chicks. Treatments included birds fed a conventional corn and soybean meal (SBM) diet while raised organically (Conv_Org), a diet adhering to NOSB guidelines and raised organically (NOSB_Org), an organic diet utilizing Brazil nut powder to provide an adequate amount of Met and raised organically (Org_Org), and a conventional corn and SBM diet raised conventionally (Conv_Conv). Ileum, liver, and *P. major* tissues were sampled at days 10 and 35. Growth and performance results suggest Brazil nut meal is a viable source of organic Met. Additionally, gene expression results corroborate performance results, suggesting Met metabolism is poor in broilers fed the NOSB and Brazil nut powder diets during the starter phase, becoming optimal during the grower and finisher phases. Additional tests are needed to further determine the effects of feed and production environment on Met metabolism.

Introduction

Methionine (Met) is required for hundreds of metabolic pathways within poultry, including DNA methylation and RNA synthesis. In addition, Met is key for the synthesis of other sulfur-containing amino acids (AA), such as cysteine (Cys), homocysteine (Hcy), and taurine (Jankowski et al., 2014). Met metabolism includes the transmethylation and transsulfuration pathways which in turn yield Cys, Hcy, and taurine, along with many other products. Met is first converted to S-Adenosylmethionine (SAM) through a reaction catalyzed by methionine adenosyltransferase (MAT). (James et al., 2008). SAM then serves as a methyl donor, transferring its methyl group to a number of molecules and compounds by a methyltransferase (MTase). Glycine N-methyltransferase (GNMT) is an MTase which synthesizes sarcosine through the methylation of glycine. GNMT is prominent in liver tissue and is vital in SAM regulation and balance, making GNMT a prominent regulator for almost all methylation reactions (Luka et al., 2009; Yen et al., 2013). In addition to sarcosine, GNMT also produces S-adenosylhomocysteine (SAH). SAH takes part in a hydrolysis reaction catalyzed by Adenosylhomocysteinase (AHCY), releasing adenosine and producing Hcy, which can be remethylated to methionine or enter the transsulfuration pathway (Mato and Lu, 2007).

The transsulfuration pathway yields thiol products such as Cys, taurine, and glutathione (GSH). The first reaction of this pathway utilizes Hcy and produces cystathionine by the enzyme cystathionine-β-synthase (CBS). Cysteine is produced in the following reaction catalyzed by cystathionine γ-lyase (CGL). Cysteine can continue through the pathway to either produce taurine or GSH (James et al., 2008; Selhub and Miller, 1992).

When the need for Met outweighs the need for transsulfuration products, Hcy is likely to be remethylated to Met. There are two pathways in which this could happen. The first is through

a pathway in which N^5-methyltetrahydrofolate (5-methyl-THF) donates a methyl group to Hcy, forming Met. This reaction is catalyzed by methionine synthase (MS). The donor 5-methyl-THF is produced by methylenetetrahydrofolate reductase (MTHFR), which oxidizes NADPH and reduces 5,10-methylene-THF, forming 5-methyl-THF (Finkelstein, 1998).

Another path in which Hcy can be remethylated is through the use of the enzyme betaine-homocysteine methyltransferase (BHMT). Betaine donates a methyl group to Hcy, forming Met and dimethylglycine (DMG). Further, DMG can be oxidized, releasing a methyl group and forming sarcosine. Sarcosine can be converted to glycine through a reversible reaction that involves SAM and SAH and the release of a methyl group from glycine (Fu et al., 2016; Zeisel and da Costa, 2009). When observing the gene expression of the various enzymes along the transmethylation, remethylation, and transsulfuration pathways, it is possible to view the effects of Met levels of differing poultry diets (Aggrey et al., 2018).

Met and Cys play an important role in poultry health due to their ability to affect humeral and cellular immunity (Wu et al., 2013). Additionally, sulfur-containing AA are vital to antioxidant function. Antioxidant compounds and AA that incorporate sulfur in their structure include Met, Cys, taurine, glutathione, lipoic acid (LA), and N-acetylcysteine (NAC) (Atmaca, 2004). These antioxidants and compounds prevent oxidative stress from reactive oxygen species (Halliwell and Gutteridge, 1999; Salami et al., 2015). For these reasons, proper Met levels are needed for poultry. However, conventional poultry diets consist of corn and soybean meal (SBM), and these two ingredients alone do not provide adequate amounts of Met to the birds. Therefore, Met must be supplemented through the use of synthetic Met. D,L-Methionine (DL-Met) consists of a 1:1 ratio of D- and L- isomers of Met. Only the L- form is biologically active, but poultry have the means

to convert the D- form to the L-isomer. (Aguirre et al., 2016; Burley, 2012; Jacob, 2013; Yodseranee and Bunchasak, 2012).

Though conventional broiler diets can incorporate appropriate amounts of DL-Met or its analogs, organic broiler diets are restricted in the amount of DL-Met used. In 2012, the National Organic Standards Board (NOSB) ruled that to be classified as organic, no more than 2 pounds of DL-Met per ton of feed are to be used to feed organic broilers (USDA, 2012). This restriction leaves organic broiler diets deficient in Met (Burley et al., 2013). This deficiency can lead to immune problems, as well as suboptimal feed efficiency and growth (Aggrey et al., 2018; Deng et al., 2007; Rubin et al., 2007; Tripathi, 2016). To combat Met deficiency, organic broiler research has pointed to several promising organic Met sources, including fish meal, Brazil nut meal, and wheat middlings (Burley et al., 2015; Jacob, 2013).

In this study, Brazil nut powder was selected as a source of Met for poultry diets because of high Met content, availability, and low health and environmental risks. The objectives of this study were to 1) examine the effects of diet type on broiler performance and gene expression and 2) examine the effects of conventional versus organic production environment on performance and gene expression of broilers.

Materials and Methods

A total of 800 Ross308 broilers were divided into 4 treatment groups of 4 replicates each with 50 birds. Birds were raised for 8 weeks. From d 1 to 14, the birds received a mash starter diet. Grower (d 15 to 28) and finisher (d 28 to 56) diets were pelleted. The conventional poultry diet (Conv_Org) was formulated using non-organic ingredients. The NOSB_Org diet was formulated using all organic ingredients and adhered to USDA National Organic Program criteria; and contained only 0.1% DL-Methionine (DLM). The Org_Org diet was formulated using Brazil nut

powder as a source of Met and organic ingredients. The Conv_Conv treatment group received the same feed as the Conv_Org treatment. The starter, grower and finisher diets of all treatments are presented in Table 4.1, 4.2 and 4.3, respectively.

At hatch, 50 chicks were placed in 1.22 m by 1.83 m conventional pens with wood shavings. The birds were brooded for 14 days. Lighting and temperature management practices were in accordance with Ross 308 standards (Avigen, 2014). At 14 days of age, Conv_Org, NOSB_Org, and Org_Org treatments were moved to an organic setting that adhered to organic production guidelines. The pens at the organic setting measured 8.23 m by 2.44 m, with pasture area measuring 13.41 m by 2.44 m (1.06 m^2 per bird). The birds were given 24-hour access to pasture, along with *ab libitum* water and feed via nipple drinkers and feeders. No artificial light was used in the organic environment. Conv_Conv was left in a conventional environment with no access to pasture and pens measuring 7.32 m by 1.83 m (0.27 m^2 per bird). Conv_Conv birds were provided light for 16 h a day and no light for 8 h and *ab libitum* water and feed via identical nipple drinkers and feeders. Temperature at the conventional environment was kept at 26.5˚C; temperatures in the organic environment varied due to daily weather variation. Weekly body weights (BW) were taken from hatch till d 56. Feed intake per pen was also measured weekly over the span of the experiment. At d 10 and 35, ileum, liver, and *P. major* tissue samples were collected from 5 birds per treatment. Sampling from Conv_Conv at d 10 was not done, however, due to the identical diet and brooding environment to Conv_Org. Tissue samples were frozen in liquid nitrogen and stored at -86˚C.

RNA extraction and RT-PCR

Tissue samples were coarsely ground in liquid nitrogen and further broken down via lysing matrix tubes (MP Biomedicals, Santa Ana, CA). Total RNA was extracted using TRIzol reagents

(Invitrogen, Carlsbad, CA), then cleaned with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The RNA was treated with RNase-Free DNase (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. The RNA was suspended in DEPC water and concentration measured on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The RNA was stored at -86˚C until further use. Ten microliters (µl) of RNA were reverse transcribed with a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's protocol. A gradient thermocycler (Eppendorf, Hauppauge, NY) was used and cycle set to: 10 minutes at 25˚C, 120 minutes at 37˚C, then 5 minutes at 85˚C. The cDNA was stored at -86˚C until further use. cDNA was diluted to a concentration of 40 nanograms per µl, measured using a NanoDrop 2000 Spectrophotometer. One µl of cDNA was used per reaction of RT-PCR, with 0.3 µl of forward primer, 0.3 µl of reverse primer, 8.4 µl of DEPC water, and 10 µl of SYBER Green Master Mix (Applied Biosystems, Carlsbad, CA). RT-PCR conditions were 50˚C for 2 minutes, 95˚C for 2 minutes, then 40 cycles of 95˚C for 15 seconds followed by 60˚C for 1 minute. A melting temperature curve was determined at the end of the 40 cycles. Each sample was run in triplicates using StepOnePlus (Applied Biosystems, Carlsbad, CA).

To examine mRNA expression within the transmethylation, transsulfuration, and remethylation pathways, expression of methionine adenosyltransferase 1 alpha (MAT1A), methionine adenosyltransferase 2 beta (MAT2B), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR), betaine-homocysteine S-methyltransferase (BHMT), glycine N-methyltransferase (GNMT), Adenosylhomocysteinase (AHCY), and cystathionine-beta-synthase (CBS) genes were measured. Analysis was done by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Beta-actin was used as a housekeeping gene.

For tissues sampled at d 10, Conv_Org was used as a control group, with NOSB_Org and Org_Org mRNA expression being expressed relatively to the control. For tissues sampled at d 35, Conv_Org was also used as a control group, with NOSB_Org and Org_Org mRNA expression being expressed relatively to the control. Additionally, at d 35, Conv_Conv was used as a control with Conv_Org mRNA expression being measured relative to that Conv_Conv, thus examining the effects of production environment.

Results

Growth and Performance

On d 14, Conv_Conv had a significantly BW ($P<0.05$) than NOSB_Org and Org_Org, while Org_Org had a significantly lower BW ($P<0.05$) than all other treatments. Body weight gain (BWG) was significantly higher ($P<0.05$) in Conv_Conv birds than NOSB_Org and Org_Org while Org_Org was significantly lower ($P<0.05$) than all other treatments. Feed conversion ration (FCR) for weeks 1 and 2 showed Conv_Org and Conv_Conv had significantly lower FCRs, while NOSB_Org and Org_Org had significantly higher FCRs ($P<0.05$). Feed intake was significantly higher for NOSB_Org ($P<0.05$) than all other treatments (Table 4.4).

The culmination of the of the grower phase showed Org_Org had a significantly lower BW ($P<0.05$) than all other treatments. Throughout the grower phase, NOSB_Org showed a higher feed intake ($P<0.05$) than all other treatments. Cumulative FCR in the grower phase showed Conv_Org had a significantly lower FCR ($P<0.05$) than all other treatments (Table 4.4).

During weeks 5, 6, 7, and 8, both Conv_Org and NOSB_Org had significantly higher BW ($P<0.05$) than Conv_Conv. Org_Org BWG was among the greatest during all four weeks of the finisher phase, being significantly greater ($P<0.05$) than Conv_Conv during week 5 and significantly greater ($P<0.05$) than all other treatments during week 8. Finisher phase FCR was

significantly lower in Org_Org ($P<0.05$) than NOSB_Org and Conv_Conv. The BW of all four treatments at the end of the study shows Conv_Conv had a significantly lower final BW ($P<0.05$) than Conv_Org, NOSB_Org, and Org_Org (Table 4.4).

Org_Org, Conv_Org, and Conv_Conv cumulative FCRs did not significantly differ ($P>0.05$). Additionally, Org_Org and Conv_Org cumulative FCRs were significantly less ($P<0.05$) than NOSB_Org. BWG over eight weeks was significantly less ($P<0.05$) for Conv_Conv than all other treatments. NOSB_Org feed intake was significantly greater ($P<0.05$) than all other treatments (Table 4.4).

Gene Expression

Day 10

Relative gene expression differences at d 10 are shown in Figure 4.1; the control group being broilers fed a conventional diet. At d 10, the genes for AHCY, GNMT, MAT1A, MAT2B, MTR, and CBS were significantly ($P<0.05$) downregulated in ileum tissue in broilers fed the NOSB diet in comparison the control. With exception of MTRR, all genes within the ileum were significantly ($P<0.05$) downregulated in the broilers fed the Brazil nut containing diet in relation to the control. In the liver at d 10, the AHCY and GNMT genes for the NOSB fed broilers were significantly ($P<0.05$) upregulated in relation to the control. The BHMT and MAT1A genes were significantly ($P<0.05$) downregulated in the Brazil nut treatment in relation to the control. Additionally, MAT2B, MTR, and CBS gene expression were significantly ($P<0.05$) upregulated in the Brazil nut treatment in relation to the control. Gene expression in the *P. major* at d 10 showed the NOSB feed treatment did not significantly differ ($P>0.05$) in any gene in relation to the control. However, BHMT, MAT2B, and CBS gene expression were all significantly ($P<0.05$) upregulated in broilers fed a diet containing Brazil nuts in relation to the control.

Day 35

Figure 4.2 illustrates gene expression of NOSB_Org and Org_Org in relation to Conv_Org at d 35 in ileum, liver, and *P. major*. In the ileum, there was significant ($P<0.05$) upregulation of the GNMT gene expression in both NOSB_Org and Org_Org in relation to Conv_Org. Additionally, MAT1A and CBS gene expression were significantly ($P<0.05$) downregulated in NOSB_Org and Org_Org in relation to Conv_Org. MTR gene expression was significantly ($P<0.05$) downregulated in Org_Org in relation to Conv_Conv. In the liver at d 35, AHCY and MAT1A gene expression were significantly ($P<0.05$) downregulated in Org_Org while CBS gene expression was significantly ($P<0.05$) upregulated in NOSB_Org and Org_Org in relation to Conv_Org. In the *P. major*, BHMT gene expression was significantly ($P<0.05$) downregulated in Org_Org. MTR and MTRR gene expression were significantly ($P<0.05$) upregulated in Org_Org and NOSB_Org, respectively, in relation to Conv_Org.

In addition to observing gene expression differences between diets, gene expression differences between production environments was analyzed. Conv_Conv was used as the control and compared to Conv_Org, observing differences in gene expression between organic and conventional husbandry environments at d 35. Figure 4.3 shows that GNMT and MTRR gene expression in the ileum were significantly ($P<0.05$) downregulated in Conv_Org in relation to Conv_Conv. MAT1A, MAT2B, MTR, and CBS gene expression were all significantly ($P<0.05$) upregulated in Conv_Org. In the liver, MAT1A gene expression was significantly ($P<0.05$) upregulated while MTR and CBS gene expression were significantly ($P<0.05$) downregulated in Conv_Org. In the *P. major*, BHMT and CBS gene expression were significantly ($P<0.05$) upregulated in Conv_Org.

Discussion

The conversion of Met to SAM is catalyzed by the MAT class of enzymes. The genes used for observing gene expression of the MAT class were MAT1A and MAT2B. MAT1A is expressed mostly in the liver and is highly expressed in the presence of high SAM levels. The gene encodes for the α_1 catalytic subunit of MATIII (dimer) or MATI (tetramer). MAT2B is expressed in all tissues and encodes for the β regulatory subunit of MATII. It is highly expressed in conditions with low SAM levels (Halim et al., 1999; Lu and Mato, 2005; Mato and Lu, 2007; Ramani et al., 2011). It has been shown mRNA expression of genes differ from tissue to tissue according to need within the tissue (Aggrey et al., 2018). When examining liver gene expression on d 10, the expression for MAT2B was highest in broilers fed the Brazil nut diet. The high expression could be due to low SAM levels. Additionally, MAT1A gene expression was lowest in that treatment, again suggesting low SAM levels. Expression of MAT1A and MAT2B in broilers fed the NOSB diet were similar to the control, indicating consistent SAM levels as compared to the control. GNMT gene expression was highest in broilers fed the NOSB diet at d 10, which is evidence of sufficient SAM levels, allowing for the transmethylation. Expression of the GNMT gene was slightly lower for the organic diet broilers, suggesting lower levels of SAM.

During times of Met deficiency, Hcy is expected to be remethylated, forming Met. There is evidence of this shown by the upregulation of the MTR gene in broilers fed the organic diet in the liver at d 10. BHMT gene expression is lower in those broilers, suggesting remethylation is happening through the one-carbon pathway, with folate being the methyl donor.

During d 10 in the liver and *P. major*, there was upregulation of the CBS gene in broilers fed the organic diet, suggesting a need for products produced along the transsulfuration pathway. Reduced SAM levels have continuously been shown to increase oxidative stress (Davis and Uthus,

2004; Ghoshal et al., 2006; Niculescu and Zeisel, 2002), increasing the need for antioxidants. Through the transsulfuration pathway, glutathione is ultimately produced (Lu, 2009). This cysteine-containing tripeptide is a part of an antioxidant system which eliminates harmful reactive oxygen species (ROS) (Atmaca, 2004). Thus, the conversion of Hcy to cystathionine to, ultimately, GSH is plausible due to low levels of SAM and an increase in oxidative stress.

BHMT gene expression levels in the *P. major* at d 10 show highest expression in broilers fed the Brazil nut containing diet. The BHMT gene is highly expressed in conditions of low Met and high choline and betaine levels (Ji et al., 2007; Slow and Garrow, 2006). This is evidence that Hcy is being remethylated to Met through the activity of BHMT, evidence of low Met levels in the *P. major* of broilers fed the organic diet at d 10. Similar regulation of MTR and MTRR genes also point to remethylation utilizing choline and betaine. MAT2B gene expression in the *P. major* was significantly higher in broilers fed the organic fed; additional evidence of low Met levels.

The results from gene expression are consistent with growth and performance results. During the starter phase, NOSB_Org and Org_Org showed sub-optimal growth and performance, with Org_Org gaining the least of all treatments over that time. Gene expression results show a possible Met deficiency within these broilers; a problem that leads to low performance and stunted growth (Aggrey et al., 2018). However, growth and performance during the finisher phase shows similar BWG and similar final BW between Conv_Org, NOSB_Org, and Org_Org (table 4). This points to sufficient amounts of amino acids, specifically Met. To corroborate these results, gene expression must be examined. Expression of the GNMT gene is consistent across the 3 treatments, suggesting SAM levels are high in all 3 of those treatments, allowing for transmethylation. MAT1A gene expression is downregulated in Org_Org, however MAT2B gene expression is not. This result points to the fact that the conversion of Met to SAM can occur through multiple MAT

enzymes. MATII also catalyzes this reaction, and its α_2 catalytic subunit is encoded by the gene MAT2A (Halim et al., 1999; Lu and Mato, 2005; Ramani et al., 2011), a gene which was not observed for gene expression. This may explain the discrepancy seen in the liver tissue. Both remethylation pathways show no difference in gene expression, suggesting sufficient Met, thus allowing Hcy to go through transsulfuration. Further evidence of this is seen by the upregulation of the CBS gene in both NOSB_Org and Org_Org, allowing for the synthesis of products along the pathway, such as Cys, GSH, and taurine.

When observing the performance results comparing husbandry environments, there was better growth and performance from Conv_Org, rather than Conv_Conv. The differences between these two treatments can be contributed to organic versus conventional production environment, as diets were identical. Further, gene expression in the liver shows upregulation of the MAT1A gene. This higher expression suggests high levels of SAM (Halim et al., 1999; Lu and Mato, 2005; Ramani et al., 2011). The CBS gene expression was significantly lower in Conv_Org broilers, which is an indication that GSH is not needed in a high amount, suggesting low oxidative stress due to normal SAM levels. In the breast muscle at d 35, the gene encoding for CBS is expressed some 80 fold higher in Conv_Org than Conv_Cov. It has been shown that the over-expression of the CBS gene is evidence of oxidative stress (Del Vesco et al., 2015). Exact reasons behind high CBS gene expression seen in this study are not completely known, and investigation into enzyme activity as well as SAM, Met, and cysteine levels are required to gain a full picture of molecular activity.

Conclusion

Results in growth and performance suggest Brazil nut powder is a viable source of organic Met, as no significant differences in week 8 body weight and cumulative performance were

observed between broilers fed synthetic Met and raised organically and broilers fed Brazil nut meal and raised organically. Additionally, broilers raised in an organic production environment out-performed broilers raised conventionally. Gene expression of important steps along the transmethylation, remethylation, and transsulfuration pathways suggest low Met levels in NOSB_Org and Org_Org broilers at d 10. However, at d 35, gene expression results show sufficient Met and SAM levels in NOSB_Org and Org_Org broilers. Gene expression results validate growth and performance results, indicating poor Met metabolism during the starter phase in NOSB_Org and Org_Org broilers, but improved Met metabolism when moved to an organic environment. Initial performance and gene expression results are evidence to an organic production environment providing optimal growth and Met metabolism compared to a conventional production environment. However, enzyme activity along with AA concentration in tissues are needed to further back results seen in this study.

Tables and Figures

Table 4.1. Nutrient composition for starter diet

	Treatments[1]			
Ingredient	Conv_Org	NOSB_Org	Org_Org	Conv_Conv
Corn (%)	54.07	38.00	61.20	54.07
Soybean Meal (%)	38.11	28.70	22.93	38.11
Soybean Oil (%)	3.00	3.50	0.00	3.00
Sunflower Meal (%)	0.00	22.05	0.00	0.00
Brazil Nuts (%)	0.00	0.00	12.00	0.00
Limestone (%)	0.68	0.00	0.00	0.68
Defluor. Phos. (%)	1.80	2.00	2.18	1.80
Sand (%)	0.00	4.27	0.00	0.00
Common Salt (%)	0.30	0.25	0.30	0.30
Vitamin Premix (%)	0.50	0.50	0.50	0.50
Mineral Premix (%)	0.08	0.08	0.08	0.08
DL-Methionine (%)	0.29	0.10	0.00	0.29
L-Lysine HCl (%)	0.17	0.35	0.52	0.17
Threonine (%)	0.99	0.20	0.29	0.99
	Composition[2]			
M.E. (Kcal/g)	3.06	3.05	3.06	3.06
Protein (%)	23.33	23.35	23.33	23.33
LYS (%)	1.28	1.27	1.28	1.28
MET (%)	0.62	0.43	0.63	0.62
CYS (%)	0.30	0.27	0.32	0.30

[1]Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically

[2]LYS, Lysine; MET, Methionine; CYS, Cysteine.

Table 4.2. Nutrient composition for grower diet

	Treatments[1]			
Ingredient	Conv_Org	NOSB_Org	Org_Org	Conv_Conv
Corn (%)	58.22	42.75	64.73	58.22
Soybean Meal (%)	34.20	25.00	20.75	34.20
Soybean Oil (%)	3.00	3.50	0.25	3.00
Sunflower Meal (%)	0.00	22.05	0.00	0.00
Brazil Nuts (%)	0.00	0.00	10.75	0.00
Limestone (%)	0.68	0.00	0.00	0.68
Defluor. Phos. (%)	1.60	1.80	1.95	1.60
Sand (%)	0.00	3.27	0.00	0.00
Common Salt (%)	0.30	0.30	0.25	0.30
Vitamin Premix (%)	0.50	0.50	0.50	0.50
Mineral Premix (%)	0.08	0.08	0.08	0.08
DL-Methionine (%)	0.25	0.10	0.00	0.25
L-Lysine HCl (%)	0.17	0.35	0.45	0.17
Threonine (%)	0.99	0.30	0.29	0.99
	Composition[2]			
M.E. (Kcal/g)	3.10	3.10	3.10	3.10
Protein (%)	21.75	21.69	21.64	21.75
LYS (%)	1.18	1.18	1.16	1.18
MET (%)	0.56	0.41	0.58	0.56
CYS (%)	0.29	0.25	0.30	0.29

[1]Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically

[2]LYS, Lysine; MET, Methionine; CYS, Cysteine.

Table 4.3. Nutrient composition for finisher diet

	Treatments[1]			
Ingredient	Conv_Org	NOSB_Org	Org_Org	Conv_Conv
Corn (%)	61.88	42.00	66.00	61.88
Soybean Meal (%)	29.90	21.25	18.00	29.90
Soybean Oil (%)	3.85	5.25	2.00	3.85
Sunflower Meal (%)	0.00	25.00	0.00	0.00
Brazil Nuts (%)	0.00	0.00	10.50	0.00
Limestone (%)	0.65	0.00	0.00	0.65
Defluor. Phos. (%)	1.45	1.60	1.95	1.45
Sand (%)	0.00	3.27	0.00	0.00
Common Salt (%)	0.27	0.30	0.25	0.27
Vitamin Premix (%)	0.50	0.50	0.50	0.50
Mineral Premix (%)	0.08	0.08	0.08	0.08
DL-Methionine (%)	0.25	0.10	0.00	0.25
L-Lysine HCl (%)	0.17	0.35	0.43	0.17
Threonine (%)	1.00	0.30	0.29	1.00
	Composition[2]			
M.E. (Kcal/g)	3.20	3.20	3.20	3.20
Protein (%)	19.97	20.22	20.07	19.97
LYS (%)	1.07	1.09	1.07	1.07
MET (%)	0.54	0.40	0.56	0.54
CYS (%)	0.27	0.24	0.28	0.27

[1]Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically

[2]LYS, Lysine; MET, Methionine; CYS, Cysteine.

Table 4.4. Mean growth and performance results of all treatments during the starter, grower, and finisher phase in addition to cumulative growth and performance results.

	Treatments[1]					
	Conv_Org (N=163)	NOSB_Org (N=166)	Org_Org (N=158)	Conv_Conv (N=176)	SEM	Pr>F
Starter Phase (0-14 d)						
BW (g), d 14	430.86^{ab}	420.87^{b}	386.98^{c}	435.71^{a}	1.89	<0.0001
BWG (g)	388.35^{ab}	378.03^{b}	345.08^{c}	393.34^{a}	7.10	<0.0001
FI (g)	465.86^{b}	510.26^{a}	465.57^{b}	467.88^{b}	6.70	<0.0001
FCR	1.20^{b}	1.35^{a}	1.34^{a}	1.19^{b}	0.02	<0.0001
Grower Phase (15-28 d)						
BW (g), d 28	1,447.34^{a}	1,446.28^{a}	1,364.73^{b}	1,419.16^{a}	6.10	<0.0001
BWG (g)	1,016.66^{ab}	1,025.19^{a}	976.49^{b}	983.78^{b}	12.72	0.0028
FI (g)	1,461.72^{b}	1,600.84^{a}	1,541.96^{b}	1,503.66^{b}	26.85	<0.0001
FCR	1.44^{b}	1.56^{a}	1.58^{a}	1.52^{a}	0.02	<0.0001
Finisher Phase (29-56 d)						
BW (g), d 56	3,960.08^{a}	3,996.36^{a}	3,984.58^{a}	3,677.55^{b}	18.02	<0.0001
BWG (g)	2,546.06^{a}	2,549.39^{a}	2,618.10^{a}	2,257.45^{b}	67.27	<0.0001
FI (g)	5,038.18^{ab}	5,285.01^{a}	4,924.95^{ab}	4,671.23^{b}	94.47	<0.0001
FCR	1.98^{ab}	2.07^{a}	1.88^{b}	2.07^{a}	0.04	<0.0001
Cumulative (0-56 d)						
BWG (g)	3,3951.06^{a}	3,952.62^{a}	3,939.67^{a}	3,634.56^{b}	66.03	<0.0001
FI (g)	6,965.75^{b}	7,396.10^{a}	6,932.49^{b}	6,642.77^{b}	91.85	<0.0001
FCR	1.76^{b}	1.87^{a}	1.76^{b}	1.82^{ab}	0.02	<0.0001

Body weight, BW; Body weight gain, BWG; Feed intake, FI; Feed conversion ratio, FRC.
[1]Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically; Conv_Conv, Conventional diet raised organically.
[a-c] Means within the same row with no common superscript differ significantly ($P \leq 0.05$).

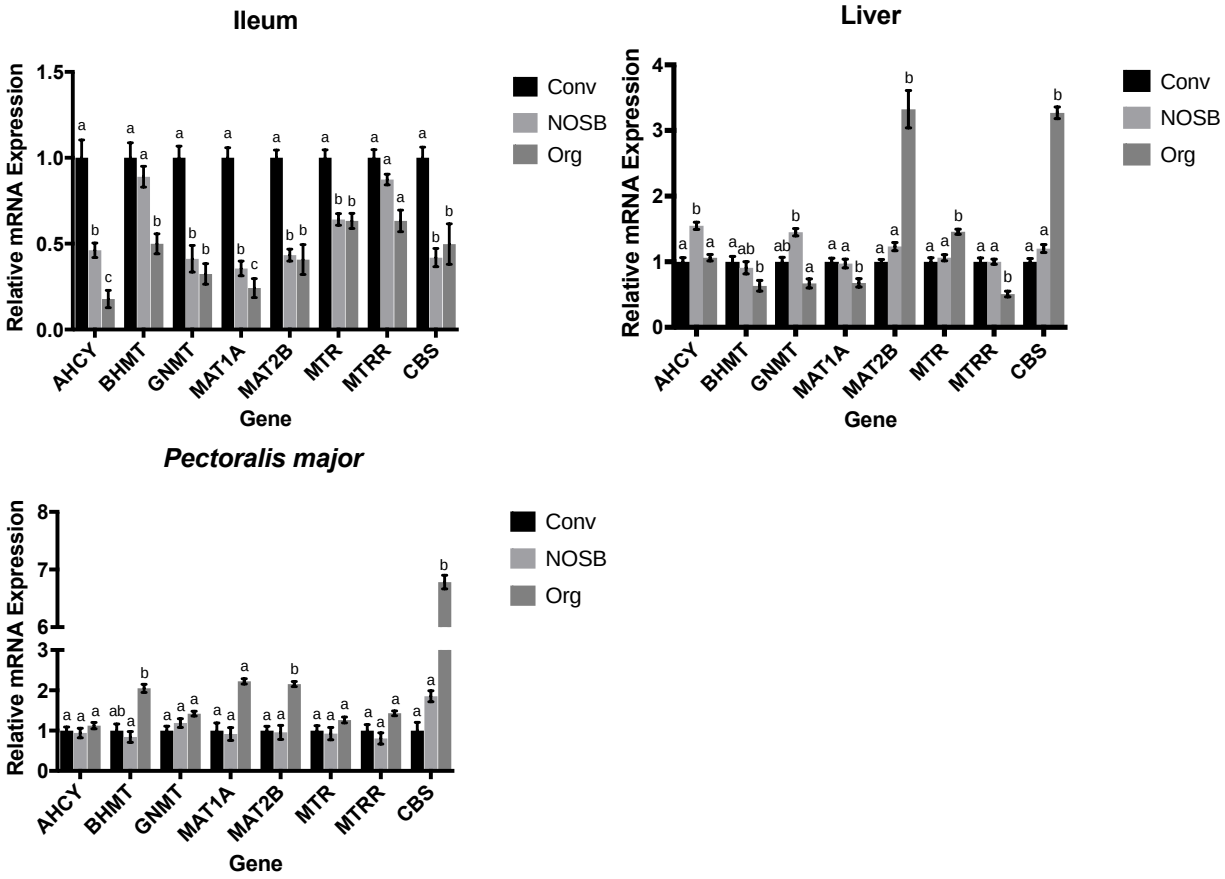

Figure 4.1. mRNA expression levels of certain genes from different tissues during the starter phase (d 10). AHCY, S-adenosyl-L-homocysteine hydrolase; BHMT, betaine-homocysteine S-methyltransferase; GNMT, glycine N- methyltransferase; MAT1A, methionine adenosyltransferase 1, alpha; MAT2B, MAT 2, beta; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; CBS, cystathionine beta synthase.
Conv, conventional diet; NOSB, NOSB National Organic Standard Board diet; Org, Brazil nut powder containing diet.
[a-c] Gene expression means within the same cluster with no common superscript differ significantly ($P \leq 0.05$).

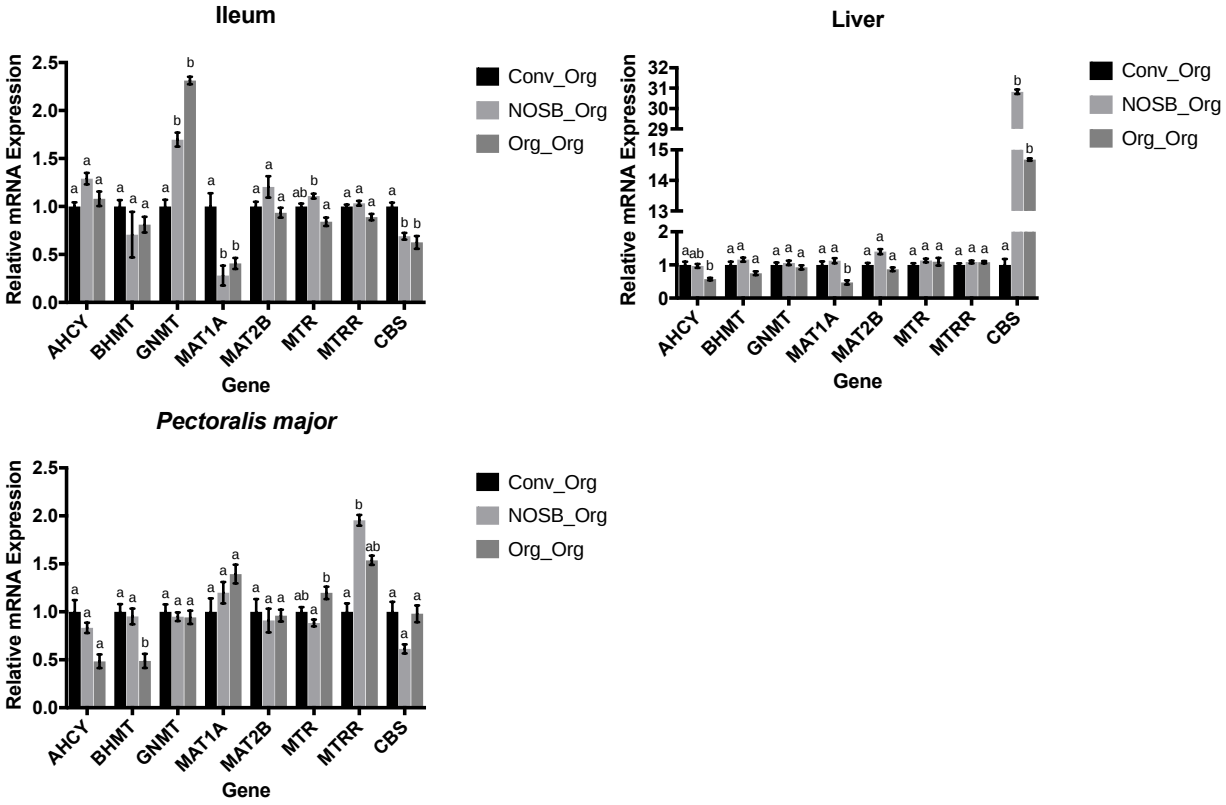

Figure 4.2. mRNA expression levels of certain genes from different tissues during the finisher phase (d 35). AHCY, S-adenosyl-L-homocysteine hydrolase; BHMT, betaine-homocysteine S-methyltransferase; GNMT, glycine N- methyltransferase; MAT1A, methionine adenosyltransferase 1, alpha; MAT2B, MAT 2, beta; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; CBS, cystathionine beta synthase.
Conv_Org, Conventional diet raised organically; NOSB_Org, National Organic Standard Board diet raised organically; Org_Org, Brazil nut powder containing diet raised organically.
[a-c] Gene expression means within the same cluster with no common superscript differ significantly ($P \leq 0.05$).

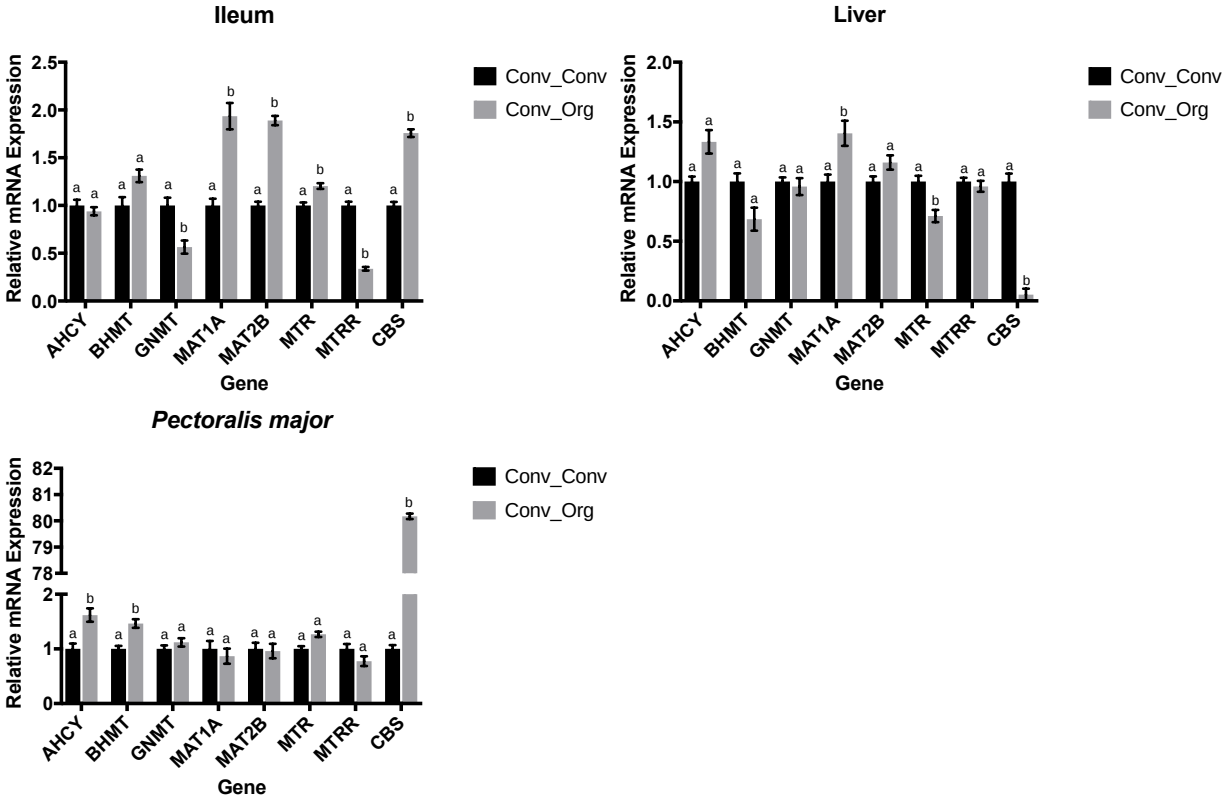

Figure 4.3. mRNA expression levels of certain genes from different tissues during the finisher phase (d 35). AHCY, S-adenosyl-L-homocysteine hydrolase; BHMT, betaine-homocysteine S-methyltransferase; GNMT, glycine N- methyltransferase; MAT1A, methionine adenosyltransferase 1, alpha; MAT2B, MAT 2, beta; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; CBS, cystathionine beta synthase.
Conv_Org, Conventional diet raised organically; Conv_Conv, Conventional diet raised conventionally.
[a-b] Gene expression means within the same cluster with no common superscript differ significantly ($P \leq 0.05$).

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CHAPTER 5

CONCLUSIONS

A few main conclusions can be formed from results observed on growth, performance, and gene expression. First, during the starter phase of broiler production, a diet adhering to NOSB guidelines of only 0.1% DL-Methionine does not provide an adequate amount of methionine to a broiler, resulting in increased feed intake and decreased growth. Additionally, broilers fed organic Brazil nut meal as a methionine source do not meet the growth of broilers fed conventional feed. These observations on growth and performance are backed by gene expression data, where genetic expression of important enzymes show a methionine deficiency. However, the conclusion that can be made over an 8-week time period, broilers fed a diet adhering to NOSB guidelines or a diet containing Brazil nut meal both meet the growth of broilers fed a conventional diet when raised organically. Broilers fed a diet containing Brazil nut meal additionally met performance of conventionally fed broilers and outperformed broilers fed a NOSB diet. Genetic expression corroborates these observations, where little differences were seen in gene expression of major enzymes in methionine metabolism.

Further, growth and performance data suggest organic production of broilers yields heavier broilers than broilers raised conventionally. Genetic data supporting this result show relative corroboration. However, investigation into enzyme activity and metabolite concentration is needed to further strengthen these claims.