Research Thesis: Comparative Effects of Two Sources and Levels of Methionine on the Performance of Broilers Under Coccidia Challenge

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Expected Graduation: Spring 2022

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Abstract

Coccidia are pervasive in the poultry industry and cause intestinal lesions, diminished growth performance, and inflammatory responses such as oxidative stress. This study hypothesized that the use of different methionine (**Met**) sources and inclusion levels to control inflammation will affect growth performance and clinical coccidiosis of chickens infected with *Eimeria maxima*. The objective of this study was to investigate the effect of two different Met sources on growth during an *E. maxima* infection. Treatment groups included inoculated control (**IC),** low Met A (**L MetA**), high Met A (**H MetA**), low Met B (**L MetB**), and high Met B (**H MetB**). Pen and feed weights were measured on d01, d8, d16, d21 and d28 to calculate body weight gain (**BWG**), percent change in body weight gain relative to IC (**%ChBWG**), and feed conversion ratio (**FCR**). On d16, all treatments received an oral dose of $8x10^4$ oocysts/bird of *Eimeria maxima* Guelph strain, with intestinal lesion scores (**LS**) recorded on d21. After d19, all mortalities were necropsied and scored as either coccidiosis-positive or coccidiosis-negative. Fresh feces were collected twice daily from each pen from d19-28 and combined to monitor average daily shedding of oocysts (**ADS**). The results showed improvement of BWG during the d16-d20 disease period, in which BWG increased for Met A and B diets compared to IC ($p<0.05$). Although no differences in mean LS were observed between treatments in either room, the distribution suggested a positive impact of Met A and Met B on reducing lesion severity. Upon evaluation of ADS, Room 1 presented a clear reduction in ADS for all Met A and Met B treatments compared to CC ($p < 0.05$), while no difference in ADS was observed in Room 2. In conclusion, this study suggests varying methionine levels can affect severity of coccidiosis, regardless of source, and may be beneficial in supporting healthier and more productive flocks.

Introduction

Eimeria Infections: Coccidiosis is an intestinal disease caused by protozoan parasites of the genus, *Eimeria* (Allen and Fetterer, 2002). It has proved pervasive in the poultry industry and has remained a leading component of health and welfare concerns. Estimates from 2016 data suggest that coccidiosis costs the US chicken industries $1.6 billion per year with an annual global cost of $14.4 billion (Blake et al., 2020). The parasite is highly transmittable in dense populations of birds (Blake and Tomkey, 2014), shown to cause diarrhea, reduced weight gain and feed consumption, and even mortality (López-Osorio, 2020). A variety of methods have been used to treat and control coccidial infections, such as ionophores and antibiotics, however, controversy over this form of treatment has brought about different alternatives that include live coccidiosis vaccinations (Chapman et al., 2010). A major problem with controlling coccidiosis was widespread anticoccidial drug resistance in multiple species of *Eimeria* (Morris and Gasser, 2006). To minimize the impact of coccidiosis, alternative strategies of treatment must be explored.

Efficacy: Common strategies of evaluating the effect of coccidiosis and treatments against it include body weight gain (**BWG**), feed conversion ratio (**FCR**), lesion score (**LS**), and oocysts per gram of feces (**OPG**) (Chasser et al., 2020). Average daily shedding of oocysts (**ADS**) is an additional strategy used to capture shedding of oocysts over a time period. Methods of evaluation such as LS are often labor intensive and subjective, and parameters such as BWG and FCR may be influenced by outside factors, making diagnosis very challenging (De Gussem, 2007). A combination of growth performance factors, such as BWG and FCR, and additional parameters, such as LS and ADS of oocysts, are necessary to understand the effectiveness of treatment strategies (Chasser et al., 2020).

Methionine Sources and the Intestinal Immune Response: Intestinal infection caused by *Eimeria* involves a complex cellular and molecular response. Methionine (**Met**) plays a major role in the cellular immune response of poultry susceptible to infection by improving health, growth, and development (Jankowski et al., 2014). Recognized as an important nutrient for the immune system and antioxidant defense system, Met also acts as the first limiting amino acid in corn and soybean meal diets of broilers (Khatlab, 2019). Methionine plays a significant role as a substrate in protein synthesis, including synthesis of other sulfur amino acids, specifically cysteine. Cysteine is an important component of the synthesis of glutathione and taurine that are crucial for host defense against oxidative stress (Métayer, 2008). Following coccidiosis infection, antibody and cell-mediated immune responses are activated and function to protect the intestinal epithelium through disease resistance (Lillehoj and Trout, 1996). Methionine can potentially be used to bolster the immune response of a coccidial infection, mitigating its impact on broilers. In this experiment, two Met sources at two different levels were implemented to monitor their efficacy in minimizing the impact of coccidiosis. Met has the potential to be implemented as a treatment strategy that will help support healthier and more productive flocks by limiting the severity of coccidiosis.

Materials and Methods

Table 1. Dietary Treatments. Throughout the experiment, birds received a control diet with no additional methionine, added methionine from source A at one of two levels, or added methionine from source B at one of two levels. On d16 all birds were orally challenged with *Eimeria maxima* Guelph at $8x10^4$ oocysts/bird to measure differences between methionine sources and levels on *Eimeria* challenged birds.

Group	Treatment	Met levels	n	Reps	d16 *E. maxima* Guelph Challenge
1	Negative control	0.35% Met	10	10	$8x10^4$ oocysts/bird
2	As 4 + 0.10% Met (0.10% Met A)	0.45% Met	10	10	$8x10^4$ oocysts/bird
3	As 4 + 0.20% Met (0.20% Met A)	0.55% Met	10	10	$8x10^4$ oocysts/bird
4	As 4 + 0.10% Met (0.11% Met B)	0.45% Met	10	10	$8x10^4$ oocysts/bird
5	As 4 + 0.20% Met (0.23% Met B)	0.55% Met	10	10	$8x10^4$ oocysts/bird

Experimental Design: A total of 500 one day old Ross 708 broiler cockerels were sourced from a local hatchery, neck tagged, randomly assigned to one of five treatments and placed on fresh pine shavings in floor pens. There were 10 replicate pens per treatment and 10 birds placed in each pen. Due to unforeseen circumstances, one pen each of H Met A and H Met B were removed from the experiment, leaving 9 pens for these groups while all others continued with 10 pens. challenge control (**CC**) received no feed additive, while other groups received additional Met from one of two different sources (**Met A** and **Met B**) incorporated at two different levels (low, **L** and high, **H**) in the diet, as shown in Table 1. Throughout the study, feed and water were provided *ad libitum* with temperature and lighting maintained at age-appropriate levels.

Preparation of Eimeria: *Eimeria* were prepared and administered using purified oocyst cultures diluted in 0.9% saline. At d16, birds were orally administered 1 ml of solution containing $8x10^4$ oocysts/mL of *Eimeria maxima* Guelph oocysts based on a pretest with a targeted 25% reduction in growth performance. Individual inoculation limited individual variation between birds in lesion scoring, growth and feed conversion ratio.

Average Daily Shedding (ADS): Twice daily, 10-12 fecal droppings were collected from each pen for a total of 10g of feces per pen per day. Feces were then diluted in 0.9% saline and combined for a collective sample across all ten days. Each collective pen sample was quantified to determine ADS.

Statistical Analysis: Prior to analysis, BW, BWG, and FCR were analyzed for room effects by t-test with at $p < 0.05$. Due to differences observed between rooms when testing these parameters, beginning d16, data was analyzed separately by room. Average BW, LS, and ADS data were expressed as mean ± standard error and subject to Analysis of Variance as a

completely randomized design using General Linear Model procedures in JMP Pro 14 software. Lesion scores were analyzed using a Proc Mixed ANOVA model in SAS 9.4. Significant differences among the means were analyzed using Tukey's Honestly Significant Difference test ($p< 0.05$).

Results

Body Weight Gain and Percent Change in Body Weight Gain: The positive impact of BWG and %chBWG of Met sources both before and during the challenge period is reflected in Tables 2 and 3. Particular improvement was noted during the challenge period, d16-20, as BWG increased for all Met A and Met B diets in both rooms compared to CC (Table 2, $p < 0.05$). In Room 1, L Met B showed the most improvement between d16-20 with %chBWG at 20.99%±3.90% compared to 0.00%±2.92% for CC (Table 3, $p= 0.0261$). Room 2 did not have any significant increases in %ChBWG, but both L Met A and H Met B showed over a 10% increase in %ChBWG compared to CC (Table 3, $p = 0.1151$ and $p= 0.1118$).

Table 2. Body weight gain. Pen weight was measured and recorded for each pen on d0, 8, 16, 21, and 28. Average bird weight per pen was then calculated based on the number of birds per pen and used to determine average body weight gain per pen. All data is presented in grams and is represented as mean ± standard error.

	0-7		8-15		16-20		21-27	
	Room 1	Room 2	Room 1	Room 2	Room 1	Room 2	Room 1	Room 2
CC[1]	122.50±1.04[a]	127.01±7.59[a]	318.13±3.21[c]	344.41±12.33[b]	193.33±4.66[b]	198.94±8.41[c]	368.25±20.75[b]	367.30±11.55[b]
L Met A	132.07±2.66[a]	136.35±4.78[a]	387.41±6.67[ab]	408.36±16.84[a]	250.60±11.35[a]	260.50±6.63[ab]	461.02±13.16[ab]	531.41±29.61[a]
H Met A	123.61±3.81[a]	141.03±2.81[a]	384.58±6.37[ab]	403.98±11.31[a]	235.05±6.92[a]	232.14±3.93[b]	477.43±23.06[a]	522.54±21.94[a]
L Met B	132.26±4.63[a]	131.87±3.07[a]	368.14±8.17[b]	392.92±4.24[ab]	265.86±5.07[a]	234.05±7.19[b]	462.60±31.49[ab]	439.00±22.90[ab]
H Met B	133.93±3.79[a]	136.22±5.57[a]	396.73±5.91[a]	417.37±9.63[a]	253.29±19.17[a]	263.48±4.91[a]	462.71±34.09[ab]	508.27±29.58[a]
SEM	2.40	2.37	14.00	12.87	12.57	11.69	19.76	31.17
p-value	0.356	0.359	0.044	0.019	0.046	0.033	0.036	0.004

*Analysis was only performed on pens that did not have birds removed between d0 and d5. Pens that were reset at d5 were analyzed separately and were not included in d0-8 data.
[1] CC = *E. maxima* Challenged Control, L Met A = *E. maxima* Low Met A, H Met A = *E. maxima* High Met A, L Met B = *E. maxima* Low Met B H Met B = *E. maxima* High Met B
[a, b, c] Mean values with different superscript letters within a column indicate a significant difference ($p<0.05$).

Table 3. Percent change body weight gain. Pen weight was measured and recorded for each pen on d0, 8, 16, 21, and 28. Average bird weight per pen was then calculated based on the

number of birds per pen. This was used to calculate the percent change in body weight gain compared to the challenged control. All data is represented as mean ± standard error.

	0-7*		8-15		16-20		21-27	
	Room 1	Room 2	Room 1	Room 2	Room 1	Room 2	Room 1	Room 2
CC[1]	0.00%±0.46%[a]	0.00%±5.79%[a]	0.00%±1.99%[b]	0.00%±2.09%[b]	0.00%±2.92%[b]	0.00%±4.98%[a]	0.00%±6.23%[a]	0.00%±0.74%[b]
L Met A	9.30%±2.62%[a]	8.15%±4.79%[a]	11.10%±1.72%[a]	7.65%±3.84%[ab]	9.95%±4.45%[ab]	13.06%±4.08%[a]	3.52%±4.79%[a]	20.40%±5.30%[ab]
H Met A	2.23%±1.61%[a]	10.21%±2.67%[a]	12.50%±1.13%[a]	6.48%±3.44%[ab]	4.63%±4.52%[ab]	1.21%±2.39%[a]	9.74%±4.94%[a]	23.21%±4.69%[a]
L Met B	9.60%±4.24%[a]	2.76%±2.08%[a]	5.62%±4.29%[ab]	9.09%±2.30%[ab]	20.99%±3.90%[a]	5.65%±3.60%[a]	3.94%±6.98%[a]	6.11%±6.48%[ab]
H Met B	10.02%±2.23%[a]	5.31%±4.21%[a]	12.01%±1.02%[a]	12.89%±2.62%[a]	8.64%±7.18%[ab]	13.14%±1.78%[a]	1.92%±7.61%[a]	14.06%±5.46%[ab]
SEM	2.12%	1.83%	2.40%	2.10%	3.50%	2.81%	1.63%	4.34%
p-value	0.346	0.451	0.029	0.033	0.026	0.112	0.777	0.036

*Analysis was only performed on pens that did not have birds removed between d0 and d5. Pens that were reset at d5 were analyzed separately and were not included in d0-8 data.
[1] CC = *E. maxima* Challenged Control, L Met A = *E. maxima* Low Met A, H Met A = *E. maxima* High Met A, L Met B = *E. maxima* Low Met B H Met B = *E. maxima* High Met B
[a, b] Mean values with different superscript letters within a column indicate a significant difference ($p<0.05$).

Feed Conversion Ratio: As shown in Table 4, FCR demonstrated some improved feed efficiency in both Room 1 and Room 2 following the challenge on d16. In Room 1, L Met B had a reduced FCR of 1.51±0.04, compared to CC at 1.72±0.03 between d16-20 (Table 4, $p=0.0178$). In Room 2, L Met A and H Met B reflected the greatest FCR improvement compared to CC with an FCR of 1.57±0.04 and 1.56±0.02 compared to 1.85±0.05 (Table 4, $p< 0.05$). Not all results were significant across the experiment, however, FCR was at least numerically reduced in all Met A and Met B treatments.

Table 4. Feed conversion ratio. Both pen weight and feed were measured and recorded for each pen on d0, 8, 16, 21, and 28. Total pen gain and feed intake were used to calculate feed conversion ratio. All data represented as mean ± standard error.

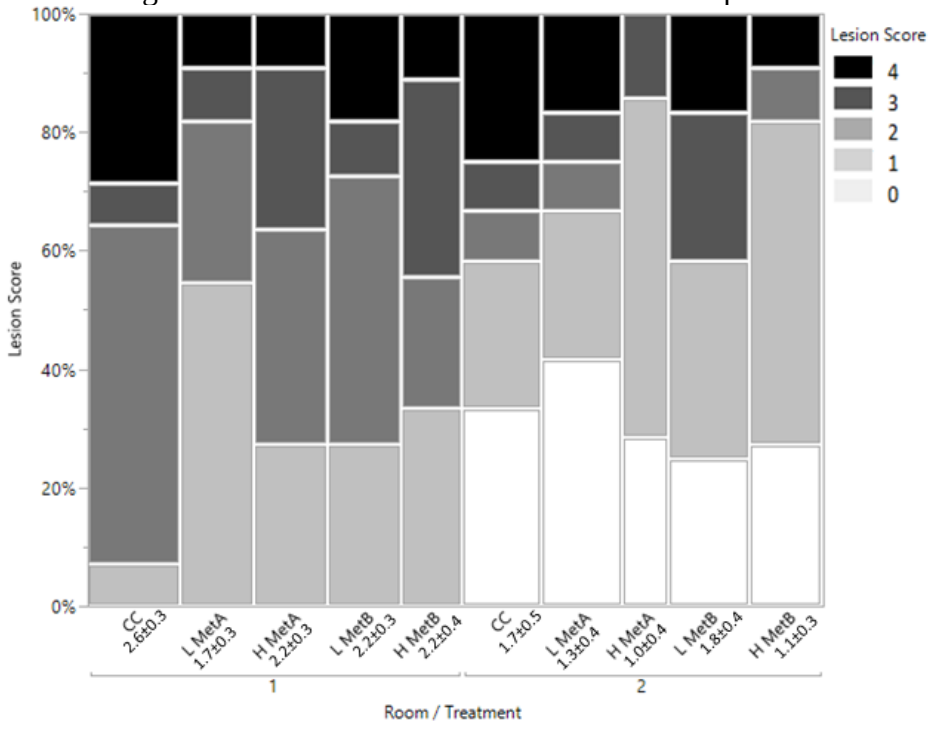
	0-7*		8-15		16-20		21-27	
	Room 1	Room 2	Room 1	Room 2	Room 1	Room 2	Room 1	Room 2
CC[1]	1.21±0.06[a]	1.21±0.06[a]	1.31±0.08[a]	1.27±0.01[a]	1.72±0.03[a]	1.85±0.05[a]	1.93±0.13[a]	1.77±0.09[a]
L Met A	1.19±0.02[a]	1.18±0.03[a]	1.17±0.01[a]	1.20±0.03[a]	1.64±0.06[ab]	1.57±0.04[b]	1.57±0.07[a]	1.50±0.06[ab]
H Met A	1.28±0.01[a]	1.20±0.02[a]	1.19±0.01[a]	1.15±0.02[a]	1.59±0.02[ab]	1.66±0.06[ab]	1.58±0.08[a]	1.40±0.04[b]
L Met B	1.22±0.05[a]	1.19±0.02[a]	1.17±0.01[a]	1.16±0.05[a]	1.51±0.04[b]	1.76±0.09[ab]	1.66±0.13[a]	1.61±0.09[ab]
H Met B	1.15±0.01[a]	1.15±0.01[a]	1.17±0.01[a]	1.17±0.03[a]	1.56±0.06[ab]	1.56±0.02[b]	1.68±0.05[a]	1.49±0.03[ab]
SEM	0.02	0.01	0.03	0.02	0.04	0.06	0.07	0.06
p-value	0.5116	0.7638	0.1169	0.1235	0.0178	0.0202	0.0993	0.0151

*Analysis was only performed on pens that did not have birds removed between d0 and d5. Pens that were reset at d5 were analyzed separately and were not included in d0-8 data.
[1] CC = *E. maxima* Challenged Control, L Met A = *E. maxima* Low Met A, H Met A = *E. maxima* High Met A, L Met B = *E. maxima* Low Met B H Met B = *E. maxima* High Met B

[a, b] Mean values with different superscript letters within a column indicate a significant difference ($p<0.05$).

Lesion Scores: On d21, LS for coccidiosis were recorded for 2 randomly preselected birds/pen. No difference in LS were observed between treatments in either room, yet an observed pattern in both rooms was evident. In Room 1, the percentage of 1's observed was greater in Met A and Met B treatments compared to CC (Figure 1). In Room 2, the percentage of 0's and 1's was greater in Met A and Met B treatments compared to CC (Figure 1). This is reflected by the L Met A mean LS in Room 1 of 1.7±0.3 compared to CC at 2.6±0.3 (Figure 1, $p=0.0908$) and the H Met A and H Met B mean LS in Room 2 of 1.0±0.4 and 1.1±0.3, respectively, compared to CC at 1.7±0.5 (Figure 1, $p=0.3372$ and $p=0.3449$). Although the LS were not significantly different between treatments, the distribution of scores reflected a positive impact of Met A and Met B on intestinal protection.

Figure 1. Coccidiosis lesion score mosaic plot. Coccidiosis lesions were observed on d21 and scored using a 0-4 scale. Any mortalities during the coccidiosis period (d19-d28) were necropsied to determine whether the mortality was associated with coccidiosis, and if so, that bird was assigned a score of 4. Mean ± standard error is reported under each column label.



Average Daily Shedding of Oocysts: In Room 1, a 60-87% reduction in ADS was observed among all Met A and B diets compared to CC (Table 5, $p<0.05$). This reduction in ADS was not observed in Room 2, however, the outcomes generally reflected the milder LS results.

Table 5. Average Daily Shedding of Oocysts from d19-28 (3-12 days post challenge). Fresh feces were collected twice daily from each pen beginning three days post challenge (**DPC**) through 12 DPC. All collected feces were diluted in 0.9% saline and combined to form an aggregate sample. Oocysts per gram of feces were quantified to determine average daily shedding of oocysts per gram of feces.

	3-12 DPC	
	Room 1	Room 2
CC[1]	7,292.70±1,550.22[a]	1,507.49±403.06[a]
L Met A	1,455.54±76.76[b]	1,008.99±430.09[a]
H Met A	2,639.36±938.84[b]	1,378.62±485.77[a]
L Met B	881.12±178.80[b]	1,892.11±543.79[a]
H Met B	1,643.36±665.92[b]	1,786.21±495.42[a]
SEM	1162.67	156.60
p-value	0.0097	0.6703

[1] CC = *E. maxima* Challenged Control, L Met A = *E. maxima* Low Met A, H Met A = *E. maxima* High Met A, L Met B = *E. maxima* Low Met B H Met B = *E. maxima* High Met B
[a, b] Mean values with different superscript letters indicate a significant difference ($p<0.05$).

Discussion

Body Weight Gain, Percent Change in Body Weight Gain, and Feed Conversion Ratio: There is a well-established connection between *Eimeria* infections and a reduction in growth parameters such as decreased BWG and feed conversion efficiency (Sharman et al., 2010; Williams, 2005). Introduction of various sources of Met help to limit the severity of *Eimeria* has been tested before, with reports showing that increased levels of Met improved BWG and FCR of broilers. The reports also suggest that increasing dietary Met beyond a certain value decreased BWG and FCR, likely due to toxicity of the sulfur amino acid (Lai, 2018). This finding was reflected in this experiment as both Met sources, Met A and Met B, resulted in a positive impact

on BWG and %chBWG following *E. maxima* challenge between d16-21, with L Met B showing the greatest improvement. In addition, FCR also improved during this period, where significant reductions were apparent in L Met B in Room 1 and L Met A and H Met B in Room 2, compared to CC. Reduced FCR mirrored the improved %chBWG for the same three treatments compared to CC. Improved performance from added Met likely buffered the impact of coccidial challenge on d16 and helped minimize a reduction in growth through d28. Improved BWG and FCR in response to the Met diets appeared to provide a buffer by increasing body weight, and thereby intestinal size that acted as a shield from the full affects during the coccidial disease period. Generally, inclusion of Met A and Met B helped limit the severity of coccidiosis and ultimately improve BW both before and during the disease period. Assessing a combination of these growth parameters in addition to quantitative parameters such as LS and ADS is essential to understand the effectiveness of Met treatment strategies during *E. maxima* challenge (Chasser et al., 2020).

Lesion Scores and Average Daily Shedding of Oocysts: Lesion scores provide insight into the degree of infection and intestinal damage as a result of the *Eimeria* species, but peak incidence of lesions is narrow leading to score variability (Chasser et al., 2020). In this study, L Met B in Room 1 and L Met A and H Met B in Room 2 resulted in the lowest mean scores of 1's and 0's compared to CC, suggesting that the Met sources provided a greater intestinal protection against lesion formation. However, LS variability makes it difficult to determine lesion severity and efficacy of the treatment, thus an additional factor such as ADS to measure coccidial infection is necessary (Barrios et al., 2017). Upon evaluation of ADS, Room 1 presented a clear reduction in ADS for all Met A and Met B treatments compared to CC ($p < 0.05$), while no difference in ADS was observed in Room 2. The mild effects of ADS in Room 2 were reflective of the milder mean lesion scores (LS) and the lack of difference between d16-21 in %chBWG

observed in Room 2 compared to Room 1. Intestinal protection most clearly appeared in the L Met A diet as shown by the lower LS in Room 1 and 2, as well as significantly reduced ADS compared to CC in Room 1 ($p<0.05$). Analysis of oocyst shedding provides a correlation to susceptibility of the pathogen as it replicates in the gastrointestinal tract (Zhu et al., 2000). The L Met A diet likely played a role in initiating the host immune response and controlling the ability of *Eimeria* to replicate within intestinal cells as explained by the lower LS and ADS. Growth performance parameters such as BWG and FCR, can be affected by other factors and require additional parameters such as LS and ADS to determine the effectiveness of the treatment strategy (Chasser, 2020; De Gussem, 2007). By including ADS and LS in conjunction with %chBWG, the influence of Met on buffering the severity of coccidial infection became apparent as the mild evaluation of ADS and LS correlated to the lack of difference in %ChBWG in Room 2.

Intestinal Immune Response: The host immune response following coccidiosis infection involves a complex interplay of cellular and molecular mechanisms. Methionine plays a major role in the cellular immune response of poultry susceptible to infection by improving health, growth, and development (Jankowski et al., 2014). Dietary Met has been associated with antibody production and cell-mediated immune responses with evidence that GSH regulates nuclear transcription factor κB pathway, T-helper cell function, and antibody and interferon-γ (IFN-γ) production under *Eimeria* challenge (Lai et al., 2018). Research has suggested that T lymphocytes respond to a coccidial infection through both cytokine production and a direct cytotoxic attack on infected cells (Lillehoj and Trout, 1996). Thus, Met interacts with the immune system through the synthesis of various antibodies and cytokines in order to provide protection against *Eimeria*. The addition of Met in the diet help broilers fight the exposure to

Eimeria infection through the stimulation of immune functions such as antibody production and cell-mediated immune responses (Lai et al., 2018). Medicated chickens that were unable to entirely defend against *Eimeria* infection who were fed Met showed decreased LS and oocyst production (Lai et al., 2018). This indicates the role Met plays on intestinal protection through activation of the host immune response.

Conclusion

Chickens fed a supplemented diet of Met exhibited greater growth performance. The greatest intestinal protection was provided by L Met A, shown by low LS in Room 1 and 2 and reduced ADS compared to CC in Room 1. The L Met A diet may have impacted the host immune response and ability of *Eimeria* to replicate within intestinal cells, which may in part explain the less severe lesions as well as lower oocyst output observed. The remaining treatments, H Met A, H Met B, and L Met A also provided protection from *E. maxima*, with the greatest impact observed in L Met A. Further investigation of the effect of Met sources and other inclusion levels on inflammation and oxidative responses is required to provide the industry with an additional treatment strategy to manage the impact of coccidiosis.

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