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Reviews

RETRACTED - Methionine: comparing methionine hydroxyl analogues for broilers, with focus on different thermal environments

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Supplementation of methionine (Met) in broiler chicken diets is essential to support productive performance and optimise carcass yield. Met is the first limiting amino acid in corn and soybean-meal based diets for poultry. The DL-Met form is the main source used in broiler diets, but other sources such as acid free hydroxy-analogous methionine (HMA-FA) are available. Studies have indicated that the molar bioequivalence of HMA-FA is approximately 88% compared with DL-Met at 99% for growth traits. However, differences in absorption and metabolism between Met sources can influence their efficacy, especially when broilers are exposed to high temperatures. The substitution of DL-Met by HMA-FA is a potential strategy to mitigate the negative effects of heat stress because it is passively absorbed in the upper portion of the gastrointestinal tract. This review highlights the effects of substituting HMA-FA for DL-Met in diets for broiler chickens reared in different thermal environments.

Keywords: carcass characteristics; methionine; performance; supplementation; thermal-environment

Introduction

For many years, amino acid nutritional requirements for poultry have been based on the supply of crude protein, which resulted in diets that overestimated or underestimated amino acid levels (Tesseraud *et al.*, 2011). However, commercial-scale production and feasible pricing of crystalline amino acids and analogue sources has enabled nutritionists

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to formulate diets that more accurately meet the nutritional requirements of broiler chickens for protein and digestible amino acids, allowing for lower diet costs and lower negative impact on environmental pollution due to lower excretion of nitrogen (N) to the environment. In this sense, analogue forms of Met are commercially available and can be used as alternatives to supply Met in poultry diets. Because hydroxyl-analogues do not contain amine groups, their supplementation in low protein diets can reduce the excess amino N in the diets and limit N excretion (Kim *et al.*, 2006), without causing any deficiency in sulphur amino acids (Zou *et al.*, 2015). In times of heat stress, poultry typically reduce their feed intake, thereby limiting Met availability for essential tissue functions. The provision of readily available Met in diets for such animals is important to maintain health and performance.

Among the essential amino acids, Met is first limiting in corn and soybean-meal based diets for poultry. This amino acid participates in protein synthesis which is required for the biosynthesis of important metabolites such as creatine, carnitine, polyamines, epinephrine, choline, melatonin and taurine (Baker *et al.*, 1996; Metayer *et al.*, 2008). Broilers have high requirements for sulphur amino acids to support feathering, which is rarely met without the proper supplementation of Met in the diets (Albino *et al.*, 1999). The HMA-FA is the main alternative source to DL-Met, due to its competitive cost. However, although HMA-FA is often economical, its bioequivalence compared to DL-Met is debated (Hoehler *et al.*, 2005).

Besides the difference among components providing Met for broiler metabolism, the thermal environment influences the absorption and bio-efficiency of each source. Microbiota is another factor influencing the bio-efficiency of each Met source used in feed (Drew *et al.*, 2003). The differences between Met sources result from their chemistry, absorption, transport and metabolism in tissues (Dibner, 2003; Lobley *et al.*, 2006; Wester *et al.*, 2006). HMA-FA can be absorbed through passive transport and diffusion in the upper part of the gastrointestinal tract. Thus, it has been suggested that HMA-FA is more efficiently used than DL-Met by birds reared at high ambient temperatures. HMA-FA possesses antimicrobial functions due to its effects on pH, benefiting colonisation by beneficial bacteria (Martín-Venegas *et al.*, 2013). Furthermore, due to the absence of an amine group, the excretion of nitrogenous compounds is reduced and thus, environmental pollution lessened.

The following review highlights the effects of substituting HMA-FA for DL-Met in diets for broiler chickens reared under various thermal environments in relation to productive performance and carcass characteristics.

Methionine sources and metabolism

Among the sources of Met, the most used in animal nutrition are the DL-Met and the HMA-FA. The DL-Met can be found in liquid form as sodium salt (DL-Met-Na), with 40% bioequivalence. The DL-Met is the only synthetic amino acid obtained by the chemical synthesis of the acrolein, hydrocyanic acid, methyl mercaptan and ammonium carbonate. Its composition varies from 88% to 99% of purity.

HMA-FA can be found in the liquid form as 2-hydroxy-4-methylthiobutiryc acid, which contains 88% of monomers and polymer forms of HMA-FA or as calcium salt in powder form (HMA-Ca) (Dibner, 2003). The difference between the two most commonly used sources, HMA-FA and DL-Met, resides basically on their molecular structure. While DL-Met has an amino acid group (NH^{+3}) in its molecule, HMA-FA has a hydroxide group (OH) and, because of this, it is not classified as amino acid. Therefore, it needs to go through a series of chemical reactions to be converted into Met by the

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animal. The HMA-FA is classified as a monocarboxylic acid with a similar chemical structure to organic formic, lactic and fumaric acids, which are widely used to substitute the promoters of growth in poultry diets.

HMA-FA has approximately 100% bioequivalence compared to DL-Met (Lemme *et al.*, 2002). Therefore, if HMA-FA is used as a substitute of DL-Met, a proportion of its inclusion must be analysed, to allow the calculation of the total cost in the diet in order to verify the financial viability of its use. However, it has been suggested that in the HMA-FA is beneficial for broilers reared under heat stress conditions due to its greater absorption in the upper digestive tract. The passive absorption of HMA-FA in the small intestine is more efficient where there is an increase of the intestinal turnover and there is a reduction of undesirable oxidative stress in animal products (Martín-Venegas *et al.*, 2013). The HMA-FA is considered to have a trophic effect on the intestinal microbiota due to acidification of the environment, benefiting bacterial competition and restricting pathogenic microorganism colonisation (Dibner and Buttin, 2002).

Some studies have demonstrated that DL-Met is quickly absorbed in the anterior region of the small intestine of broiler chicken, being less significant in more distal regions. The absorption of DL-Met occurs with energy expenditure, through dependent and independent carriers of the Na and L-systems (Martín-Venegas *et al.*, 2009). Contrary to DL-Met, the absorption of the isomers of the HMA-FA occurs in a passive way, by diffusion down the concentration gradient (Meirelles *et al.*, 2003) *via* a system a specific proton-dependent transporter named MCT1 (Mono Carboxylic Transporter-1) normally dedicated to monocarboxylic acids such lactic acids (Martín-Venegas *et al.*, 2014). The low pH of the upper part of the gastrointestinal tract of the poultry, due to HCl in the chyme derived from the proventriculus and gizzard, increases the non-dissociated form of the HMA-FA molecule, facilitating its absorbency by diffusion in the duodenum. However, Richards *et al.* (2005) observed that absorption of HMA-FA was higher than 70% when it was digested in the proventriculus and 85% was absorbed before the duodenum. Suida (2006) highlighted that, contrary to DL-methionine, the absorption of HMA-FA occurs along all the small and caecum intestine, where it can be absorbed or utilised by the caecal microbiota.

Although the transport mechanisms of the DL-Met and of the HMA-FA present differences in relative speed of absorption, it is not totally clear that these can limit the exploitation of the component (Martín-Venegas *et al.*, 2006). This difference of absorption can be related to the acid form, in other words, they can be in the form of monomers, dimers or oligomers. Boebel and Baker (1982), determined, using high-performance liquid chromatography, the relationships as follows: 64 to 66% of monomers, 15 to 19% of dimers, trimers absent, 1 to 3% of oligomers and 12% of water. Viana *et al.* (2009) suggested that the HMA-FA was a mixture of approximately 65% of monomers, 20% of dimers and 3% of trimers. Once the concentrated product is in feed, the equilibrium shifts in the direction of monomers, resulting in the formation of HMA calcium salts. The amount of non-monomeric forms of HMA-FA is highest in the supplement and decreases in feed and after ingestion (Dibner, 2003). Several *in vitro* and *in vivo* techniques have been used to elucidate the hydrolysis and fate of the HMA-FA non-monomeric forms by pancreatic intestinal enzymes, intestinal epithelial cells (Dibner 2003) and everted sacs during *in vivo* intestinal perfusion and growth performance studies (Martín-Venegas *et al.*, 2006). These studies concluded that the HMA-FA polymer fractions that accurately represented those found in the product are subject to hydrolysis to monomers by intestinal enzymes and mucosa and are not a limiting factor in the absorption or conversion of HMA-FA.

After being absorbed, the L-Met and D-Met isomers, derived from the DL-Met, as well

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as from the HMA-FA, are transported to the liver, mainly through the portal vein, and a small quantity is transported through the lymphatic system *via* the liver. The main metabolism of the methionine occurs in the liver, and is responsible for most of the conversion of HMA-FA and D-Met to its biochemical active form, L-Met. In the extra-hepatic tissues, L isomers are converted into other metabolites or are linked to a specific RNA transporter for protein synthesis in the ribosome.

Preliminary considerations when evaluating different methionine sources

The first consideration regarding Met sources are the different ingredients, characteristics, and their potential for variation in biological responses. Each source presents different compositions and chemical structures, and hence, different modes of absorption, transport and metabolism in animal tissues (Dibner and Knight, 1984; Lobley *et al.*, 2006; Wester *et al.*, 2006). The main misconceptions when comparing Met sources, include arbitrary equivalences (no conclusive scientific basis) of 65% bioefficacy of Met-analogue sources relative to DL-Met; use of purified diets (including crystalline amino acids) or diets formulated based on crude protein content; consideration of total sulphur amino acid in diets and, in particular, the statistical methods applied to establish the relationship between Met sources and performance.

Establishing 65% bioefficacy for other Met sources does not take into account their composition and or compare them an equimolar basis. Hence, conclusions can be misleading and inconclusive in dose-response studies. Both equimolar concentrations and dietary inclusion levels of Met influences free plasma content, and consequently, feed intake and growth rate (Vázquez-Añón *et al.*, 2006; Gonzalez-Esquerra *et al.*, 2007). Therefore, the results of such studies favour sources that have higher equimolar basis of Met.

The use of purified experimental diets with inclusion of crystalline amino acids and the establishment of practical levels of total sulphur amino acids influence the performance response when comparing Met sources for broilers (Vázquez-Añón *et al.*, 2006). After all, this affects the total and essential-N balance, and, in particular, free Met plasma content. Plasma Met level is a parameter used to determine the efficacy of Met uptake by tissues (Lobley *et al.*, 2006; Wester *et al.*, 2006). These authors reported that HMA-FA was more readily used by peripheral tissues and presented greater metabolisation in the kidneys, resulting in lower plasma concentrations. This stimulated feed intake and increased growth rate of broilers fed HMA-FA. Gonzalez-Esquerra *et al.* (2004) observed lower feed intake in broilers fed diets containing DL-Met compared with those fed HMA-FA. According to Elkin *et al.* (1988), this can be explained by the higher concentration of the D-isomer, which favours the increase in Met plasma levels in broilers fed DL-Met.

Thermal environment and heat on broilers

The maximum expression of genetic potential in birds requires a thermoneutral zone for rearing broilers, in which the thermoregulatory system of the broiler is not triggered to produce or dissipate heat. However, tropical climates and inadequacy of housing and facilities to ensure appropriate temperature often leads to heat stress, impairing productivity. This implies difficulty of expression of ethological behaviours inherent to the species, affecting the welfare of broilers (Dalólio *et al.*, 2015).

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During exposure to heat stress, poultry use mechanisms to increase heat dissipation and decrease heat production (Sahin *et al.*, 2009). Feed intake is one of the most important mechanisms for reducing thermogenesis. Concomitantly, a reduction of circulating levels of thyroid hormone is observed in order to reduce metabolic heat production (Bahrami *et al.*, 2012). In addition, heat stress increases mortality, corticosterone concentration, acute enteritis in the mucosa of the jejunum, capillary mucosal haemorrhage and reduces the relative weights of bursa and spleen (Martindale 2005; Quinteiro-Filho *et al.*, 2010). This way, diet composition, density and components can be adjusted to attenuate the negative effects of heat stress. The interaction between nutrition and ambient temperature is essential to understand and determine the nutritional requirements of the animals.

Interaction of thermal environment and methionine sources; effects of HMA-FA in thermal stress

The use of HMA-FA in liquid form has shown benefits during periods of heat stress. The supply of HMA-FA for broilers up to 47-d-old raised under high temperatures (30.3°C average temperature) resulted in higher feed intake (+ 7.07%), weight gain (+ 4.98%), better feed conversion ratio (2.3 *vs.* 3.1) and reduction in mortality when compared to DL-Met (Gonzalez-Esquerra and Leeson, 2006). This can be related to the dynamics of absorption, because, in periods of high stress, more sloughing of the epithelial cells in the intestine occurs, which facilitates passive absorption, by diffusion, of HMA-FA (Dibner *et al.*, 1992). Heat stress increases blood osmolarity, caused by the respiratory alkalosis (Borges *et al.*, 2007). This increase in pH preserves the protonated gradient in the microvillus of the intestine and can benefit the diffusion of the HMA-FA from the intestinal lumen.

Swick *et al.* (1990) and Dibner *et al.* (1992), when raising broiler chicken kept in environment at 32°C, observed beneficial effects of HMA-FA over the DL-Met in feed, justifying its superiority in terms of transport and absorption compared with DL-Met. However, Mitchell and Hunter (1996) did not observe more efficient absorption of the DL-Met in comparison to HMA-FA.

Ribeiro *et al.* (2001) and Ribeiro *et al.* (2005), when evaluating different sources of HMA-FA, DL-Met or L-Met on performance of broiler chickens reared under heat stress (35°C, Ribeiro *et al.* (2001); 31°C and 85% relative humidity, Ribeiro *et al.* (2005)) and slaughtered at 42 days, did not observe any influences of sources for weight gain, FCR, carcass yield or feathering. However, Bunchasak *et al.* (2006) observed better weight gains (+ 2.62%) in broilers when supplemented with HMA-FA in the diet compared to DL-Met during the period of 1-21 days. However, no significant effects were observed in weight gain, feed intake, feed conversion ratio, mortality rate or uniformity in the total growing period (1 to 42 days).

Montanhini Neto *et al.* (2013) compared HMA-FA and DL-methionine supplementation in broiler chickens kept under heat stress (32°C), and observed better FCR (1.97 *vs.* 2.03), higher carcass yield (+ 4.17%) when the broilers were supplemented with HMA-FA. This had no influence on cloacal bursa, lymphoid tissue, lymphoid clusters or antibody levels. However, Swennen *et al.* (2011) evaluated two levels of crude protein (18.2 low CP and 23.2 high CP), and showed higher concentration of total hepatic glutathione, reflected in lower lipid peroxidation, in broilers fed with diet containing HMA-FA with low protein content. Willemsen *et al.* (2011) observed an increase in total hepatic glutathione and oxidised total hepatic glutathione in broilers kept under heat stress (32°C) and fed with HMA-FA compared to DL-Met. Martín-Venegas *et al.* (2013) reported a reduction in hepatic glutathione caused by HMA-FA

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as a source of Met. According to these authors, this occurs due to the process of trans-sulphuration of HMA-FA, producing taurine and glutathione, which function as antioxidants because they reduce the tumour necrosis factor, producing less peroxides (H_2O_2).

Effects of HMA-FA in a thermoneutral environment

Meirelles *et al.* (2003), when analysing the DL-Met or the L-Met on performance of broiler chicken slaughtered at 47 days, did not observe any influence of sources on performance, carcass yield or feathering. Similarly, Daenner and Bessei (2003) did not observe any influence of Met source on performance of broilers from 1 to 33 days old, when comparing the effect of the HMA-FA and DL-Met. Hoehler *et al.* (2005) studied the effect of Met supplementation by means of the inclusion of DL-Met and of HMA-FA and did not observe differences between sources on weight gain, feed intake and FCR of broiler chicken aged 1 to 21 days old.

In contrast, Swennen *et al.* (2011) reported greater weight gain (+ 11.51%) and higher relative weight of liver (+ 8.19%) for broilers fed diets supplemented with HMA-FA in comparison to DL-Met, with no effect on relative heart weight, abdominal fat or breast muscle. Nonetheless, Sangali *et al.* (2014) and Sangali *et al.* (2015) did not find any effect through substitution of DL-Met by HMA-FA in relation to performance and carcass characteristics for broilers aged 22 to 42 days or 1 to 21 days old, respectively.

Some factors need to be taken into account to explain the divergence between studies, including age, environmental factors (temperature, health challenges, and management practices), nutritional management (feeding programs and nutritional requirement references), feedstuffs included in the diet and the best methionine + cystine: lysine ratio that maximises productivity when comparing each methionine source. When using analogue methionine sources, this ratio still needs to be established for broilers, warranting further studies.

Zhao *et al.* (2018) observed the bio-efficacy of HMA-FA compared to DL-Met for performance, carcass traits, feather growth, and redox status of Cherry Valley ducks. The DL-Met and HMA-FA had equal biological value for growth performance, carcass traits, and feather growth. However, antioxidant capacity was better with HMA-FA in the meat during *post-mortem*, contributes to a better duck meat quality and shelf life.

Vázquez-Añón *et al.* (2006) and Gonzalez-Esquerra *et al.* (2007) evaluated HMA-FA and DL-Met in broiler and turkey diets, formulated to be either deficient or optimised for total sulphur amino acid (TSAA) levels. Both authors observed that DL-Met and HMA-FA promoted performance when included in TSAA-deficient diets based on sorghum, corn and soybean meal. However, Agostini *et al.* (2016) observed that, in pelleted diets based on wheat, maize and soybean meal, with different levels of TSAA to lysine (0.62 to 0.73), there was an increase in performance of broilers up to 28 days of age that received HMA-FA compared to DL-Met. There is a need of further studies to establish the optimal digestible methionine + cystine to lysine ratio when HMA-FA is used as a dietary Met source.

Effects of the HMA-FA on intestinal microbiota

Studies *in vitro* and *in vivo* have demonstrated that intestinal microbiota and the ambient temperature influence the bioavailability of Met sources (Drew *et al.*, 2003; Martín-Venegas *et al.*, 2006). The absence of the amine radical (NH_2) is the main difference

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between Met and its analogues which, when in the liquid state, are presented as acid (2-hydroxy-4-methylbutiryc acid) with a hydroxyl in the amine position (Dibner and Buttin, 2002). According to Martín-Venegas *et al.* (2013), the HMA-FA, being a monocarboxylix acid, possesses a chemical structure similar to other organic acids which have been used in place of antibiotic growth promoters in poultry and pigs, like formic, lactic and fumaric acids. Nonetheless, Salary *et al.* (2015) do not observe any effect of HMA-FA on intestinal pH. Supplementation with HMA-FA increased antibody titres for Newcastle disease, influenza and avian infectious bronchitis, improved immunity and reduced the undesirable bacteria in the intestinal microflora (Salary *et al.*, 2015). Met, when properly available, acts directly on antibody production (Mirzaaghatabar *et al.*, 2011). The oxidation of Met residues has been well-documented in terms of production of therapeutic proteins such as alpha-1 antitrypsin, granulocyte colony-stimulating factor, coagulation factor VIIa, and, in particular, immunoglobulin gamma antibodies (IgGs) (Khor *et al.*, 2010).

It is known that the main mechanism of action of organic acids is due to the reduction of pH in the interior of the bacterial cell, leading to a higher antimicrobial activity (Van Immerseel *et al.*, 2006). The non-dissociated form of the acid is fat-soluble (Pickler *et al.*, 2012). In this form, it has the capacity of passing through the cell membrane in a passive way and, in an environment with a pH higher than its pKa, the acid dissociates itself, alternating the cytoplasmic pH of the bacterial cell, acting as a bactericide. Knowing the bactericidal property of organic acids, as well as their capacity for acidifying the environment, which will make it more propitious to the proliferation of the beneficial microbiota, it is interesting to evaluate the effect of HMA-FA on the composition of intestinal microbiota in broiler chickens.

Conclusions

HMA-FA can be used as a substitute for DL-Met with the objective of ensuring the proper contribution of amino acids for performance and carcass characteristics in broiler chickens. For the proper indication of use of each source, it is necessary to analyse the housing environment of the poultry as well as the cost benefit. There is evidence that, during heat stress, HMA-FA compared with DL-Met may have greater absorption. Furthermore, HMA-FA may have a positive effect on health of broilers due to its action as an organic acid against pathogens. As such, further research to evaluate these sources for poultry in different thermal environments is necessary to more accurately assess optimal inclusion and economic viability for diets formulated for broiler chicken.

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