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Effect of dietary methionine hydroxy analogue-free acid (MHA-FA) supplementation levels on growth performance, blood metabolites and immune responses in broiler chickens

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Abstract

Background: Broiler diets are commonly supplemented by liquid methionine hydroxy analogue-free acid (MHA-FA) containing as product with 88% of active substance to meet sulphur amino acid (SAA) requirements.

Objectives: Two experiments were conducted to assess the effects of different dietary MHA-FA levels on growth performance, blood metabolites and immune responses in broilers.

Methods: In experiments 1 and 2, 432 and 360 male broilers were fed either a basal corn–soya bean meal diet that met the energy and nutrient requirements with the exception of SAAs or the basal diet supplemented with 5 graded levels of MHA-FA (+0.9 and +0.8 g/kg), for 1–11 and 11–24-day ages, respectively. Chicks were vaccinated with inactivated H9N2/Newcastle disease (ND) and live-ND on days 5 and 11, respectively.

Results: Responses in both experiments had similar tendency at corresponding dietary MHA-FA levels. By increasing dietary MHA-FA level, weight gain (WG), feed efficiency (FE), relative breast weight (RBW) and immunity against ND and avian influenza virus inoculation improved with quadratic trend. Blood serum triglyceride, low-density lipoprotein, alanine aminotransferase and alkaline phosphatase concentration were affected in response to dietary MHA-FA levels with quadratic trends. By using broken-line regression analysis, the optimum dietary MHA-FA levels for optimized WG, FE and RBW during 1–10-day age were obtained at 2.20, 3.31 and 3.33 g/kg, respectively; based on MHA-FA content of Met equivalent, the digestible SAA requirements were estimated 0.81%, 0.91% and 0.92%, respectively. Similarly, for the 11–24-day age period, the optimum dietary MHA-FA supplementation levels were obtained 1.79, 2.21, 2.41 and 2.53 g/kg, with the digestible SAA requirements estimated 0.75%, 0.79%, 0.80% and 0.81% for optimized WG, FE, RBW and immune responses, respectively.

Conclusions: Supplemental levels of MHA-FA that optimize performance, breast meat and immunity in broilers fed corn–soya bean meal diet, varied from 0.18% to 0.33%, correspond to age and what traits are considered for optimization.

KEYWORDS
broiler chickens, growth performance, immunity, methionine hydroxy analogue-free acid, regression model

1 | INTRODUCTION

It is agreed that the diets used in broiler nutrition based on plant sources are deficient in methionine (Met) and cystine (Cys), known as sulphur amino acids (SAAs) (Faridi et al., 2016). Met is an initiator amino acid in the protein synthesis (Pontin et al., 2018) and is coupled to a specific initiator, methionine tRNA, by methionyl-tRNA synthetase (Chattapadhyay et al., 1990). In the living system, Met plays a vital role in many metabolic reactions (Brosnan & Brosnan, 2006) and energy production (Rehman et al., 2019). Met deficiency in broiler chickens' diet causes reduced growth, metabolic disorders and impaired immunity (Bunchasak, 2009; Rehman et al., 2019). The SAA requirements of the broiler chickens were previously determined by numerous researchers. Considering that experiments were conducted in different environmental conditions, basal diets with variation in feed ingredients and dietary energy and nutrient concentration, Met source, bird's genetic lines and age, estimation methods and traits are taken into consideration for optimization, but there are inconsistent results in the estimation (Pontin et al., 2018; Robbins et al., 2006).

Broiler diets are commonly supplemented by dry DL-methionine (DL-Met) containing about 99% of active substance and/or liquid DL-Met hydroxy analogue-free acid (MHA-FA; chemically known as 2-hydroxy-4-methylbutanoic acid) containing as product with 88% of active substance to meet the bird's SAA requirements (Agostini et al., 2016; Zhao et al., 2018). MHA-FA is chemically different from DL-Met because it has a hydroxyl group at the asymmetric carbon atom, whereas DL-Met has an amino group. This chemical difference lowers the bioavailability of MHA compared to DL-Met (Rehman et al., 2019). Both methionine sources allow for accurate balancing of the dietary amino acid profile; however, information about the relative biological efficiency of MHA-FA compared with DL-Met is a relevant factor for cost-effective purchasing, feed formulation and animal production (Agostini et al., 2016; Pontin et al., 2018; Powell et al., 2017; Zhao et al., 2018; Zou et al., 2015). Considering that most of the studies were carried out in order to determine the broiler SAA requirements by using DL-Met supplements and there are a few studies using MHA-FA, the current study was done to find out the effects of different doses of dietary MHA-FA supplementation on growth performance, serum biochemical profile, liver functional enzyme and immunity of broiler chickens during the starter (1–11 days of age) and grower (11–24 days of age) periods. In addition, the suitability of the regression method was assessed to find an optimal dietary MHA-FA level based on growth, carcass and immunity parameters. Corresponding to this, our hypothesis was to find an optimal dietary digestible SAA level at the situation where MHA-FA is the sole supplemental for supply broiler chickens with SAA (Met + Cys) requirements.

2 | MATERIALS AND METHODS

2.1 | Housing and management

Two experiments (Ex.) were designed on the base of comprehensive guides of animal welfare. The house temperature for day-olds was set at the range of 32–34°C and gradually decreased (0.5°C/day) to reach at the range of 20–22°C, which was kept constant thereafter. During the whole of rearing period, the relative humidity was 50%–60% and a lighting schedule of 18L:6D was imposed throughout the experimental period. All birds were reared in floor pens with wood shavings as litter. Metabolizable energy, protein and digestible amino acids of the feedstuffs used for experimental diet formulation were determined by using the Near Infra red (NIR) analysis (Evonik Nutrition & Care GmbH, an agent in Tehran, Iran). The values were used to create basal starter and grower diets in a minimum cost equation by user-friendly feed formulation done again software, University of Georgia, Athens, GA, United States (UFFDA, 1992). Basal diets were designed to meet or exceed broiler strain recommendations with the exception of digestible SAAs (Tables 1 and 2).

2.2 | Birds and experimental design

In Ex.1, newly hatched feather-sexed male Ross-308 chicks (n 432, Ross 308, mean weight 50 ± 1.60 g/birds) were obtained from a local hatchery. Chicks were randomly allotted into one of 36 pens (12b/pen) and arranged in a completely randomized design with 6 treatments and 6 replicates/treatment. A batch of basal corn–soya bean meal diet was made which met the nutrient and energy requirements of broilers during the starter period (Aviagen, 2022) with the exception of SAAs (Met + Cys) and then divided into six equal portions that were supplemented with MHA-FA at the rate of 0, 0.9, 1.8, 2.7, 3.6 and 4.5 g/kg in expense of filler (corn starch) and mixed to provide six experimental diets. Similarly, in Ex.2, newly hatched feather-sexed male chicks were obtained and fed a commercial starter diet (22% CP

TABLE 1 Ingredients and nutrients composition of the basal diets.

Items	Experiment 1 (days 1–11)[a]	Experiment 2 (days 11–24)[b]
Ingredient, g/kg as-fed basis		
Corn (ME = 3498 kcal/kg, CP = 7.8%)	519.4	551.9
Soya bean meal (ME = 2800, CP = 45.22%)	413.3	376.5
Soya bean oil (ME = 8820 kcal/kg)	20.1	29.0
Limestone	11.6	10.6
Dicalcium phosphate	18.7	16.6
Sodium chloride	4.3	4.4
Vitamin premix[c]	2.5	2.5
Mineral premix[d]	2.5	2.5
L-Lysine-HCL	1.3	1.2
L-Threonine	1.8	0.8
Filler (corn starch)	4.5	4.0
Determined nutrients composition[e], as-fed basis		
Metabolizable energy, kcal/kg	3000	3100
Crude protein, %	23.0	21.5
Calcium, %	0.96	0.87
Available phosphorus, %	0.48	0.43
Sodium, %	0.20	0.20
Digestible lysine, %	1.28	1.15
Digestible methionine, %	0.32	0.30
Digestible Sulphur amino acids, %	0.62	0.59
Digestible threonine, %	0.86	0.77

Abbreviations: ME, metabolizable energy; CP, crude protein.

[a]The experimental diets were provided in such a way that a batch of basal diets (without methionine supplementation) was made which met the nutrient and energy requirements of broiler chickens during the starter period with the exception of SAAs and then divided into six equal portions and were supplemented with methionine hydroxy analogue-free acid (MHA-FA) at the rate of 0, 0.9, 1.8, 2.7, 3.6 and 4.5 g/kg in expense of filler (corn starch) and mixed to provide six experimental diets.

[b]The experimental diets were provided in such a way that a batch of basal diets (without methionine supplementation) was made which met the nutrient and energy requirements of broiler chickens during the grower period with the exception of SAAs and then divided into six equal portions and were supplemented with methionine hydroxy analogue-free acid (MHA-FA) at the rate of 0, 0.8, 1.6, 2.4, 3.2 and 4.0 g/kg in expense of filler (corn starch) and mixed to provide six experimental diets.

[c]Vitamin premix supplied the following per kilogramme of diet: vitamin A (all-trans-retinol), 12,000 IU; vitamin D3 (cholecalciferol), 5000 IU; vitamin E (α-tocopherol), 18 IU; vitamin K3 (menadione), 2.65 mg; vitamin B1 (thiamin), 2.97 mg; vitamin B2 (riboflavin), 8.0 mg; vitamin B3 (niacin), 57.42 mg; vitamin B5 (pantothenic acid), 17.86 mg; vitamin B6 (pyridoxine), 4.45 mg; vitamin B9 (folic acid), 1.9 mg; vitamin B12 (cyanocobalamin), 0.02 mg; vitamin H2 (biotin), 0.18 mg; choline chloride, 487.5 mg; and antioxidant 1.0 mg.

[d]Mineral premix supplied the following per kilogramme of diet: Zn (zinc sulphate), 110 mg; Mn (manganese sulphate), 120.6; Fe (iron sulphate), 40.5; Cu (copper sulphate), 16.1; I (calcium iodate), 1.26; Se (sodium selenite), 0.31; choline chloride, 474.0.

[e]The determined ingredient analysis was used to calculate nutrient composition (crude protein, calcium and sodium were measured by the AOAC [2002] methods; metabolizable energy, digestible amino acids and available phosphorus were measured by NIR analysis).

and 3000 kcal ME/kg) until 10 days of age. At 11 days of age, the chickens (n 360, Ross 308, mean weight 253 ± 8.14 g/birds) were assigned to six dietary treatments, six replicates/treatment and 10b/replicate. The experimental diets were provided in such a way that a batch of basal corn–soya bean meal diet for grower stage (11–24 days of age) was made which met the nutrient and energy requirements of broiler chickens with the exception of SAAs (Aviagen, 2022), then divided into six equal portions, and were added MHA-FA at the rate of 0, 0.8, 1.6, 2.4, 3.2 and 4.0 g/kg in expense of filler (corn starch) and mixed to provide six experimental diets.

2.3 | Data collection and sampling

2.3.1 | Growth performance traits

The chicks of each pen were weighed in groups at the beginning (1- and 11-day old) and at the end (11- and 24-day old) of Ex. 1 and 2, respectively. In order to minimize the error resulting from the digestive tract contents weight, the birds were starved for 4 h before weighing. The feed consumption of each pen was calculated by subtracting the amount of feed remaining at the end of each experiment from the

TABLE 2 Experimental design.

Experiment 1 (days 1–11)			Experiment 2 (days 11–24)		
Addition of MHA-FA, g/kg of diet	Addition of Met equivalents[a], %	Dietary DSAAs level, %	Addition of MHA-FA, g/kg	Addition of Met equivalents[a], %	Dietary DSAAs level, %
Basal diet (non-supplemented)		0.62			0.59
0.9	0.08	0.70	0.8	0.07	0.66
1.8	0.16	0.78	1.6	0.14	0.73
2.7	0.24	0.86	2.4	0.21	0.80
3.6	0.32	0.94	3.2	0.28	0.87
4.5	0.40	1.02	4.0	0.35	0.94

Abbreviations: DSAAs, digestible sulphur amino acids; Met, methionine; MHA-FA, methionine hydroxy analogue-free acid.
[a]Based on that MHA-FA content of 88% of Met equivalent in the commercial product.

total feed given during the experimental period and adjusted for mortality. The growth performance indices, such as final live body weight (LBW), weight gain (WG) and feed intake (FI), were calculated. The feed efficiency (FE) was calculated by dividing the WG by the amount of FI.

2.3.2 | Blood sampling and serum biochemical profile

Blood samples were collected on days 11 (Ex.1) and 24 (Ex.2) from two randomly selected birds/replicate (12 birds/treatment) from the wing vein with helping disposable syringe (22-gauge needles). Blood samples were centrifuged at 1900 *g* for 5 min at 4°C to extract serum. Serum samples were analysed for uric acid (UA), creatinine (Cr), total protein (TP), albumin (Alb), total fat, triglyceride (TG), total cholesterol (Chol), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase (ALP) concentrations using an automated multi-pathway system autoanalyzer test (Cobas Bio, Roche) with kits from Pars Azmoon Company.

2.3.3 | Vaccine inoculation and immune responses

In Ex.2, on day 5, all birds were injected with the inactivated bivalent vaccines of Newcastle disease (ND) and avian influenza (AI) by subcutaneous route in the back of the neck (H9N2/ND 0.3 mL/bird, Razi Vaccine & Serum Research Institute, Iran). Moreover, on day 11, all birds were inoculated via the oral route with a commercial live-ND vaccine (Avinew NeO, Boehringer Ingelheim). Blood samples were collected at 24 days of age from two randomly selected birds/replicate (12 birds/treatment) for antibody analysis against ND virus (NDV) and AI virus (AIV). An immunological evaluation was carried out using HI kits (IDEXX, Labs Inc.) to test antibodies against NDV. Moreover, AIV antibody titre was performed using commercially available ELISA kits (IDEXX, Labs Inc.). ELISA absorbance was measured at 650 nm using an ELISA reader (BioTek Instruments Inc. ELX 800) according to standard procedures. The antibody titres were expressed as the log2 of the inversion of the highest serum dilution that could inhibit the haemagglutination of red blood cells completely.

2.3.4 | Carcass and visceral organs relative weight

At the age of 11 (Ex.1) and 24 (Ex.2) days, one bird from each repetition related to each treatment (six birds/treatment), which was close to the average weight of the repetition, was selected, weighed and slaughtered. After slaughtering, the visceral organs and pectoral and femoral muscles were dissected and weighed (digital weighing scale, 0.001 g, model GF 400, A&D Weight). The relative weight of cut parts was calculated as a percentage of live weight (g/100 g LBW).

2.4 | Statistical analysis

The experimental data were analysed for normality via the univariate procedure and then analysed by ANOVA using general linear model procedure procedures of SAS 9.4 software (SAS, 2014) appropriate for treatments in a randomized complete design. Means were compared using the Tukey test at the level of 0.05 probability. Orthogonal polynomial contrast coefficients were used to determine linear and quadratic effects of increasing MHA-FA supplementation levels on all measurements. To estimate the optimized responses, liner broken-line (LBL) and quadratic BL (QBL) models were used. The iterative procedure makes repeated estimates for coefficients and minimizes residual error until the best fit lines are achieved (Robbins et al., 2006).

3 | RESULTS

3.1 | Experiment 1

The effects of different MHA-FA supplementation levels in the starter (1–11 days of age) diet on growth performance traits, carcass and

TABLE 3 Effect of dietary liquid methionine hydroxy analogue-free acid (MHA-FA) supplementation on growth performance and carcass part relative weight in male Ross-308 broiler chickens during the starter period (1–11 days of age) Ex. 1[a].

		Growth performance				Carcass and cut relative weight[b] (g/100 g of live body weight)								
Addition of MHA-FA, g/kg of diet	Dietary DSAAs level, %	Live body weight, g	Feed intake, g	Weight gain, g	Feed efficiency	Carcass	Breast	Legs	Frame	Liver	Bursa of fabricius	Heart	GIT	Cover
Basal diet (non-supplemented)	0.62	248^{c}	349	197^{c}	0.566^{b}	49.36	14.00^{c}	16.02	19.35	3.29	0.25	0.81	16.38	5.98
0.9	0.70	255^{bc}	355	204^{bc}	0.574^{bc}	49.73	14.76^{bc}	15.43	19.55	3.22	0.27	0.78	15.11	6.68
1.8	0.78	267^{ab}	351	215^{ab}	0.611^{ab}	50.58	16.11^{abc}	15.92	18.55	3.23	0.24	0.83	14.75	6.20
2.7	0.86	265^{ab}	348	214^{ab}	0.616^{ab}	51.25	18.11^{a}	15.34	18.52	3.38	0.29	0.89	15.73	6.46
3.6	0.94	260^{abc}	346	211^{abc}	0.608^{ab}	52.40	17.72^{ab}	15.55	19.13	3.18	0.26	0.77	14.37	6.42
4.5	1.02	256^{ab}	350	205^{bc}	0.585^{bc}	49.28	15.87^{abc}	15.90	17.52	3.26	0.28	0.80	16.54	5.94
SEM		3.41	1.67	3.48	0.011	0.959	0.786	0.393	0.702	0.129	0.030	0.046	0.986	0.320
MHA-FA dose response														
Linear		0.001	0.742	0.001	0.001	0.357	0.001	0.318	0.057	0.924	0.528	0.889	0.959	0.777
Quadratic		0.001	0.892	0.001	0.002	0.097	0.008	0.332	0.654	0.941	0.967	0.370	0.142	0.164
Estimated dietary optimum MHA-FA level, g/kg		2.14	-	2.20	3.31	-	3.33	-	-	-	-	-	-	-

Note: In each column, values with different superscripts (a–c) are significantly different ($p < 0.05$).
Abbreviations: Dig SAAs, digestible sulphur amino acids; DL-Met, DL-methionine.
[a]The values are means of six replicates.
[b]Carcass obtained by feathers, head, feet, skin and viscera removed; frame is the carcass without the breast and legs; gastrointestinal tract (GIT) is the whole digestive organs (crop, proventriculus, gizzard and small and large intestines); cover (skin + feathers).

visceral organ relative weight are shown in Table 3. Responses to dietary MHA-FA levels during the starter period were significant (quadratic effect, $p < 0.05$) for final LBW, WG, FE and breast relative weight (BRW). The FI was not affected by dietary MHA-FA supplementation ($p > 0.05$). The LBW and WG improved as dietary MHA-FA level increased up to 1.8 g/kg. Birds fed diets containing 1.8 g/kg MHA-FA performed the highest LBW and WG, which were significantly ($p < 0.05$) higher (7.66% and 9.14%, respectively) than those fed non-Met-supplemented diets. Similarly, FE and BRW improved as dietary MHA-FA level increased up to 2.7 g/kg. Birds fed diet containing 2.7 g/kg MHA-FA performed the highest FE and BRW, which were significantly ($p < 0.05$) higher (8.83% and 29.35%, respectively) than those fed non-supplemented diet.

The effect of dietary MHA-FA levels on blood metabolites and liver functional enzymes is shown in Table 4. Responses to dietary MHA-FA level were significant (quadratic effect, $p < 0.05$) for blood metabolites and liver function enzymes. The lowest TG, Chol, LDL-C, TP, Alb, ALT and ALP concentrations were observed in the birds fed diet containing 2.7 g/kg MHA-FA, and the highest concentrations of these metabolites belong to birds fed non-supplemented diet. By increasing dietary MHA-FA supplementation level up to 2.7 g/kg, the blood metabolites, including TG, Chol, LDL-C, ALT and ALP concentrations, decreased. However, with increasing dietary MHA-FA supplementation to 3.6 and 4.5 g/kg, those blood metabolite concentrations increased.

3.2 | Experiment 2

The effects of different dietary MHA-FA levels during the grower period (11–24 days of age) on growth performance traits, carcass and visceral organ relative weights are shown in Table 5. Responses to dietary MHA-FA levels during the grower period (11–24 days of age) were significant (quadratic effect, $p < 0.05$) for final LBW, WG and FE and (linear effect, $p < 0.02$) for BRW. The FI was not affected by dietary MHA-FA supplementation ($p > 0.05$). LBW and WG improved as dietary MHA-FA levels increased up to 1.6 g/kg. Birds fed diet containing 1.6 g/kg MHA-FA in the grower period performed the highest LBW and WG, which were significantly ($p < 0.05$) higher (12.75% and 15.43%, respectively) than those fed non-Met-supplemented diets. Similarly, FE improved as dietary MHA-FA level increased up to 2.4 g/kg. Birds fed diet containing 2.4 g/kg MHA-FA performed the highest FE, which was significantly ($p < 0.05$) higher (19.79%) than those fed non-supplemented diet.

The effect of grower diet MHA-FA supplementation levels on blood metabolites, liver functional enzyme and immunity is shown in Table 6. Responses to dietary MHA-FA level were significant (quadratic effect, $p < 0.05$) for blood metabolites and liver function enzymes. The highest TG, LDL-C, UA, Cr, ALT and ALP concentrations were observed in the birds fed the non-supplemented diet. By increasing dietary MHA-FA

TABLE 4 Effect of dietary methionine hydroxy analogue-free acid (MHA-FA) supplementation on blood metabolites in male Ross-308 broilers on day 11 Ex. 1[a].

Addition of MHA-FA, g/kg of diet	Dietary DSAAs level, %	(mg/dL)						(g/dL)		(U/L)		
		TG	Chol	HDL-C	LDL-C	UA	Cr	TP	Alb	ALT	AST	ALP
Basal diet (non-supplemented)	0.62	56^{ab}	168^{a}	75	76^{a}	7.85	0.48	2.83	1.35	10.75^{a}	248	16,772^{ab}
0.9	0.70	58^{ab}	156^{ab}	62	70^{ab}	7.73	0.53	2.88	1.33	4.50^{bc}	225	16,806^{ab}
1.8	0.78	56^{ab}	143^{ab}	71	59^{bc}	7.35	0.40	2.20	1.13	4.75^{bc}	168	15,200^{b}
2.7	0.86	52^{b}	141^{b}	71	55^{c}	7.48	0.40	2.60	1.15	6.75^{b}	277	15,051^{b}
3.6	0.94	63^{ab}	145^{ab}	64	74^{a}	6.73	0.38	3.03	1.43	3.40^{c}	197	17,116^{ab}
4.5	1.02	65^{a}	169^{a}	67	79^{a}	7.43	0.45	2.90	1.53	6.30^{b}	222	19,414^{a}
SEM	2.91	5.91	2.84	3.34	0.39	0.06	0.13	0.07	0.61	12.62	905.90	
MHA-FA dose response												
Linear		0.042	0.001	0.439	0.001	0.149	0.201	0.132	0.103	0.001	0.490	0.012
Quadratic		0.041	0.001	0.643	0.001	0.480	0.322	0.113	0.101	0.013	0.387	0.002

Note: In each column, values with different superscripts (a–c) are significantly different ($p < 0.05$).
Abbreviations: Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Chol, whole cholesterol; Cr, creatinine; Dig SAAs, digestible sulphur amino acids; DL-Met, DL-methionine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; TP, total protein; UA, uric acid.
[a]The values are means of six replicates.

TABLE 5 Effect of dietary methionine hydroxy analogue-free acid (MHA-FA) supplementation on growth performance and carcass part relative weight in male Ross-308 broiler chickens during the grower period (11–24 days of age) Ex. 2[a].

Addition of MHA-FA, g/kg of diet	Dietary DSAAs level, %	Growth performance				Carcass and cut relative weight[b] (g/100 g of live body weight)								
		Live body weight, g	Feed intake, g	Weight gain, g	Feed efficiency	Carcass	Breast	Legs	Frame	Liver	Bursa of Fabricius	Heart	GIT	Cover
Basal diet (non-supplemented)	0.59	800^{b}	1119	557^{b}	0.480^{b}	58.57	19.52^{c}	18.64	20.41	2.78	0.24	0.67	10.44	7.94
0.8	0.66	851^{ab}	1121	607^{ab}	0.567^{a}	59.25	21.43^{bc}	17.59	20.23	2.40	0.20	0.64	10.01	8.29
1.6	0.73	902^{a}	1126	643^{ab}	0.571^{a}	60.77	22.43^{b}	18.11	21.00	2.78	0.19	0.65	9.00	7.02
2.4	0.80	895^{a}	1101	631^{ab}	0.575^{a}	61.12	22.89^{b}	18.36	19.66	2.74	0.22	0.64	9.97	7.59
3.2	0.87	889^{ab}	1094	628^{ab}	0.574^{a}	61.70	22.95^{b}	18.84	20.30	2.63	0.21	0.60	9.11	7.83
4.0	0.94	884^{ab}	1094	635^{ab}	0.581^{a}	60.94	24.82^{a}	18.21	19.67	2.59	0.23	0.61	8.83	7.51
SEM		23.23	29.01	21.58	0.018	0.847	0.455	0.42	0.63	0.15	0.03	0.04	0.34	0.21
MHA-FA dose response														
Linear		0.004	0.351	0.016	0.001	0.087	0.001	0.612	0.364	0.835	0.836	0.296	0.230	0.094
Quadratic		0.019	0.844	0.044	0.014	0.781	0.022	0.632	0.638	0.874	0.302	0.936	0.706	0.192
Estimated dietary optimum MHA-FA level, g/kg		1.95	-	1.79	2.21	-	2.41	-	-	-	-	-	-	-

Note: In each column, values with different superscripts (a–c) are significantly different ($p < 0.05$).
Abbreviations: Dig SAAs, digestible sulphur amino acids; DL-Met, DL-methionine.
[a]The values are means of six replicates.
[b]Carcass obtained by feathers, head, feet, skin and viscera removed; frame is the carcass without the breast and legs; gastrointestinal tract (GIT) is the whole digestive organs (crop, proventriculus, gizzard and small and large intestine); cover (skin + feathers).

supplementation level up to 2.4 g/kg, the blood metabolites, including TG, Chol, LDL-C, ALT and ALP concentrations, decreased. However, with increasing dietary MHA-FA supplementation to 3.2 and 4.0 g/kg, those blood metabolite concentrations increased. Immune response to ND and AI vaccination was affected by a significant and quadratic dietary MHA-FA level ($p < 0.01$).

3.3 | Estimated optimum dietary MHA-FA level

The current study results showed that with increasing MHA-FA supplementation levels, WG and FE were increased (quadratic; $p < 0.01$) during the starter and grower phases. This indicates that the basal diets were deficient in SAAs, which enabled us to determine the

TABLE 6 Effect of dietary methionine hydroxy analogue-free acid (MHA-FA) supplementation on blood metabolites and immune responses (antibody titre) to Newcastle disease, and avian influenza vaccination in male Ross-308 broilers on day 24 Ex. 2[a].

Addition of MHA-FA, g/kg of diet	Dietary DSAAs level, %	(mg/dL)						(g/dL)		(U/L)			Immune responses, log_2	
		TG	Chol	HDL-C	LDL-C	UA	Cr	TP	Alb	ALT	AST	ALP	NDV	AIV
Basal diet (non-supplemented)	0.59	94[a]	209[a]	79	88[a]	5.45	0.43	3.30	1.50	3.67[b]	278	13,838[a]	5.75[ab]	3.25[b]
0.8	0.66	91[ab]	191[ab]	81	83[a]	4.58	0.25	4.08	1.75	3.25[b]	266	10,262[b]	5.75[ab]	3.50[b]
1.6	0.73	79[b]	182[ab]	73	78[ab]	3.68	0.27	3.85	1.93	2.75[b]	260	10,329[b]	6.25[ab]	4.25[ab]
2.4	0.80	82[ab]	172[bc]	76	66[bc]	3.15	0.30	3.08	1.83	2.75[b]	253	9590[bc]	7.75[a]	5.50[a]
3.2	0.87	90[ab]	163[bc]	65	61[c]	3.45	0.30	3.50	1.60	3.50[b]	252	8568[cd]	5.75[ab]	3.75[ab]
4.0	0.94	87[ab]	150[c]	75	68[bc]	4.30	0.25	3.48	1.75	5.82[a]	243	8174[d]	4.25[b]	4.00[ab]
SEM		2.87	7.53	2.28	2.94	0.20	0.05	0.13	0.14	0.33	15.74	282.71	0.58	0.43
MHA-FA dose response														
Linear		0.009	0.006	0.069	0.001	0.131	0.112	0.412	0.082	0.001	0.417	0.001	0.007	0.006
Quadratic		0.017	0.158	0.303	0.076	0.121	0.158	0.518	0.284	0.006	0.696	0.002	0.002	0.015
Estimated dietary optimum MHA-FA level, g/kg													1.8	2.53

Note: In each column, values with different superscripts (a–c) are significantly different ($p < 0.05$).
Abbreviations: AIV, avian influenza virus vaccination; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Chol, whole cholesterol; Cr, creatinine; Dig SAAs, digestible sulphur amino acids; DL-Met, DL-methionine; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NDV, Newcastle disease vaccination; TG, triglyceride; TP, total protein; UA, uric acid.
[a]The values are means of six replicates.

optimum dietary MHA-FA supplementation level and estimate broiler digestible SAA requirements when using MHA-FA as the sole source of dietary SAA supplemental. The optimization model was solved using the NLIN SAS 9.1 programme procedure. First, it should be noted that the FI response did not fit the regression model in the starter and grower phases. Fitted regression models during starter and grower periods for the WG, FE, relative breast weight (RBW) and immunity responses as a function of dietary MHA-FA supplementation level are shown in Figures 1–4, respectively. The QBL configuration model was selected based on the highest adjusted R^2 and the lowest root means square error (RMSE) and Akaike's information criterion (AIC) to achieve the best dietary MHA-FA supplementation level for WG during starter period (Figure 1A) and grower period (Figure 1B), FE during starter period (Figure 2A) and grower period (Figure 2B), RBW at the end of starter period (Figure 3A) and at the end of grower period (Figure 3B) and immunity responses to ND (Figure 4A) and AI vaccination (Figure 4B).

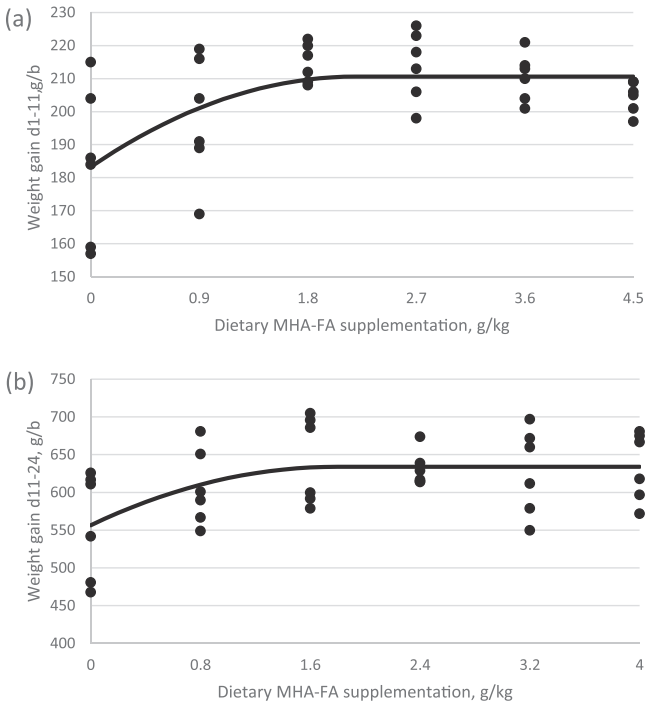

FIGURE 1 Weight gain (*Y*, in g/b) as a function of dietary methionine hydroxy analogue-free acid (MHA-FA) level (*X*, in g/kg of diet). (A) Ex 1; fed 1–11 days of age. Quadratic broken-line, $Y = 210.6 - 5.65(2.2\text{-}X)^2$, $p < 0.001$, $R^2 = 0.38$; the break point occurred at 2.2 g/kg MHA-FA supplementation. (B) Ex 2; fed 11–24 days of age. Quadratic broken-line, $Y = 633.9 - 24.23(1.79\text{-}X)^2$, $p < 0.008$, $R^2 = 0.26$; the break point occurred at 1.79 g/kg MHA-FA supplementation.

4 | DISCUSSION

4.1 | Growth performance

The growth performance traits, including final LBW, daily WG and feed conversion ratio of broilers, were significantly increased ($p < 0.05$) in the starter (Ex.1; days 1–11) and grower (Ex.2; days 11–24) periods due to the effect of MHA-FA. However, FI was not affected by dietary MHA-FA supplementation. These findings on growth

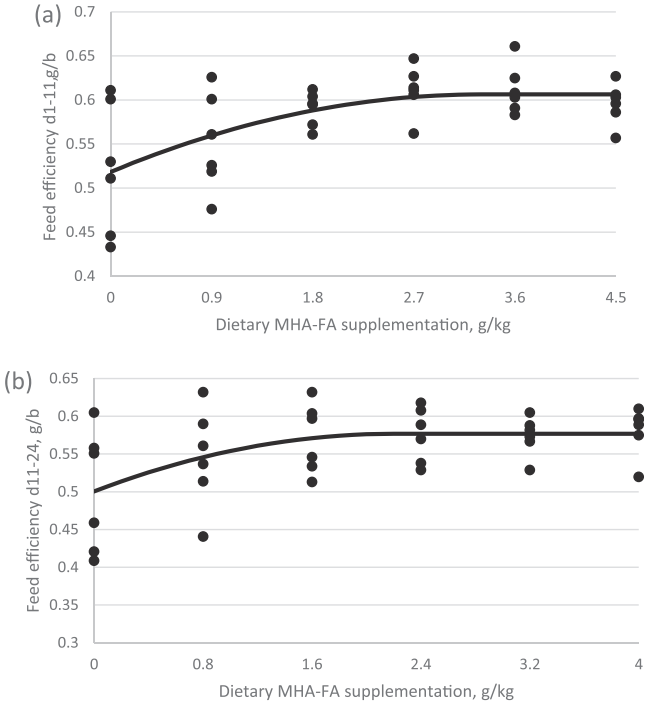

FIGURE 2 Feed efficiency (*Y*, g weight gain [WG]/100 g feed intake [FI]) as a function of dietary methionine hydroxy analogue-free acid (MHA-FA) level (*X*, in g/kg of diet). (A) Ex 1; fed 1–11 days of age. Quadratic broken-line, $Y = 0.606 - 0.008(3.3\text{-}X)^2$, $p < 0.001$, $R^2 = 0.39$; the break point occurred at 3.3 g/kg MHA-FA supplementation. (B) Ex 2; fed 11–24 days of age. Quadratic broken-line, $Y = 0.577 - 0.016(2.21\text{-}X)^2$, $p < 0.007$, $R^2 = 0.26$; the break point occurred at 2.21 g/kg MHA-FA supplementation.

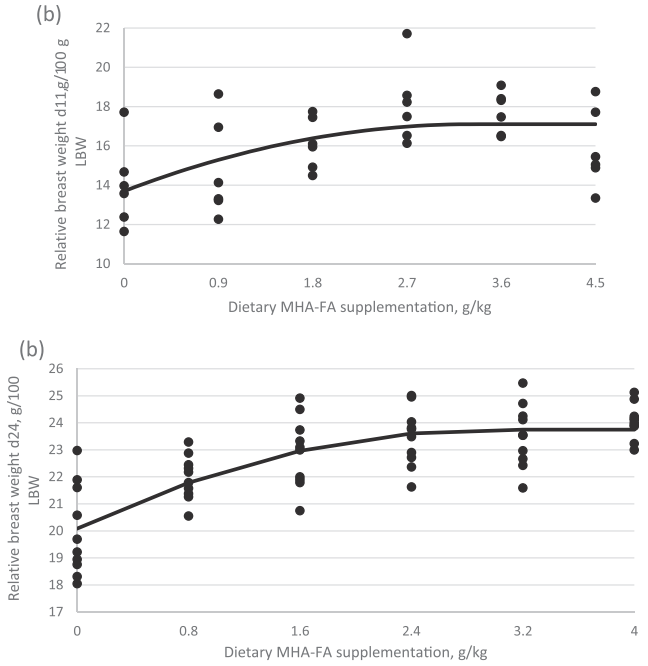

FIGURE 3 Breast relative weight (*Y*, in % of live body weight) as a function of dietary methionine hydroxy analogue-free acid (MHA-FA) level. (A) Ex 1; fed 1–11 days of age. Quadratic broken-line, $Y = 17.11 - 0.31(3.33\text{-}X)^2$, $p < 0.003$, $R^2 = 0.30$; the break point occurred at 3.33 g/kg MHA-FA supplementation. (B) Ex 2; fed 11–24 days of age. Quadratic broken-line, $Y = 23.74 - 0.41(2.98\text{-}X)^2$, $p < 0.001$, $R^2 = 0.59$; the break point occurred at 2.41 g/kg MHA-FA supplementation.

performance confirmed the reports of earlier researchers who studied the effect of different dietary methionine source supplements, including L-Met, DL-Met and MHA-FA, on growth performance and observed no differences among L-Met, DL-Met and DL-MHA-FA for WG and feed efficiency. It is reported that in the broiler chickens, growth production indexes increased with an increased level of MHA-FA in diet (Zou et al., 2015). Although not certain, it is generally accepted that MHA-FA has a low biological efficiency compared to DL-Met. For example, Sauer et al. (2008) indicated that the relative efficiency of MHA-FA was estimated at 81% and 79% of the values for DL-Met, on an equal basis, in broiler chickens. Similarly, low efficiency of MHA-FA (73% on an equivalent basis) compared to DL-Met has been reported (Hoehler et al., 2005). In contrast, the bioefficacy of MHA-FA compared to DL-Met in broiler chicks has also been reported. Nevertheless, it is common practice in the feed industry to add MHA-FA sources to poultry diets, although bioavailability compared to DL-Met is still in dispute (Conde-Aguilera et al., 2016; Liu et al., 2006; Swennen et al., 2011). The FI in the starter and grower periods remained unchanged by the effect of dietary MHA-FA level; this is supported by other researchers (Dilger & Baker, 2007; Goulart et al., 2011; Ohta & Ishibashi, 1995; Rehman et al., 2019). In contrast, it has been shown that broilers increase FI when fed diet contained insufficient Met (Moran & Edwin, 1994).

4.2 | Carcass and visceral organs relative weight

The levels of MHA-FA had a significant ($p < 0.05$) effect on RBW measured at the end of starter and grower periods; as the dietary MHA-FA level increased, the RBW improved ($p < 0.01$) with a linear trend. This finding confirms previous studies (Lemme et al., 2002; Liu et al., 2006; Schutte et al., 1997). Over the past decade, commercial broilers have continued to reach target market weight within shorter growing time. In these fast-growing broiler chickens, at slaughter weight, first 24 days of rearing period (starter and growing periods) encompass an estimated 60% of their whole life span. From the initial week of life alone, broilers can increase their body weight by at least threefold. In addition, more developmental changes were observed in muscle mass (Jin et al., 1998). Our results confirm the previous studies that muscle growth significantly improves with SAA supplementation in broiler chickens (Rehman et al., 2019; Schutte et al., 1997; Yoo et al., 2017). The present study showed that the relative weight of the body coat (skin + feathers) remained unchanged with dietary MHA-FA supplementation (Tables 3 and 5). However, there are several reports that cystine deficiency reduces the growth rate of the body coat (Kalinowski et al., 2003).

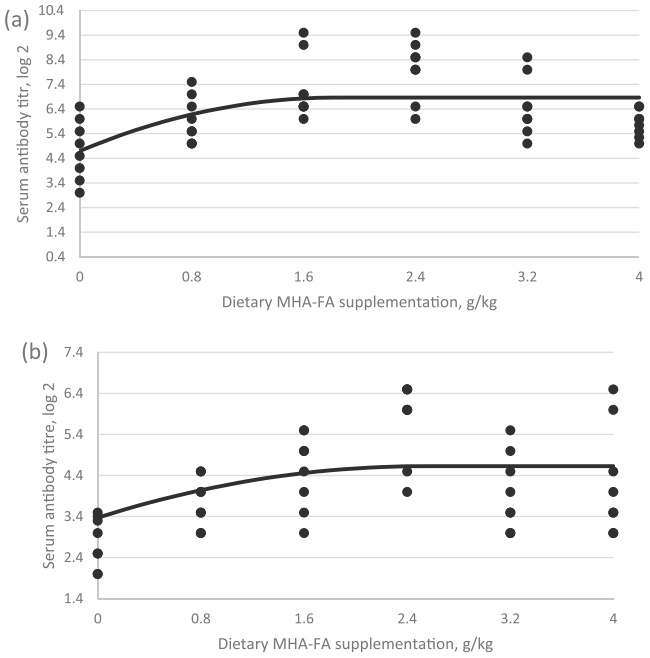

FIGURE 4 Serum antibody titre (*Y*, in log2) as a function of dietary methionine hydroxy analogue-free acid (MHA-FA) level. (A) In response to Newcastle disease vaccination. Quadratic broken-line, $Y = 6.87 - 0.65(1.83\text{-}X)^2$, $p < 0.001$, $R^2 = 0.30$. The break point occurred at 1.83 g/kg MHA-FA supplementation. (B) In response to avian influenza vaccination. Quadratic broken-line, $Y = 4.63 - 0.20(2.53\text{-}X)^2$, $p < 0.048$, $R^2 = 0.13$. The break point occurred at 2.53 g/kg MHA-FA supplementation.

4.3 | Serum biochemical profile

In general, in both experiments, chickens fed non-supplemented diet had significantly higher blood serum TG and cholesterol compared to chickens fed different levels of MHA-FA supplementation diets. Methionine is the precursor of L-carnitine and plays an essential role in lipid metabolism (Sigolo et al., 2019). It plays a crucial role in the production of energy by transporting fatty acids into mitochondria to be burned. Reportedly, L-carnitine has hypolipidemic effects, leading to a reduction in blood concentrations of TG, free fatty acids, total cholesterol and very high LDL-C (Diaz et al., 2000). The current experiment showed that liver functional enzymes were affected by dietary MHA-FA levels in a quadratic trend. The blood serum concentrations of ALT and ALP were the highest in the birds that were fed un-supplemented diets; by increasing dietary MHA-FA levels, the blood serum ALT and ALP concentrations decreased with quadratic trend (Tables 4 and 6). It was reported that liver functional enzymes are involved in amino acid catabolism (Muramatsu et al., 1971). The concentration of these enzymes in the blood serum decreases when the liver is working healthy (Rezaeipour et al., 2016). The increased serum ALT level may be due to the decreased antioxidative enzymes and reactive oxygen species (ROS) at the cellular level (Rehaman et al., 2017). Decreased activity of antioxidative enzymes alters the balance between the production of ROS and the antioxidant system, which affects serum metabolites (Chand et al., 2018). It is reported that SAAs can improve antioxidant properties by participating in glutathione (GSH) production (Afkhami et al., 2020).

4.4 | Immune responses

The present experiment was done to study whether the graded level of MHA-FA in diet could affect immune responses to mediators included with ND and AI vaccines. Results showed dietary MHA-FA supplementation improved immunity with a quadratic trend. However, there is a lack of references that agree or disagree with the current study. According to the current study, reported Met deficiency may adversely effect on the immune system (Rehman et al., 2019). A higher antibody titre in response ND and AI vaccination was observed in the broilers which fed on the diets containing up to 2.4 g/kg of MHA-FA, but adding more doses did not cause a higher antibody titre. Deficiency of dietary protein or amino acids has been reported to affect immune system activity and increase the susceptibility of animals and humans to infectious diseases (Afkhami et al., 2020).

4.5 | Estimated optimum dietary MHA-FA level

In general, the WG, FE and RBW of broilers gradually increased with increasing dietary MHA-FA levels in both experiments, thus demonstrating a clear SAA deficiency of the basal diets. The present data suggest that the optimum dietary MHA-FA during 1–11 days of age (starter period) to optimize WG, FE and RBW were 2.2, 3.31 and 3.33 g/kg of diets, respectively. Moreover, during 11–24 days of age (grower period) to optimize WG, FE, RBW and immunity responses to ND and AI vaccination, dietary MHA-FA supplementation was achieved at 1.79, 2.21, 2.41, 1.80, and 2.53 g/kg, respectively. Few experiments using graded levels of MHA-FA as the sole source of dietary Met supplemental are presented in the literature. Comparison of the results of these studies is also confounded by the statistics used to estimate the supplement levels that will optimize the responses evaluated. Reported maximum BWG and breast meat yields by using 0.15% MHA-FA in male broilers from 7 to 41 days (Esteve-Garcia & Llaurado, 1997). It has been reported to increase the gain of broiler chickens by adding 0.24% 2-hydroxy-4-(methylthio) butanoic acid (Lemme et al., 2002), 0.2% (Dibner et al., 2004) and 0.32% (Vedenov & Pesti, 2010) MHA-FA to corn–soya bean meal diet. Moreover, 0.32% MHA-FA supplementation provided best BWG and FCR in 21-day-old turkeys (Gonzales-Esquerra et al., 2007).

4.6 | Estimated digestible SAA requirements

According to the basal diet digestible SAA level (Table 2) and estimated dietary optimum MHA-FA levels (Tables 3 and 5 and Figures 1–4) based on an MHA-FA content of 88% of Met equivalent in the commercial product, the broiler digestible SAA requirements during the starter

period (1–11 days of age) were calculated 0.81% (optimize WG), 0.91% (optimize FE) and 0.92% (optimize RBW). Similarly, during the grower period (11–24 days of age), the broiler digestible SAA requirement was calculated 0.75% (optimize WG), 0.79% (optimize FE), 0.80% (optimize RBW) and 0.81% (optimize immune response). These estimates are clearly higher than the recommended values (NRC, 1994) and lower than the recommended values (0.95 and 0.87% for 1–10 and 11–24 days of ages, respectively) of the strain (Aviagen, 2022). Digestible SAA requirements during the first half (1–24 days) of broiler chicken rearing period were reported to be pretty variable, ranging from 0.68% (Faridi et al., 2016) to 1.0% (Ren et al., 2020). Aviagen (2022) recommended 0.92%–1.00% digestible SAAs in practical diets for Ross 308 broilers, depending on the age of the birds. In terms of SAA dose responses, it has been shown that requirements for maximum FE are often greater than requirements for WG (Faridi et al., 2016; Goulart et al., 2011; Pontin et al., 2018), which corroborates the current finding. Moreover, in the current study, the effects of graded levels of MHA-FA were particularly specific on the RBW trait. These results confirmed the results of other researchers that Met levels to increase RBW seem to be higher than requirements to obtain an optimal WG (Kalinowski et al., 2003; Pontin et al., 2018). To support the achievement of the genetic potential of today's broiler, so that breast muscle growth is increased, an increase in dietary AA concentration is required (Pontin et al., 2018).

5 | CONCLUSION

In conclusion, in the current study, increasing dietary MHA-FA supplementation increased growth performance, improved breast muscle deposition and increased the immunity of broilers fed corn–soya bean meal diets deficient in Met. In the broiler chickens during 1–10 days of age, fed a basal diet with 0.62% digestible SAAs, the optimum dietary MHA-FA levels for optimized WG, FE and RBW were obtained 2.20, 3.31 and 3.33 g/kg (0.22%, 0.31% and 0.33%, respectively); based on an MHA-FA content of 88% Met equivalent, the digestible SAA requirements were estimated 0.81%, 0.91% and 0.92%, respectively. Similarly, during 11–24 days of age, fed a basal diet with 0.59% digestible SAAs, the optimum dietary MHA-FA supplementation levels were obtained 1.79, 2.21, 2.41 and 2.53 g/kg (0.18%, 0.22%, 0.24% and 0.25%), with the digestible SAAs requirements estimated 0.75%, 0.79%, 0.80% and 0.81% for optimized WG, FE, RBW and immune responses, respectively. Overall, in male broilers fed corn–soya bean meal diet during 1–24 days of age, the optimum dietary supplemental levels of MHA-FA for optimizing growth performance, breast meat and immune response varied from 0.18% to 0.33% according to age period and what production parameter was taken into consideration for optimization; correspondingly, the digestible SAA requirements were estimated 0.75%–0.92%.

AUTHOR CONTRIBUTIONS

Heydar Zarghi designed the experimental trail. Saeed Ghavi carried out the experimental trail and lab analysis. Heydar Zarghi performed the statistics, tabulated the data and wrote the draft paper.

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CONFLICT OF INTEREST STATEMENT

No potential conflicts of interest were reported by the authors.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes and feed legislation.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are included in this published article.

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PEER REVIEW

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DECLARATIONS

The authors declare that all of the authors listed in the manuscript are employed at an academic or research institution where research or education is the primary function of the entity. Moreover, this manuscript is independently submitted by the authors.

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