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Physiological responses to DL-methionine and L-methionine supplementation in the early life of TETRA-SL LL hybrid and Hungarian Partridge Colored Hen genotypes

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List of abbreviations and acronyms

18S rRNA	18S ribosomal RNA
AA	Amino acid
ACTB	Actin beta
ADG	Average daily gain
AHW	Absolute heart weight
ALH	Absolute liver heart
ALT	Alanine aminotransferase
ANOVA	Analysis of variances
AST	Aspartate aminotransferase
BD	Basal diet
BW	Body weight
CAT	Catalase
CP	Crude protein
Cys	Cysteine
DL-Met or DLM	DL-methionine
FRAP	Ferric reducing ability of the plasma
GHR	Growth hormone receptor
GPX	Glutathione peroxidase
GPX1	Glutathione peroxidase 1
GRAN	Granulocyte
GSH	Glutathione
GST3	Glutathione S-transferase alpha 3
Hb	Haemoglobin
H&E	Hematoxylin-Eosin
HBW	Hatching body weight
HMTBA	DL-2-hydroxy-4-(methylthio) butanoic acid
HPC	Hungarian Partridge-colored hen breed
HSP70	Heat shock protein 70
Ht	Hematocrit
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor

IL1-β	Interleukin-1 beta
IL-6	Interleukin 6,
JNK	c-Jun N-terminal kinase
LD1	Linear discriminate function 1
LDA	Linear discriminator analysis
L-Met or LM	L-methionine
LOOD	Lipid peroxides
LYM	Lymphocyte
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume of red blood cell
MD2	Tricellulin (also referred to as MARVEL domain containing 2)
MDA	Malondialdehyde
Met	Methionine
Met-Cys	Methionine plus cysteine
MHA	Methionine hydroxyl analog
MHA-FA	DL-methionine-hydroxyl analog-free acid
MID	Mid-range cells
MS	Methionine supplementation
Msr	Methionine sulfur reductase
MsrA	Methionine sulfur reductase A enzyme
MUC2	Mucin-2
NLCPAR	Nonlinear common plateau asymptotic regression technique
NRC	National Research Council
NRF2	Nuclear factor, erythroid 2 like 2
OCLN	Occludin
RBC	Red blood cells
RBE	Relative biological efficacy
rGSH	Reduced glutathione
RHW	Relative heart weight
RLW	Relative liver weight
RMSE	Root square of mean of the standard error
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
RT–PCR	Real-time polymerase chain reaction
SAH	S-adenosylhomocysteine
SAHH	SAH hydrolase
SAM	S-adenosylmethionine
SID	Standardized ileal digestible
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
TGSH	Total glutathione
TJ	Tight junction
TJP2	Tight junction protein 2
TLR4	Toll-like receptor 4
TrxR	Thioredoxin reductase
TSAA	Total sulfur-containing amino acid
TSL	TETRA-SL LL layer hybrid
WBC	White blood cells
ZO1	Zonula oocluden 1

1. INTRODUCTION

The main challenges for the coming century in livestock farming systems include meeting the high predicted demand for meat due to the exponential growth of humans. To overcome that challenge, farmers must maximize animal performance by enhancing knowledge of the animal requirements and affecting factors (EL-TARRAS *et al.* 2019). Methionine (Met) is the most limiting amino acid for birds due to its demand for muscle and feather metabolism and feed composition (KIM *et al.* 2019; REHMAN *et al.* 2019; CHEN *et al.* 2020), therefore methionine supplementation can maximize the bird's performance (MAJDEDDIN *et al.* 2019). Protein supplementation in the bird's diet traditionally involved soybean meal along with cereal maize (corn) (ZHANG 2016). The amino acid methionine (Met) is often the first limiting one in soybean meal-corn-based poultry diets, especially during feathers development. Dietary supplementation with limiting amino acids in the poultry diets is required to reduce incorporation level of protein-rich feedstuffs while maintaining performance and reducing nitrogen excretion by more effective protein synthesis (MANDAL *et al.* 2004; SHEN *et al.* 2015). Traditionally, Met is the only amino acid obtained by chemical synthesis, resulting in a racemic mixture of D and L forms (DL–Met). Met can be supplemented in the feed (ULLRICH *et al.* 2019), in drinking water (CADIRCI & KONCAGUL 2014), as well as application of *in-ovo* feeding is also possible (CHEN *et al.* 2020). DL–Met is the most common form of sulfur amino acid supplementation for birds and it has been used for ages. In addition a number of trials have also been conducted with DL-2hydroxy-4-(methylthio) butanoic acid (DL-HMTBA) (AGOSTINI *et al.* 2016; ZHANG 2016). However, recently, L-Met (produced by fermentation) became widely available and registered as an additive feed for poultry (MILLECAM *et al.* 2020).

These sources differ in physical and chemical characteristics; hence their bioavailability and physiological function are also different depending on their metabolism in poultry (SANGALI *et al.* 2014; WAN *et al.* 2017), therefore differing in their effect on performance. Both L-methionine and D-methionine are actively transported against a concentration gradient, while methionine hydroxy analogues (MHA) are passively absorbed by diffusion from high to low concentration (MEIRELLES *et al.* 2003). Animal cells cannot utilize D-isomer amino acids; conversion to L-isomer is necessary for protein synthesis (MILLECAM *et al.* 2020). DL-Met and HMTBA must be converted to L-Met to be utilized in the body, mainly by the liver and kidneys (SANGALI *et al.* 2014; ZHANG *et al.* 2017).

Some older research with low genetic capacity broiler chicks showed similar biological efficacy. Still, little is known concerning laying hens and dual-purpose old Hungarian chicken breeds. Studies with rats indicated a positive antioxidant response to L-Met supplementation during oxidative stress. Other studies with L-Met, betaine, and choline showed interacting effects and L-Met also had a liver-protecting impact (PARK *et al.* 2018).

Few studies have investigated the effect of DL-Met and L-Met sources on growth performance, intestinal development, and health and antioxidant status of poultry via dietary supplementation but not during embryonic development. The dietary supplementation revealed that either DL-Met or L-Met affected the growth performance of ducks in the starter phase and enhanced the small intestinal morphology and feather development (ZHANG *et al.* 2019). However, they reported that L-Met was more effective than DL-Met, with a range of 120% to 140% for the growth performance parameter at the starter phase and 153% for the feather traits at the finisher phase. The same trend was reported in broiler chicks, where L-Met improved the redox status and intestinal development compared with DL-Met (SHEN *et al.* 2015). Furthermore, L-Met showed a positive effect on the intestinal development, immune responses and antioxidant system of broilers challenged with *Eimeria spp.* (TENG *et al.* 2023). Contrary to this, another current study has shown that different Met sources (DL-Met, L-Met, and MHA) similarly affected intestinal barrier function and microbiota in broilers (BAREKATAIN & KLUENEMANN 2023).

Limited information is available on the effect of Met sources on layer genotypes at early and embryonic stages. In modern commercial hatcheries, newly hatched chicks are often denied access to feed and water for about 24 to 48 hours, depending on the hatching window, hatchery treatment, and rearing unit's location (DANG *et al.* 2022a). This forces the chicks to rely only on the nutrients present in the yolk sac reserves during this period, which is frequently insufficient to provide the nutrients needed for rigorous growth and metabolism, and these chicks are prone to oxidative stress due to the increased production of free radicals (UNI *et al.* 2005; ELWAN *et al.* 2019). Antioxidants are an essential line of defense against free radicals. On the other hand, freshly laid eggs contain relatively low levels of antioxidants. This means that the embryo is constantly subjected to the negative consequences of oxidative stress. A balanced antioxidative condition during

incubation is essential for hatchling viability. The balance between pro-oxidants and antioxidants during embryonic development plays a role in the rearing period of chickens.

The *in-ovo* injection of nutrients can overcome these issues, bridge the gap between hatching, and provide a tool to overcome the imbalances between antioxidants and pro-oxidants (KADAM *et al.* 2013). Studies have shown that the amnion is an effective site for applying the *in-ovo* injection, and the embryo ingests the amniotic fluids before pipping. Therefore, this procedure is sometimes called *in-ovo* feeding (DANG *et al.* 2022b). *In-ovo* feeding provides nutrients to chicks at the critical stage, facilitating embryo development and post-hatch growth performance (JHA *et al.* 2019). The high demand for protein for growth and to reduce the negative effect of oxidative stress during late embryonic development is shown to be achieved by the *in-ovo* feeding of specific nutrients (DANG *et al.* 2022a). *In-ovo* feeding of methionine has been shown not only to alleviate oxidative stress (BHANJA *et al.* 2012; ELWAN *et al.* 2019; DANG *et al.* 2022a) but also to be used as a source of amino acids for protein synthesis and, hence to decrease protein-based gluconeogenesis in hatchlings (COSKUN *et al.* 2018).

The TETRA-SL LL hybrid (TSL) is a brown egg layer hybrid that is suitable for both intensive industrial and extensive systems, either kept in a cage and alternative systems. They are known for efficient and persistent egg production with an extended life cycle, high livability, feed efficiency (5.7 -6.1 kg between 0-17 weeks of age and 110-120 g/day feed consumption between 18 and 100 weeks of age), and high hatchability due to genetic improvement. TSL produces eggs with excellent internal and external egg quality. The average egg weight is 64.0 to 65.5 g for 52-week-old TSL hens (BÁBLONA 2020). The most important characteristic of TSL is its outstanding livabilty. They have low mortality rate ranging between 2 -8% through the lying cycle (17-100 weeks), this minimizes the chick losses and ensures a higher number of hens reaching peak production. The hens reach up to 50% production at a relatively low age (140-150 days) and quickly ramp up to 90% production within 160-170 days of age. Throughout their laying cycle, TSL hens produces a total of 479-491 eggs per bird, with a total egg mass of 31.0 -31.8 kg. The TSL body weight at17 weeks of age is 1.35 – 1.46 kg, while at 100 weeks of age is 1.90-2.05 kg (BÁBLONA 2020). Hungarian Partridge-Colored hen (HPC) is among the indigenous chicken breeds that have been conserved in the gene reserves by the Hungarian government since 1973. They are known for their dual-purpose function and

palatable meat. HPC has good adaptability, scavenging ability, and disease resistance capacity (LAN PHUONG *et al.* 2014; LUGATA *et al.* 2022c). The HPC flocks egg production can reach 140 to 150 pieces per hen per production cycle (LAN PHUONG *et al.* 2014). These differences are due to the variations in the alleles and genes and the interaction between their phenotype and the environment and nutrition. There is a scarcity of information on the effect of nutrient manipulation in the eggs of the TSL and HPC on the antioxidant status and gene expression related to growth and immunity.

1.1. Objectives of the PhD program

In general, this Ph.D. program aimed to evaluate the physiological responses of newly hatched chicks to *in-ovo* supplementation of DL-Met and L-Met in **TETRA-SL LL hybrid (TSL)** and Hungarian partridge colored hen breed (**HPC)** layer genotypes. Additionally, this study aimed to assess the effects of dietary supplementation with DL-Met and L-Met on the growth performance and hematological parameters of TSL chicks during the starter phase

The specific objectives of the thesis are as follows:

- ✓ To determine the effect of *in-ovo* injection of DL and L-Met on blood biochemical parameters (AST, ALT, uric acid, FRAP) and jejunum morphology of TSL and HPC chicks.
- ✓ To examine the influence of *in-ovo* injection of methionine sources on antioxidant status (GSH, TAC) in the liver, intestine, and pectoral muscles of the newly hatched chicks of the two genotypes.
- ✓ To evaluate the effect of *in-ovo* feeding of methionine sources on the gene expression related to growth, antioxidant status, and intestinal tight-junction proteins of the newly hatched chicks of TSL and HPC.
- ✓ To assess the biological efficacy of DL and L-Met supplementation on growth performance, feather development and hematological parameters of the TSL chicks from day 1 to 28 days of life.

2. LITERATURE REVIEW

2.1. Bio-efficacy of dietary methionine sources and levels in poultry

Several studies have been conducted on the effect of dietary Met sources especially DL-Met and HMTBA on poultry performance for over 50 years (SEKIZ *et al.* 1975; YALCIN *et al.* 1999). Several studies have demonstrated no significant difference between DL-Met and HMTBA as methionine sources for broiler chickens fed traditional corn-soybean meal diets in terms of daily gain and feed efficiency (SANGALI *et al.* 2014; ZHANG *et al.* 2017). However, the two dietary Met sources' relative biological efficacy (RBE) remains controversial despite these studies. Although previous studies presented that the two Met sources showed similar levels of Met efficacy (BIRD 1952), HMTBA has lower efficiency compared to L-Met or DL-Met in diets that have low levels of total dietary sulfur-containing amino acids (TSAA). This is because HMTBA metabolized slower than the L-Met and DL-Met (SMITH 1966; SAUNDERSON 1985). Dose response trials are commonly used to estimate the RBE, and based on the achieved plateau the relative efficacy can be calculated (LIU *et al.* 2004). The contradicting conclusions can be accounted for by the statistical models used (AGOSTINI *et al.* 2016). Different models have been proposed to estimate the RBE of DL-Met and HMTBA, such as the nonlinear regression model with plateau (NLCPAR) (LITTELL *et al.* 1997), exponential model (LEMME *et al.* 2002), linear, quadratic, and exponential model (VÁZQUEZ-AÑÓN *et al.* 2006). KRATZER & LITTELL (2006) have questioned the first two models. The two products, HMTBA and DL-Met, have different absorption and metabolic pathways, which means they don't have the same dose-response profile. In experiments on male chickens for 6-7 weeks, HMTBA treated groups had better performance than DL-Met at TSAA deficient levels (VÁZQUEZ-AÑÓN *et al.* 2006). However, they further pointed out that the differences in responses to Met sources were dose-dependent, and HMTBA may perform better than DL-Met at levels to achieve the maximum response. Still, DL-Met may outperform HMTBA at suboptimal intake (VÁZQUEZ-AÑÓN *et al.* 2006). Furthermore, AGOSTINI *et al.* (2016) showed that practical HMTBA treatment groups had better performance than DL-Met supplemented animals, when dietary supplementation was above the required levels. In contrast, females exhibited the opposite response when dietary TSAA level was below the requirement.

A study conducted on young male turkeys indicated similar results: feeding DL-HMTBA source for 21 days reduced growth performance compared to DL-Met at deficient TSAA

levels (GONZALES-ESQUERRA *et al.* 2007). This suggests that predictions should be developed independently for each Met source, and the dose-response should be determined based on predicted differences. The calcium salt of DL-HMTBA had an average RBE of 63% compared to DL-Met in male Ross 308 and Cobb 500 chickens consuming a TSAA deficient diet (ELWERT *et al.* 2008). A study conducted by MANDAL et al. (2004) found that the efficacy of liquid DL-methionine-hydroxy analog-free acid (MHA-FA) for growth performance was 62.1%, 64.8%, and 63.9% and for feed efficiency was 63.0%, 67.7% and 64.0% at weeks 0-3, 3-6 and 0-6 respectively. SAUER *et al.* (2008) reported that DL-HMTBA had 79% and 87% RBE over DL-Met for the average daily gain and feed conversion ratio based on the analyses of forty trials, using NLCPAR model. Economic analysis of these meta-analyses revealed that the relative economic values were between 81%-86% depending on the market prices of broilers and the cost of feed including DL-Met (VEDENOV & PESTI, 2010). It is worth noting that these results were questioned by the authors because the profit-maximizing levels of DL-Met and DL-HMTBA were quite above the levels used in most experiments. No differences were found in RBE between DL-Met and L-Met in various types of diets with low-protein but varying TSAA contents fed to broilers from 8 to 20 days of age (DILGER & BAKER 2007). Supplementing 0.2% L-cysteine in a TSAA-deficient diet improved feed efficiency, but anorexic behavior was observed in the chickens, which was modt probably caused by the unique nutritional imbalance. A reduced feed intake resulting in an improved gain-to-feed ratio is a rare phenomenon in animal nutririon experiments (DILGER & BAKER 2007). More research is needed to provide a physiological basis for this phenomenon, especially in the different chicken genotypes. Also, most of these studies have not considered the effects of genotypes on the RBE of methionine sources.

2.2. The effect of dietary methionine sources and levels on the growth performance of poultry

The dietary methionine sources' effect on growth/production performance has been measured by different parameters ranging from bodyweight gain and egg production per day to feed intake. These factors are affected differently by dietary Met sources and levels at different phases. It has been reported by MEIRELLES *et al.* (2003) that DL-Met promotes bird growth more effectively in the starter phase than MHA-FA. However, in the grower phase, weight was affected by Met levels, while in the finisher phase, no significant effect

was seen for weight gain due to sources or levels. Additionally, several studies have shown that dietary methionine sources improved the weight of birds in the starter phase, but not in the growing and finisher phase (AGOSTINI *et al.* 2016; REHMAN *et al.* 2019; WICKRAMASURIYA *et al.* 2019). It has been suggested that the feed conversion ratio differs between Met sources.

Methionine levels have indicated improvement in the performance of birds. However, there is controversy regarding the ideal level of dietary Met for poultry. Several scholars have reported Met levels ranging from 0.3% to 1.2% during the starter phase and 0.3% to 0.9% during the growth phase, which is higher than the recommended levels (reviewed by LUGATA, *et al.* 2022a). MURAWSKA *et al.* (2018) investigated two levels (L-Met: 0.54% low, 0.82% high; DL-Met: 0.58% low, 0.78% high; and MHA 0.55% low, 0.79% high) for a feeding period of weeks 1 to 4, 5 to 8, 9 to 12 and 13 to 16 respectively in hybrid turkeys and revealed increased body weight in diets with high Met levels. Furthermore, WANG *et al*. (2019) had two levels (0.05% low and 0.25% high of DL-Met and MHTBA) and presented that high Met levels improved BW, ADG and FCR of broiler chickens. MEIRELLES *et al.* (2003) studied four different levels of Met: 0.41%, 0.47%, 0.53%, and 0.59% for the starter diet; 0.35%, 0.41%, 0.47% and 0.53% for the grower diet; and 0.30%, 0.36%, 0.42%, and 0.48% for the finisher diet and reported an increase in weight gain with increasing levels of methionine in all phases. They also noted that feed intake was not affected except in the final stage, and the feed conversion ratio was influenced by both the methionine source and dietary level. Slow and fast-growing broilers, weight gain was not affected between 3-6 weeks of age, but the feed conversion ratio (FCR) was improved with the highest methionine content (0.50%) (KALINOWSKI, 2003). Moreover, PARVIN *et al.* (2010) also studied the impact of dietary levels of Met (3.5, 4.5, 5.0, 5.5, and 6.0 g/kg with ~ 230 g/kg crude protein and 12.12 MJ/kg metabolizable energy) in the growth performance of Japanese quails from 0 to 35 days of age. They reported that live weight at day 35 did not improve further at higher levels, while feed intake and FCR were improved at 5.5 and 5.0 g/kg Met levels, respectively. In addition, different dietary Met supplementation levels (0.45% and 0.40%, 0.60% and 0.51%, 0.71% and 0.57% in weeks 1 – 4 and 5 – 8, respectively) significantly increased body weights of turkeys at 56 days of age (KUBIŃSKA *et al.* 2015). Similarly, adding DL-HMTBA at different levels (0.143%, 0.286% and 0.429%) and DL-Met at 0.093%, 0.186% and 0.279% to the broilers' diet improved their daily gain, feed conversion ratio, and relative weights of breast and abdominal fat compared to those with basal diet only

(SANGALI *et al.* 2014). Dietary methionine affects the growth performance under normal and infection conditions. LAI *et al*. (2018) showed that increasing dietary Met levels from 0.45% to 0.56% and to 0.68% resulted in higher weight gain and better feed conversion ratio of broilers treated against coccidiosis but not in vaccinated broilers.

The study by DRAŻBO *et al.* (2020) demonstrated that dietary Met levels (0.04%, 0.08%, 0.16% and 0.24%) significantly improved the average daily intake, feed intake, feed conversion ratio, and final body weight as compared to control regardless Met resources. In addition to affecting regular growth performance traits, DL-HMTBA and DL-Met may also differ in their effects on fat deposition (BUNCHASAK & SILAPASORN 2005; JARIYAHATTHAKIJ *et al.* 2018). It has been recommended that broilers do not require more than 0.50% and 0.38% Met in starter and grower diets to achieve optimum feed efficiency and gain. However, higher doses of methionine are necessary to improve immune response. Higher levels of dietary Met content decreased feed intake and weight gain as a result of higher plasma homocysteine concentration (XIE *et al.,* 2004). During the process of D-Met conversion to L-Met other amino acids are deaminated, which may cause differences in homocysteine production when different Met sources uused. In the study of RIBERIO *et al.* (2005) L-Met supplementation to a broiler diet was more effective in improving FCR than DL-Met and MHA.

Increased dietary intake of DL-Met and L-Met with different levels of dietary Met + Cys improved daily egg production, egg mass, egg weight, FCR and mortality of laying hens under tropical conditions in low protein diets (14% CP- containing 0.26, 0.30, 0.38, or 0.44% Met). The same was reported for laying duck breeders; where increasing dietary Met levels improved production parameters, hatchability, day old duckling weight, and OVAL mRNA level (RUAN *et al.,* 2018). Furthermore, REDA *et al*. (2020) reported that egg number, egg weight, and egg mass were higher with the addition of all DL-Met levels (0, 0.5, 1.5, 2.5, and 3.5 g/kg) than that of the control group of quail breeders. Additionally, SWENNEN *et al.* (2011) showed low crude protein impaired body weight, but HMTBA supplements in low CP diets improved body weight compared to DL-Met supplemented diets**.**

The effect of Met sources on chicken growth and performane depends on the genotypes, breeds or strains of chicken used. For example, different broiler strains showed different responses to dietary Met sources, with some strains having higher weight gain and feed efficiency than others (WEN *et al.* 2017). Similarly, native chickens had better body weight gain compared to broilers when fed with different Met sources (EL-TARRAS *et al.*

2019). Furthermore, dietary Met source effects are influenced by environmental factors. The study by WICKRAMASURIYA *et al.* (2019) revealed that dietary Met sources significantly impacted body weight gain in tropical conditions than in room temperatures. Moreover, the feed intake was significantly reduced on day 21 (starter period 1-21 days) and throughout the experiment (1-35 days) due to acute heat stress as compared to the thermoneutral treatment. Furthermore, the feed efficiency was also observed to decrease during the 21 and 28 days, independently of methionine isomers present in the diet.

2.3. Poultry antioxidants system

Stress management is a crucial aspect of poultry production, as it can be caused by several factors, including the management, nutrition, technology, environment and internal stressors. Most stresses are linked to excess production of free radicals, which result in oxidative stress at the molecular level. To combat this, animals have developed antioxidant defence systems that protect them in stressful environments. Reactive oxygen species (ROS) are crucial signaling molecules that regulate stress adaptation in living organisms or cells in small concentrations, but they can also cause damage to cells when produce in large amounts. There are several lines of antioxidant defence in the antioxidant defence network. The first line consists of antioxidant enzymes that detoxifying the superoxide radical (the major biological radical) and its metabolic products. This includes superoxide dismutase, glutathione peroxidases (six different types in avian species), and catalase (HORVÁTH & BABINSZKY 2018; SURAI *et al.* 2019). Additionally, metal-binding proteins are also included in the first line of antioxidant defence, as some metals play a significant role in catalyzing free radical production, such as free iron and copper (SURAI *et al.* 2019). Chain-breaking antioxidants such as vitamin E, carotenoids, ascorbic acid, glutathione, and uric acid, are responsible for limiting and ending chain oxidation reactions in the second line of antioxidant defence (SURAI *et al.* 2019).

The third level of the antioxidant defence system is made of specific enzymes that deal with the damages caused by free radicals and their hazardous byproducts. Some examples of these enzymes include methionine sulfoxide reductase (Msr), DNA-repair enzymes, phospholipases, and proteasomes) (HORVÁTH & BABINSZKY 2018; SURAI *et al.* 2019). The fourth line of antioxidant defence involves protective protein modifications that prevent inactivation, such as glutathionylation and other modifications. Heat shock protein (HSP) play a critical role defence line against oxidative stress by scavenging ROS,

repairing damaged molecules and activating antioxidant enzymes. Few HSP32, HSP70 and HSP90 s have been recognized as important components of the poultry antioxidant system network (SURAI & KOCHISH 2017). Heat shock proteins (70,90 and 32) proved to be protective in various stress-related conditions in poultry production (SURAI & KOCHISH 2017). Notably, apoptosis can be included in the antioxidant defence network to cope with terminally damaged cells that cannot be restored. In general, the main goal of the integrated antioxidant defence network is to maintain optimum redox status, which refers to the balance between the process of production and inactivation/detoxification of ROS/reactive nitrogen species (RNS) (HORVÁTH & BABINSZKY 2018; SURAI *et al.* 2019). To achieve the above goal of the antioxidant defence system, commercial food for animal and poultry production requires external assistance, such as dietary supplementation of traditional antioxidants like vitamin E or other nutrients with regulatory functions in the antioxidant defenses, such as taurine, carnitine, branched-chain amino acids, and others.

2.3.1. Oxidative stress responses

The physiological parameters studied in poultry in response to dietary methionine include oxidative stress, antioxidants, blood biochemical parameters, blood profiles and serum hormones. Several scholars have shown that dietary Met sources have an impact on different aspects of the physiology of poultry. Oxidative stress is the imbalance between the production of reactive species and antioxidants, causing damage to the components of cells such as protein, lipids and DNA. Lipids are most susceptible to oxidative stress damage by lipid peroxidation. Lipid peroxidation (measured as plasma thiobarbituric acid reactive substances (TBARS) levels) in the plasma of 4 weeks broilers were significantly affected by methionine sources as it was highest in DL-Met supplemented than HMTBA (SWENNEN *et al.* 2011). In contrast to turkey fed diets supplemented with MHA, JANKOWSKI et al. (2018) reported contradictory results, observing reduced levels of malondialdehyde (MDA) in the small intestinal wall of turkeys receiving DL-Met supplemented diets. Furthermore, L-Met supplemented diets were more effective in reducing oxidative stress (MDA) in the liver of turkeys during the first 28 days of age as compared to DL-Met, this indicates that L-Met is more beneficial than DL-Met (PARK *et al.* 2018). However, JANKOWSKI et al. (2017) found similar effectiveness in relation to MDA concentration in turkey’s small intestinal wall between L-Met and DL-Met-

supplemented treatments. Nevertheless, in a study conducted by JANKOWSKI et al. (2018), it was found that MDA content in the small intestine of a young turkey infected with the hemorrhagic enteritis virus was affected by both Met sources and levels. The DL-Met reduced the MDA content than MHA, and high Met levels significantly decreased MDA content. Nevertheless, additional research revealed that the dietary levels of Met did not have any effect on the MDA content in the liver or brain of the newly hatched ducklings (RUAN *et al.* 2018). These differences in the responses of the oxidative stress marker (MDA) could be due to the differences in the tissues, poultry species, and experimental conditions. For example, KALVANDI *et al.* (2019) reported that the quail's breeder in the heat stress group had higher plasma and liver MDA content than those in the thermoneutral group. However, they demonstrated that adding 1.15 times the NRC (1994) recommended Met requirement to the diet decreased MDA concentrations in heat-stressed quails to a level similar to those under thermoneutral conditions. In addition, the same was observed in *Eimeria spp* challenged broilers, which had high levels of oxidative substances such as nitrite and TBARS with no effect of Met supplementation on the same (KHATLAB *et al.* 2019). ZHAO *et al.* (2018) observed that increasing Met levels led to increased MDA concentrations, and there was no significant difference between DL-Met and DL-HMTBA.

2.3.2. Antioxidant responses

Several parameters related to redox capacity and antioxidant status have been studied as responses to dietary methionine in broilers (SWENNEN *et al.* 2011; LAI *et al.* 2018; KHATLAB *et al.* 2019; WANG, YIN, *et al.* 2019; MAGNUSON *et al.* 2020), turkeys (JANKOWSKI *et al.* 2017; ZDUŃCZYK *et al.* 2017; JANKOWSKI *et al.* 2018; MURAWSKA *et al.* 2018; PARK *et al.* 2018), quail (REDA *et al.* 2020), ducks (RUAN *et al.* 2018) and in piglets (ZEITZ *et al.* 2019) (Table 1). The most common parameters related to antioxidant status studied in poultry tissues are total antioxidant capacity (TAC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH). Several studies have shown that the antioxidant status is affected differently by different methionine sources, in which the HMTBA source has showed better antioxidant status than DL-Met in the broilers (SWENNEN *et al.* 2011; WANG *et al.* 2019) as well as in turkeys (JANKOWSKI *et al.* 2017; ZDUŃCZYK *et al.* 2017). In addition, a high level of methionine is believed to strengthen the antioxidant system by enhancing SOD activity in the duodenum, CAT activity in the ileum, and GPx activity as well as the total

antioxidant capacity in the serum (JANKOWSKI *et al.* 2017; JANKOWSKI *et al.* 2018; WANG *et al.* 2019).

The activities of antioxidant enzymes in the blood, such as SOD and GPx, in relation to Met levels and sources as a marker of redox status have been studied widely. However, the results are not consistent. In the study of CHEN *et al.* (2013) higher dietary Met levels increased serum SOD, but decreased GPx activity. Another study found that increased Met levels resulted in higher GPx activity but did not affect plasma SOD activity (KUBIŃSKA *et al.* 2016). Regarding the Met sources, JANKOWSKI et al. (2017) found that turkeys given MHA-supplemented diets had lower plasma SOD activity than those fed DL-Met or L-Met diets, and that compared to DL-Met, L-Met, and MHA, the consumption of other dietary sources tended to increase the plasma GPx activity while decreasing SOD activity in the small intestine. Different levels and sources of dietary Met resulted in different activities of SOD and CAT in small intestinal and liver samples. However, the observed differences were difficult to interpret (Table 1). FOUAD *et al.* (2016) observed that high methionine levels increased the GSH synthetase and GPx gene expression levels. This is similar to what was observed by ZDUŃCZYK *et al.* (2017) that increased levels of SOD activity, total GSH and FRAP values in the blood plasma of turkeys were found only in higher methionine level fed groups. Furthermore, dietary protein is believed to influence the redox status of broilers regarding dietary methionine sources (SWENNEN *et al.* 2011). Apart from dietary protein, other factors that influence redox status include high stocking density (which is the case in intensive poultry production), which causes high stocking density stress (MAGNUSON *et al.* 2020) as well as high ambient temperature (RIBERIO *et al.* 2005; WILLEMSEN *et al.* 2011; ZEITZ *et al.* 2018; ZEITZ *et al.* 2020). However, it is worth noting that the effects of these factors on oxidative stress are inconsistent. MAGNUSON et al. (2020) pointed out that the high stocking density (16 birds/m^2 in grower or 12 birds/m^2 in finisher) employed in their study failed to alter the major oxidative stress-related biomarkers. Nevertheless, the stock density considered as high in their study was within the acceptable stocking density for broilers (SUGIHARTO 2022).

Table 1

Effect of Met sources on poultry oxidative stress/antioxidant status at different supplementation levels

Met Sources/Poultry Species	Met Levels	Main Results	References	Remarks
DLM and LM and HMTBA/male Cobb 500 broiler	MS = 0.22% DLM, 0.22% LM or 0.31% HMTBA	In breast muscles: ↑ TGSH and rGSH in LM and HMTBA were observed. ↮ by met source for MAD, FRAP, and the ratio of rGSH: GSSG and GSSG: TGSH.	(ZHANG *et al.* 2018)	Met + Cys deficiency did not compromise the antioxidant capacity of chickens
LM, DLM, and MHA Hybrid Converter turkey	BD = 0.40%, 0.34%, 0.29%, and 0.26% Met for 1–4, 5–8, 9–12 and 13–16 weeks respectively; MS = NRC 1994 –Low and 40% extra NRC recommendation-High	Higher Met content ↑ SOD; CAT activity, Vit C concentration, and ↑ plasma FRAP, TGSH, and ↓ MDA ↮SOD, Vit C, MDA, or LOOH in the small intestinal or liver of turkey-fed diets with different Met levels. MHA ↑ TGSH and ↓ SOD, and MDA in the plasma compared with DLM. LM ↑ TGSH compared with DLM In the small intestine, SOD; DLM > LM > MHA ↮ MDA and ↑ LOOH in LM compared with other Met sources. In liver: ↮ Vit C, LOOH, and SOD but interaction for SOD, CAT, and LOOH.	(JANKOWSKI *et al.* 2017)	A higher Met level improved the indicators of redox status MHA lowered plasma and intestinal SOD more than DLM or M. LMH and LM decreased plasma and hepatic MDA and increased plasma glutathione levels.
DLM, LM, and MHA Hybrid converter turkey	Low = 100% and High = 150% of NRC (1994) recommendations for each feeding phase	Met source and Met level; ↮ vit C, LOOH, and MDA Higher Met Level ↓ CAT, ↑ SOD DLM: ↑ SOD CAT: LM > DLM > MHA	(MURAWSKA *et al.* 2018)	MHA reduced the CAT activity in the breast meat compared with LM and DLM sources.
DLM and L-Met Hatched turkey.	basal diet (BD), the BD + 0.17 or 0.33% DL-Met or L-Met (60, 75, and 90% for SAA of NRC)	L-Met ↑ GSH and ↓ MAD in the liver than DLM during 28 days. MS regardless of the source ↓ MAD in the duodenal mucosa. ↮ PC, TAC on day 7 but day 28 MS irrespective of the source ↓ PC and ↑ TAC in the duodenum than BD	(PARK *et al.* 2018)	L-Met was more effective in reducing oxidative stress and improving glutathione in the liver than DL-Met.
MHA and DLM Female Hybrid converter turkey	0.15% and 0.37% in weeks 1–4 of age, and 0.0% and 0.1% in weeks 5–8 of age added to	Higher Met level ↑ SOD, GSH + GSSG, and FRAP values in the blood. ↮ MAD, GPx, Vitamin C, and CAT.	(ZDUŃCZYK *et al.* 2017)	DLM improved the activities of SOD, CAT, and GSH +GSSG levels

Met Sources/Poultry Species	Met Levels	Main Results	References	Remarks
	BD (0.40 and 0.35% Met at 1–4 and 5–8 weeks of age, respectively)	MHA ↓ SOD, CAT, and GSH + GSSG in the blood and ↑ MDA		
DLM and HMTBA Male Ross 308	0.25% of DLM or HMTBA was added to 18.3 or 23.2% of CP diets.	DLM ↑ plasma TBARS and FRAP compared with HMTBA at 4 weeks of age. At 6 weeks of age: interaction of protein and met source for FRAP and TBARS. MHA ↑ SOD and reduced GSH but ↔GSH synthetase, glutathione reductase, or Msr-A gene expression in the liver at 6 weeks old chicken.	(Swennen *et al.* 2011)	MHA presented a more pronounced antioxidant effect than DLM
DLM and LM 1-d-old Ross 308	basal diet (BD), the BD + 0.095% LM or DLM, the BD + 0.190% LM or DLM, and the BD + 0.285% LM or DLM (representing 60, 70, 80, and 90% of the Met + Cys requirement)	0.285% of LM ↑ GSH and TAC but ↓ PC in the duodenum compared to DLM at the same level.	(Shen *et al.* 2015)	L-Met served better in reducing protein oxidation and increasing antioxidant status in the duodenum/gut of chicks than DLM.
DLM and MHA Cherry Valley ducks	0.04, 0.12, 0.16, and 0.20% of Met equivalents for the grower phase	Booth dietary Met source and level affected the TAC < GPx, GSSG, and MDA in pectoralis major muscles on day 42. MHA ↑ TAC and GPx in pectoralis major muscles and GSH in the breast muscles compared with DLM. ↑ Met level; ↑ GSH, GSSG, and MDA regardless of the Met source.	(Zhao *et al.* 2018)	MHA improved the antioxidant status of cherry valley pectoralis major muscles compared with DLM (increased antioxidants capacity markers (TAC, GPX, and GSH)

↔ Represents no difference. ↑ Represents increased or high or upregulated. ↓ Represents decreased or low, or downregulated. DLM—DL-methionine, LM—L-methionine, MHA/HMTBA—methionine hydroxyl analogue/DL-2-hydroxy-4-(methylthio)butanoic acid; TGSH—total glutathione, rGSH—reduced glutathione, GSSG, GPx—glutathione peroxidase, MDA—malondialdehyde, GSH/GSSG—glutathione and glutathione disulfide ratio, FRAP—ferric reducing the ability of plasma, TBARS—thiobarbituric acid reactive substances, MsrA—methionine sulfur reductase A, SOD superoxide peroxidase, CAT—catalase, LOOD—lipid peroxides, Vit C—vitamin C, TAC—total antioxidant capacity, PC—protein carbonyl

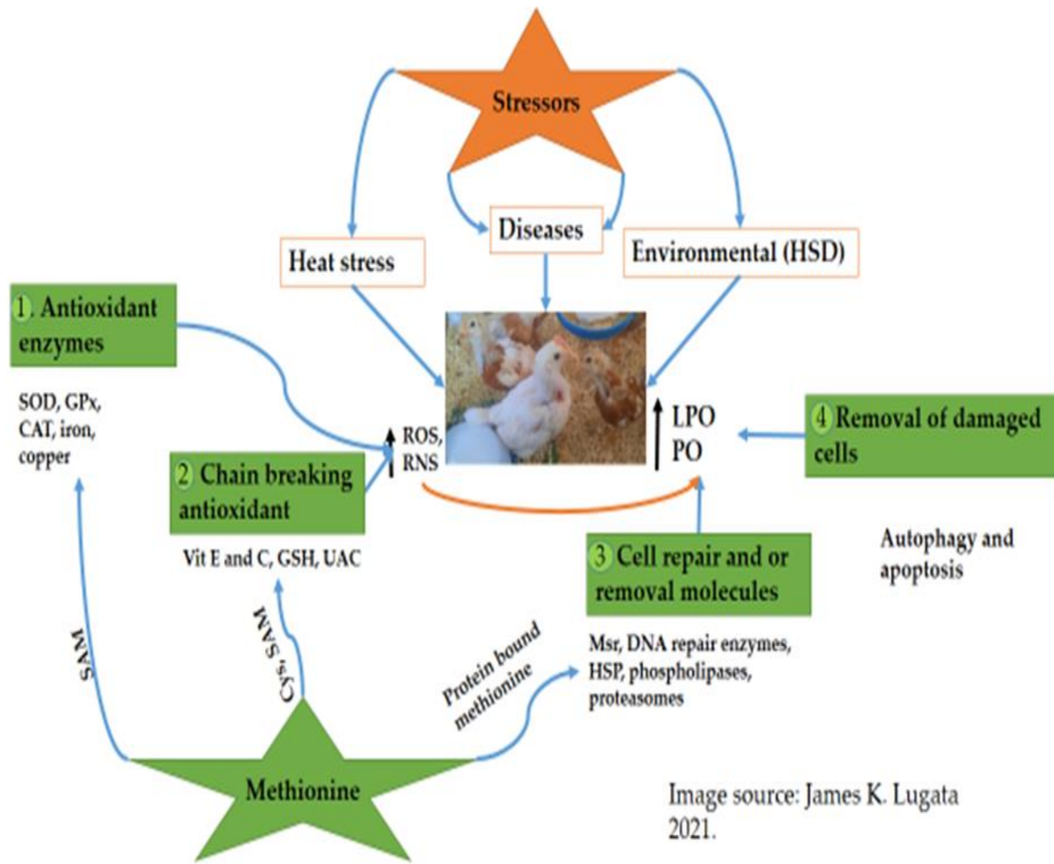
2.3.3. How antioxidants network work under oxidative stress

When the production of ROS and RNS exceeds the antioxidant defence system's capacity, oxidative stress damages biological compounds, including polyunsaturated fatty acids, proteins, and DNA (MISHRA & JHA 2019). In response to oxidative stress, the antioxidant defense system employs several strategies to restore normalcy (SURAI *et al.* 2019) (Figure 1). Firstly, antioxidants decrease the production of free radicals by inhibiting the activity of enzymes such as NADPH oxidase and xanthine oxidase that are involved in ROS/RNS formation. Antioxidants also prevent the formation of new free radicals by acting on iron and copper bound to protein. Secondly, preserving mitochondrial integrity is crucial, as it is the primary source of free radicals in the biological system. Thirdly, scavenging free radicals using vitamins E, C, coenzyme Q, and GSH, and detoxifying or decomposing free radicals and non-radical toxic compounds using enzymes like SOD, GPx, and catalase are essential components of an antioxidant defense strategy. Fourthly, the vitamin E recycling mechanism, which involves ascorbic acid, thioredoxin reductase (TrxR), vitamins B1, and B2, can boost its biological antioxidant efficacy. Fifthly, the anti-stress strategy mainly involves redox signaling, transcription factor (Nrf2), vitagene activation, and the creation of protective molecules with antioxidant and detoxifying capabilities. Sixthly, the repair of damaged molecules through enzymatic mechanisms such as heat shock proteins (HSP), methionine sulfoxide reductase (Msr), DNA repair enzymes, etc. followed by removing the damaged molecules using phospholipases, phospholipid hydroperoxide GPX (PH–GPx), proteasomes, etc. is essential to prevent accumulation. Lastly, the antioxidant defense network includes apoptosis, autophagy, and other processes that remove terminally wounded cells and prevent damage from spreading to other cells/tissues (SURAI *et al.* 2019).

Recent research suggests that all antioxidants in the body work together as a team to maintain a balanced internal environment, known as adaptive homeostasis (DAVIES 2016). There are cooperative interactions in this team when one member assists another in working more efficiently. Antioxidant defense systems are present in all cell compartments, including mitochondria, nucleus, and cytoplasm, and are expressed in a tissue-specific manner. Antioxidants are produced both internally, such as AO enzymes, GSH, CoQ, uric acid, carnitine, and taurine, and consumed through the diet, such as vitamin E, carotenoids, synthetic antioxidants, carnitine, and silymarin. Stress can induce the expression and activity of many antioxidant enzymes, including SOD, GPx, and other selenoproteins (SURAI *et al.* 2019; LUGATA *et al.* 2023, In press).

Figure 1

Overview of stressors' effect on poultry's antioxidant system and the role of methionine



(1) Detoxifies free radicals at the start of their formation using enzymes like super oxidase dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and chelating metals like iron and copper;
(2) Scavenges the free radicals using vitamin E and C, glutathione (GSH), and uric acid (UAC);
(3) Cell repairs and removes damaged molecules, including methionine sulfoxide reductase (Msr), heat shock proteins (HSPs), and DNA-repairing enzymes;
(4) Deals with removing damaged cells and preventing the damage from spreading (autophagy and apoptosis; methionine has a direct and indirect influence on these processes)
(based on: LUO & LEVINE 2008; ZHANG 2016; SURAI *et al.* 2019).

2.3.4. Role of Methionine on Oxidative Stress/Status of Poultry under Heat Stress

Table 2 summarizes the impact of methionine (Met) supplementation through various methods, such as *in-ovo*, water, and dietary, on the oxidative stress and antioxidant status of poultry under both heat and cold stress conditions. Heat stress (HS) is the primary stressor that causes significant issues in poultry production (WILLEMSEN *et al.* 2011). In chickens, heat stress has several detrimental effects, including impaired antioxidant enzyme activity, increased lipid peroxidation and ROS formation, decreased vitamin concentration, and disruption of mitochondrial function (HORVÁTH & BABINSZKY 2018).

It has been demonstrated that heat stress, both acute and chronic, alters the antioxidant system in chickens. Generally, animals generate reactive oxygen species due to a variety of physiological and non-physiological processes, including the Fenton reaction, cellular respiration, mitochondrial dysfunctions, infections, diseases, neutrophils, phagocytes, and stress (MARTÍNEZ *et al.* 2017).

Table 2

The effects of dietary Met supplement (*in-ovo*, diet, water) on the oxidative stress on poultry under heat stress and cold stress conditions

Met Sources/Poultry Species	Met Levels	Main results	References	Remarks
DLM and HMTBA Cornish Cross cockerels 31 ˚C from 14 to 42 days	BD = 0.276% MS (DLM = 0.309% and 0.404% in grower; 0.277% and 0.360% in finisher correspond to 100% or 130% required Met, respectively) MS (HMTBA = 0.719% and 0.934% in grower; 0.644 and 0.837%) (88% DLM and 58% HMTBA used as sources of Met)	130% ↑ hepatic GSH, GSSG in grower, and plasma FRAP finisher. 130% ↓ GPx, GST, and SOD in grower adipose tissues and ↓ plasma GSH, hepatic GSSG but ↑breast GSSG in the finisher broiler. In grower; DLM ↑ breast GSH and thigh GSH and GSSG than HMTBA 130% ↓ hepatic GPx and adipose tissue GPx and GST activities of the finisher broiler. In grower broiler: DLM; ↓ activities of breast GPx, thigh GR, adipose GST and all assayed antioxidant enzymes in the liver as compared with HMTBA	(LIU *et al.* 2019)	Chronic heat stress 30% extra Met improved the antioxidant status of broilers under HS No effect of MS (either source or levels) on BW, average daily gain and feed conversion of birds throughout the study. DLM increased the FI in finisher birds as compared with HMTBA.
DLM DL-HMTBA HS 32 ˚C until 6 weeks of age Male Ross broiler	BD = 0.28% Met MS = 1.0 or 1.2 g/kg for each source (0.10% or 0.12% of either DLM or DLHMTBA added to basal diet)	In week 4: ↩ FRAP on HS and MS ↓ MAD, but ↑UA in HS chicks In week 6: ↩ plasma FRAP, SOD, UA by either HS or MS HS ↑ MDA in DLM but not DL-HMTBA ↑ hepatic ratios of reduced GSH to total GSH and reduced GSH to GSSG in HMTBA at HS	(WILLEMSEN *et al.* 2011)	Chronic HS (4 weeks exposure): 0.1% and 0.12% of either DLM or HMTBA did not affect the antioxidants of broilers. HMTBA improved growth performance (FI and BW) at 5 and 6 weeks of age
DL-Met Male Cobb broilers for 42 days	Met deficient (MD)-without Met supplementation (MS) Control (MD + 0.24% Met) in starter diet, (MD+ 0.12% Met) in grower diet)	Met deficiency leads to oxidative stress in both the liver and kidney of growing chicks ↑ MDA ↓ SOD, CAT, GSH-PX, and GSH	(SONG *et al.* 2021)	Met-deficient (MD) causes injury to the kidney and liver. MD depressed body weight (BW) from 14 to 42 days
MHA Broilers	Basal diet + 0.46%, 0.36% and 0.32% in starter, grower, and finisher, respectively	The antioxidant activity was improved by MHA supplementation	(ERFANI *et al.* 2021)	0.2% of vitamin C supplemented together with MHA, no effect on BW

Met Sources/Poultry Species	Met Levels	Main results	References	Remarks
Free Met DL-Met, and Met dipeptide (DL-MM Male cobb broilers, from 21 to 42 days	MD-without MS DL-M (MD + 0.27%) DL-MM (MD + 0.28%) (MD = 0.543, DL-M = 0.810, DL-MM = 0.809 digestible Met + Cys)	Heat stress increased the expression of GSS and GPX. Met supplementation (MS) reduced the expression of GPX	(SANTANA *et al.* 2021)	Thermoneutral group 27–18 ˚C; Heat stress continuous exposure 30 ˚C, 60% RH from 21 to 42 days MS increased BW
DL-Met Male cobb 500 Heat stress (HS) 38 ˚C for 24 h.	Starter: MD-, DL1 = MD + 0.295%; DL2 = MD + 1% Grower: MD-; DL1 = MD + 0.275%; DL2 = MD + 1%	Broilers reared in HS and fed DL1 and DL2 increased expression levels of GSSG and GPx7 in both feeding phases.	(DEL VESCO *et al.* 2015)	Acute heat stress MS increased BW, DL2 reduced FI
DL-2-hydroxy-4-methylthiobutanic acid (DL-HMTBA) HS 38 ˚C for 24 h at 41 day Male cobb 500	Starter MD; DL-HMTBA: = 0.45% Grower DL-HMTBA = 0.42% (MD = 0.58, and 0.54; MS = 0.88 and 0.81 in starter and grower digestible Met + Cys, respectively)	↑ TRxR1, SOD, and MsrA in starter under HS and fed DL-HMTBA diet. Interaction of the HS and MS observed In grower: HS ↑ SOD, TRxR1, and MsrA; MS also ↑ levels of expression than MD but not the interaction between temperature and diet in a grower phase.	(GASPARINO *et al.* 2018)	Acute heat stress HS lowered the feed intake (FI), and BW gain MS did not influence FI and BW gain
DL-Met Male Cobb 500 broilers HS 27.4 ˚C ± 0.3 ˚C, and mean relative humidity was 63.7 ± 0.6% from 21 days until day 35	Control diet = 0.50%, 0.42%, and 0.39% in starter, grower, and finisher DLM 1 = 0.19, 0.16 and 0.15% added in starter, grower, and finisher diets, respectively. DLM2 = 0.37, 0.32, and 0.29% added in starter grower and finisher diets, respectively.	HS: ↓ plasma tocopherol, GSH: GSSG and ↑ GSSG, ↑ hepatic GSH, GSSG and ↓ Vit C than the thermoneutral group. High Met levels ↑ GSH and GSSG levels in the liver and thigh muscle of broilers subjected to HS High Met levels also ↑ tocopherols in the liver and plasma of heat-exposed ⥊ TBARS, GSH: GSSG	(ZEITZ *et al.* 2020)	Chronic heat stress HS affected broilers' performance negatively. MS did not improve the birds' performance under HS.
In-ovo Met –cysteine (Cys) injection HS(39.6 ˚C for 6 h/d) from day 10 until day 18	5.90 mg L-Met plus 3.40 mg L-cysteine	↑ TAC and GSH in serum and tissues of newly hatched chicks in Met + Cys injected group	(ELWAN *et al.* 2019)	Met-Cys injection elevated the antioxidant capacity of chicks exposed to HS during incubation. No effect on hatchability

In-ovo Met-Cys injection Ross broilers HS (39.6 ˚C for six h daily) between 10 and 18 days of the incubation	5.90 mg L-Met plus 3.40 mg L-cysteine	↓ HSP70 and ↑GSH-Px expression in the liver, jejunum, cardiac muscles, and pectoral muscle tissues ↑ T-SOD in serum and tissues ↑ GSH-Px and GSH/GSSG ratio in serum and tissues ↓ MDA in serum and tissues ↬CAT except in pectoral muscles	(ELNESR *et al.* 2019)	*In-ovo* injection of Met + Cys improved the antioxidant status of the newly hatched chicks exposed to heat stress during incubation.
DLM and HMTBA Peking ducks HS (summer temperature range (mean of highest) 32.1 ˚C to 24.7 ˚C (mean of lowest)	Basal diet (BD) = 0.45 and 0.40% of Met in starter and grower period 0.05%, 0.2% and 0.35% of either DLM or HMTBA were added to the basal diet during starter and grower periods.	0.35% ↑ HSP70 mRNA expression of the intestine and liver ↑ MDA by 0.35% of DLM on day 16. 0.35% DLM ↑ MDA on day 35. 0.35% HMTBA ↑ HSP70 expression compared with other treatment groups.	(GUO *et al.* 2018)	HMTBA improved the antioxidant status of the liver and intestine on day 35. Growth performance was not reported.
DLM and HMTBA HS 35 ˚C and TN 24 ˚C Male Ross 308.	MS; DLM = 0.31 and 0.51% = starter, 0.26 and 0.43% for grower) HMTBA = 0.34 and 0.57% for starter, 0.29 and 0.49% for grower diet.	TGSH and GSH were higher in the HS group. DLM had lower TBARS in the acute phase. ↬ GPx. ↓ GSSG in super-adequate digestible sulfur amino acid (DSAA). GSH: GSSG ratio was higher than inadequate DSAA.	(BOLEK 2013)	MS did not have significant influences on the antioxidants or the bird performance.
DLM HS (38 ˚C for 24 h, starting on the sixth day TN (25 ˚C) Male meat quails.	MD, MS = 0.27% (MD = 0.57% MS = 0.84% Met + Cys digestible)	HS induced ↑ GPx, ↑UA, ↑ H_2O_2 production, ↓GSH, ↓ MAD levels on MS diet. Interaction between HS and diet on GPx and CAT	(DEL VESCO *et al.* 2014)	MS can mitigate ROS-induced damage by increasing the activities of antioxidants. MS did not affect feed consumption and weight gain.
DLM. Breeder Japanese quail	BD = 0.70% Met + Cys; BD + MS with DLM to provide proportions concentrations of 1.15, 1.30 and 1.45 times the quail requirements per NRC (1994) recommendations.	MS ↑ plasma and liver SOD, CAT, GPx, and ↓ MAD. 1.15 times the NRC ↑ plasma and liver SOD, CAT, GPx and ↓ MAD in HS quails. MS ↑ plasma TAC compared to HS and TN groups.	(KALVANDI *et al.* 2019)	Met could improve quails' performance, immunity, and antioxidant status by reducing the adverse effects of HS. MS improved the productive performance of birds.

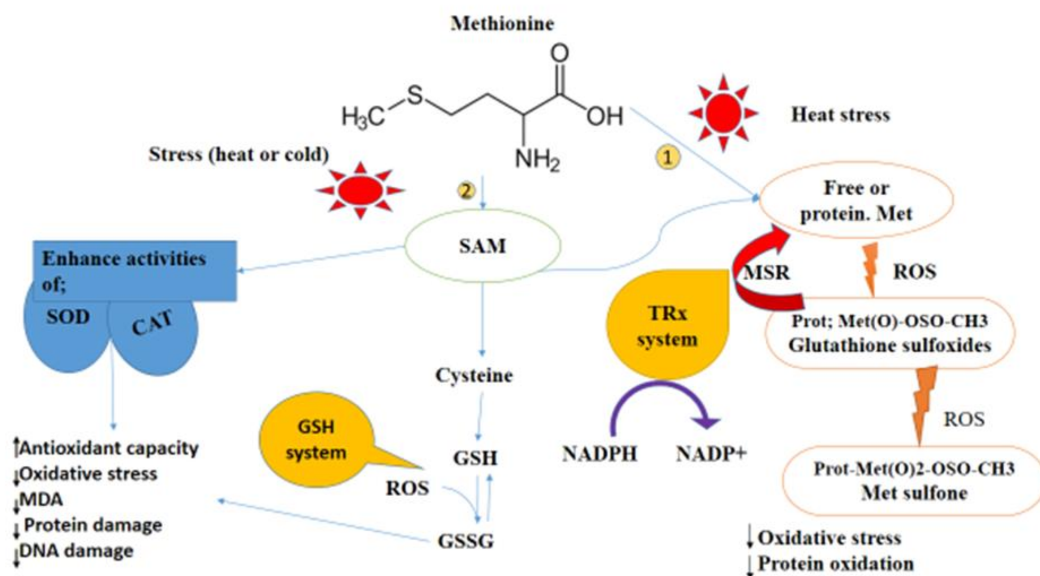
DL-HMTBA Broilers. [Low (12 to 14 ˚C) vs. control temperature (thermoneutral, 24 to 26 ˚C)] from 8 to 28 days of age.	BD = 0.32% Met MS = 0.17% or 0.51% of DL-DL-HMTBA added to BD (HMTBA containing 88% of active substance)	0.51% ↑ hepatic GSH and GSH-Px and lung SOD activity of broiler. Low temperature reduces the expression of GSH synthetase and increases GSH reductase gene expression. Higher DL-HMTBA induced ↑ GST in the lung, GSH synthetase in the liver and lung, and ↓ GSH reductase in the lung at low temperatures.	(Wang, Yang, *et al.* 2019)	At low temperatures, 0.51% MS ↑ GSH synthesis gene expression hence increases the antioxidant capacity in the liver and lung No effect of MS on the growth performance
DL-HMTBA Broilers. 12–14 and 24–26 ˚C for Low ambient temperature (LAT) and normal ambient temperature (NAT)	BD = 0.32% Met as-fed basis MS (0.17, 0.34, 0.51, and 0.68% DL-HMTBA added to BD)	LAT ↑ serum MAD and PC on day 21; ↓ serum GSH, GSH-Px and TAC on days 21 and 28. MS ↑ serum GSH content, GSH-Px activity, TAC and SOD at day 28, and SOD and GSH-PX activities and ↓ PC at day 21 under LAT conditions.	(Yang *et al.* 2016)	MS improved the serum antioxidant activities in a dose-dependent manner under LAT conditions. LAT ↓ BWG and ADFI at days 7–14 and 15–21 of the experiment.
Zinc- Met Male cobb 500 The average daily temperature ranged from 33 ˚C to 36 ˚C	BD = 0.25, 0.23 and 0.15% Met for starter, grower, and finisher diets, respectively. MS = 0, 0.025, 0.05, and 0.10% ZnMet (98% ZnMet purity)	The higher level of Zn-M ↑ plasma GPx activity and ↓ muscles MDA in broilers reared in higher ambient temperature	(Saleh *et al.* 2018)	Chronic stress ZnM supplementation increased the BWG and BW and improved FCR under HS

⥃ Represents no difference, or similar or no effect. ↑ Represents increased or high or upregulated. ↓ Represents decreased or low, or downregulated. Abbreviations: BD— basal diet, MS—methionine supplementation, MD—methionine deficient, HS—heat stress, DL—DL-methionine, MHA/HMTBA—methionine hydroxyl analogue/DL-2-hydroxy-4-(methylthio)butanoic acid; T-SOD—Total superoxide dismutase, TAC—total antioxidant capacity, GPx—glutathione peroxidase, PC—protein, MDA—malondialdehyde, GSH/GSSG—glutathione and glutathione disulfide ratio, FRAP—ferric reducing the ability of plasma, SOD—superoxide dismutase, CAT—catalase, GSH—glutathione, TRxR1—thioredoxin receptor 1, MsrA—methionine sulfoxide reductase A enzyme, HSP70—heat shock protein 70, UA— uric acid.

Met provides two distinct mechanisms of protection against oxidative stress (Figure 2): first, Met acts as an indirect source of antioxidants (precursors of cysteine and glutathione) (SANTANA *et al.* 2021). Secondarily, Met acts as direct source of antioxidants during cell repair, when a number of reactive oxygen species (ROS) react with Met residues in proteins and with Met sulfoxide, which scavenges the reactive species (LUO & LEVINE 2008). All organisms, including bacteria and mammals, have enzymes called met sulfoxide reductases (MSRs) that lower reactive oxygen species (ROS). To achieve this, each protein molecule's eight Met residues are oxidized while the individual protein's enzymatic integrity is preserved. By lowering the oxidized MSR, thioredoxin (TRx) gets the enzyme ready for a new catalytic cycle. Reactive nitrogen and oxygen species can also be scavenged by NADPH (LUO & LEVINE 2008). This pathway, wherein the genes TRxR1 and MsrA were substantially expressed in heat-stressed broilers and supplemented with DL-Met, demonstrates how heat stress and Met supplementation (MS) affect the antioxidant potential of DL-HTMTA (GASPARINO *et al.* 2018).

Figure 2

The function of free/protein-bound Met and dietary Met in the antioxidant system of chickens during heat stress



1. Reactive oxidative species (ROS) oxidize the sulphur side chain of methionine. If met sulfoxide is exposed to ROS for an extended period, it may undergo further oxidation to create methionine sulfone. Although it is unable to convert methionine sulfone, methionine sulfoxide reductase can convert methionine sulfoxide back into Met.

2. Met metabolites cause the antioxidant enzymes SOD and CAT to become active and produce GSH, which in turn reduces oxidative damage brought on by heat stress.

Several studies have been conducted to examine the effect of Met supplementation on the function of glutathione in heat-stressed chickens. This is because Met plays a role in synthesizing cysteine, S-adenosylmethionine, and glutathione (Table 2, Figure 2). Many of these studies have shown that broiler chickens who received Met supplementation had higher levels of glutathione when exposed to heat stress (ELNESR *et al.* 2019; ZEITZ *et al.* 2020; SANTANA *et al.* 2021). In another study involving DL-Met on the enzymes (cystathionine β-synthase, glutathione synthetase, and glutathione peroxidase) participating in glutathione synthesis, higher expression was observed in heat-stressed broilers (DEL VESCO *et al.* 2015). Increasing the expression of these enzymes results in the effective synthesis of these most potent antioxidants and, thus, a significant contribution to the scavenging system's ability to alleviate the ROS effect (SWENNEN *et al.* 2011). *In-ovo* Met injection enhanced GSH in serum and tissues of newly hatched chicks exposed to heat stress from d10 to d18 (ELWAN *et al.* 2019), as well as *GPx* gene expression, and activity of GPx and GSH/GSSG in serum and tissues of newly hatched chicks (ELNESR *et al.* 2019). MS has demonstrated its ability to offer protection in both hot and cold weather conditions. The hepatic GSH and GSH-Px at high levels of MS were enhanced in the investigations including HMTBA at varied levels at low temperatures (12 to 14 °C) (YANG *et al.* 2016; WANG, YANG, *et al.* 2019).
The impact of methionine supplementation (MS) on multiple enzymatic antioxidant systems, such as the activity of SOD and CAT in heat-stressed poultry, in addition to TRx and glutathione systems, has been thoroughly investigated (WILLEMSEN *et al.* 2011; GASPARINO *et al.* 2018; ELNESR *et al.* 2019). Superoxide free radicals are produced during a dismutation reaction, and SOD is a crucial enzyme for their removal. Catalase-catalyzed activities or the glutathione system eliminate the generated H_2O_2 (FANG *et al.* 2002). Conversely, HS causes the generation of H_2O_2, which MS reduced by raising the activity of CAT in the quail's liver and plasma (DEL VESCO *et al.* 2015; KALVANDI *et al.* 2019). Numerous studies on the impact of MS effects on SOD under various heat-stress scenarios and poultry life stages have been conducted. The majority of these investigations verified that, in heat-stressed poultry, MS considerably raises SOD regardless of the tissues (BOLEK 2013; DEL VESCO *et al.* 2014; LIU *et al.* 2019). Furthermore, even in broilers raised in thermoneutral environments, Met deficiency has been shown to reduce SOD and CAT (LUO & LEVINE 2008). Additionally, MS affects non-enzymatic antioxidants (vitamin C and tocopherols), total antioxidant capacity, ferric lowering the power of

plasma, and other antioxidants in heat-stressed chickens (WILLEMSEN *et al.* 2011; ELNESR *et al.* 2019; KALVANDI *et al.* 2019; ZEITZ *et al.* 2020).

2.4. Effect of methionine supplementation on blood biochemical parameters

Blood biochemicals, blood profiling, and serum hormones have been investigated as the physiological responses to dietary methionine in poultry. A blood profile has been used as an indicator of acute stress. ZHANG *et al*. (2017) observed no effect of methionine supplementation on white cell differential count, which meant no stress was exerted by methionine supplements in birds. However, KALVANDI et al. (2019) indicated that methionine supplementation led to stress by observing higher lymphocyte and lower heterophil and heterophil/lymphocyte ratios in quail that received methionine supplements than in basal diets. Several studies suggest that blood biochemicals (total protein, globulin, ALT, and AST as an indicator of hepatic function; a uric acid indicator of renal function) are used as an indicator of the health status of poultry. Previous studies indicated that dietary methionine levels led to high levels of globulin but no changes in the concentration of total protein (HADINIA *et al.* 2014; ZHANG *et al.* 2017). JARIYAHATTHAKIJ *et al.* (2018) observed that methionine supplementation in low crude protein diets increased total plasma protein and albumin levels compared to those with a low CP diet without supplementation. They further pointed out that these blood parameters in broilers (total protein, uric acid, and albumin) were significantly affected by dietary treatments at 25 days but not at 42 days. However, JANKOWSKI *et al.* (2018) observed no significant difference in the blood biochemical parameters of turkey concerning methionine levels and sources (DL-Met and MHA), except for plasma uric acid levels, which increased with the increasing dietary methionine levels. YODSERANEE & BUNCHASAK (2012) reported that plasma uric acid was higher in chicks fed MHA than those provided DL-Met supplements. Uric acid concentration (a potent antioxidant) is inversely correlated with oxidative activity in poultry (YODSERANEE & BUNCHASAK 2012). All this work does not explain a clear trend in blood biochemical parameters regarding dietary methionine sources and levels. Therefore, further research is needed to know the blood parameters as a response to the L-Met and DL-Met methionine in the poultry diet in relation to the different dietary conditions as well as genotypes.

Studies have been conducted on meat quality regarding the effect of dietary Met sources and levels and revealed that Met sources have no impact on meat quality (pH, freshness

during storage, redness) (DRAŻBO *et al.* 2020). In contrast, some indicated that different Met sources at different levels or concentrations affect the meat quality of poultry (MURAWSKA *et al.* 2018; DRAŻBO *et al.* 2020). In summary, more research has been done on the effects of dietary methionine levels on the metabolism of poultry, methionine requirements, and production. Different dietary conditions and environmental factors influence the dietary methionine sources' effect on poultry production (ZEITZ *et al.* 2020). However, there is scant information on the influence of methionine sources on blood biochemical parameters at different stages of development in laying type chickens. Therefore, there is a need to investigate the impact of dietary methionine sources and levels of supplementation on the physiology parameters and bioefficacy in different layer genotypes during the embryonic development period and early period of life.

2.5. Dietary methionine effect on gene expression in poultry

Many studies have investigated the effect of different dietary supplemental Met sources on the alteration of gene expression in poultry (DEL VESCO *et al.* 2013; WAN *et al.* 2017; ZHANG *et al.* 2018; LIU *et al.* 2019; FAGUNDES *et al.* 2020) either in relation to the dietary condition (SWENNEN *et al.* 2011; JARIYAHATTHAKIJ *et al.* 2018) or environmental factors such as temperature (DEL VESCO *et al.* 2015; LIU *et al.* 2019). Most of the genes studied concerning the effects of dietary methionine sources and levels include the expression of inflammatory genes (ZEITZ *et al.* 2017; LIU *et al.* 2019; MAGNUSON *et al.* 2020), oxidative stress and antioxidants related genes (DEL VESCO *et al.* 2015; RUAN *et al.* 2018; WANG, YIN, *et al.* 2019), as well as insulin-like growth factor I (*IGF1*) (DEL VESCO *et al.* 2013; WEN *et al.* 2017; EL-TARRAS *et al.* 2019).

The above studies have shown no clear trend in how dietary methionine alters the expression of selected genes. Some authors showed that dietary levels had no significant effects on the expression of the selected genes. For example, SWENNEN *et al.* (2011) reported that hepatic methionine reductase A (Msr-A) gene expression was not significantly affected by dietary treatment. In addition, DEL VESCO (2013) reported that Met levels did not affect the gene expression of growth hormone receptor (*GHR*) and *IGF1* in muscle tissue. However, in the liver, the expression of these genes was concentration-dependent, as it was revealed that *GHR* and *IGF1* mRNA levels were higher in broilers fed 0.24% DL-Met than in 0.08% and control. The study by WEN *et al.* (2017) also showed that high methionine levels increased *IGF1* in both strains of broilers (fast and slow-growing broilers). RUAN *et al.* (2018) studied the expression of ovalbumin

and antioxidant-related genes in laying ducks at six DL-Met levels (2.00, 2.75, 3.50, 4.25, 5.00, and 5.75 g/kg). They revealed that dietary level had a linear effect on some gene expression and a quadratically increased expression of other genes in the liver of a duck. Some experiments with the *in-ovo* injection of methionine and cysteine indicated positive effects on embryonic development, *IGF1*, and toll-like receptor 4 (*TLR4*) gene expression in broilers (ELWAN *et al.* 2019).

However, some experiments revealed a significant difference in the expression of hepatic genes related to methionine between DL-Met and HMTBA (WAN *et al.* 2017). The authors further pointed out that the expression of methionine synthase, adenosine deaminase (ADA), SAH hydrolase (SAHH), and methionine adenosyltransferase 2A (MAT2A) changed quadratically as HMTBA levels increased, while the expression of glycine N-methyltransferase (GNMT) and SAH hydrolase (SAHH) changed quadratically as DL-Met levels increased. LIU *et al.* (2019) also showed that mRNA levels of HSP 90 and c-Jun N-terminal kinase (JNK) in the liver were higher in DL-Met-supplemented broilers compared to the HMTBA-fed birds. WANG *et al*. (2019) pointed out that DL-Met is an effective precursor of glutathione synthesis to improve antioxidant functions. In contrast, HMTBA facilitates S-adenosylmethionine (SAM) synthesis and stimulates antioxidant-related gene expression.

In some experiments, Met supplements interact with the environment (temperature) to alter the expression levels of the selected genes (uncoupling proteins (UCP), betaine–homocysteine methyltransferase (BHMT), cystathionine b-synthase (CBS), glutathione synthetase (GSS), and glutathione peroxidase 7 (GPx7) in both the starter and grower periods of the broiler (DEL VESCO *et al.* 2015). LIU *et al.* (2019) reported the interaction effect between the concentration and sources of dietary methionine on the mRNA levels of selected inflammatory genes in the liver. EL TARRAS *et al.* (2019) reported that dietary Met supplementation influenced the transcriptional regulation and activity of Met oxidases in tissue in an age-specific manner. Therefore, as explained above, there is no clear expression pattern for selected genes in different studies. The wide range of Met levels and other factors like environmental factors, such as temperature and an organism's dietary condition and tissues, influence gene expression.

2.6. Role of Methionine on intestinal development, health and function

The performance of poultry at all stages is significantly influenced by the development and maturation of the intestine (LUGATA *et al.* 2022b). The functional capacity of the intestinal development is facilitated by major morphological and physiological changes following the amniotic fluid ingestion by the embryo after 17 days of embryonic development (UNI *et al.* 2003; GIVISIEZ *et al.* 2020). Methionine is known for preserving the intestinal barrier's functional and structural integrity (CHEN *et al.* 2014), enhancing the renewal and generation of intestinal epithelium, and stimulating stem cell proliferation (SAITO *et al.* 2017; GONG *et al.* 2023).

Several studies have been conducted on the effects of Met sources and levels via dietary supplementation, drinking water, or in-ovo feeding on intestinal development, health, and function at different stages of poultry (Table 3). Dietary or drinking water-supplemented Met studies mainly focus on maintaining intestinal health and function. Met has been observed to increase beneficial gut bacteria and reduce pathogenic bacteria in chickens (DAHIYA *et al.* 2007; NAVA *et al.* 2009; GONG *et al.* 2023). In addition, due to the involvement of Met in immune functions, several studies have been conducted on the role of methionine on the intestinal immune barrier under challenging conditions with pathogens. L-Met maintained the intestinal integrity of the broilers compared to DL-Met under *Eimeria* infection (TENG *et al.* 2023). Regarding the Met levels, Met deficiency has been shown to enhance intestinal integrity under infection (Table 3) (CASTRO *et al.* 2020; REN *et al.* 2020; TENG *et al.* 2023). This supports the ongoing rebuilding process of intestinal integrity due to Met deficiency. Similar findings have been reported in another animal species with Met restriction, which increases tight junction protein expression (RAMALINGAM *et al.* 2010). Tight junctions are the protective barrier that restricts pathogenic molecules and bacteria from entering. The expression of the tight junction proteins is considered another indicator of intestinal health.

Intestinal size and structure reflect its functions. Increased intestinal weight and villus height are typically linked to an increase in the small intestine's ability for digestion and absorption, which in turn enhances the animals' health. Met sources and levels effects on the intestinal development and morphology are limited and contradictory. HMTBA increased the ileal villus height and villus height to crypt depth ratio in broilers (GONG *et al.* 2023). In addition, L-Met increased the villus height, villus width, and decreased crypt depth more than DL-Met on 7-day broilers (SHEN *et al.* 2015), while both DL-Met and

L-Met had similar effects on villus height, area, and height /crypt depth ratio in ducks (ZHANG *et al.* 2019) and domestic pigeons (ZHONG *et al.* 2022). Furthermore, limited studies have also shown how Met could enhance intestinal morphology through in-ovo feeding (Table 3).

Table 3

Effect of Met source and levels supplemented via diet, drinking water or in-ovo on the intestinal development, health and function of poultry

Met sources	Met levels	Poultry species	Main finding on intestinal development, health and function	References
HMTBA DL-Met	2.4 DL-Met or 2.66 HMTBA g/kg	Male Ross chickens	HMTBA ↑ cysteine and taurine content in the chicken enterocytes than DL-Met	(MARTÍN-VENEGAS *et al.* 2006)
MHA-FA DL-Met	0, 0.2, 0.4, or 0.8% DL-Met or 0, 0.227, 0.0454 and 0.908% MHA-FA	Ross 308 broilers	Both Met source ↓ *Coliforms perfringens* at high concentrations and therefore, minimize the risk of necrotic enteritis	(DAHIYA *et al.* 2007)
L-Met and DL-Met	Basl diet (BD) + 0.095% L-Met or DL-Met, the BD + 0.190% L-Met or DL-Met, and the BD + 0.285% L-Met or DL-Met (representing 60, 70, 80, and 90% of the Met + Cys requirement)	Ross 308 chickens	Chicks fed 0.285% L-Met ↑ villus height compared to those fed basal diet, ↑ villus width, ↓ crypt depth than those fed with 0.285% DL-Met on day 7. On day 21, 0.285% L-Met ↑ villus height than and ↑ villus width observed in chicks received 0.285% of either L-Met or DL-Met compared to basal diet.	(SHEN *et al.* 2015)
Zinc methionine (Zn-Met)	40 and 80 mg Zn-Met	Hyline laying hens	80 mg Zn-Met diet ↑ villus height, villus area and villus height/crypt depth ratio, ↓ crypt depth in the jejunum	(LI *et al.* 2019)
L-Met DL-Met	Starter phase: 0.05, 0.10, 0.15, or 0.20% of either source. Grower phase: 0.04, 0.08, 0.12, or 0.16% of either source.	Pekin male ducks	DL-Met or L-Met ↑ villus height of the ileal mucosal of the ducks at days 14 and 35 DL-Met ↓ ileal crypt depth than a basal diet.	(ZHANG *et al.* 2019)
L-Met	0, 0.15 and 0.31% of L-Met representing 70, 85 and 100% TSAA	Cobb 500 male broilers challenged with *Eimeria spp.*	↔ on the intestinal histomorphology and tight junction protein gene expression among the Met levels. Broilers fed with the 100% TSAA ↑ GSH levels at 12 days post-infection. The positive effects were observed in either 85% or 100% of TSAA levels. The supplementation of L-Met up to either of these levels was sufficient to ensure good performance and intestinal health in birds, regardless of their exposure to Eimeria spp.	(CASTRO *et al.* 2020)

Met sources	Met levels	Poultry species	Main finding on intestinal development, health and function	References
Met plus Cysteine	0.6, 0.8, 0.9, or 1.0% standardized ileal digestible (SID) M+C	Male broilers Challenged with *Eimeria spp.*	Met +Csy at 0.8% SID ↑ the levels of jejunum total IgA. After Eimeria challenge, broilers fed 0.8% SID M+C had increased ($P < 0.05$) levels of jejunum luminal anti-*Eimeria* IgA compared to broilers fed diets containing 0.6 and 1.0% SID M+C. Collectively, in 11- to 21-D broilers.	(REN *et al.* 2020)
DL-Met, DL-methionyl-DL-methionine (Dl-Met-Met)	Basal diet + 0.3% of either Met source. Crude protein (CP = 15%)	Domestic pigeons (*Columba livia*)	Both Met source ↑ intestinal morphology and structure in the squabs by ↑ relative intestinal weight, villus height and villus height to crypt ratio. Met supplementation ↑ tight junction proteins (ZO-1 and Claudin-1) expression in the jejunum	(ZHONG *et al.* 2022)
L-Met DL-Met	0.19 and 0.38 % of either source (80 and 100% of TSAA recommended)	Cobb 500 broilers, challenge with *Eimeria spp.* infection	Eimeria infection ↓ growth performance, antioxidant status, tight junction genes expression and immune cytokines. L-Met ↓ gut permeability than DL-Met on 5 days post infection (DPI). 80% ↑ ZO1 expression than 100% at 6 DPI.	(TENG *et al.* 2023)
DL-Met, L-Met MHA-FA	(80, 100 and 120% of the recommendation	Ross 308	Met supplementation regardless of the source and levels ↑ the Claudin 2 gene expression in the jejunum of the broiler at day 21. ↪ on the mRNA expression of TJ, IL1-β, MUC2, and GPx1 of either Met source or levels. Met levels and source did not change the bacteria in the digesta.	(BAREKATAIN & KLUENEMANN 2023)
HMTBA DL-Met	80, 100, and 120% of Arbor Acre TSAA recommendation	Arbor Acre male broiler chicks	HMTBA promotes the intestinal barrier by regulating claudin-1 gene expression and serum diamine oxidase. Compared to DL-Met, HMTBA ↓ inflammatory cytokine IL-6 gene expression improved intestinal morphology. Low-TSAA ↑ claudin-1 and zonula occluden-1, *IL- 1β* and *IL-6* on day 14. 100% TSAA ↑ Lactobacillus and intestinal morphology	(GONG *et al.* 2023)

Met sources	Met levels	Poultry species	Main finding on intestinal development, health and function	References
HMTBA in drinking water	0.02-0.2% of the blend of HMTBA and acidifier	Mixed-sex Arbor Acre broilers	0.10, 0.15 and 0.20% acidifier ↓ gut pH and ↑ duodenal weight, length, villus height and the ratio of the villus height to crypt depth 0.20% acidifier ↑ probiotics and ↓ pathogenic bacteria	(GUO *et al.* 2022)
***In-ovo* methionine –cysteine injection**	5.90 mg L-methionine plus 3.40 mg L-cysteine	Ross 308 broiler	↑ villus height, width, area and crypt depth observed in in-ovo Met-Cys compared to other groups	(ELNESR *et al.* 2019)
***In-ovo* of L-Met**	5, 10, and 20 mg injected on embryonic day 9.	Yellow-feathered broiler chicks	*In-ovo* of L-Met ↑ relative weight of duodenum, jejunum and ileum, villus height and crypt depth. 10 mg L-Met ↑ transepithelial electrical resistance of the jejunum. 10 and 20 mg ↑ the expression of the tight junction proteins (ZO-1 and Claudin-1)	(CHEN *et al.* 2021)
***In-ovo* injection of Met and or disaccharide (DS)**	Met injection (5 g/L Met + 7.5 g/L NaCl), DS injection (25 g/L maltose + 25 g/L sucrose + 7.5 g/L NaCl), or DS plus Met injection (25 g/L maltose + 25 g/L sucrose + 5 g/L Met + 7.5g/L NaCl), respectively at 23 day of incubation.	Jilin White geese	*In-ovo* feeding of Met increased small intestine weight, jejunum alkaline phosphatase activities, and jejunum villus height and surface area. Met plus DS injection synergistically enhanced jejunum villus height and surface area.	(DANG *et al.* 2022c)
***In-ovo* injection of DL-Met**	Met levels (0.5, 1.0, 1.5, 2.0 and 2.5%) + 0.5% NaCl,	Rhode Island red	↑ gizzard weight and length of the duodenal loop in 0.5% Met, ↓ colon length with increasing levels of Met ↑ density of the goblet cells in the villi and Lieberkühn in the small and large intestine ↑ mucous tunic of small intestine by 1.0% Met	(FARIAS *et al.* 2023)

↽ Represents no difference, or similar or no effect. ↑ Represents increased or high or upregulated. ↓ Represents decreased or low, or downregulated. Abbreviations: BD— basal diet, MS—methionine supplementation, TSAA—total sulphur amino acids, SID—standardized ileal digestible, CP—crude protein, DL—DL-methionine, MHA/HMTBA—methionine hydroxyl analogue/DL-2-hydroxy-4-(methylthio) butanoic acid; DPI—days post-infection, IgA—Immunoglobulin A, *ZO-1*—zonula occluden-1, TJ—tight junction protein, *IL1-β*—interleukin-1 beta, *MUC2*—mucin-2, F *IL-6* —interleukin 6, GSH—glutathione, Met-Cys—methionine plus cysteine

The following conclusions can be drawn from the literature:

Methionine (Met) is crucial for keeping poultry's antioxidant status intact. Met, both attached to proteins and free in food, has the ability to act as an antioxidant and mitigate oxidative stress brought on by a range of physiological and environmental stresses.

Research comparing Met's antioxidant value to National Research Council (NRC, 1994) guidelines has demonstrated that raising Met inclusion ratios above NRC guidelines improves antioxidant status but has little to no impact on production or development. The ideal Met concentration needed to activate vital antioxidants like GSH, SOD, CAT, and GPX, which are components of poultry's first line of defence against free radicals, is nevertheless, not well understood. Consequently, more research needs to be done to ascertain the optimal Met concentration needed to activate these vital antioxidants.

It is crucial to evaluate the antioxidant system before determining whether increasing the quantities of Met (or total sulphur amino acids) in poultry diets is appropriate. Special attention should be given to layers due to the significant changes in their antioxidant status as the required levels of Met in their diets increase. Additionally, there is a lack of studies examining the antioxidant responses of laying-type chickens to different inclusion rates of Met during the early stages of their lives, including embryonic development.

Met has shown the potential to affect the expression of tight junction proteins, which are essential for maintaining intestinal barrier function. These proteins play a crucial role in regulating nutrient absorption and preventing the passage of pathogens and harmful molecules. The expression of tight junction proteins serves as an indicator of intestinal health, and Met restriction has been found to increase their expression.

It's important to note that limited research has explored the influence of Met on intestinal development through *in-ovo* feeding. Moreover, none of the studies have directly compared the effects of DL-Met and L-Met *in-ovo* feeding on the intestinal development, health, and function of different laying-type genotypes, including commercial and native genotypes. The results from these studies emphasize the complex and multifaceted nature of Met's influence on intestinal health in poultry. The effects vary depending on factors such as the Met source, levels, and the specific context of the research. Further investigation, especially comparative studies involving DL-Met and L-Met in different poultry genotypes, would provide a more comprehensive understanding of the practical implications of these findings for poultry production and health management.

3. MATERIALS AND METHODS

All experiments were conducted at the Kismacs Experimental Station of Animal Husbandry of the University of Debrecen under the Institute of Agricultural Research and Educational Farm between June and July 2021. The experimental protocol was according to international, national, and institutional guidelines for the use of animals. All the experimental procedures adhered to the ethical standards of the institution and received approval from the National Authority, including the National Scientific Ethical Committee on Animal Experimentation, the local veterinary ethics committee, and the institutional animal care and use committee (6/2021/DEMÁB). Two experiments were performed to fulfil the objectives of the dissertation: *in-ovo* feeding and dietary feeding experiments.

3.1. Experiment I*:* Effect of *in-ovo* application of different Met sources and dosages on the physiological parameters of layer chicks

3.1.1. Hatching eggs

A total of 570 hatching eggs were obtained; 360 eggs for the TETRA SL LL (TSL) genotype were procured from TETRA-Bábolna Ltd. (Bábolna, Hungary). We collected 210 freshly laid eggs (not older than four days) from the Hungarian Partridge-Colored hen (HPC) flock that were 52 - 58 weeks old at the Kismacs Experimental Station (University of Debrecen, Kismacs, Hungary). All the eggs were marked with T for TSL and H for HPC, respectively, and the eggs were fumigated before incubation.

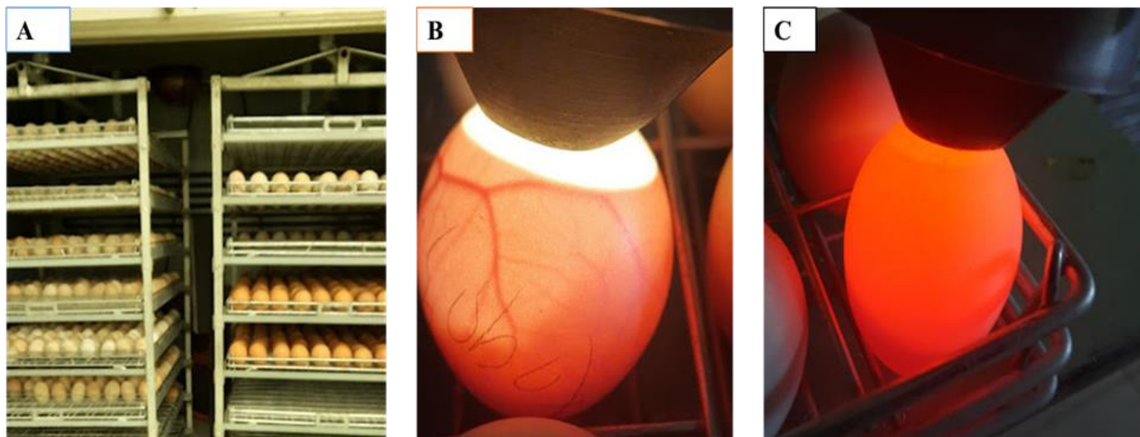
3.1.2. Incubation and experimental setup

All eggs were first stored at 21 ˚C for 24 h and subsequently incubated at 37.8 ˚C with a relative humidity of 60% until 17.5 d of incubation (FASENKO 2007). From 17.5 to 21 days of incubation, the relative humidity was raised to 65-70%. The eggs were set and incubated in an automatic egg-turning incubator (PLM 3600, PL Maschine Kft., Budapest, Hungary), where every hour they were automatically turned. The eggs were candled on the tenth day of incubation, and non-fertile eggs and dead embryos were removed (Picture 1). A total of 34 infertile eggs and 11 early-dead embryos were removed from the incubator for HPC, while 21 infertile eggs and 7 early-dead embryos were removed for TSL. The unfertilized and dead embryos were sorted based on illumination (CHEN *et al.* 2020). On the 17.5th day of embryonic development (17.5 days of

incubation), eggs were candled and randomly allotted to *in-ovo* treatments. A total of 400 embryonated eggs were distributed among 16 groups (8 groups for each genotype), with 30 eggs for TSL and 20 eggs for HPC genotype. The treatment groups were non-injected (Control), saline solution (Saline) injected (0.5 ml), 5, 10, or 15 mg DL-Met or L-Met injected (in 0.5 ml saline solution) for both HPC and TSL genotypes (Figure 3). The treatment consisted of a 2x2x3 factorial arrangement based on two sources of Met (DL and L-Met), 2 genotypes of layers (TSL and HPC breeds), and 3-doses of Met for each source.

Picture 1

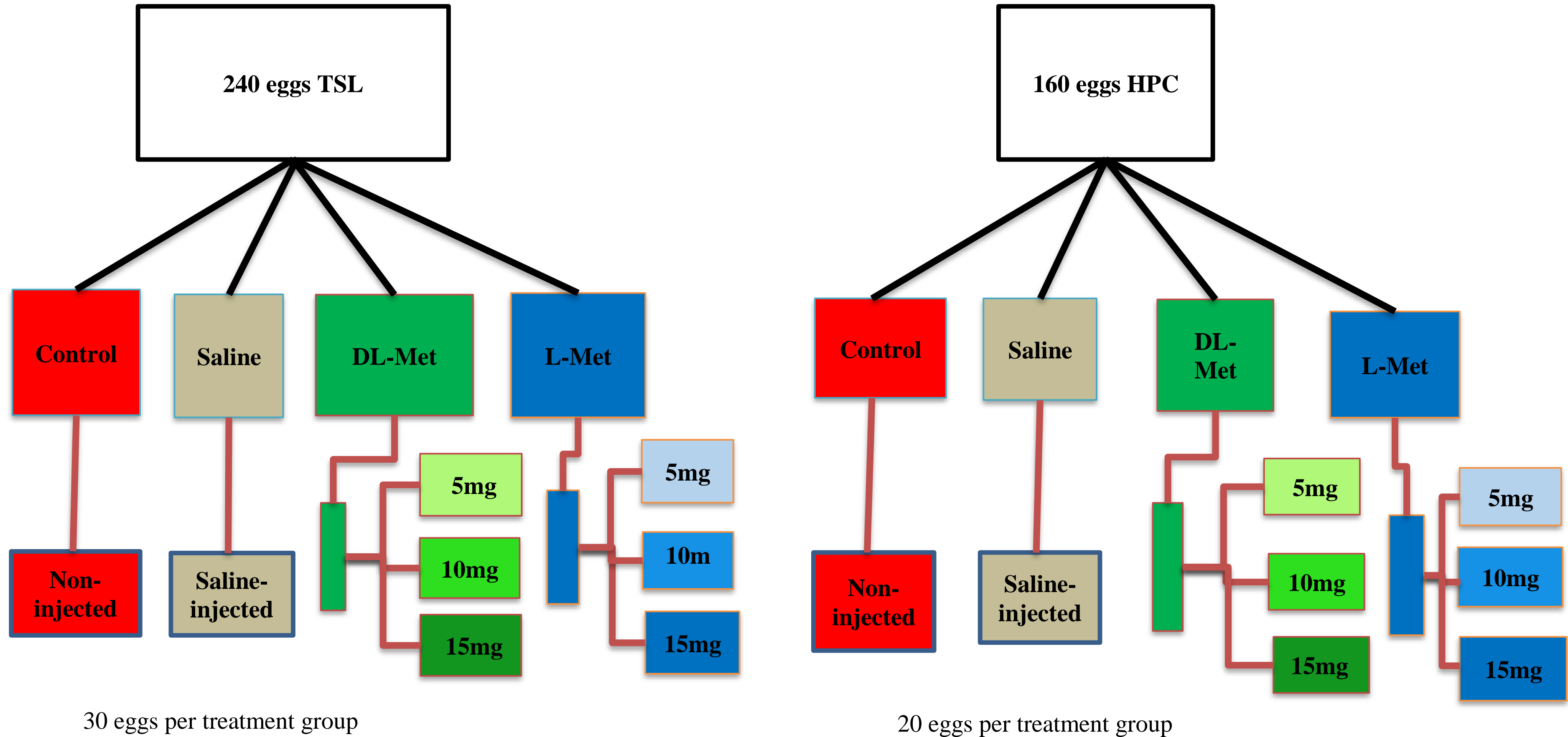
Egg setting in the incubator and -egg candling on the 10th day of incubation



A- Egg setting in the incubator, B-egg candling on the 10th day of incubation with developing embryo. C – Egg candling on the 10th day of incubation showing non-fertile eggs that were removed from the incubator

Figure 3

Experimental design (TSL: TETRA-SL layer hybrid, HPC: Hungarian partridge colored hen breed)



3.1.3. Preparation of Met solutions and *in-ovo* injection procedure

L-Met and DL-Met were purchased from Sigma-Aldrich, Merck KGaA, Darmstadt, Germany. We prepared 5, 10, and 15 mg Met/0.5 mL of 0.75% normal saline solution from DL-Met (DL-Methionine, No. M9500, purity ≥ 99%, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and L-Met (L-Methionine, No. 64319, BioUltra, purity ≥ 99.5%, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The *in-ovo* injection of the Met solution procedure was performed according to CHEN *et al.* (2020) and TOMBARKIEWICZ *et al.* (2020). Briefly, the surface of every egg was disinfected with a 70% ethanol-soaked cotton ball before injection. Next, a small hole (0.5 mm diameter) was drilled at the lager end of the egg. The egg was injected with a 0.5-mL solution containing Met at a dose of 5, 10, and 15 mg of either L or DL-Met into the amniotic sac by using a 23-gauge needle (Picture 2). After injection, the holes were immediately sealed with hot paraffin and eggs were transferred to hatcheries trays and returned to the incubator. All eggs were maintained outside the incubator for less than 30 min during the injection. Embryonated eggs that were not subjected to *in-ovo* injection were also returned to the incubator and hatched, and the chicks were used for a dietary Met supplementation trial.

Picture 2

***In-ovo* injection at day 17.5 of incubation**

3.1.4. Sample collection

The weight of the chicks on the day of hatching and one day after hatching (sampling day) were recorded (Picture 3). The hatchability percentage was calculated as hatched chicks per fertile eggs multiplied by 100. The groups injected with 10 and 15 mg of Met had low hatchability and were excluded from blood and tissue sampling. Eight chicks (one-day-old) were then euthanized by cervical dislocation for blood and tissue sampling from the remaining groups. In addition to chick live weight, liver and heart weight were also recorded during tissue sampling. The blood sample (1 to 1.5 mL) was collected from the jugular vein into ethylenediaminetetraacetic acid (EDTA-coated) tubes. The blood samples were centrifuged at 3,000 x g for 10 min at room temperature to separate the plasma, and the plasma samples were stored at -80 ˚C until further analysis. Also, the tissues (liver, small intestine, and pectoral muscle) were extracted and snap-frozen in liquid nitrogen before being transported to the laboratory and stored at -80 ºC until further analysis. About 1 cm of the jejunum was taken (5 chicks per treatment) and washed with phosphate-buffered saline before being fixed in 10% formalin for histology examination.

Picture 3

Sample collection

A and B; weight measurement of TSL hybrid and HPC hen chicks, respectively, C- Tissue sampling.

3.1.5. Plasma biochemical analysis

3.1.5.1. Ferric-reducing ability of plasma (FRAP) analysis

The ferric reducing ability of plasma (FRAP) was determined according to the manufacturer kit protocol (Ferric Antioxidant Status Detection Kit Catalog Number EIAFECL2; Thermo Fisher Scientific, Life Technologies Corporation, Carlsbad, USA). Briefly, this method is based on the oxidized form Fe ^{III} being reduced to Fe ^{II} in acidic conditions, with a blue-colored complex ferrous-tripyridyltriazine being formed. The intensity of the color change then determines the antioxidant capacity. The assay buffer

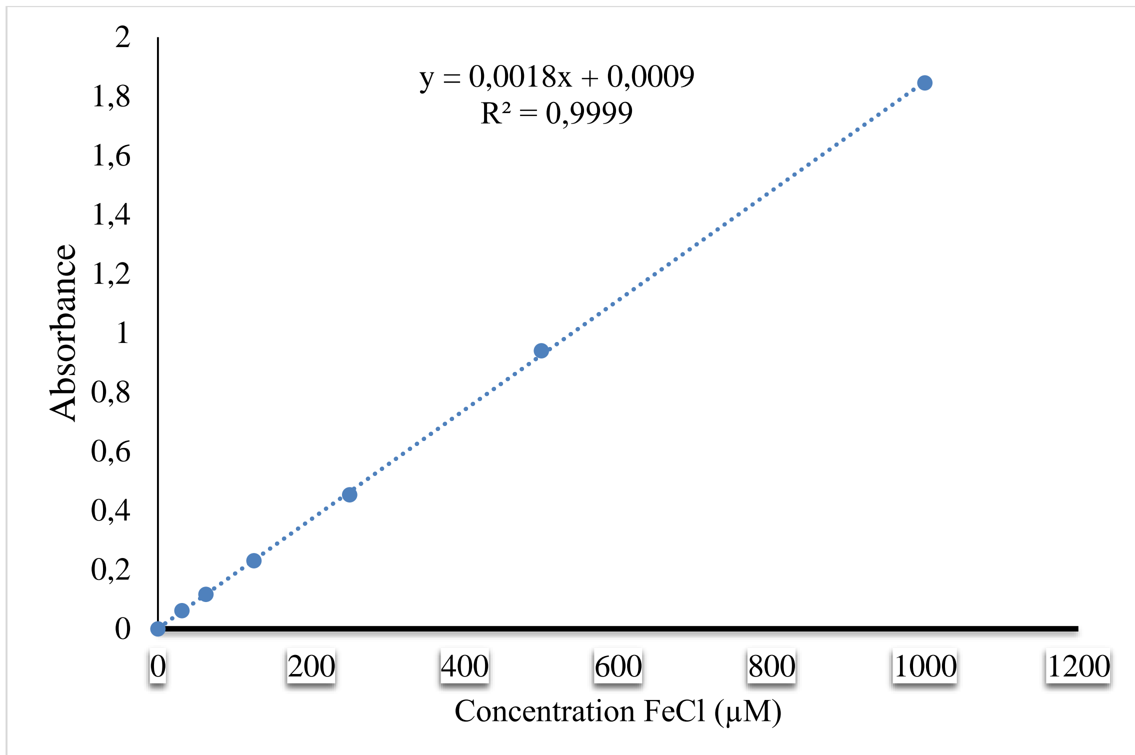
concentrate (10X acetate buffer with stabilizers and preservatives) was used to prepare the 1X assay buffer by diluting 7 mL of assay buffer 10X with 63 mL of deionized water. Samples of plasma were diluted by taking 20 µL of plasma into 40 µL of 1X assay buffer to make a total volume of 60 µL. Then, after dilution, the sample was mixed well by hand, followed by centrifugation for about 1 to 2 minutes to check if there were bubbles. All samples were used within 2 hours after dilution. Standards were diluted as follows: 20 µL of 10 mM ferrous chloride standard was added to one tube containing 180 µL 1X assay buffer and labeled 1,000 µM $FeCl_2$. Then 100 µL 1X assay buffer was added to each of the 6 tubes and labeled as 500, 250, 125, 62.5, 31.25, and 0 µM $FeCl_2$. Thereafter, a serial dilution of the standards was made, as follows: 100 µL of 1000 µM $FeCl_2$ solution into 500 µM $FeCl_2$ tube, then 100 µL from 500 µM $FeCl_2$ solution into 250 µM $FeCl_2$ tube, followed by taking 100 µL from 250 µM $FeCl_2$ solution into 62.5 µM $FeCl_2$ tube, and lastly 100 µL from 62.5 µM $FeCl_2$ tube into 31.25 µM $FeCl_2$ tube. The solution was thoroughly mixed between steps and was used within two hours of dilution. The FRAP color solution was prepared by taking 12.5 mL of 1X assay buffer, 1.25 mL of FRAP reagent A, and 1.25 mL of FRAP reagent B to make a total volume of 15 mL of the solution.

3.1.5.2. FRAP assay procedure

First, 75 µL of FRAP color solution was added to each well. Then, 20 µL of standard or diluted samples were added to the appropriate wells. Mix gently by hand, followed by centrifuging for about 1 to 2 minutes in the platefuge, then incubating for 30 minutes at room temperature. The absorbance was read at 560 nm, and a standard curve was generated by four-parameter curve fitting, and linear regression curves provided the best standard curve fit. However, blank-corrected absorbance was used before plotting. The concentrations of unknown samples were obtained from the standard curve and expressed as µM $FeCl_2$ equivalent (Figure 4).

Figure 4

Standard curve of FRAP used for calculating the concentration of the unknown sample



3.1.5.3. Liver enzymes and kidney status indicator (AST, ALT and Uric acid) assay

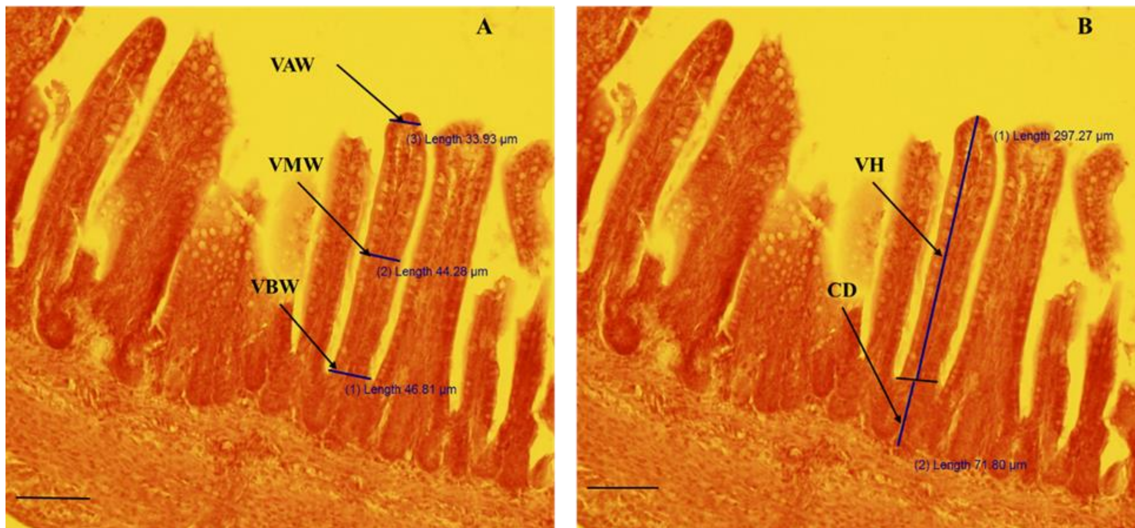
The plasma samples were analyzed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and uric acid using an auto-analyzer (Lab-Analyse 10261, OrvosTechnika Kft., Budapest, Hungary) and its accompanying kits. In brief, the measurement was performed at room temperature, and it was made sure that the samples and reagents were at room temperature before and during the measurement. Three different technical replicates were performed, depending on the parameter, according to kit manufacturer instructions. The first measurement is the endpoint (uric acid measurement), wherein a 12 µl sample was pipetted into uric acid reagents. The respective mixed sample with the reagent was then incubated for 5 minutes. For AST and ALT, the measurement was kinetic, in which distilled water was used as a blank. In this method, 50 µl of sample was pipetted into the AST and ALT reagents, gently mixed, and measured directly. The units used for the parameters measured were µmol/L for uric acid and U/L for AST and ALT, respectively.

3.1.6. Histology analysis

A routine histological laboratory approach was carried out, dehydrated in increasing grades of ethanol, cleared in xylene, and embedded in paraffin. To overcome bias resulting from sectioning and further histological procedures, we placed all 5 samples from the same treatment group in one paraffin block, resulting in 4 paraffin array blocks from the different groups (Control, Saline, DL-Met, and L-Met). Tissue sections (10µm thick) were cut with a conventional vibratome (HM 335E, Microm, Germany), and the sections were mounted on microscope slides. After deparaffinizing with xylene and rehydrating in decreasing grades of ethanol, the sections were stained with hematoxylin-eosin (H&E) stain according to the supplier's protocol (Vector Laboratories, New York, CA, USA). For examining the villi of the jejunum, each sample was captured by a digital camera (DP71, Olympus, Japan) attached to the transmitted light microscope (BX61, Olympus, Japan) using a 20x objective lens after the adjustment of the illumination. 10 vertical villi per chick were chosen randomly from the cross-section per individual for measurement. Furthermore, the villus height (VH) was determined from the villus tip to the villus-crypt junction; the crypt depth (CD) was also measured as the depth of invagination between two villi. The villus height, width, area, and crypt depth were determined according to NAZEM *et al.* (2017) (Picture 4). The villus width was measured at three points: at the base of the villus, the middle, and the tip of the villi height (villus apical width), while the calculation of the villous height and width at the half-height resulted in the villus surface area (VA) (UNI *et al.* 2003). The villus surface area was calculated by the following formula: $2\pi \times VH \times (VW/2)$. Measurements were performed with an Olympus light microscope using Olympus CellSens software.

Picture 4

Villi measurements at 200x magnification and scale bars = 100 µm



A: villus width was measured: VBW-villus base width, VMW-villus middle width and VAW-villus apical width. B: The villus height –VH (villus tip to crypt opening), crypt depth -CD (crypt opening to the base of the crypt just before the lamina propria), and. The ratios of Villus height to crypt depth were then computed.

3.1.7. Tissues Antioxidants analysis

3.1.7.1. Total Glutathione (GSH) content determination in tissues

Samples were collected, and snap-frozen in liquid nitrogen, and stored at -80 ˚C prior to analysis. The tissues (liver, intestine, and pectoral muscles) were homogenized by grounding under liquid nitrogen in a cooled mortar and pestle, and 20 mg were transferred into a new tube, then placed in a mini-cooler (-20 ˚C) (TIPPLE & ROGERS 2012). Then 500 µL of 5% SSA (1 of SSA into 20 mL of water) were added, and vortexing was used to mix the sample before incubation for 10 minutes on ice. The homogenate was centrifuged at 13300 RPM (VWR Micro-Star 17R) for 10 minutes at 4 ˚C to precipitate proteins (Picture 5). The supernatant was transferred into a new tube and frozen at -80 ˚C until further analysis. Prior to analysis, different optimal dilutions were determined for each tissue, and the optimal dilution factor was 40x, 20x, and 10x for the liver, pectoral muscles, and intestine, respectively. For liver samples, first, a 5x dilution was made by taking 50 µL of sample and 200 µL of assay buffer, then 50 µL of the diluted sample (5x) was added to 350 µL of the sample diluent to make an 8x dilution; the final dilution was 40x. The same procedure was performed for pectoral and intestine tissues with their respective dilution factors.

Picture 5

Sample preparation

A- homogenization by using mortar and pestle under liquid nitrogen) for GSH content determination. B- Sample chilled in a mini Cooler during homogenization. C- sample incubated on ice for 10 mins after adding 5% SSA, D- centrifugation for supernatant extraction.

The GSH concentration was determined using the glutathione colorimetric detection kit-EIAGSHC (Invitrogen, ThermoFisher Scientific Carlsbad, CA 92008 USA). The standard and diluted samples (50 µL) were added to 25 µL of colorimetric detection reagents and 25 µL of the reaction mixture, prepared according to kit instructions, in duplicate in a 96-well plate. Then, the plate taps on the side to mix and is incubated at room temperature for 20 min. The absorbance was read on a plate reader (Synergy HT Multi-Mode Microplate Reader-SN 1712214, BioTek Instruments, Inc., 100 Tigan Street, Winooski, USA). The concentration of GSH in each sample was extrapolated from the equation derived from the absorbance and concentration of the standard. The concentration of the GSH was expressed as millimoles of GSH/mg of tissue, and the assay's sensitivity was 0.634 µM of GSH.

3.1.7.2. Total antioxidant capacity determination in the tissues

Total antioxidant capacity highlights the cell's ability to combat reactive oxygen species and free radicals. The liver, intestine, and pectoral muscles were homogenized by grounding under liquid nitrogen in a cooled mortar and pestle. About 25 mg of fine-ground tissues were weighed and suspended in 250 µL of ice-cold phosphate-buffered saline (PBS) (1:9, wt./vol). The homogenate was centrifuged at 15000 x g at 4 ˚C for 10 min, and the supernatants were transferred into the new tubes and stored in the freezer (-80 ˚C) for further analysis. The TAC was determined using the total antioxidant capacity assay kit (MAK187, Sigma-Aldrich, 3050 Spruce Street, St. Louis, MO 63103, USA) based on the manufacturer’s instructions. Briefly, 1 to 100 µL of samples were added to the microplate wells, and the water was added to bring the final volume to 100 µL. Then,

100 µL of Cu2+ working solution was added to all standard and sample wells. The microplate was mixed by pipetting and incubated for 90 minutes at room temperature. Finally, the absorbance was measured at 570 nm using a microplate reader (Synergy HT Multi-Mode Microplate Reader-SN 1712214, BioTek Instruments, Inc., 100 Tigan Street, Winooski, USA) in duplicate. The Trolox standard solution of 0, 4, 8, 12, 16, and 20 µL of the 1 mM (1 nmole/L) generating 0 (blank), 4, 8, 12, 16, and 20 nmole/well by adding water to the final volume of 100 µL was prepared. The absorbance was corrected by subtracting the blank (water plus working solution) from the samples and the standard absorbance. The standard graph was plotted to determine the TAC concentration. The concentrations of the TAC were calculated as Trolox equivalents and expressed in mM Trolox equivalents per mg of tissue.

3.1.8. Gene expression analysis

3.1.8.1. Sample preparation and total RNA isolation

The liver and intestine samples were homogenized using an ultraturax homogenizer (D1000 handheld homogenizer, Benchmark Scientific, Inc., Sayreville, NJ, USA). The total RNA was isolated using TRIzol reagents according to the Direct-zol RNA Miniprep (R2052, Zymo Research Orange, CA, USA) kit protocol. The sample was centrifuged at 16,000 x g for 1 minute, and the supernatant was transferred to a new tube. An equal volume of ethanol (96%) was added to a sample and mixed thoroughly. The mixture was then transferred into a Zymo-Spin IICR column in a collection tube and centrifuged at 16,000 x g for 30 seconds. The column was then moved into the new collection tube, treated with DNase I, and incubated at room temperature (20 to 30 ˚C) for 15 minutes. After incubation, 400 µl of direct-zol RNA PreWash was added to the column and centrifuged at 16000 x g for 30 seconds. A 700 µl RNA wash buffer was added to the column and centrifuged at 16000 x g for 1 minute. This step was repeated, then the column was carefully transferred into an RNase-free tube, and 70 µl of DNase/RNase-free water was added to elute the RNA. Sample preparation and centrifugation were performed at 4 ˚C Celsius to minimize RNA degradation from the muscles (low-yield tissue). The quantity and purity of RNA were determined using 50 µl in the microplate reader (Synergy HT Multi-Mode Microplate Reader-SN 1712214, BioTek Instruments, Inc., 100 Tigan Street, Winooski, USA). The absorbance was read with the help of Gen5 microplate and imager software (BioTek version 3.03). The quality and RNA integrity were checked by the Qubit RNA IQ assay kit (# Q33222, Thermo Fisher Scientific, Carlsbad, CA 92008

USA) using the Qubit 4 flurometer (Invitrogen by Thermo Fisher Scientific). The RNA IQ number (which indicates the RNA sample integrity and quality) ranged from 8.7 to 10. The total RNA was either used directly to synthesize cDNA or stored at -80 ˚C for less than four days before cDNA was made.

3.1.8.2. cDNA synthesis and RT-qPCR

About 1 µg of the total RNA was reverse-transcribed into complementary DNA. The cDNA synthesis was done using the LunaScript RT SuperMix Kit (New England Biolabs, Inc., E3010L). In brief, the cDNA synthesis was made using 1µg of total RNA through the reaction process of 2 minutes at 25 ˚C, 10 minutes at 55 ˚C and lastly, 1 minute at 95 ˚C by the PCR^{max} Alpha Thermal Cycler (Cole-Parmer Ltd., UK). The cDNA was diluted in a 1:5 ratios (200 ng) for qPCR analysis. The cDNA was stored at -80 ˚C until the RT-qPCR assay. The cDNA samples were amplified according to the manufacturer's instructions using the 5x HOT FIREPol EvaGreen qPCR Mix Plus kit (Solid BioDyne, Tartu, Estonia). In brief, a PCR reaction with a total volume of 10 µL consisting of 2 µL of cDNA template, 2 µL of 5x HOT FIREPol EvaGreen qPCR mix plus 0.2 µL of 100 nM of each primer, and 5.6 µL of distilled water. An AriaMx Real-Time PCR system performed real-time polymerase chain reaction (PCR) (Agilent Technologies-Applied Biosystems, Carlsbad, CA, USA). The samples were run in duplicate in a 96-well plate, and no template control primer was used. The PCR procedure included a pre-run at 95 ˚C for 12 minutes, followed by 40 cycles of denaturation at 95 ˚C for 15 seconds, an annealing and extension step at 60 ˚C for 20 seconds, and a final elongation step at 72 ˚C for 20 seconds. The 18sRNA was selected as the reference gene (for liver and intestine tissue), and its stability was tested with an algorithm. The target gene mRNA expression was normalized with the selected reference gene, and the relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ model (Livak & Schmittgen, 2001). The mRNA expression of insulin-like growth factor 1 (*IGF1*), insulin-like growth factor 1 receptor (*IGF1R*), growth hormone receptor (*GHR*), toll-like receptor 4 (*TLR4*), superoxide dismutase 1 (*SOD1*), tricellulin (*MD2*), glutathione S-transferase alpha 3 (*GST3*), glutathione peroxidase 1 (*GPX1*), nuclear factor, erythroid 2 like 2 (*NRF2*), occludin (*OCLN*), and tight junction protein 2 (*TJP2*) were analyzed. Melting curves revealed nonspecific product or primer dimers, suggesting the accuracy of mRNA transcript identification by displaying *IGF1, IGF1R, GHR, TLR4, SOD1, MD2, GST3, GPX1, NRF2, OCLN*, and *TJP2*-specific primers suitable for RT-PCR (Table 4).

Table 4

Primer details utilized in our study

Genes[1]	**Primer sequence (5' - >3')**	**GenBank accession No.**	**Product length (bp)**
GST3	F: GCCGAATGGAATCAGTACGCTGG	NM_001001777.2	114
GST	R: ACAGCAGGGATCCATCTGACTT		
GPX1	F: CGGCTTCCCCTGCAACCAATTCG	NM_001277853.3	55
GPX1	R: GATCTCCTCGTTGGTGGCGTTCT		
SOD1	F: AGCGCAGGTGCTCACTTCAATCC	NM_205064.2	87
SOD1	R: CACATTGCCGAGGTCACCCAC		
OCLN	F: AAGCCAACATCTACTGGGACCG	XM_025144247.2	87
OCLN	R: TAGCCCCATCCGCCACGTT		
TJP2	F: ACAGCTATACGTCACGCCCAAAG	XM_025144668.3	120
TJP2	R: CCCATATCAGCTCTTCCATGCCT		
MD2	F: AGGCACAGGGAGCAGATGGAAC	XM_424965.8	110
MD2	R: AGCTGCCTGTAAGTGACCTCT		
NRF2	F: CCCCGCACCATGGAGATCGAG	XM_046943472.1	72
NRF2	R: TGGCGCTGCGAAAAAGCACCT		
IGF1	F: CAC TAT GCG GTG CTG AGC TGG TT	XM_015867574.2	118
IGF1	R: ATC CCC TTG TGG TGT AAG CGT CT		
IGF1R	F: TAC AAC TAC CGC TGC TGG ACC AC	XM_015873184.2	107
IGF1R	R: AGG CAC TCA GGA TGG CAA CAC		
GHR	F: GGC ACT GGT CTG TGT GAA TGA CT	XM_032441512.1	89
GHR	R: CCA GCT CAG GTG ATC TGC ACT T		
TLR4	F: ACCCGAACTGCAGTTTCTGGAT	NM_001030693.1	120
TLR4	R: AGGTGCTGGAGTGAATTGGC		
18S rRNA	F: CTC TTT CTC GAT TCC GTG GGT	AF173612.1	96
18S rRNA	R: CAT GCC AGA GTC TCG TTC GT		

[1] *GST3*: glutathione S-transferase alpha 3, *GPX1*: glutathione peroxidase 1, *SOD1*: superoxide dismutase 1, NRF2: nuclear factor, erythroid 2 like 2, *MD2*: tricellulin (also referred as MARVEL domain containing 2), *TJP2*: tight junction protein 2, *OCLN*: occludin, *IGF1*: insulin-like growth factor 1, *IGF1R*: insulin-like growth factor 1 receptor, *GHR*: growth hormone receptor, *TLR4*: toll-like receptor 4, 18S rRNA**:** 18S ribosomal RNA.

3.2. Experiment II: Early development of TSL chicks as affected by dietary methionine supplementation

3.2.1. Experimental animals, housing, and dietary treatments

The Tetra SL LL layer hybrid (TSL) eggs were obtained from Bábolna Tetra Ltd. (Bábolna, Hungary) and incubated at standard temperature and humidity (37.8 ˚C and 50% Rh, PLM 3600, PL Machine KFT Budapest-Hungary). After hatching, a total of 96 one-day-old chicks were weighed and distributed to six treatments, with four replicates having four chicks (125 cm^2/bird) per pen. The experimental design was a complete randomized block with a 2 x 3 factorial arrangement (2 Met sources x 3 Met levels). The chicks had similar initial body weights for each treatment, and pen, feed, and water were provided ad libitum. The pens were bedded with wood shavings. Infrared lamps (Optima Plus II 175 W) provided extra heating in the pens, and the temperature in the pen was maintained according to the breeder's recommendation (BÁBOLNA TETRA LTD. 2020). The chicks were fed ad-libitum of a standard mash diet that was formulated according to the manual guidelines of TSL, except for the Met levels, for 28 days. The diets were formulated to include 90, 100, and 110% of either DL (MetAmino, feed grade 99%, Evonik GmbH, Wesseling, Germany) or L-Met (L-Met 100, feed grade 99%, CJ Europe GmbH., Schwalbach/Taunus, Germany) of the nutrient requirements of the breeders (Table 5).

Table 5

Composition (%) and nutrient content (g/kg) of experimental diets formulated with different methionine inclusion levels compared to the recommendation*

		DL-Met			L-Met		
		90%	100%	110%	90%	100%	110%
Corn		59.79	59.84	59.88	59.79	59.84	59.88
Soybean meal, 46%		27.99	27.92	27.86	27.99	27.92	27.86
Fishmeal 65%		5	5	5	5	5	5
Sunflower oil		3.64	3.62	3.6	3.64	3.62	3.6
Limestone		1.13	1.13	1.13	1.13	1.13	1.13
MCP		1.61	1.61	1.61	1.61	1.61	1.61
Salt		0.3	0.3	0.3	0.3	0.3	0.3
L-Lys		-	-	-	-	-	-
DL-Met		0.04	0.08	0.12	-	-	-
L-Met		-	-	-	0.04	0.08	0.12
L-Thr		-	-	-	-	-	-
L-Trp		-	-	-	-	-	-
Vit. and min. premix[a]		0.5	0.5	0.5	0.5	0.5	0.5
Total		100	100	100	100	100	100
	Requirement*	Calculated nutrient content					
AMEn, MJ/kg	**12.35**	12.35	12.35	12.35	12.35	12.35	12.35
CP[b]	**20**	21.5	21.3	21.8	22.2	21.0	21.4
sidLys	**1.0**	1.008	1.007	1.005	1.008	1.007	1.005
sidMet	**0.4**	0.36	0.4	0.44	0.36	0.4	0.44
sidThr	**0.63**	0.65	0.65	0.65	0.65	0.65	0.65
sidTrp	**0.2**	0.42	0.42	0.42	0.42	0.42	0.42
Ca	**1.0**	1.0	1.0	1.0	1.0	1.0	1.0
available P	**0.48**	0.48	0.48	0.48	0.48	0.48	0.48
Na	**0.17**	0.17	0.17	0.17	0.17	0.17	0.17
sidMet/sidLys	**0.4**	0.357	0.397	0.438	0.357	0.397	0.438
sidThr/sidLys	**0.63**	0.647	0.647	0.647	0.647	0.647	0.647
sidTrp/sidLys	**0.2**	0.413	0.414	0.414	0.413	0.414	0.414

* TETRA-SL LL Commercial Layer Management Recommendation Guide.

[a] 1 kg premix provided: 3,000,000 NE vitamin A, 600,000 NE vitamin D3, 14,700 mg/kg vitamin E, 600 mg vitamin K3, 450 mg vitamin B1, 150 mg vitamin B2, 3600 mg Ca-d-Pantothetane, 1200 mg vitamin B6, 7 mg vitamin B12, 33 mg biotin, 7507 mg niacin, 180 mg folic acid, 84,000 mg choline chloride, 19,800 mg Zn, 2880 mg Cu, 14,418 mg Fe, 19,800 mg Mn, 270 mg I, 63 mg Se, 18 mg Co

[b] analysed value

3.2.2. Measurements

All chicks in each pen were included in measuring the length of the fourth primary feather and body weight weekly for four weeks (ZENG *et al.* 2015). The length of the feather was measured in millimeters using a Vernier-calliper with 0.01 mm accuracy. Due to the immeasurable amount of feed waste and the fact that the experiment utilized layer genotype, therefore the average feed intake and feed conversion ratio were not calculated in this study. No mortalities were observed for the entire experimental period.

3.2.3. Blood sampling and hematological analysis

At the end of the trial (28 days of life), 2 chicks from each pen (8 birds per treatment group) were randomly selected. Blood (approximately 0.5 mL) was collected from the cutaneous ulnar vein, also known as the brachial wing vein, into EDTA-coated tubes following the procedures of KELLY & ALWORTH (2013). The blood samples were placed on ice immediately and transferred to the laboratory. Before analysis, blood samples were allowed to come to room temperature and gently mixed. A pre-diluent method was used (according to the Urit-3000Vet Plus automated hematology analyzer operation manual), where 20 μL of blood was pippeted and diluted to 1 mL of dilution buffer. Hematological parameters such as the number of red blood cells (RBC, 10^{12}/L), hemoglobin (Hb, g/dL) concentration in the blood, hematocrit (Ht; %), the number of white blood cells (WBC, 10^{9}/L), and platelet count were determined. In addition, the following mean corpuscular volume of red blood cells (MCV, fL), mean corpuscular hemoglobin (MCH, pg), mean corpuscular hemoglobin concentration (MCHC, g/dL), lymphocyte percentage and count (LYM% and LYM#), mid-range (eosinophil + basophil) percentage and number (MID% and MID#), and granulocyte percentage and number (GRAN% and GRAN#) were obtained. The hematological parameters were analyzed using an automated hematology analyzer (URIT-3000 Vet Plus, Orvostechnika Ltd., Budapest). Reading was done in triplicate, and the average of the runs was taken.

3.3. Statistical analysis

The statistical analyses were performed using R-version 4.2.2 (R CORE TEAM 2022). In the data from the *in-ovo* feeding experiment, the individual bird (chick) was considered an experimental unit for all parameters except for the histology data. Data were analyzed using a two-factor analysis of variance (ANOVA), and a general linear model was appropriate for evaluating the fixed effects (the genotypes and Met sources) and their interactions. When the interaction effect was significant, the treatment effect was analyzed for genotypes separately. Tukey post hoc test was performed to compare the mean difference between the treatments. Data were presented as means, and the significance level for differences was set at $P < 0.05$.

Dietary Met supplementation experiment data analysis was performed with R-version 4.2.2. The pen was considered an experimental unit for breeders. The data were checked for normality by using the Shapiro-Wilrk test. Data were analyzed by using analysis of variance (ANOVA) and a general linear model appropriate for evaluating the main effects (methionine sources and dietary methionine levels). The experimental unit for ADG was the pen ($n = 4$/treatment), while for BW, FL ($n = 16$/treatment), and hematological parameters ($n = 8$/treatments), the individual birds were considered the experimental unit. Tukey's multiple comparison test was performed when analyses of variance indicated a significant difference.

4. RESULTS AND DISCUSSION

4.1. Effect of *in-ovo* injection of different methionine sources on physiological parameters of TETRA SL hybrid and Hungarian partridge-colored chicks post-hatch

4.1.1. Hatchability

The *in-ovo* injection method has been broadly used in poultry studies to improve the growing embryo's physiological status (ELNESR *et al.* 2019). Hatchability decreased after injection with increasing levels of both Met sources (Table 6). All injected eggs showed lower hatchability than the non-injected eggs. The highest hatchability (100%) was found in the control (non-injected eggs) for the TSL genotype, whereas the HPC control group had approximately 90% hatchability. In ovo injection with only saline solution decreased hatchability by 11 and 5 percentage points for the TSL and HPC genotypes, respectively. Injecting 5 mg of DL-Met into TSL eggs reduced hatchability by 10%, while 5 mg L-Met reduced hatchability by 24.1% compared to control. In the HPC genotype, the 5 mg injected groups showed a 16.9% decrease in hatchability in both Met sources. Higher doses (10 and 15 mg) of either Met source were detrimental to both the genotypes. This result corroborates the results reported by SOGUNLE *et al.* (2019), who reported 2.70% hatchability in 10 mg L-methionine-injected eggs from indigenous chickens. COŞKUN *et al.* (2014) similarly reported low hatchability when DL-methionine was injected into fertile broiler eggs compared to the control. However, the hatchability of the eggs injected with 5 mg of Met sources in our trial ranged from 72% to 90%, which is similar to what has been reported in the literature (percentages between 70% and 80%) that *in-ovo* injected Met (COŞKUN *et al.* 2014; COŞKUN *et al.* 2018; ELWAN *et al.* 2021). Furthermore, a recent study also reported that injection of Met at levels more than 1.0% (10 mg/egg) proved to be toxic to embryos due to sharp decreases in hatchability and increased embryonic mortality (FARIAS *et al.* 2023). Generally, these findings are similar to those reported for the in-ovo injection of other amino acids (COSKUN *et al.* 2018; PEEBLES 2018). The increase in amino acid concentration in the solution employed for in-ovo injection leads to a proportional increase in the osmolarity of the solutions, which causes an osmotic imbalance between the injected solution and embryonic medium mortality (FARIAS *et al.* 2023). The low hatchability observed at high concentrations may be attributed to the osmolality difference caused by the injected solution and the limited physiological ability of the embryo to absorb Met.

Table 6

Effect of *in-ovo* injection different amount of Met at 17.5th day of embryo development on hatchability of the TSL and HPCgenotypes (%)[a]

Hatchability	**Control**	**Saline**	**DL-Met**			**L-Met**		
Genotype			5 mg	10 mg	15 mg	5 mg	10 mg	15 mg
TSL	100.0	88.9	90.0	30.0	10.0	75.9	44.8	23.1
HPC	89.1	84.2	72.2	31.6	22.2	72.2	11.8	17.6

[a] Hatchability (%) is calculated as the percentage of eggs hatched over the number of fertile eggs subjected to the respective treatment. TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed, Control: non-injected group, Saline: saline injected group, DL-Met: DL-Met injected group, L-Met: L-Met injected group.

Due to the very low hatchability of higher dose in-ovo injection of Met sources it was decided that blood and tissue sampling was carried out only on control and 5 mg supplemented groups.

4.1.2. Hatching body weight and relative liver and heart weight

Examining all samples together (i.e, control, saline, and experimental) as a pooled effect, a significant difference was observed between the two genotypes in the following values: hatching body weight, absolute heart weight, and relative heart weight (Table 7, $P < 0.05$). The hatching body weight and heart weight values were significantly higher for the TETRA-SL LL hybrid and lower for the Hungarian Partridge Colored hen breed layer genotypes. In contrast, the relative liver weight was significantly higher in HPC chicks than in TSL chicks. However, there was no significant interaction effect between treatment and genotype, except for the absolute liver weight (Table 7, $P = 0.0279$). The 5 mg DL-Met and control groups had significantly higher hatching weights compared to the 5 mg L-Met treatment, but not significantly higher than that of the saline treatment. Additionally, in the TSL genotype, 5 mg of L-Met treatment significantly reduced hatching weight *($P = 0.006$)* compared to all treatment groups, while no significant effect was observed in the HPC genotype.

Table 7

Effect of *in-ovo* injection of 5 mg DL and L-Met in eggs of TSL and HPC genotypes at 17.5 days of incubation on hatching performance parameters

Parameter		HBW (g)	ALW (g)	AHW (g)	RLW (%)	RHW (%)
Pooled effects						
Genotype	TSL	41.2 [a]	1.27	0.37 [a]	2.84 [b]	0.90
	HPC	35.7 [b]	1.15	0.33 [b]	2.97 [a]	0.92
Treatment	Control	39.7 [a]	1.17	0.36 [a]	2.99	0.92
	Saline	38.2 [ab]	1.17	0.34 [ab]	3.05	0.90
	DL-Met	39.5 [a]	1.21	0.37 [a]	3.08	0.95
	L-Met	36.5 [b]	1.10	0.32 [b]	3.02	0.88
P - values	Genotype	**0.0001**	0.6365	**0.0289**	**0.0321**	0.1641
	Treatment	**0.0350**	0.5025	**0.0380**	0.9011	0.5653
	Interaction	0.2186	**0.0279**	0.3487	0.0631	0.3736
RMSE		3.291	0.229	0.015	0.578	0.132
Treatment effect by genotype						
TSL	Control	43.6 [c]	1.20	0.38	2.78	0.88
	Saline	40.7 [c]	1.29	0.37	3.15	0.92
	DL-Met	41.6 [c]	1.12	0.37	2.71	0.89
	L-Met	38.3 [d]	1.08	0.34	2.81	0.88
	P-value	**0.0061**	0.2209	0.2898	0.3348	0.8756
	RMSE	2.7041	0.2113	0.0465	0.5145	0.1144
HPC	Control	35.69	1.11	0.35	3.16	0.98
	Saline	35.48	1.00	0.31	2.84	0.89
	DL-Met	37.71	1.33	0.37	3.51	0.99
	L-Met	34.81	1.12	0.31	3.22	0.88
	P-value	0.4673	0.0909	0.0732	0.2323	0.3059
	RMSE	3.7875	0.2452	0.0557	0.6355	0.1467

[a,b] Means within the column of the main effect with similar superscript letters are not significantly different and [c,d] means with similar superscript letters are not signicant different within column of TSL genotype ($P > 0.05$). HBW: Hatching body weight, ALW: absolute liver weight, AHW: absolute heart weight, RHW: relative heart weight, RLW: liver relative weight. TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed, control: non-injected, saline: saline injected, DL-Met: DL-Met injected, L-Met: L-Met injected (5 mg of Met /0.5 mL of NaCl).

OHTA *et al.* (2001) observed that inoculating solutions based on amino acids *in-ovo* resulted in higher hatching rates and live birth weights. However, unlike to our study, their solutions comprised a mixture of 18 amino acids. In a study on the inoculation of solutions containing different amino acids (*in-ovo)*, JOHRI (2004) found that the amino acid solution resulted in a higher starting weight of the chicks. As a result, the presence of a variety of amino acids, rather than just one, in the inoculating fluid may have influenced the positive outcome. According to TONA *et al.* (2003), the quality of day-old chicks is proportional to the quality of the incubation. The injection with 30 mg of lysine

had the lowest average score (84.77) compared to the control group (89.16), methionine 20 mg (89.13), methionine 30 mg (89.10), and lysine 20 mg (88.05), which had the highest average scores. UNI *et al.* (2005) found that feeding late-term embryos *in-ovo* injection with carbohydrates enhanced hatching weight by 5 to 6% compared to controls. *In-ovo* administration of all 20 AAs, chick weight increased by 3.6 percent and 2.1 percent, respectively, according to OHTA *et al.* (2001) and BHANJA *et al.* (2004a). *In-ovo* administration of AA is thought to increase AA usage and, as a result, decrease in AA degradation by the embryo. In rat hepatocytes, free AAs also reduce protein breakdown (VENERANDO *et al.* 1994). On the other hand, the relative liver weight was significantly influenced by *in-ovo* treatment and genotypes. DL-Met injection positively influenced relative liver weight in the Hungarian genotype. This result concurs with the findings of COŞKUN *et al.* (2018), who found that relative liver weight was positively influenced by *in-ovo* injection of methionine. L-Met *in-ovo* feeding resulted in a decrease in hatching weight; this might be due to physiological properties and utilization by the growing embryo being different from that of DL-Met. These findings indicate that L-Met *in-ovo* feeding at 5 mg/egg might not benefit the laying chicken genotypes compared to DL-Met *in-ovo* feeding.

4.1.3. Jejunum histomorphometry

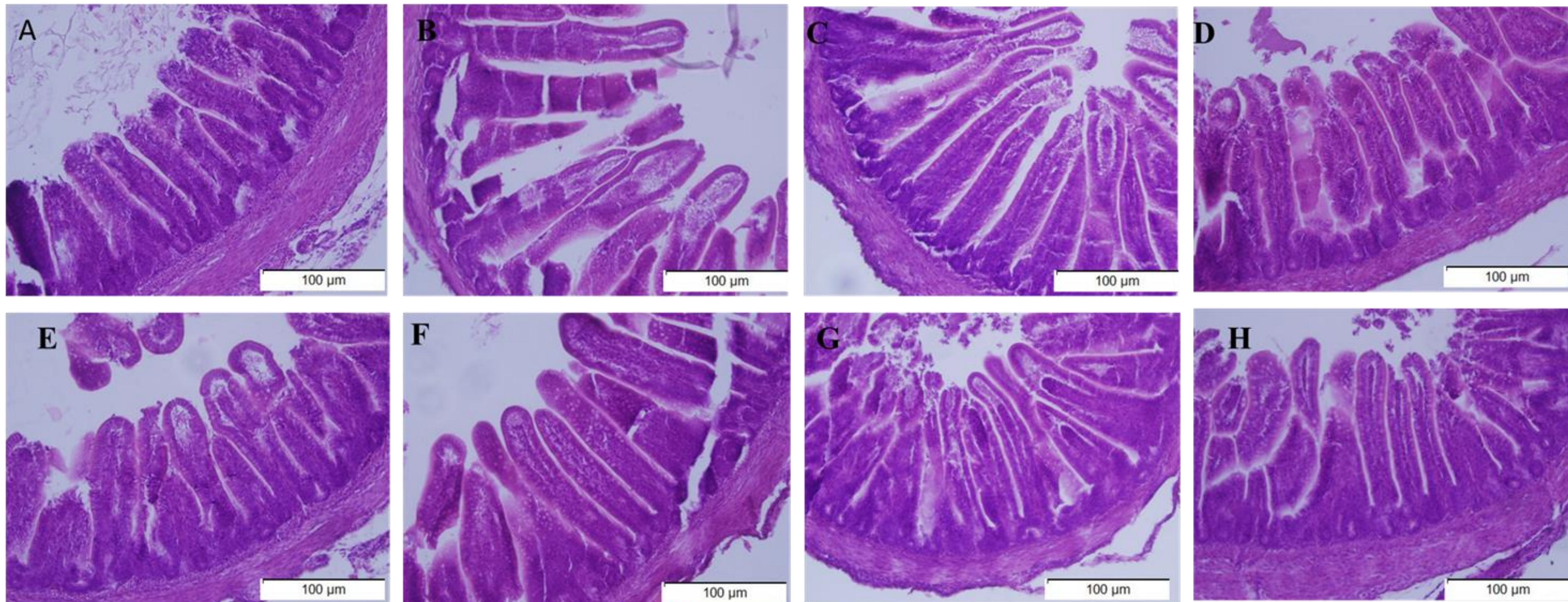
Upon examination with H&E staining (see representative images in Picture 6), it was revealed that the jejunum villi height was higher in the DL-Met group than in both the L-Met group and the control treatment (Table 8). However, saline group had a significantly increased villus height compared to the other groups. The genotype influenced villus width at the basal and middle, and villus area ($P < 0.05$). TSL genotype had reduced basal and middle villus widths and villus area compared to HPC (Table 8). The *in-ovo* injection of DL-Met significantly increased all the jejunum morphology parameters except crypt depth when compared to the control (Table 8, $P < 0.05$), but had a similar effect as the saline group in villus width at the basal and villus area. Both L-Met and saline supplementation were effective in increasing villus apical width and crypt depth compared to the DL-Met and control groups. Therefore, the DL source of Met was more effective in improving the villus height/crypt depth ratio and villus area, while L-Met increased apical width and crypt depth, with effects comparable to those of saline treatment. The interaction effect of genotype and *in-ovo* injection treatment was significant for all measured parameters except villus crypt depth and villus height crypt

ratio (Table 8, $P < 0.05$); therefore, data were evaluated separately for each genotype. In the TSL genotype, the villus basal and middle width, and villus area decreased by *in-ovo* feeding of L-Met compared other treatment groups. In addition, DL-Met in-ovo injection increased the villus height to crypt depth ratio and decreased the crypt depth compared to all other treatments. Moreover, both Met sources increased the jejunum surface area and width at the basal and middle of the villus in the HPC genotype when compared to the control, but the difference was not significant when compared with the saline group (Table 8). Saline significantly increased villus height compared to all other treatments in the HPC genotype.

Picture 6

Cross sections of the jejunum from newly hatched TSL and HPC chicks as responses to *in-ovo* injected with methionine sources

(representative images: Scale bar = 100 µm, 200x magnification)



A to D representative images of jejunum villi of HPC chicks hatched from A) non-injected. B) saline-injected group. C) Injected with 5mg of DL-Met group. D) Injected with 5 mg of L-Met group. E to H representative images of jejunum villi of TSL chicks hatched from E) non-injected group, F) saline-injected group, G) DL-Met-injected group, H) L-Met injected group. TSL= TETRA SL layer hybrid, HPC= Hungarian Partridge colored hen breed.

Table 8

Effect of *in-ovo* injection with 5 mg of DL and L-Met on intestine histology parameters of newly hatched chicks from TSL and HPC genotypes

Parameter		Villus basal width µm	Villus apical width µm	Villus middle width µm	Villi height µm	Crypt depth µm	Villus Height/ Crypt depth ratio	Villus area (x10^{3} µm^{2})
Pooled effects								
Genotype	TSL	74.72 ^{b}	61.63	72.75 ^{b}	349.9	61.89	5.89	80.09 ^{b}
	HPC	82.47 ^{a}	61.06	79.79 ^{a}	345.2	61.45	5.73	87.05 ^{a}
Treatment	Control	72.79 ^{b}	54.72 ^{c}	73.35	321.9 ^{c}	60.43 ^{b}	5.60 ^{b}	74.69 ^{b}
	Saline	77.25 ^{ab}	63.76 ^{ab}	75.26	378.9 ^{a}	69.25 ^{a}	5.59 ^{b}	89.92 ^{a}
	DL-Met	80.85 ^{a}	61.52 ^{b}	80.62	349.3 ^{b}	53.64 ^{c}	6.62 ^{a}	89.10 ^{a}
	L-Met	77.50 ^{ab}	66.68 ^{a}	73.52	339.3 ^{bc}	64.96 ^{a}	5.31 ^{b}	77.89 ^{b}
***P*-values**	Genotype	0.0001	0.861	0.001	0.577	0.522	0.714	0.013
	Treatment	0.0001	0.001	0.065	0.001	0.001	0.001	0.001
	Interaction	0.0001	0.001	0.001	0.001	0.001	0.145	0.001
	RMSE	15.48	10.66	13.66	48.4	9.30	1.32	21.27
Treatment effects by genotypes								
TSL	Treatment							
	Control	80.14 ^{d}	57.07 ^{e}	79.65 ^{d}	332.0 ^{e}	62.38 ^{d}	5.59 ^{e}	83.05 ^{d}
	Saline	71.23 ^{de}	58.04 ^{de}	71.71 ^{d}	359.8 ^{d}	67.43 ^{d}	5.46 ^{e}	81.35 ^{d}
	DL-Met	80.82 ^{d}	64.50 ^{d}	76.75 ^{d}	347.9 ^{de}	52.59 ^{e}	6.71 ^{d}	84.31 ^{d}
	L-Met	66.10 ^{e}	66.40 ^{d}	63.26 ^{e}	354.0 ^{d}	66.94 ^{d}	5.43 ^{e}	70.52 ^{e}
	P-value	0.0001	0.0024	0.0001	0.0009	0.0001	0.0041	0.0450
	RMSE	14.51	10.62	12.73	42.6	9.61	1.43	20.90
HPC	Treatment							
	Control	65.03 ^{e}	52.33 ^{e}	64.04 ^{e}	303.5 ^{f}	59.70 ^{ef}	5.14 ^{e}	61.22 ^{f}
	Saline	85.10 ^{d}	66.15 ^{d}	81.25 ^{d}	411.1 ^{d}	72.34 ^{d}	5.81 ^{de}	104.38 ^{d}
	DL-Met	84.99 ^{d}	58.35 ^{e}	84.49 ^{d}	350.7 ^{e}	54.68 ^{f}	6.54 ^{d}	93.89 ^{de}
	L-Met	88.65 ^{d}	68.21 ^{d}	83.39 ^{d}	325.1 ^{f}	63.06 ^{de}	5.20 ^{e}	84.98 ^{e}
	P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
	RMSE	14.62	10.71	14.62	54.1	8.94	1.17	21.69

^{a,b,c} Means with similar superscript letters are not significantly different within the column of main effect and ^{d,e,f} means with similar superscript letters are not signicant different within column of TSL and HPC genotype respectively ($P > 0.05$), TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed. Control: non-injected group, Saline: Saline injected group, DL-Met: DL-Met injected group, L-Met: L-Met injected group, RMSE - the root of the Mean Square Error.

Our results indicated that the injection of saline and DL-methionine on day 17.5 of embryonic development increased the villus surface area compared to the L-Met and control groups. These results suggest an improved jejunal absorption rate (NAZEM *et al.* 2019). DL-Met injection improved intestinal development compared with the control in all but one measured parameter (Picture 6 and Table 8). This indicates that DL-Met is

more efficient in stimulating the proliferation and differentiation of enterocytes than L-Met (CHEN *et al.* 2021). Studies have shown that chicken embryo enterocytes respond to the nutrient (Met) by proliferating, differentiating and facilitating intestinal epithelium renewal (NAZEM *et al.* 2017; COSKUN *et al.* 2018; CHEN *et al.* 2021). In our study, the L-Met *in-ovo* feeding and saline *in-ovo* injection increased the villus crypt depth while the DL-Met supplementation significantly decreased it relative to the control group, indicating rapid tissue expansion. This corroborates the results reported when the sulfur amino acids were *in-ovo* injected on the first day of incubation, increasing the ileal crypt depth at hatching (GAMBOA GONZALES *et al.* 2022). Few studies have reported the effects of *in-ovo* feeding of Met on the jejunum, the primary site for nutrient absorption. Their findings align with our experimental results; *in-ovo* feeding of Met enhances the jejunum villus height and width, improving the production performance post-hatch (NAZEM *et al.* 2017; CHEN *et al.* 2021). However, there are limited studies on the effects of the *in-ovo* feeding of the two Met sources on intestinal development. Our experiment demonstrated that DL-Met *in-ovo* feeding as well as saline *in-ovo* injection increased villus height, facilitating the villus's tissue differentiation and absorption capacity. Unexpectedly, saline *in-ovo* showed positive effects on the villus height and comparable effects with *in-ovo* feeding of Met sources on villus basal width and villus area. This indicates that saline may have a positive effect on the jejunum morphology and it may be attributed to the moisturization. In addition, L-Met *in-ovo* feeding increased the cellular turnover of the tissue, as indicated by deeper crypt depth (CHEN *et al.* 2021; ELWAN *et al.* 2021). A recent study reported that Met deficiency suppressed intestinal organoid formation and size. In contrast, the Met hydroxyl analogue promoted intestinal stem cell regeneration while decreasing cell differentiation compared to L-Met (WANG *et al.* 2022). These findings indicate that the cells' bioavailability and utilization of the Met source might differ, hence, different physiological functions. Embryonic intestinal development is not only affected by nutrients but also by intrinsic factors like genotypes. In this study, the commercial layer genotype has a small absorptive surface area, as shown by decreased villus width compared to the native genotype. Lastly, the difference observed in this study might be the function of the age of the studied animal, as these findings do not agree with the experiments in turkey, where 0.33% supplementation of DL and L-Met did not affect the small intestinal morphometry on days 7 and 28 of life (PARK *et al.* 2018). In addition, experiments with piglets that received DL-Met or L-Met for 3 weeks did not differ in their gut morphology or their expression of tight junction genes. Furthermore, two Met

sources' effect on the morphology via feed supplementation has been reported in Pekin ducks, where dietary DL-Met or L-Met supplementation increased ileal villus height at days 14 and 35 (ZHANG *et al.* 2019).

4.1.3. Liver enzymes and kidney status indicators (AST, ALT and Uric acid) and Ferric reducing ability of the plasma (FRAP)

The genotypes did not significantly influence plasma parameters except for AST: ALT, uric acid, and FRAP (*P < 0.05,* Table 9). However, the TSL genotype has a higher uric acid and FRAP levels and a lower AST:ALT ratio than the HPC genotype (*P < 0.05).* Treatment with *in-ovo* feeding of 5 mg Met sources significantly influenced the plasma uric acid, AST, ASL/ALT ratio, and FRAP (*P < 0.05*). In addition, treatment tends to influence the ALT levels in the plasma of chicks (*P = 0.0563).* The interaction effect of in-ovo feeding and genotype was observed in all measured parameters except for the AST:ALT ratio and ALT level (*P < 0.05).* Treatment and genotype interaction tended to have an effect on the ALT levels (*P = 0.0772). In-ovo* feeding with L-Met significantly lowered the levels of FRAP (*P < 0.05)* in the plasma compared with the other treatment groups. In addition, the L-Met group had a significantly lowered uric acid content than the control (*P < 0.05)* but was not significantly different from the DL-Met and saline groups (*P > 0.05).* The saline group had a significantly higher AST level and ASL/ALT ratio than all other groups (*P < 0.05,* Table 9*).*

In the HPC genotype, treatment significantly influenced all the plasma parameters measured. A comparable effect of control, DL-Met and L-Met is observed in all parameters except for FRAP. The DL-Met group exhibited significantly higher (*P < 0.05*, Table 9) plasma FRAP levels than L-Met but was comparable with the other experimental groups in the HPC genotype. Saline *in ovo* injection significantly elevated the AST level and AST:ALT ratio compared to all other treatment groups (*P < 0.05).* In the other hand, it significantly reduced the ALT level compared to the control group in the HPC genotype (*P < 0.05).* However, in the TSL genotype, no significant effect of treatment was observed in either AST or ALT levels or the AST:ALT ratio (*P > 0.05*). DL-Met significantly increased the uric acid content in the TSL chicks compared to L-Met (*P < 0.05),* but not better than the other groups (Table 9*).*

Table 9

The effect of *in-ovo* injection of 5 mg DL and L-Met on the plasma biochemical parameters of one-day-old TSL and HPC chicks

Plasma parameter		AST (U/L)	ALT (U/L)	AST/ ALT	Uric acid (µmol/l)	FRAP (µM $FeCl_2$ equivalent)
Pooled effects						
Genotype n = 32						
	TSL	93.63	57.00	1.64 ^{b}	301.73^{a}	78.96 ^{a}
	HPC	98.08	59.50	2.02 ^{a}	267.67^{b}	70.27 ^{b}
Treatment n = 8						
	Control	93.20 ^{b}	63.63	1.42 ^{b}	323.1 ^{a}	81.36 ^{a}
	Saline	109.13 ^{a}	49.90	2.81 ^{a}	283.99 ^{ab}	73.01 ^{a}
	DL-Met	88.11 ^{b}	63.59	1.43 ^{b}	284.22 ^{ab}	84.80 ^{a}
	L-Met	93.12 ^{b}	55.89	1.66 ^{b}	247.49 ^{b}	59.30 ^{b}
P- Values	Genotype	0.2750	0.6820	**0.0342**	0.0391	**0.0171**
	Treatment	**0.0017**	0.0563	**0.0001**	**0.0110**	**0.0002**
	Interaction	**0.0007**	0.0772	**0.2331**	**0.0010**	**0.0021**
RMSE		15.17	16.35	0.82	59.74	14.88
Treatment effect by genotype n = 8						
TSL	Control	102.22	57.23	1.71	329.44 ^{de}	90.12 ^{d}
	Saline	94.09	57.56	1.56	365.36 ^{d}	87.39 ^{d}
	DL-Met	87.54	63.47	1.42	276.59 ^{e}	78.21 ^{d}
	L-Met	91.19	49.73	1.87	235.54 ^{f}	60.12 ^{e}
	P-value	0.3200	0.3584	0.2361	**0.0075**	**0.0069**
	RMSE	15.97	15.024	0.4422	61.93	16.89
HPC	Control	84.18 ^{e}	70.02 ^{d}	1.12 ^{e}	316.78 ^{d}	72.60 ^{e}
	Saline	124.16^{d}	42.23 ^{e}	4.06 ^{d}	202.61 ^{e}	58.62 ^{e}
	DL-Met	88.67 ^{e}	63.71 ^{de}	1.44 ^{e}	291.85 ^{d}	91.38 ^{d}
	L-Met	95.06 ^{e}	62.03 ^{de}	1.45 ^{e}	259.43 ^{de}	58.48 ^{e}
	P-value	**0.0001**	**0.0267**	**0.0001**	**0.0035**	**0.0001**
	RMSE	14.26	17.58	1.0761	57.81	12.34

^{a,b} Means with the same superscript letters are not significantly different within the main effect column and ^{d,e,f} means with similar superscript letters are not signicant different within column of TSL and HPC genotype respectively (*P > 0.05)*. TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed, Control: non-injected, Saline: saline-injected: DL-Met: DL-Met injected, L-Met: L-Met injected. AST: aspartate aminotransferase, ALT: alanine aminotransferase, AST/ALT: aspartate aminotransferase to alanine aminotransferase ratio, FRAP: ferric reducing ability of the plasma.

The findings of the *in-ovo* trial indicated that DL-Met improved the liver status, as indicated by the lowest mean of AST (88.11 U/L). The increase in the AST liver enzyme level above 230 U/L has been used to indicate hepatic damage in birds (CHITTURI & FARRELL 2013). The AST/ALT ratio and the AST and ALT are used to demonstrate liver damage in mammals and birds (BONA *et al.* 2018). AST is considered a sensitive avian indicator of hepatic damage and muscle injury (HARR *et al.* 2005), while ALT is a non–specific cell damage indicator (HOCHLEITHNER *et al.* 1994). The enzyme activities of AST and ALT can also be used as oxidative stress markers. The experiment with broilers exposed to heat stress reported increased activities of AST and ALT. However, Met supplementation to the birds under heat stress resulted in similar levels of AST and ALT as those birds under a thermal comfort zone (GASPARINO *et al.* 2018).

The plasma uric acid concentration was affected by *in-ovo* treatment, genotype, and their interaction ($P < 0.05$) (Table 9). The TSL chicks had higher uric acid concentrations as compared with their counterpart. At the same time, the chicks from the non-injected group also had higher uric acid, followed by the saline-injected and DL-Met group, and L-Met had the lowest level (Table 9). Uric acid is birds' important nitrogen metabolic end product (DONSBOUGH *et al.* 2010). Also, it plays an important role in the plasma's total antioxidant capacity; hence, it is used as an indicator of renal function and status (SONG *et al.* 2021). In our experiment, *in-ovo* injection of DL and L-Met in the eggs significantly reduced the uric acid content in the circulating blood of one-day chicks compared to the control. This result agrees with the findings by WANG *et al.* (2019), which found a linear decrease of the uric acid content with the supplementation of either DL or L-Met, which reflected the better utilization of dietary nitrogen in broilers due to Met addition (DONSBOUGH *et al.* 2010). This high uric acid concentration in non-injected could result from increased liver metabolic processes due to imbalanced amino acids in chicks (DONSBOUGH *et al.* 2010).

The ferric-reducing ability of the plasma (FRAP) involves antioxidants providing electrons to reduce ferric ions to the ferrous form. The higher the FRAP value, the stronger the antioxidant capability (KHAJALI *et al.* 2010), reflecting the plasma's water-soluble antioxidants (ZDUŃCZYK *et al.* 2017). The plasma FRAP is known to be composed of 60% uric acid, 15% ascorbic acid, 10% protein-SH groups, and 5% tocopherols (VOSSEN *et al.* 2011; ZDUŃCZYK *et al.* 2017). In our trial, the *in-ovo* injection of L-Met reduced the antioxidant power of the chicks as compared to control and DL-Met injected

chicks. This corroborates with the experiment by JANKOWSKI *et al.* (2017), which found no significant effect of increasing the L-Met level from 100% of the NRC to 150% of the recommendations, while in the other sources (MHA and DL-Met), the effects were significantly observed on FRAP. The reduction of FRAP by *in-ovo* injection of L-Met as compared to DL-Met might be because L-Met was better utilized by enterocyte cells and used as a more efficient substrate for protein metabolism than DL-Met (SHEN *et al.* 2014). However, this is supported by the plasma uric acid results, since more than 50% of plasma antioxidant power is due to uric acid. Also, given the fact that L-Met is readily available for utilization by cells (protein synthesis, intra-kinases, and oxidative stress) as compared to DL-Met, in which the D-isomer must be converted to L-isomer in the liver or kidney to be used by the cell (SHEN *et al.* 2015), because the key enzyme is available in the liver or kidney. Therefore, D-Met is not readily available for gastrointestinal cells. In addition, some research has also indicated that the expression of this key enzyme is very low in young animals. Moreover, in another experiment, the Met source, as well as the interaction of the source and the dose, has been indicated on FRAP (DL-Met Vs MHA), with DL-Met elevating the plasma FRAP (JANKOWSKI *et al.* 2018).

4.1.4. Tissues antioxidants

4.1.4.1. Tissues total GSH content

The *in-ovo* Met treatment and genotypes significantly affected the liver GSH content of the one-day-old chicks ($P < 0.001$), but a tendacy effect of their interaction was noted (Table 10, $P > 0.05$). The chicks hatched from non-injected eggs had a higher GSH content than those from injected groups (Figure 5A). The TSL genotype had a higher GSH content than its counterpart genotype in all treatments (Figure 5A). In the TSL genotype, the liver GSH concentration was in the following order: Non-injected > Saline-injected > DL-Met=L-Met. The GSH content was significantly reduced in the in-ovo injected groups than in the control group of TSL genotype (*$P < 0.05$).* However, HPC responds differently to the *in-ovo* treatments in the following order: Non-injected > DL-Met >L-Met = saline injected (Figure 5). The saline group had a significantly reduced GSH content of the liver as compared to the control ($P = 0.0236$) but not significantly different from other groups (*$P > 0.05$).* Additionally, L-Met tends to decrease the GSH content as compared to the control (*$P = 0.0665$).*

In the muscles, the GSH content was significantly influenced by genotype alone ($P < 0.05$). However, treatment tended to influence the muscle's GSH content (Table 10, $P =$

0.0751). The DL-Met supplementation significantly reduced the muscle GSH content in the HPC genotype compared to the control group (Figure 5B, $P < 0.05$). Furthermore, the saline group tended to have low muscles GSH when compared to the control group (*P = 0.0829).* The muscle GSH content of the TSL genotype was not affected by the treatments. In the intestine, no significant effect of treatment was observed in both genotypes (Figure 5C, $P > 0.05$).

Moreover, the genotype effect was significant on the GSH content of all tissues; the HPC genotype had higher GSH content in the muscles and lower in the liver and intestine than the counterpart genotype (Table 10, Figure 5A-C).

Table 10

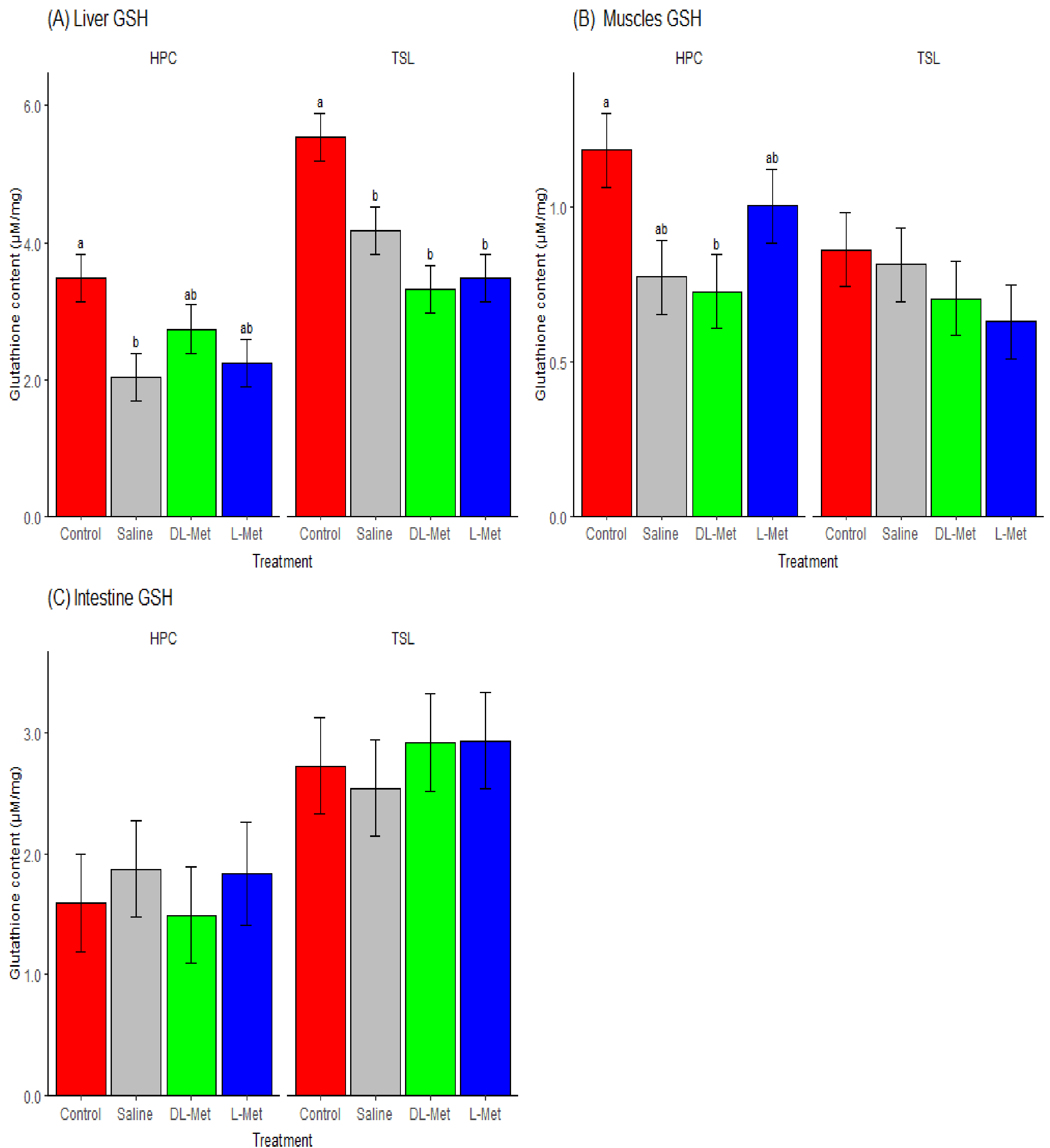
Summary of analysis of variance of the effect of genotype, treatement and their interaction on the tissues glutathione content in the chicks

Factor/Tissues GSH		Liver	Muscle	Intestine
P-values	Genotype	**0.0001**	**0.0488**	**0.0004**
	Treatment	**0.0001**	0.0751	0.9179
	Interaction	0.0926	0.2173	0.8208
RMSE		0.9829	0.3374	1.1313

GSH – glutathione, RMSE –root mean square error.

Figure 5

The effect of *in-ovo* injection of 5 mg DL and L-Met on tissues GSH (µM/mg of tissue) of 1d post-hatch chick of the TSL and HPC genotypes



A) Liver GSH in TSL and HPCchicks. B) Muscle GSH content in TSL and HPC genotypes. C) No significant effect of *in-ovo* feeding of Met sources on the Intestine GSH content in TSL and HPC genotypes.

[a,b] Means with similar letters are not significantly different within the genotype ($P > 0.05$). Data are presented as estimated means and SEM ($n = 8$). TSL: TETRA –SL layer hybrid, HPC: Hungarian Partridge colored hen breed, Control: non-injected, Saline: saline-injected, DL-Met: DL-Met injected and L-Met: L-Met injected.

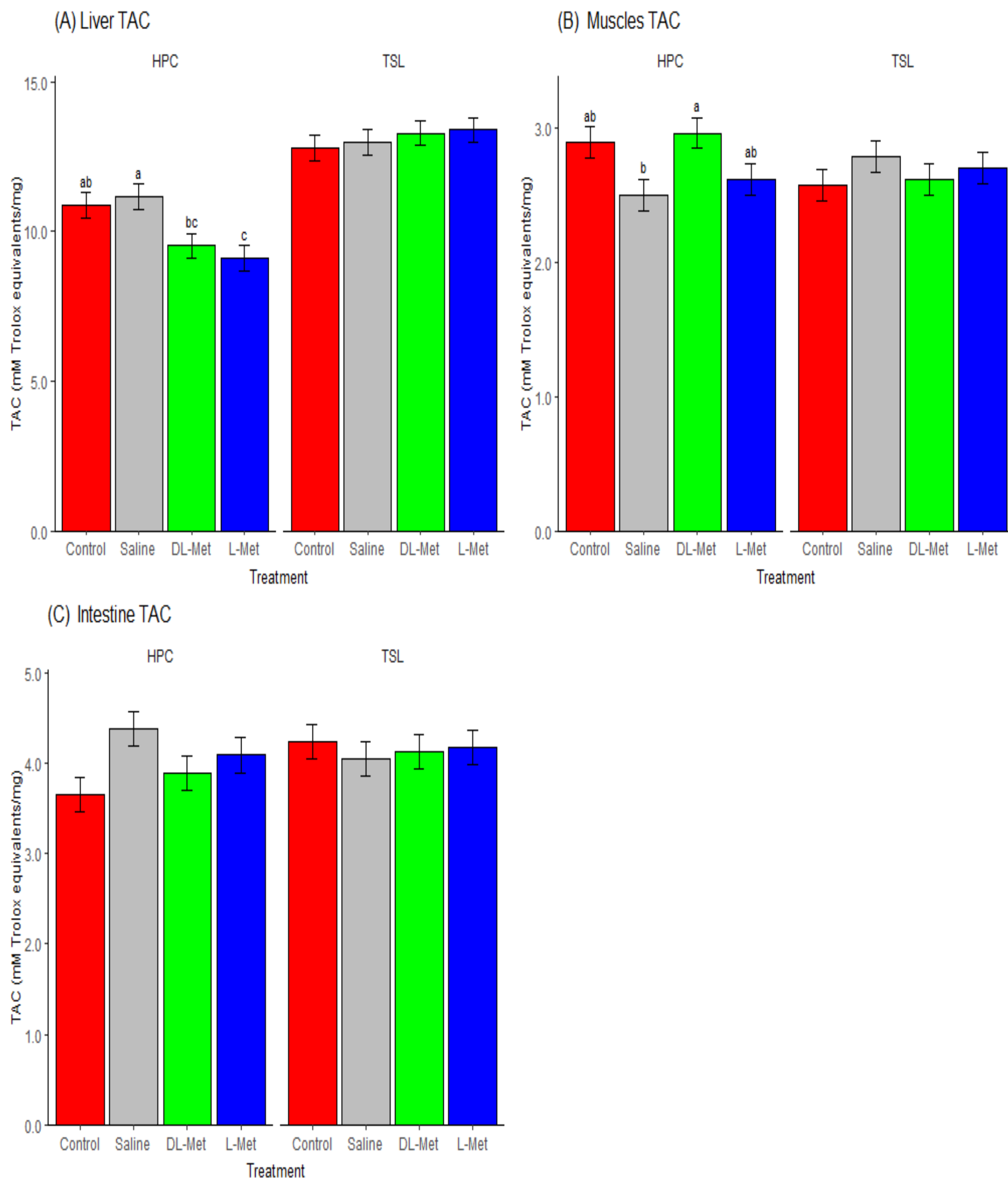
Glutathione (GSH) is one of the major/potential antioxidants that play a great role in removing free radicals such as peroxide hydroxyl radicals as well as play a part in maintaining the thiols from membrane proteins, in addition to acting as the substrate for GPx and glutathione reductase (NAIK & PANDA 2007; VARDI *et al.* 2008). The hypothesis here was that *in-ovo* injection with L-Met would positively enhance the redox status (GSH content) of the chicks as compared to DL-Met and control, since the L-Met could be immediately converted to Cys and then to GSH in the cytoplasm of the hepatocytes. However, the result of this experiment does not concur with our hypothesis and does not corroborate with other studies (ELNESR *et al.* 2019; ELWAN *et al.* 2019). Given the fact that it has been established that the first-Met-pass metabolism by the gut of a broiler affects its redox and growth performance (SHEN *et al.* 2015), in this experiment, the first-pass metabolism of Met by the gut of TSL and HPC chicks did not affect or improve their redox status by increasing the total GSH content as it was expected.

4.1.4.2. Total antioxidant capacity (TAC) in tissues

The genotype ($P = 0.0001$) and the interaction of the genotype and treatment ($P = 0.0069$) significantly influenced the total antioxidant capacity (TAC) of the liver tissue (Table 11). The interaction of genotype and treatment was more evident between the control and Met groups. In the HPC genotype, L-met reduced TAC significantly to the saline (*P = 0.006)* and control (*P = 0.0234)* groups, while DL-Met decreased it only compared to the saline-injected group (*P = 0.0378,* Figure 6A), but not in the TSL genotype. No significant effect of the Met treatments or genotype was observed in the muscle TAC. The DL-Met significantly increased the muscle TAC content compared to the saline-injected group in the HPC genotype but not in the TSL genotype (Figure 5B, $P < 0.05$). In the intestine, the *in-ovo* injection of Met did not influence the TAC in both genotypes ($P > 0.05$, Figure 6C). However, a tendency of interaction effect between the genotype and treatment was observed in intestine TAC content (*P = 0.1281).* The difference between the genotypes in the TAC level was only observed in the liver tissue, where TSL had higher levels than the HPC genotype (Table11, Figure 6A-C).

Figure 6

Effect of *in-ovo* feeding of 5 mg DL and L-Met on the TAC of day-old chicks tissues from the two genotypes



A) Liver TAC responses to the treatment in respective genotypes. B) Muscle TAC responses to treatment in the two genotypes. C) Intestine TAC in both genotypes.

[a,b,c] Means with similar letters are not significantly different within the genotype ($P > 0.05$). Data are presented as estimated means and SEM ($n = 8$). TSL: TETRA –SL layer hybrid, HPC: Hungarian Partridge colored hen breed. Control: non-injected group, Saline: Saline injected group, DL-Met: DL-Met injected group, L-Met: L-Met injected group.

Table 11

Summary of analysis of variance of the effect of genotype, treatement and their interaction on the tissues total antioxidant capacity in the chicks

Factor/Tissues TAC		Liver	Muscle	Intestine
P-values	Genotype	**0.0001**	0.3586	0.2873
	Treatment	0.1985	0.5718	0.4880
	Interaction	**0.0069**	**0.0224**	0.1281
RMSE		1.1900	0.3295	0.5342

TAC- Total antioxidant capacity, RMSE –root mean square error.

The TAC highlights the cell's ability to combat ROS and free radicals. The *in-ovo* feeding affected the TAC in a genotype-specific manner and in tissues (LUGATA *et al.* 2023). The previous study reported that *in-ovo* injection of Met-Cys improved both GSH and TAC in newly hatched broiler different tissues, including the small intestine (ELWAN *et al.* 2019). Furthermore, the genotype-specific differences were significant in the GSH contents across all tissues and only in the hepatic TAC level (Figure 6A-C). The TSL had higher GSH and TAC contents than the HPC; this might be due to the difference in the utilization of the Met by the two genotypes.

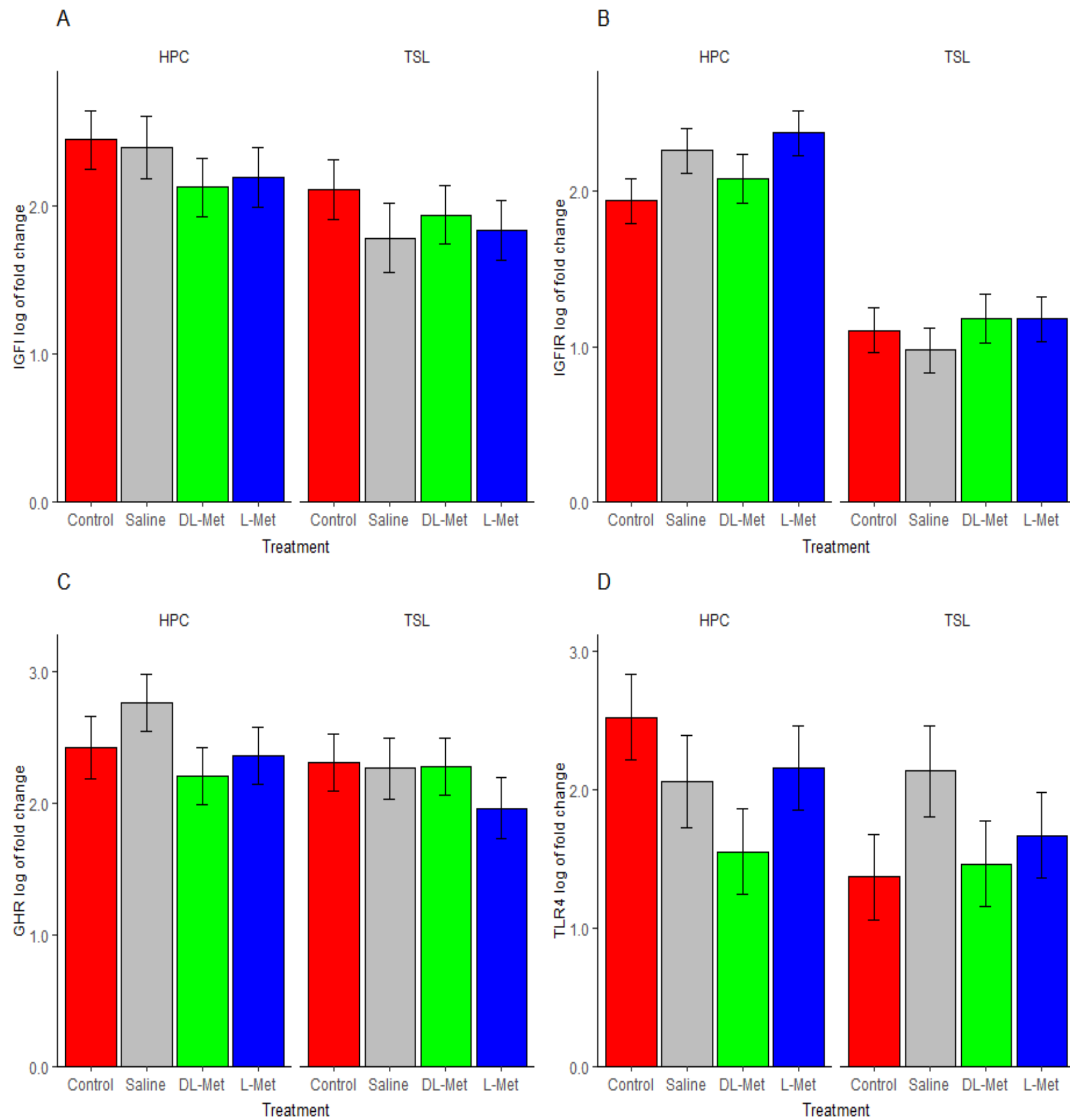
4.1.5. Hepatic gene expression

The genotype significantly influenced the expression of the *IGF1* and *IGF1R* genes (Figure 7A and B, $P < 0.05$), and tended to influence the *TLR4* (Figure 7D, $P = 0.0579$) and *GHR* gene expression (Figure 7C, $P = 0.1505$). There is no interaction effect of genotypes and *in-ovo* feeding on the gene expression (Table 12).

There were no significant influences on the expression of studied hepatic genes of one-day-old chicks by the *in-ovo* feeding of methionine treatments (Figure 7, $P > 0.05$). However, the chicks responded differently to the *in-ovo* feeding of Met sources within the genotypes. In the HPC genotype, *in-ovo* feeding of L-Met tended to upregulate the expression of *IGF1R* (Figure 7B, $P = 0.16$). In contrast, DL-Met tended to downregulate the expression of *TLR4* (Figure 7D, $P = 0.12$) compared to the control. No significant effect or tendency in the expressions of the genes (*IGF1, IGF1R, GHR*, and *TLR4*) is noted for TSL genotype chicks (Figure 7A-D).

Figure 7

Effect of *in-ovo* feeding of 5 mg DL and L-Met on the liver gene expression related to growth and immunity of TSL and HPC chicks



(A) *IGF1* gene expression. (B) *IGF1R* gene expression. (C) *GHR* gene expression. (D) *TLR4* gene expression.
TSL: TETRA-SL layer hybrid, HPC: Hungarian Partridge colored hen breed. Control: non-injected group, Saline: saline injected group, DL-Met: DL-Met injected group, L-Met: L-Met injected group. *IGF1*: insulin-like growth factor 1, *IGF1R*: insulin-like growth factor 1 receptor, *GHR*: growth hormone receptor, *TLR4*: toll-like receptor 4.

Table 12

Summary of analysis of variance of the effect of genotype, treatement and their interaction on the growth related and TLR4 gene expression in the chicks

		es			
		IGF1	IGF1R	GHR	TLR4
P-values	Genotype	**0.0165**	**0.0001**	0.1505	0.0579
	Treatment	0.5414	0.3860	0.4230	0.2862
	Interaction	0.7978	0.3492	0.5624	0.2194
RMSE		0.5647	0.4133	0.6160	0.8734

IGF1: insulin-like growth factor 1, *IGF1R*: insulin-like growth factor 1 receptor, *GHR*: growth hormone receptor, *TLR4*: toll-like receptor 4. RMSE –root mean square error.

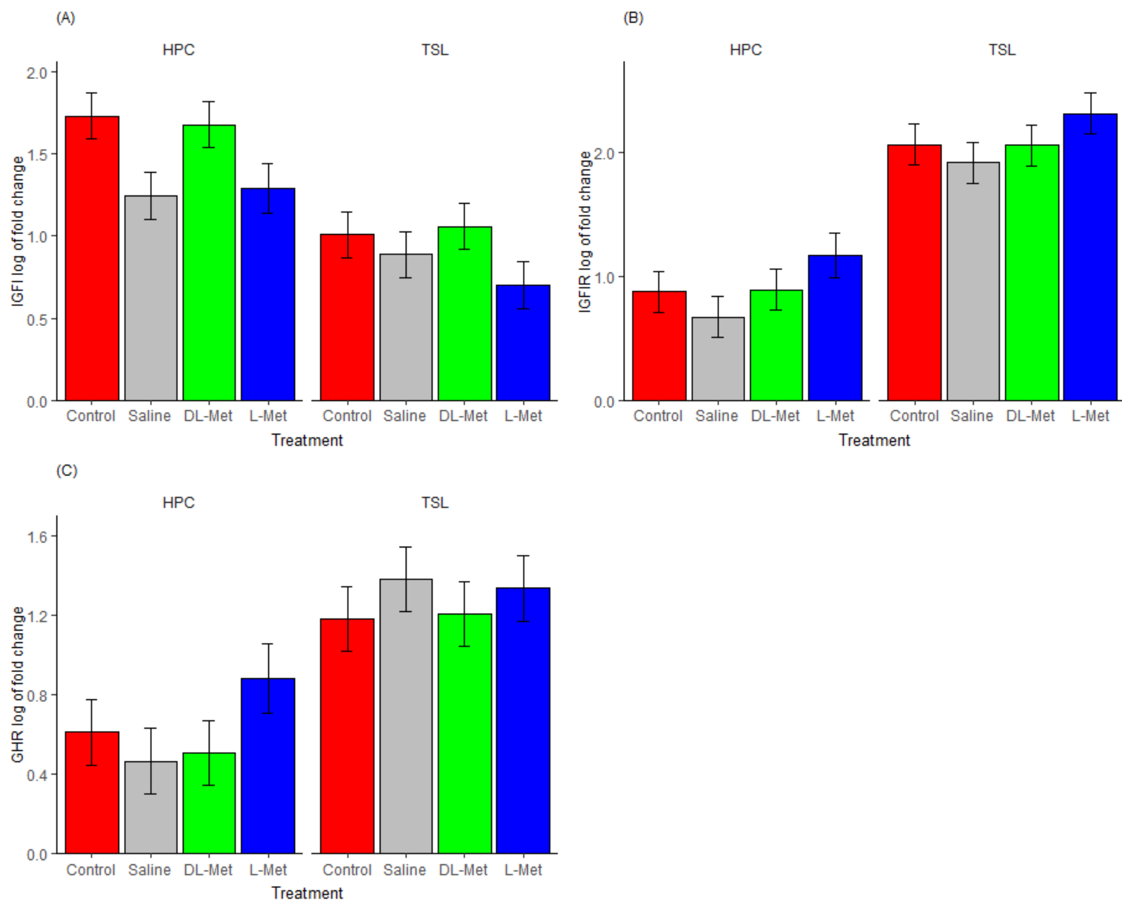
4.1.6. Intestinal gene expression related to growth, antioxidants, and tight junction

4.1.6.1. Jejunum growth-related genes

Generally, *in-ovo* injection of Met significantly affected the expression of *IGF1* (P = 0.0108) and *IGF1R* mRNA expression ($P = 0.0454$). In the HPC genotype, saline tended to downregulate the *IGF1* expression ($P = 0.0829$) when compared to the control. Regarding the two sources, the L-Met injection tends to decrease the expression of the *IGF1* gene compared to the control in the HPC genotype (Figure 8A, $P = 0.1521$). The same pattern is not observed in the TSL genotype. On the other hand, both L-Met and DL-Met had similar effects on the expression of the *IGF1R* and *GHR* mRNA in both genotypes (Figure 8B & 8C, respectively). The effect of the genotype was significantly evident in the *GHR*, *IGF1*, and *IGF1R* mRNA expression (Table 13), with the TSL genotype having highly expressed *IGF1R* and *GHR* (Figure 8B and C, $P < 0.0001$) than their counterpart genotype. Unexpectedly, the HPC genotype had upregulated the expression of *IGF1* when compared to the TSL genotype (Figure 8A, $P < 0.0001$).

Figure 8

Effects of *in-ovo* injection of 5 mg DL and L-Met on jejunum relative mRNA expression levels of growth-related genes in TSL and HPC chicks at one day of age



(A) *IGF1* gene expression. (B) *IGF1R* gene expression. (C) *GHR* gene expression.
TSL = TETRA-SL layer hybrid, HPC= Hungarian Partridge colored hen breed, Control = non-injected group, Saline = saline injected group, DL-Met = DL-Met injected group, L-Met = L-Met injected group. *GHR* = growth hormone receptor, *IGF1* = insulin-like growth factor 1, *IGF1R* = insulin-like growth factor 1 receptor. Data are presented as estimated marginal means and SEM (n = 8).

Table 13

Summary of analysis of variance of the effect of genotype, treatement and their interaction on the jejunum growth related gene expression in the chicks

	Factor	**Genes**		
		IGF1	IGF1R	GHR
P-values	Genotype	**0.0001**	**0.0001**	**0.0001**
	Treatment	**0.0108**	**0.0454**	0.3949
	Interaction	0.6222	0.9910	0.5445
RMSE		0.3980	0.4686	0.4613

IGF1 = insulin-like growth factor 1, *IGF1R* = insulin-like growth factor 1 receptor, *GHR* = growth hormone receptor. RMSE –root mean square error.

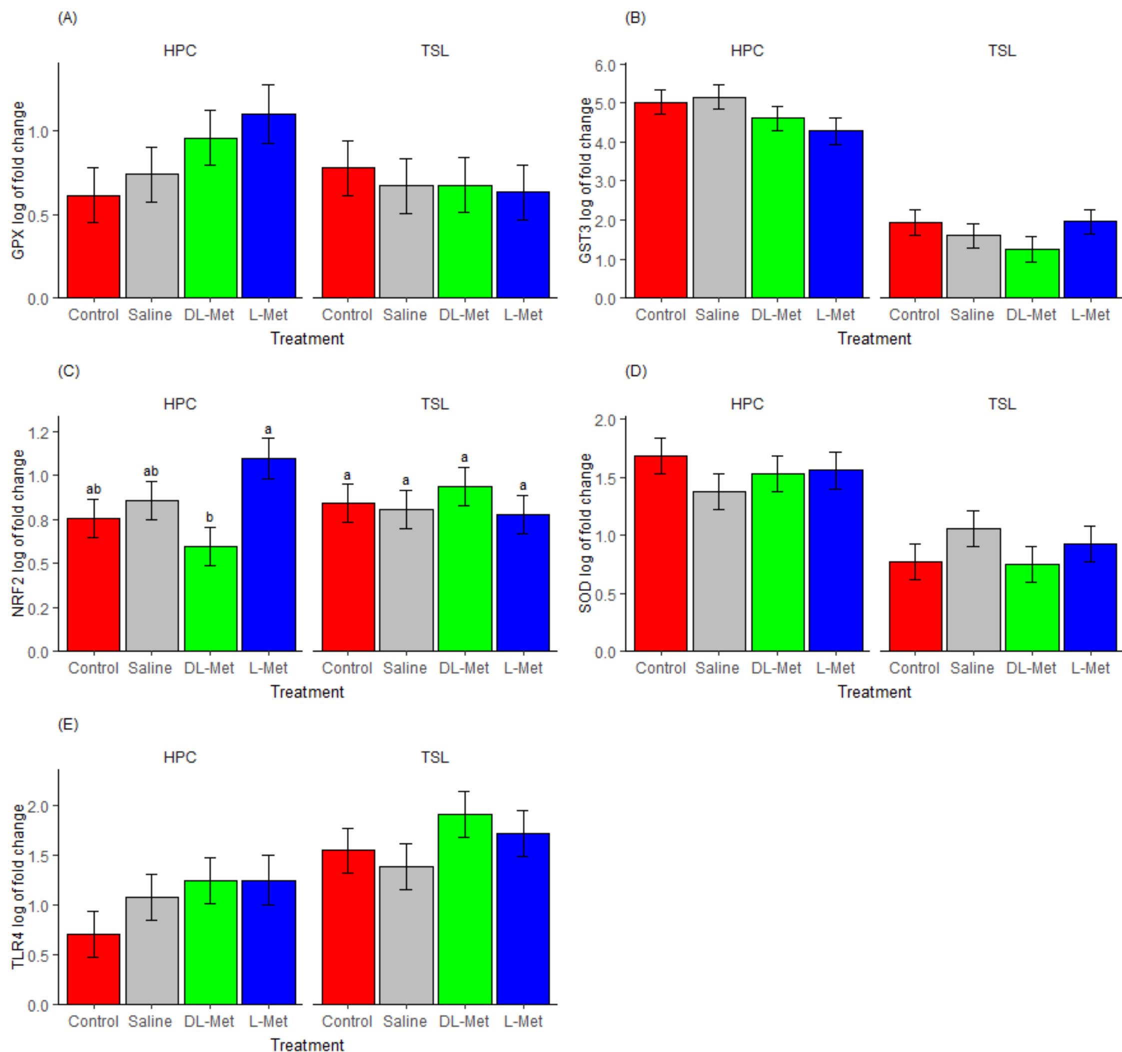
Chicken growth hormone (GH) regulates the growth and development of chickens, primarily through the insulin-like growth factor-I (IGF1) hormone. IGF-1 hormone is synthesized in the liver under GH control and secreted into the circulation. The gene expression of *GH, IGF1,* and *IGF1R* was influenced by the genotype, while the treatment affected only the expression of *IGF1* in the jejunum tissues. The TSL genotype has higher expressed *IGF1R* and *GHR* mRNA levels than the HPC. These results imply that the difference in growth and performance production may be attributed to genotype responses to the Met treatment. In contrast, the expression of *IGF1* mRNA was significantly higher in the HPC than in the TSL genotype. This finding contradicts previous results that reported significant differences in *IGF1* mRNA expression between fast-growing and slow-growing chickens in breast muscles during embryonic development (LU *et al.* 2009). The amino acids, particularly sulfur amino acids, significantly increase broilers' growth by affecting protein synthesis and changing growth-related gene expression (ELWAN *et al.* 2021). In the current trial, *in-ovo* injection of L-Met increased the expression of *GHR* and *IGF1R* genes in the small intestine tissue compared to the control and DL-Met. This indicates an efficient way to improve chick embryonic intestinal development and growth, as well as how genotypes respond to the diet differently. Better intestinal development characterized by highly expressed growth-related genes may be associated with the chicks' intestinal health and antioxidant status.

4.1.6.2. Jejunum antioxidant-related genes

No treatment effect was noted on the antioxidant-related genes (Figure 9 A, B, D, & E) except for *NRF2* for the HPC genotype (Figure 9C, $P = 0.01$), where DL-Met injection significantly reduced the expression of the respective genes. The genotype influenced the expression of the studied antioxidant genes, except for *GPX1* and *NRF2* (Table 14, Figure 9A & C, respectively, $P > 0.05$). The HPC genotype had higher mRNA expression of the *SOD1* and *GST3* genes than the TSL genotype ($P < 0.01$, Figure 9B & C respectively). In addition, the mRNA expression of *TLR4* was downregulated in the HPC genotype compared to the TSL genotype (Figure 9E, $P < 0.05$). Moreover, the interaction effect was noted on the expression of the *NRF2* mRNA ($P = 0.0375$), with L-Met significantly increasing the expression on the HPC genotype and decreasing its expression on the TSL as compared to the control and DL-Met (Figure 9C, $P = 0.02$).

Figure 9

Effects of *in-ovo* injection of 5 mg DL and L-Met on jejunum relative mRNA expression levels of antioxidant-related genes in TETRA-SL layer hybrid (TSL) and Hungarian partridge colored hen breed (HPC) chicks at one day of age



(A) *GPX1* expression in TSL and HPC chicks (B) GST3 expression in TSL and HPC chicks (C) *NRF2* gene expression in TSL and HPC chicks (D) *SOD1* expression in TSL and TSL chicks (E) *TLR4* gene expression in TSL and HPC chicks.

[a, b] Means that having similar superscript letters are not significantly different ($P < 0.05$).

GPX: glutathione peroxidase 1, *SOD*: superoxide dismutase 1, *GST3*: glutathione S-transferase alpha 3, *NRF2*: nuclear factor, erythroid 2 like 2, *TLR4*: toll-like receptor 4. Control: non-injected group, Saline: saline injected group, DL-Met: DL-Met injected group, L-Met: L-Met injected group. Data are presented as estimated marginal means and SEM ($n = 8$).

Table 14

Summary of analysis of variance of the effect of genotype, treatement and their interaction on the antioxidant related and TLR4 gene expression in the chicks

	Factor	**Genes**				
		GPX1	GST3	NRF2	SOD1	TLR4
P-values	Genotype	0.1790	**0.0001**	0.7990	**0.0001**	**0.0010**
	Treatment	0.7184	0.2488	0.5248	0.9319	0.1776
	Interaction	0.6222	0.2419	**0.0375**	0.2554	0.6754
RMSE		0.4611	0.8815	0.3097	0.4315	0.6503

GPX1: glutathione peroxidase 1, *SOD1*: superoxide dismutase 1, *GST3*: glutathione S-transferase alpha 3, *NRF2*: nuclear factor, erythroid 2 like 2, *TLR4*: toll-like receptor 4. RMSE –root mean square error.

Met has been shown to modulate the immune response in poultry, including activating the Toll-like receptors (TLRs). However, limited information is available on its effect on embryonic chicks (ELWAN *et al.* 2019). TLRs are critical in recognizing and responding to pathogen-associated molecular patterns on the surface of bacteria and other pathogens (PAUL *et al.* 2013; KANNAKI *et al.* 2015). In our experiment, the results indicate that *in-ovo* injection of Met slightly upregulated the expression of *TLR4* mRNA relative to the control group in both genotypes. Unlike the previous finding that reported overexpression of *TLR4* on newly hatched chicks, it was clear that it was triggered by heat stress during the incubation (ELWAN *et al.* 2019). *TLR4,* together with lysozyme, plays a critical role in binding and detoxifying lipopolysaccharide (TAKADA *et al.* 1994) and, therefore, participates in protecting against major gram-negative bacteria in cases of *ovo* contamination (KANNAKI *et al.* 2015). The upregulation of *TLR4* mRNA expression by *in-ovo* injection of Met at normal conditions indicates the protection of the developing embryo *in-ovo* and newly hatched chick against pathogens (KANNAKI *et al.* 2015).

The antioxidant status of chicks during late embryonic development and newly hatched chicks is affected by a range of factors, including temperature, humidity, carbon dioxide fluctuations, and delay in collecting them from the hatcher and hatching window, to mention a few (SURAI & KOCHISH 2019). The late embryonic development and hatching period are critical stages in poultry, and the chicks are subjected to stressful conditions that could lead to excessive production of reactive oxygen species (ROS). This causes late embryonic and chick post-hatch mortality. The nuclear factor erythroid 2 like 2 (*NRF2*) modulates the expression of several genes that code for vital aspects of the glutathione- and thioredoxin-based antioxidant systems at homeostasis and in stressful conditions. The results indicated the interaction effect of the treatment and genotype on

the *NRF2* mRNA expression in the jejunum of one-day-old chicks. The gene expression of *NRF2* was increased by L-Met injection but not by DL-Met in HPC compared to the TSL genotype. In developing rats, L-Met has been shown to activate the NRF2-ARE pathway to stimulate endogenous antioxidant activity (SOD, GSH, and GPx) and mitigate the effect caused by ROS (WANG, LIANG, *et al.* 2019). In addition, the gene expression of *SOD1, GST3*, and *GPX1* was also highly expressed in the HPC genotype; this means that the old genotype is well protected and enhanced for survival in a harsh environment than the improved genotype (TANG *et al.* 2019; LUGATA *et al.* 2022c). The development of the intestinal antioxidant system is crucial for intestinal development and chicks' growth due to the diet change from yolk to plant-based protein. SOD and GSH play a critical role in protecting the intestine and facilitating the high-rate proliferation of intestinal tissue (TANG *et al.* 2019; CHEN *et al.* 2021).

4.1.6.3. Jejunum tight junction-related genes

No significant treatment effects were found on tight junction genes in both genotypes (Table 15, $P > 0.0.5$, Figure 10A and C) except for the *OCLN*, where a significant effect and a tendency of reduction were noted in the TSL genotype due to *in-ovo* injection of DL-Met ($P = 0.02$), saline ($P = 0.07$), and L-Met ($P = 0.12$) when compared to control (Figure 10B). The study revealed significant genotype effects on the expression of *OCLN, TJP2*, and *MD2* ($P < 0.001$, Figure 11). *OCLN and MD2* mRNA expression were higher in the HPC genotype compared to the TSL genotype ($P < 0.001$, Figure 10 A&B respectively), while the TSL genotype exhibited increased expression of *TJP2* ($P < 0.001$, Figure 10C).

Table 15

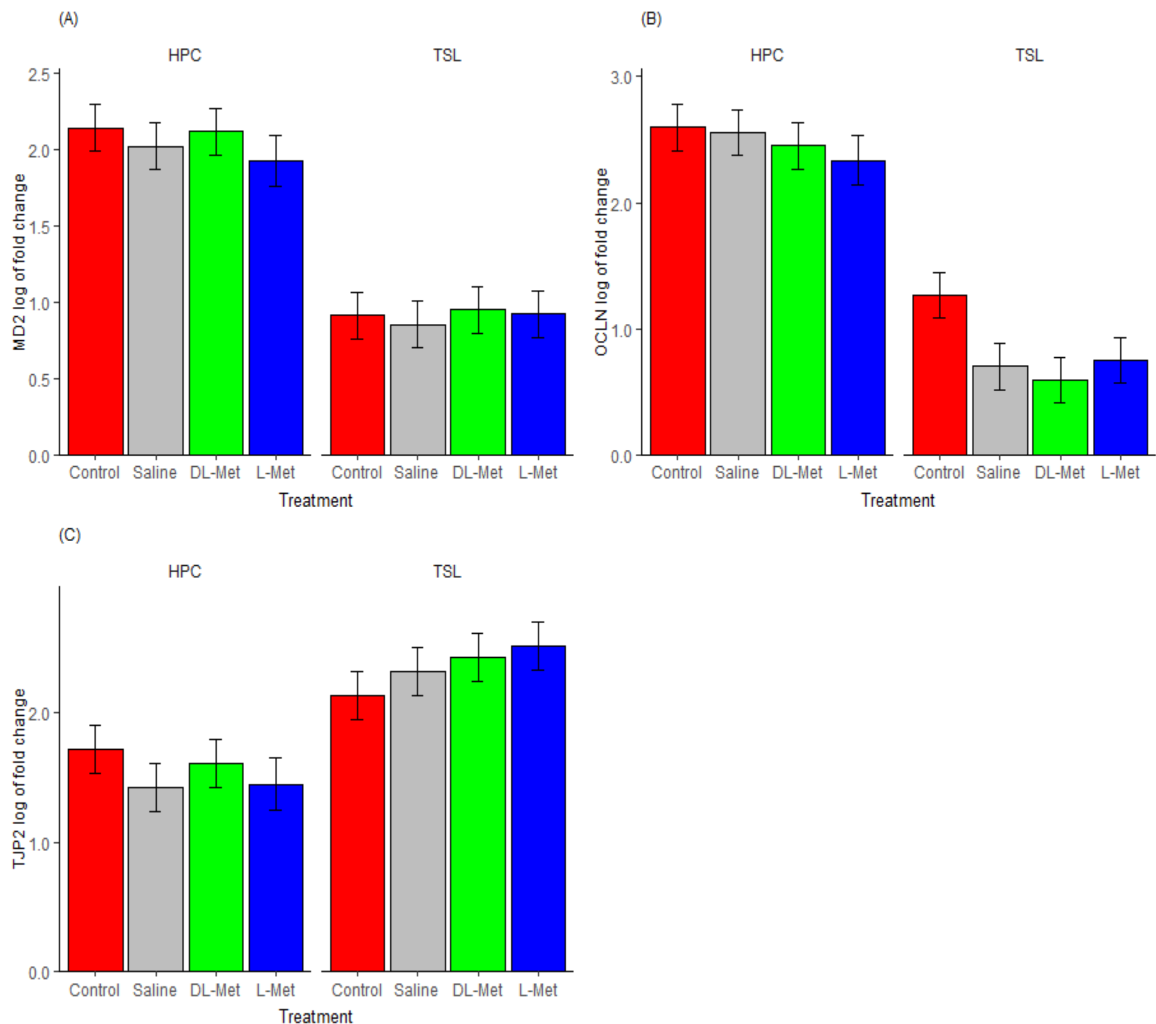
Summary of analysis of variance of the effect of genotype, treatement and their interaction on the tight junction related gene expression in the chicks

	Factor	**Genes**		
		MD2	OCLN	TJP2
P-values	Genotype	**0.0001**	**0.0001**	**0.0001**
	Treatment	0.7463	0.0778	0.8314
	Interaction	0.9030	0.4156	0.3474
RMSE		0.4324	0.5167	0.5255

MD2: tricellulin, *TJP2*: tight junction protein 2, *OCLN*: occludin. RMSE –root mean square error.

Figure 10

Effects of *in-ovo* injection of 5 mg DL and L-Met on jejunum relative mRNA expression levels of tight junction-related genes in TETRA-SL layer hybrid (TSL) and Hungarian partridge colored hen breed (HPC) chicks at one day of age



(A) *MD2* gene expression in TSL and HPC chicks (B) *OCLN* expression in the TSL and HPC (C) *TJP2* gene expression in the TSL and HPC chicks.
MD2: tricellulin, *TJP2*: tight junction protein 2, *OCLN*: occludin. Control: non-injected group, Saline: saline-injected group, DL-Met: DL-Met injected group, L-Met: L-Met injected group. Data are presented as estimated marginal means and SEM (n = 8).

Tight junctions (TJ) are specialized protein structures essential to maintaining the integrity and barrier function of epithelium and epithelium layers in various organs, including the intestinal epithelium (GONZÁLEZ-MARISCAL *et al.* 2003). TJ plays a critical role in regulating paracellular permeability by blocking the passage of pathogens, toxins, and luminal antigens while permitting the transit of ions and solutes. These proteins are dynamic, complicated, and strictly regulated (BAREKATAIN *et al.* 2023). Occludin

(*OCLN*) is among the TJ proteins that prevent paracellular permeability (VON BUCHHOLZ *et al.* 2021). The TJ protein tricellulin (*MD2*) function in chicken is not yet clearly known; however, it is believed to play a critical role in preventing microbe passages by sealing the tricellular junction between three neighboring cells (KRUG *et al.* 2009; VON BUCHHOLZ *et al.* 2021). Our results indicate that treatment did not influence the expression of TJ-related genes. This means that the integrity of the epithelium of the jejunum was maintained by the injection of Met, as there were no changes in the mRNA expression of *OCLN*. However, the genotype influenced the tight junction mRNA expression; the HPC overexpressed the *OCLN* and *MD2* mRNA compared with the TSL genotype. This suggests that the HPC genotype might have a better TJ strand network and enhanced barrier function than the TSL genotype. The study has reported that *MD2* and *OCLN* regulate the development and/or maintenance of TJ-strand branching points that support the integrity of the epithelial barrier (SAITO *et al.* 2021). Unlike the *OCLN* and *MD2* genes, the *TJP2* gene was more overexpressed in the jejunum of the TSL than in the HPC genotype. The TJP2 has strongly been suggested to contribute to paracellular calcium uptake in the intestine of layers (GLOUX *et al.* 2019). Improving the TSL genotype for high egg production may explain the overexpression of *TJP2*, a pore-forming cytosolic protein responsible for ions and water permeability.

4.2. Effect of methionine source and levels of supplementation on TETRA-SL chicks at early life (28 d)

4.2.1. Growth performance

Generally, neither Met source nor Met levels influenced the body weight of the chicks for the whole period of the experiment, except for the interaction effect on week four (Table 16). Moreover, the dietary Met source and Met level did not significantly influence the ADG except for the fourth week of age, where the interaction was noted. In week 4, the highest ADG value (14.6 g/day) was obtained in the DL-Met group with a supplementation level of 110%, which increased by 7.6% from the control (DL-Met at 100%) (Table 16). Growth performance has been used to determine the bioavailability (digestion, absorption, and utilization) of amino acids, particularly methionine, the first limiting amino acid in maize and soybean meal-based poultry diets (SHEN *et al.* 2014; SHEN *et al.* 2015).

Table 16

Effects of different Met sources and levels on growth performance in TETRA –SL layer hybrid chicks

Traits		BW on day 1, g	BW on day 7, g	ADG, g/day	BW on day 14, g	ADG, g/day	BW on day 21, g	ADG, g/day	BW on day 28, g	ADG, g/day	ADG, g/day
Pooled effects				Week 1		Week 2		Week 3		Week 4	Overall
Met source	DL-Met	42.39	78.22	5.11	124.05	6.50	205.01	11.57	271.05	10.34	8.39
	L-Met	42.38	76.77	5.04	117.86	6.03	197.37	11.41	265.85	9.48	8.02
Met level	90%	42.36	77.58	5.02	121.88	6.75	203.69	11.69	260.83	8.38	7.99
	100%	42.40	78.27	5.21	120.59	5.97	198.27	10.67	263.15	9.50	8.04
	110%	42.40	76.71	4.98	120.55	6.16	202.04	12.15	281.30	11.74	8.59
***P*-values**	Met sources	0.9835	0.4031	0.7826	0.1198	0.2599	0.3035	0.8669	0.6377	0.4046	0.5035
	Met levels	0.9981	0.6758	0.6921	0.9476	0.2170	0.8386	0.3880	0.2398	0.0643	0.6065
	Interaction	0.9993	0.2813	0.3508	0.5582	0.4419	0.4851	0.8128	**0.0130**	**0.0174**	0.0667
	RMSE	2.96	7.81	0.58	18.84	0.87	34.85	2.05	51.07	2.62	1.27
Effect among all treatment combinations											
DL-Met	90%	42.35	77.86	5.04	125.63	6.69	205.59	11.42	250.46 ^{a}	8.22 ^{a}	7.88
	100%	42.41	77.68	5.04	120.76	6.16	197.85	11.02	255.49 ^{ab}	8.23 ^{a}	7.61
	110%	42.41	79.06	5.24	125.78	6.67	211.61	12.26	306.87 ^{b}	14.56 ^{b}	9.68
L-Met	90%	42.37	77.36	5.00	118.13	6.84	201.67	12.04	270.5 ^{ab}	8.52 ^{ab}	7.90
	100%	42.37	79.00	5.39	120.40	5.71	198.78	10.20	271.33 ^{ab}	10.76 ^{ab}	8.47
	110%	42.38	74.03	4.72	115.19	5.65	191.84	12.00	255.72 ^{ab}	8.91 ^{ab}	7.49
	P-value	1.000	0.5631	0.6905	0.5803	0.3178	0.7042	0.7922	**0.0400**	**0.0218**	0.2136
	RMSE	2.95	7.81	0.58	18.84	0.87	34.85	2.05	51.07	2.62	1.27

[ab] Means with similar letters superscript do not differ ($p > 0.05$). The result was presented as mean and RMSE (n = 4/treatment for ADG and n = 16/treatment for BW). 90% 100% and 110% supplementation levels of the recommendation of methionine. BW-Body weight, ADG- average daily gain, RMSE –root mean square error.

Our findings suggest that TSL young chicks have the same ability to utilize both DL-Met and L-Met for their body weight, feather growth, and body weight gain in the first four weeks of their age. These results are consistent with several other studies that have shown that chicks can effectively use DL-Met in the same manner as L-Met for weight gain (DILGER & BAKER 2007). Moreover, the study by JANKOWSKI et al. (2017) on turkey did not find any differences between dietary Met sources on growth performance. However, SHEN *et al.* (2015) found that L-Met was utilized better than DL-Met for the intestinal development and growth of the young broiler. They reported that the overall average daily gain (ADG) resulted from 100 units of L-Met; the chick required 138 units of DL-Met. Additionally, PARK *et al*. (2018) reported that L-Met outperformed DL-Met in young turkeys from day 0 to 28 of age, with a relative bioavailability of 160% for weight gain. Another study by SHEN *et al*. (2014) showed that young nursery pigs at days 26 to 46 of age resulted in 144% better ADG when fed L-Met than DL-Met. However, the results of our study at the fourth week of age show that the chicks who received DL-Met with an inclusion level of 110% had higher BW and ADG in the treatment groups. In comparison to the control group in the DL-Methionine source, the highest inclusion level increased the body weight of the chicks by approximately 23%. However, in the L-Met source, the growth was reduced by 7% at the highest level of inclusionwhen compared to the control group. Generally, varying the dietary methionine levels by ±10% of the layer's requirement does not have any significant effect on the body weight and average daily gain of the TSL chicks from day 1 to 28 of age.

4.2.2. Feathers development

During the first three weeks of rearing, the feather length development of the chicks was not affected by either Met source or Met levels. However, in the fourth week, we observed an interaction between source and level (Table 17). These findings are consistent with a previous study by CHEN *et al*. (2020), which reported that DL-Met and L-Met supplementation did not affect the featherweights of broiler chicks. Similarly, other studies by ZHAO *et al.* (2018) and ZENG *et al.* (2015) also found that DL-Met and DL-HMTBA had similar efficiency for feather growth in Cherry Valley ducks and Pekin ducks, respectively. Our findings suggest that, similar to body weight, dietary DL and L-Met sources have comparable efficacy on the feather development of TSL chicks up to 28 days of age.

Table 17

Effects of different dietary Met sources and levels of supplementation on feather development of TSL chicks from 1-28 days of age

		Feather length on day 7 (mm)	Feather length on day 14 (mm)	Feather length on day 21 (mm)	Feather length on day 28 (mm)
Pooled effects					
Met source	DL-Met	38.58	57.50	73.80	84.94
	L-Met	36.67	55.99	72.74	84.24
Met level	90%	36.32	56.46	72.98	84.98
	100%	39.71	57.88	73.80	83.87
	110%	36.85	55.92	73.08	84.96
P-values	Source	0.1898	0.1609	0.1709	0.4310
	Level	0.1250	0.3108	0.6245	0.5215
	Interaction	0.0720	**0.0325**	**0.0265**	0.0625
RMSE		7.07	5.24	3.83	4.34
Effect among all treatment combinations					
DL-Met	90%	35.37	55.76	73.37	84.96
	100%	40.38	58.13	73.09	83.14
	110%	39.99	58.62	74.96	86.72
L-Met	90%	37.26	57.20	72.54	85.01
	100%	39.04	57.62	74.51	84.59
	110%	33.71	53.23	71.07	83.09
	P-value	0.0528	0.0516	0.0797	0.1865
	RMSE	7.07	5.24	03.83	4.34

TSL: TETRA-SL layer hybrid. The result was presented as mean and pooled RMSE (n = 16/treatment). 90%, 100%, and 110% supplementation levels of the recommended methionine

4.2.3. Hematological parameters

The blood parameters of chicks were influenced by Met source and levels both separately and interactively (Table 18). Met source had a significant effect on the number of red blood cells (RBC) in the blood of chicks (P = 0.0039), as did Met concentrations (P = 0.0156), but there was no significant interaction between Met source and levels and the number of RBC (Table 12). Hb concentration differed significantly among Met sources (P = 0.0006) and levels (P = 0.0125), but there was no interaction effect on chicks' blood. The Ht value was significantly affected by both Met source (P = 0.0045) and levels (P = 0.0068), with a demonstration of the interaction of Met source and Met levels (P = 0.0244). However, no significant differences in MCV, MCH, and MCHC values were observed due to either dietary Met sources, levels, or their interaction (Table 18).

The effect of dietary Met source on platelet count in blood liters was found to be statistically significant (P = 0.0005). The results showed higher counts in the L-Met as compared to the DL-Met group. No significant effects of Met levels or interactions of Met sources and Met levels were revealed. The highest value of platelet count (10.44 × 10^9/L) was recorded in the L-Met group with 110% inclusion, while the lowest (2.33 × 10^9/L) was in the DL-Met group at the same dose (Table 18).

The WBC count in the blood liter was influenced by both Met sources (P = 0.0030), Met levels (P = 0.0093), and their interaction (P = 0.0014). Simalarly, the percentage of lymphocytes was significantly affected by both dietary Met source and dietary Met levels, as well as their interaction. The number of LYM cells was only influenced by Met sources (P = 0.0006) and levels (P = 0.0010) without an interaction effect being observed. Meanwhile, the MID percentage was not influenced by the source or the levels, but the MID number was significantly affected by both factors but not by their interactions (Table 18).

The effect of Met sources and levels on the percentage of GRAN % was statistically significant (P = 0.0140 and P = 0.0116, respectively). However, there was no significant interaction effect between Met sources and levels on GRAN%. Additionally, the GRAN cell count was significantly affected by both Met-sources (P= 0.0045) and Met levels (P = 0.0006), as well as their interaction (P = 0.0017). Unexpectedly, the highest levels of dietary inclusion in the L-Met group resulted in the lowest number and percentage (26.18 × 10^9/L and 37.63%, respectively) of GRAN as compared to other dietary inclusion levels in both sources (Table 18).

It should be noted that there is a scarcity of studies on the effect of Met sources and levels on the TSL genotype. However, in the literature, few studies are available, especially regarding the effect of varying levels of Met, particularly when chelated with zinc. These studies have indicated non-significant changes in the hematological profile of layer chickens under different doses of zinc-methionine (BELIH 2016; CHEN et al. 2018). Additionally, the same results have been reported in quail breeders supplemented with varying dietary DL-Met levels, with no effect on the hematological parameters (REDA *et al.* 2020). While hematological tests are commonly used in mammals to assess the animal's health status, they are less commonly used in avian species due to a lack of generally accepted reference values. However, hematology diagnostic tools are commonly used in veterinary and human medicine due to the ease blood sampling procedure (Ameha et al. 2019).

Table 18

Effects of different dietary Met-sources and levels of supplementation on the hematological parameters of TSL chicks from 1 to 28 d of age

		RBC (10^{12}/L)	Hb (g/dL)	Ht (%)	MCV (fL)	MCH (pg)	MCHC g/dL	Platelet (10^{9}/L)	WBC (10^{9}/L)	LYM (%)	MID (%)	GRAN (%)	LYM (10^{9} cells/L)	MID (10^{9} cells/L)	GRAN (10^{9} cells/L)
Pooled effects															
Met source	DL Met	2.99 ^{b}	11.91 ^{b}	38.42 ^{b}	127.66	39.5	30.76	2.70 ^{a}	84.49 ^{b}	45.09 ^{a}	13.04	41.77 ^{b}	38.35 ^{b}	11.06 ^{b}	36.15 ^{b}
	L Met	2.77 ^{a}	10.99 ^{a}	35.67 ^{a}	127.70	39.11	30.84	8.00 ^{b}	77.39 ^{a}	47.45 ^{b}	12.97	40.05 ^{a}	36.06 ^{a}	9.92 ^{a}	31.85 ^{a}
Met level															
	90%	2.94 ^{ab}	11.4 ^{ab}	36.56 ^{ab}	126.79	38.62	30.70	5.54	78.82 ^{a}	46.50 ^{b}	13.17	40.47 ^{ab}	35.41 ^{a}	10.02 ^{a}	32.52 ^{a}
	100%	2.97 ^{b}	11.96^{b}	38.77 ^{b}	129.21	39.69	30.68	3.86	86.67 ^{b}	44.57 ^{a}	12.85	42.36 ^{b}	39.21 ^{b}	11.27 ^{b}	38.01 ^{b}
	110%	2.76 ^{a}	11.05 ^{a}	35.62 ^{a}	126.55	39.56	31.01	6.76	78.94 ^{a}	47.61 ^{b}	13.02	39.97 ^{a}	37.04 ^{ab}	10.22 ^{ab}	31.69 ^{a}
P-values	Source	**0.0039**	**0.0006**	**0.0045**	0.9806	0.3649	0.7146	**0.0005**	**0.0030**	**0.0006**	0.512	**0.0140**	**0.0442**	**0.0039**	**0.0045**
	Level	**0.0156**	**0.0125**	**0.0068**	0.1699	**0.0487**	0.4701	0.3101	**0.0093**	**0.0010**	0.124	**0.0116**	**0.0234**	**0.0150**	**0.0006**
	Inter-action	0.2395	0.0864	**0.0244**	0.2585	0.8934	0.0727	0.1241	**0.0014**	**0.0204**	0.586	0.0904	0.1464	0.0650	**0.0017**
RMSE		0.21	0.77	2.55	3.68	1.05	0.73	4.09	6.69	1.95	0.39	2.13	3.27	1.36	4.08
Effect among all treatment combinations															
DL Met	90%	2.97 ^{a}	11.49^{a}	36.14 ^{ab}	126.53	38.85	30.69	2.52	76.54 ^{ab}	46.30 ^{a}	13.18	40.59 ^{ab}	35.32	10.05 ^{a}	31.17 ^{ab}
	100%	3.09 ^{a}	12.51^{a}	40.17 ^{b}	128.03	39.73	30.98	3.22	92.34 ^{c}	43.56 ^{a}	12.97	42.90 ^{b}	40.13	11.75 ^{b}	40.15 ^{c}
	110%	2.91 ^{ab}	11.68^{a}	37.99 ^{b}	128.14	39.77	30.60	2.33	86.83 ^{bc}	45.36 ^{a}	13.00	41.65 ^{b}	39.59	11.22 ^{b}	36.42 ^{bc}
L Met	90%	2.91 ^{ab}	11.32^{ab}	36.81 ^{ab}	126.95	38.42	30.71	9.06	81.11^{abc}	46.68 ^{a}	13.15	40.36$^{a\ b}$	35.48	9.99 ^{ab}	33.87 ^{bc}
	100%	2.85 ^{ab}	11.31^{ab}	37.14 ^{b}	130.58	39.64	30.39	4.50	82.13 ^{bc}	45.57 ^{a}	12.71	41.72 ^{b}	38.29	10.70 ^{ab}	35.52 ^{bc}
	110%	2.54 ^{b}	10.15 ^{b}	32.31 ^{a}	125.28	39.31	31.43	10.44	69.73 ^{a}	50.24 ^{b}	13.04	37.63 ^{a}	34.50	9.06 ^{a}	26.18 ^{a}
P-value		**0.0059**	**0.008**	**0.0007**	0.2816	0.1820	0.2254	**0.0039**	**0.0002**	**0.0001**	0.357	**0.0057**	**0.0156**	**0.0015**	**0.0001**
RMSE		0.21	0.77	2.55	3.68	1.05	0.73	4.09	6.69	1.95	0.39	2.13	3.27	1.09	4.08

^{abc} Means with similar superscript letters in a column are not significantly different ($P > 0.05$). The result was presented as the mean and pooled RMSE (n = 8/treatment). RBC: red blood cells, Hb: hemoglobin, Ht: hematocrit, MCV: mean corpuscular volume of red blood cells, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, WBC: white blood cells, LYM: lymphocyte, MID: mid-range, GRAN: granulocyte, TSL: TETRA-SL layer hybrid. 90% 100% and 110% supplementation levels of the recommended methionine

The assessment of RBC number is an essential element in hematological diagnostics, which detects the malfunctioning of the circulatory system (TALEBI *et al.* 2005). The study revealed that high levels of methionine may cause changes in the complete blood count (TOUE *et al.* 2006; TOMBARKIEWICZ *et al.* 2020). In this study, the RBC parameters were within the reference range regardless of the Met source and dietary Met levels. The chicks who received the high dose of either source methionine had a slightly lower RBC number (Table 18) that was statistically significantly different from other groups. However, the RBC counts of TSL chicks in DL-Met ranged from 2.82 – 3.42 x 10^{12}/L and for L-Met went from 2.49- 3.02 x 10^{12}/L, which is within the recommended upper limit range of 2.5 – 3.5 x 10^{6}/µL for healthy chickens by MAOBA et al. (2021). Moreover, these results corroborate SIMARAKS *et al.* (2004) and DING *et al.* (2019) that the RBC counts are in the range of 2.0 -3.0 x 10^{6}/µL for Thai indigenous chicken and China Haidong chickens, respectively. In addition, TOMBARKIEWICZ *et al.* (2020) reported that the number of RBCs varied with age, ranging from 1.70 – 2.37 x 10^{6}/µL, 1.80- 1.96 x 10^{6} /µL, and 2.72 – 2.91 x 10^{6}/µL on the first, seventh, and 35th days of age of broilers, respectively. The higher Hb levels confirm the observed effect of Met source and levels on RBC number. However, the Hb values of TSL chicks amongst the six treatments ranged from 9.63 to 13.80 g/dL and are within the reference range of 7.0 to 13.0 g/dl of healthy chickens (MOHANTY & GAYATRIACHARYA 2020). The Ht estimates the percentage of RBC in the individual's body, also called packed cell volume. The Ht value was lower in the 90% dose and 110% dose of either DL or L-Met than in 100%. Generally, the Ht value ranged from 30.10 to 44.53%, which was affected by both (CAMPBELL 2012). The elevation of RBC parameters that include MCV, MCH, and MCHC is believed to be due to a disorder of blood cell maturation, with the symptoms being megaloblastic and hemolytic anemia, few to mention. However, in this study, MCV and MCH were not influenced by either Met source or Met level. This corroborates the study by CHEN *et al.* (2018), where the hematological parameters in adult birds were not affected by either Met alone or in combination with zinc. MCH values in this study ranged from 38.42 to 39.73 pg, within the normal values range of 33-47 pg for healthy chickens (MAOBA *et al.* 2021). In blood samples, MCH, the mean volume of hemoglobin per RBC, is mostly used to indicate the extent of anemia (CHUKWUDI *et al.* 2017), while MCHC is used to estimate the concentration of hemoglobin in RBC, which was within the range of 30.39 to 31.43 g/dL, which was similar to the accepted range (CAMPBELL 2012; ODUNITAN-WAYAS *et*

al. 2018; MAOBA *et al.* 2021). The platelet number in this study ranged from 2.33 to 10.44 x 10^9/L and was influenced by dietary Met sources.

Several studies have shown that WBC counts in adult chickens range from 12 - 30 x 10^3/μL (TALEBI *et al.* 2005) or 9 – 32 x 10^3 /μL (CAMPBELL 2012), but in this experiment, 28-d-old chicks were used, and the WBC ranged from 69.73 – 92.34 x 10^9/L. This result correlates with the findings of TOMBARKIEWICZ *et al*. (2020) in young chickens with WBC counts ranging from 17 – 107 x 10^3 /μL in response to the injection of methionine in the eggs. This indicates the immunological status of the chicks as the influence of the methionine, as the 100% inclusion level had the highest WBC number than either 90% or 110% in both Met sources. The higher value of WBC indicates the ability of the chicks to fight or resist diseases, which is a reflection of adequate methionine (ADEYEMO *et al.* 2010; MAOBA *et al.* 2021). Furthermore, the study by ADEYEMO et al. (2010) indicated the influence of Met on hematological parameters, including WBC, in the first four weeks of rearing broilers. On the contrary, ZHANG *et al*. (2017) showed that Met supplementation on broiler diets did not affect WBC differential count. The percentage of lymphocytes in a total WBC count was noted be higher (46.3%) in the Met-deficient diet (90%) for the DL-Met source, while for the L-Met was observed to be higher (50.24%) in the Met-excess diet (110%). This may indicate that L-Met, when used in high doses, improved the ability to fight diseases and the recovery process in the chicks (MAOBA *et al.* 2021). Granulocytes (GRAN), which also play a great role in fighting pathogens, were influenced by Met source and levels. DL-Met resulted in a higher number and percentage of GRAN than L-Met, while the highest Met level of inclusion had the lowest percentage and number than other levels. This means that the ability of the TRTRA-SL chicks to fight infection was altered by dietary Met source and levels. Chicks received L-Met, and a higher level of inclusion was immunologically stable.

5. CONCLUSIONS AND RECOMMENDATIONS

In conclusion, *in-ovo* feeding of 5 mg DL-methionine, compared to 5 mg L-methionine, significantly enhances the hatching weight and heart weight of layers regardless of the genotype, but not significantly better than non-injected treatment. *In-ovo* feeding of 5 mg DL-methionine, compared to 5 mg L-methionine, results in considerably (17%< difference) better hatchability in the TETRA-SL LL hybrid than the Hungarian Partridge colored hen genotype, but not better than the non-injected group.

The *in-ovo* feeding of DL-methionine has advantages over L-methionine in promoting higher hatching weight, heart weight, and antioxidant capacity (FRAP) in the plasma. However, these advantages are not superior to those observed in the non-injected and saline groups. Both DL-methionine and L-methionine exhibit similar effects on liver enzyme levels (AST, ALT), glutathione content, and plasma uric acid levels, which are relatively less or equal to the saline group. This suggests that both Met sources can effectively support liver health and metabolism, and the benefits of methionine on GSH content may not be justified.

The commercial genotype TSL appears to have improved the antioxidant defense system and enhanced hatching body weight, liver health, and function compared with the indigenous HPC genotype. This result demonstrates the difference in growth performance and metabolism between the two genotypes. The practical implications of these findings are significant for poultry management and nutrition and could lead to enhanced breeding and production practices.

Furthermore, *in-ovo* feeding of DL-Met and L-Met sources has been shown to enhance intestinal development in the late-term embryonic development of chicks. This study found that DL-Met injection exhibited higher efficacy in promoting intestinal development by increasing the villus height, villus height crypt ratio, and villus surface area compared to L-Met injection, but not when compared to saline in-ovo injection. Therefore, the positive effect of in-ovo injection on intestinal morphology may be attributed to moisturization rather than Met supply. Nevertheless, more extensive research is needed to evaluate the benefits of saline in-ovo injection on the gut morphology of layers.

Moreover, it is noteworthy that the effectiveness of *in-ova* injection of L-Met and DL-Met on the expression of tight junction genes (*OCCLN, MD2, and TJP2*), antioxidant

defense (*GST3, GPX1, SOD1, and NRF2*), immune response (*TLR4),* and growth-related genes (*IGF1R, GHR, and IGF1*) was comparable. However, in comparison with the control group, neither treatment demonstrated significant effectiveness. Our results also suggest that the HPC genotype displays beneficial characteristics regarding the antioxidant defense system and tight junction barrier function compared to the TSL genotype. On the other hand, the TSL genotype demonstrates better hatching body weight, *TLR4, TJP2, GHR, and IGF1R* gene expression than the HPC genotype. These findings suggest potential impacts on intestinal physiology and function and highlight the importance of genotype-specific gene expression patterns for optimizing breeding strategies and enhancing poultry productivity and health.
Regarding the Met sources in each genotype, in-ovo feeding of L-Met proved to be better source than DL-Met and was utilized well in the Hungarian Partridge Colored chicks than in the TETRA SL chicks for muscles GSH, increased the intestinal NRF2 gene expression and decreased plasma uric acid content. In the other hand DL-Met exhibited promising potential as source of in-ovo feeding in TETRA SL chicks than L-Met by improving the intestinal development.

Lastly, dietary supplementation of the pre-starter diet with 90% of the Met requirements can maintain the growth performance and support feather growth equivalent to 100% and 110% Met levels of either DL or L-Met from day 1 to 28 days of TSL chicks age. Generally, DL-Met improved the hematological parameters of TSL chicks reared for 28 days compared to L-Met supplementation. This research suggests that DL-Met and L-Met have similar effects on early TSL chicks' development and feather growth. This implies they may have the same biological efficacy for average daily gain and body weight, but different utilization for the response of hematological parameters.

6. NEW SCIENTIFIC RESULTS

1. *In-ovo* feeding of 5 mg DL-methionine facilitates intestinal development (*$P < 0.05$)* by increasing the villus height (7.8%), villus apical width (11.1%), villus surface area (16.0%) and villus height/crypt ratio (15.4%) while decreasing the crypt depth (11.2%) in the jejunum of newly hatched (1d) chicks of TETRA-SL and Hungarian partridge colored hen when compared to the non-injected group.
2. *In-ovo* feeding with 5 mg of methionine sources improves the liver and renal functions of the newly hatched TETRA –SL and Hungarian partridge-colored hen chicks compared with the control but is not better than saline injection.
3. *In-ovo* injection of 5 mg DL-methionine improves ($P < 0.05$) the ferric-reducing ability of the plasma in Hungarian partridge-colored chicks, but not in TETRA SL chicks. Additionally, the Hungarian partridge-colored genotype had a better intestinal antioxidant defence system and tight junction barrier function than the TETRA-SL layer hybrid genotype ($P < 0.05$).
4. In layers, 90% of the recommended dietary sidMet level either in the form of DL or L-methionine could effectively support the feather's growth and growth performance ($P > 0.05$) as 100% (0.40% Met of TSL recommended) or more of the Met supplementation in early life.
5. Our results suggest that dietary DL-methionine supplementation in pre-starters is more beneficial in maintaining the physiological status by improving (increased by 4 -12%) the hematological parameters ($P < 0.05$) of TETRA-SL LL hybrid layer chicks than L-methionine.

7. PRACTICAL RESULTS

I. *In-ovo* feeding of methionine sources affects hatchability and antioxidant status. Poultry farmers should weigh the pros and cons of DL-methionine vs L-methionine for their flocks. Understanding specific needs optimizes supplementation.

II. The study shed light on the potential of 5mg DL-Met to improve the antioxidant status of chicks and how both Met sources enhanced intestinal development and support liver health, which are critical for nutrient absorption and overall health, respectively. In practice, this information may be used to design the *in-ovo* feeding programme to promote intestinal development and maximize nutrient absorption and overall production performance in later life.

III. The study revealed the significance of genotype-specific responses; hatcheries should consider the genotype-specific variation if they consider *in-ovo* feeding programs to improve their flocks' growth performance and overall health.

IV. The 90% dietary supplementation of methionine, particularly DL-Met, is enough to support the feather's development and growth performance and maintain the health status of the mid-heavy layers at a young age. This implies that 0.36% methionine in the diet is as effective as the 0.40% methionine required in the early life of the layers. The findings of this study can be a useful resource for poultry farmers and nutritionists to improve the nutritional content of diets for TSL chicks. By considering the quality and quantity of methionine, they can enhance healthy growth, efficient utilization of resources, and the birds' overall well-being. The study also emphasizes the significance of monitoring hematological parameters in poultry health management.

8. SUMMARY

Methionine is the first limiting amino acid in the commercial starter poultry diet, as it is critical for feather development. Mainly, it has been commonly supplemented via feed and less via water. However, the development of *in-ovo* technology has allowed *in-ovo* manipulation of nutrient content in the egg during incubation. The dietary supplementation of methionine has been well established in poultry, mainly due to its roles in growth performance, antioxidant capacity enhancement, immunomodulation, and involvement in feather development—currently, three commonly available methionine sources; DL-methionine, methionine hydroxyl analogue, and L-methionine. The latter has recently been known as a feed supplement and is believed to be readily available to be utilized by the animal cell. The literature has contrasting findings regarding the efficacy of methionine sources. To our best knowledge, there are limited studies on the biological efficiency of the methionine sources, particularly DL-methionine and L-methionine, on mid-heavy production performance layers and native breeds. Therefore, this study had the following specific objectives:

I. To determine the effect of *in-ovo* injection of DL and L-Met on blood biochemical parameters (AST, ALT, uric acid, and FRAP) and jejunum morphology of TETRA –SL and Hungarian partridge colored hen chicks.

II. To examine the influence of *in-ovo* injection of methionine sources on antioxidant status (GSH, TAC) in the liver, intestine, and pectoral muscles of the newly hatched chicks of the two genotypes.

III. To evaluate the effect of *in-ovo* feeding of methionine sources on the gene expression related to growth (IGF1, IGF1R, and GHR) and immunity (TLR4) in the liver, intestine, and pectoral muscles of the newly hatched chicks of TSL and HPC.

IV. To examine the effect of *in-ovo* feeding of methionine sources on intestinal development and health (jejunum morphology) and intestinal antioxidant gene-related and tight junction protein genes on both genotypes.

V. The biological efficacy of DL and L-methionine on the TETRA SL layers' performance, feather development, and hematological parameters at an early stage of life.

The research objectives above were achieved by setting up two different experiments. Experiment I, investigates the effects of *in-ovo* feeding of methionine sources on the development, health status, and hatching performance of the chicks from the two genotypes. The second experiment investigated the impact of dietary supplementation with methionine sources on the early life of TETRA SL chicks from day 1 to 28 days of age. The first experiment used 400 embryonated eggs; the *in-ovo* feeding was carried out on 17.5 days of incubation. Chicks were weighed on the day of hatching, and after one day (day of sampling), the chicks were sacrificed and heart and liver weights were taken. In addition, blood was sampled, and tissues such as the liver, intestine, and pectoral muscles were extracted. The plasma was used to analyze the liver and kidney health indicators, the plasma's antioxidant capacity, and amino acid metabolism. The tissues were used to determine the tissue's antioxidant capacity, gene expression, and jejunum development and function. The second experiment of dietary Met supplementation used a total of 96-day-old TETRA SL chicks, factorial arranged among the six treatment groups with four replicates of four birds each. The six treatments were two methionine sources with three methionine levels designed to supplement 90%, 100%, and 110% of their recommendations. The data was collected weekly for the feather measurement, body weight, and average daily gain. On the last day of the experiment, two birds per replicate were randomly selected for blood collection.

The results from the *in-ovo* feeding trial indicated that the hatchability ranged from 10% to 100%; the *in-ovo* feeding of methionine resulted in low hatchability. *In-ovo* application of DL-methionine resulted in relative better hatchability than L-methionine, particularly in the TETRA-SL chicks. Additionally, high concentrations of DL and L-methionine drastically reduced hatchability in both genotypes. However, the *in-ovo* feeding of DL-methionine significantly improved the hatching weight ferric-reducing ability of the plasma (FRAP) compared to L-methionine ($P < 0.05$), but not better than the saline group. Both methionine sources enhanced the chicks' liver health and antioxidant status. Moreover, the *in-ovo* feeding of either DL-methionine or L-methionine improved intestinal development by increasing the villus width, height, and surface area compared to the control in both genotypes ($P < 0.05$). Unexpectedly, saline *in-ovo* showed positive effects on villus height and comparable effects with *in-ovo* feeding of Met sources on villus basal width and villus area. The genotype responses to the *in-ovo* feeding of methionine sources were also noted; in the TETRA SL (TSL) genotype, the *in-ovo* feeding of DL-methionine significantly decreased the crypt depth, jejunum glutathione

(GSH), intestinal glutathione S-transferase 3 alpha (GST3), and occludin (OCLN) gene expression compared to L-methionine (P < 0.05).

We analyzed the influences of the genotypes on different parameters; the genotype's differences were significant in most of the parameters examined. The TSL genotype had improved hatching weight, heart weight, high hepatic GSH total antioxidant capacity (TAC), and FRAP compared to the HPC genotype (P < 0.05). While the HPC genotype had significantly higher muscle GSH and TAC levels, it upregulated hepatic *IGF1* and *IGF1R* receptors more than the TSL genotype (P < 0.05). The HPC genotype also overexpressed intestinal *IGF1*, tricellulin (*MD2*), *OCLN*, superoxide dismutase 1 (*SOD1*), and *GST3* genes more than the TSL genotype (P < 0.05).

The results from the dietary methionine supplementation indicated no significant effect of methionine sources and levels on the body weight, average daily gain, or feather development of the TETRA SL chick up to 28 days of age. However, on the last week of the experiment (day 28), the interaction of methionine source and levels was significant in the body weight and average daily gain (P < 0.05). In addition, the feather development (feather length) was a function of both methionine source and levels, as it was significant from the fourth week of the experiment (P < 0.05). Furthermore, both methionine sources and levels influenced the hematological parameters: red blood cells (RBC), hemoglobin (Hg), white blood cells (WBC), lymphocytes (LYM), mid-range (MID), and granulocytes (GRAN) values (P < 0.05). The interaction of methionine source and levels was significant on the hemotrict (Ht), WBC, LMY %, and GRAN (P < 0.05). The dietary supplementation of DL-methionine at 100% of the recommendation showed favorable results, with the highest WBC, RBC, Hb, and Ht values among all treatment groups. Contrary to that, the 90% DL-methionine-supplemented group had the lowest WBC, regardless of the methionine sources. The L-methionine-supplemented group had twofold higher platelet values than the DL-methionine group (P < 0.05). No significant effect of either methionine source or levels was observed on the mean corpuscular volume of red blood cells (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) (P > 0.05).

In conclusion, the findings from the *in-ovo* feeding trial demonstrate the difference in growth performance and metabolism between the two genotypes. The practical implications of these findings are significant for poultry management and nutrition and could lead to enhanced breeding and production practices. In addition, these findings (*in-*

ovo feeding trial) suggest potential impacts on intestinal physiology and function and highlight the importance of genotype-specific gene expression patterns for optimizing breeding strategies and enhancing poultry productivity and health.

This result on dietary supplementation suggests that DL-Met and L-Met have similar effects on early TSL chicks' development and feather growth. This implies they may have the same biological efficacy for average daily gain and body weight, but different utilization for the response of hematological parameters.

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10. PUBLICATIONS IN THE FIELD OF RESEARCH

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List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (1)

1. **Lugata, J. K.**, Ozsváth, X. E., Ndunguru, S. F., Kidane, G. R., Knop, R., Angyal, E., Oláh, J., Szabó, C.: Effect of genotype on the hematological parameter of TETRA-SL and Hungarian Partridge coloured chickens at young age.
Agrártud. Közl. 2022 (1), 99-104, 2022. ISSN: 1587-1282.
DOI: http://dx.doi.org/10.34101/actaagrar/1/10395

Foreign language scientific articles in international journals (4)

2. **Lugata, J. K.**, Ndunguru, S. F., Reda, G. K., Gulyás, G., Knop, R., Oláh, J., Czeglédi, L., Szabó, C.: In ovo feeding of methionine affects antioxidant status and growth-related gene expression of TETRA SL and Hungarian indigenous chicks.
Sci. Rep. 14 (1), 1-14, 2024. EISSN: 2045-2322.
DOI: http://dx.doi.org/10.1038/s41598-024-54891-3
IF: 4.6 (2022)

3. **Lugata, J. K.**, Ndunguru, S. F., Reda, G. K., Ozsváth, X. E., Angyal, E., Czeglédi, L., Gulyás, G., Knop, R., Oláh, J., Mészár, Z. M., Varga, R., Csernus, B., Szabó, C.: Methionine sources and genotype affect embryonic intestinal development, antioxidants, tight junctions, and growth-related gene expression in chickens.
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5. **Lugata, J. K.**, Ortega, A. D. S. V., Szabó, C.: The Role of Methionine Supplementation on Oxidative Stress and Antioxidant Status of Poultry-A Review.
   Agriculture-Basel. 12 (10), 1-20, 2022. EISSN: 2077-0472.
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   IF: 3.6

Foreign language abstracts (3)

6. **Lugata, J. K.**, Oláh, J., Czeglédi, L., Mészár, Z. M., Varga, R., Szabó, C.: Influence of in-ovo injection of methionine on the histomorphometric analysis of jejunum in layer genotypes newly hatched chicks.
   In: ASD 2022 : Book of Abstracts / Vladimir Brajković, Ivana Držaić, Mario Shihabi, Ino Čurik, University of Zagreb Faculty of Agriculture, Croatia, 83, 2022. ISBN: 9789538276361
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   In: Book of Abstracts : 29th Animal Science Days International Symposium, Hungarian University of Agriculture and Life Sciences, Gödöllő, 33, 2021.

List of other publications

Foreign language scientific articles in international journals (2)

9. Szabó, C., **Lugata, J. K.**, Ortega, A. D. S. V.: Gut health and influencing factors in pigs.
   Animals (Basel). 13 (8), 1-28, 2023. EISSN: 2076-2615.
   DOI: http://dx.doi.org/10.3390/ani13081350
   IF: 3 (2022)
10. Brassó, D. L., Komlósi, I., **Lugata, J. K.**, Rózsáné Várszegi, Z., Massányi, P., Knop, R.: Haematological parameters of Hungarian farmed ostriches.
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    IF: 0.8 (2022)

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Foreign language conference proceedings (1)

11. Ortega, A. D. S. V., Xayalath, S., **Lugata, J. K.**, Szabó, C.: Effects of heat stress-induced oxidative stress on the reproduction of sows and its alleviation by dietary antioxidants: a review.
In: XXV. Tavaszi Szél Konferencia 2022 : Tanulmánykötet I.. Szerk.: Molnár Dániel, Molnár Dóra, Nagy Adrián Szilárd, Doktoranduszok Országos Szövetsége, Budapest, 13-25, 2022. ISBN: 9786156457134

Foreign language abstracts (1)

12. Ortega, A. D. S. V., Xayalath, S., **Lugata, J. K.**, Szabó, C.: Effects of heat stress-induced oxidative stress on the reproduction of sows and its alleviation by dietary antioxidants: a review.
In: XXV. Tavaszi Szél Konferencia 2022 : Absztraktkötet. Szerk.: Molnár Dániel, Molnár Dóra, Doktoranduszok Országos Szövetsége, Budapest, 72-73, 2022. ISBN: 9786158205481

Total IF of journals (all publications): 21,3
Total IF of journals (publications related to the dissertation): 17,5

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

19 March, 2024

11. STATEMENTS

STATEMENT

I wrote this thesis in the framework of the University of Debrecen Doctoral School of Animal Science for the purpose of obtaining a doctoral degree (Ph.D.) at the University of Debrecen.

Debrecen, 2024.03.22

....

James Kachungwa Lugata

PhD candidate

STATEMENT

I hereby certify that the doctoral candidate **James Kachungwa Lugata** has carried out his work under my supervision within the framework of the above-mentioned Doctoral School between 2020-2024 The candidate has made a decisive contribution to the results of the thesis through his independent creative work, and the thesis is the candidate's independent work. I recommend that the thesis be accepted.

Debrecen, 2023.03.22

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Dr. Csaba Szabó

supervisor

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