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Original Research Article

Multi-carbohydrase enzymes improve feed energy in broiler diets containing standard or low crude protein

Sosthene Musigwa [a], Natalie Morgan [a], Robert A. Swick [a], Pierre Cozannet [b], Sarbast K. Kheravii [a], Shu-Biao Wu [a, *]

[a] School of Environmental and Rural Science, University of New England, Armidale, NSW, 2351, Australia
[b] Adisseo France SAS, 92160, Antony, France

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ABSTRACT

This study evaluated the effect of multi-carbohydrase (MC) on energy and nitrogen (N) balance and gene expression in broilers fed diets with different crude protein (CP) contents. The study employed a 2 × 2 factorial arrangement of treatments. The factors were presence or absence of MC, and standard (SCP) or low (LCP) dietary CP concentration. A 3-phase feeding program was used, including starter (0 to 7 d), grower (8 to 17 d) and finisher (18 to 28 d) phases. The study was undertaken in closed calorimetry chambers. Each of the 4 dietary treatments was replicated 8 times in total across 2 runs, with 2 birds per replicate ($n = 64$). Data for energy partitioning and N balance were collected from d 25 to 28. On d 28, birds were euthanized to collect muscle and intestinal tissue samples for gene expression. The results showed that the MC increased apparent metabolizable energy (AME, $P < 0.01$) and net energy (NE, $P < 0.05$), and reduced the feed conversion ratio (FCR, $P < 0.01$) in all diets. The proportion of energy retained as fat per total energy retention (REf/RE) was positively correlated with feed AME and NE ($r = 0.541$, $P < 0.01$ and $r = 0.665$, $P < 0.001$, respectively), suggesting that feed energy augmented with increased fat gain. Muscle ATP synthase subunit alpha (*ATP5A1W*) gene expression had a positive correlation with REf/RE and feed NE ($r = 0.587$, $P < 0.001$ and $r = 0.430$, $P < 0.05$, respectively). Similarly, muscle peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PGC-1A*) expression was negatively correlated with weight gain and positively correlated with FCR ($r = -0.451$, $P < 0.05$ and $r = 0.359$, $P < 0.05$, respectively). These correlations show that over-expressions of muscle genes related to energy production reduce bird performance. This study demonstrated that MC increase dietary energy utilization, regardless of dietary CP concentration. However, the energy released by the enzymes increases feed energy-to-CP ratio, meaning there is excess energy that is then deposited as body fat. This suggests that supplemental MC in broiler feeds is beneficial if diets are formulated to contain marginal energy levels.

1. Introduction

Livestock manure is a substantial contributing factor to global emissions of ammonia and nitrogen (N) excreted in the environment. For example, about 65% of feed N consumed by pigs is excreted in the form of faeces or urine (Morse, 1995; Aletor et al., 2000; Hou et al., 2015). Moreover, protein is the most costly component of feed, and its high dietary concentration may increase water consumption, causing wet litter issues (Francesch and Brufau, 2004). One of the current approaches taken by the poultry industry in an attempt to tackle this issue is lowering crude protein (CP) content in diets. Evidence has shown that feeding low-

* Corresponding author.
E-mail address: shubiao.wu@une.edu.au (S.B. Wu).
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CP (LCP) diets to broiler chickens results in heightened N efficiency and reduced N excretion, thus reducing the environmental impacts and carbon footprint of poultry production (Aletor et al., 2000; Bregendahl et al., 2002; Gomide et al., 2011; Belloir et al., 2017). However, altering CP levels in isoenergetic diets is associated with changes in energy-to-protein ratios, which can have major impacts on body composition, such as body fat content (Buyse et al., 1992; Nieto et al., 1997; Collin et al., 2003; Swennen et al., 2004). In fact, diets containing a high energy-to-CP ratio can result in heightened body fat deposition, whereas diets containing a high CP-to-energy ratio result in the lean muscle (Buyse et al., 1992; Nieto et al., 1997; Collin et al., 2003; Swennen et al., 2004).

The use of carbohydrase to improve feed energy has become a common practice, where more than 80% of the global market of carbohydrase enzymes is counted for by xylanase and β-glucanase (Adeola and Cowieson, 2011). Previous studies have demonstrated that supplementing multi-carbohydrase (MC) in poultry diets, such as xylanase, β-glucanase and arabinofuranosidase, can improve energy and protein utilization, nutrient digestibility and growth rates (Ravindran, 2013a; Cozannet et al., 2017; Saleh et al., 2019). The main mechanism behind this is that these exogenous enzymes improve nutrient availability by reducing intestinal digesta viscosity, through the degradation of non-starch polysaccharides. For instance, they can hydrolyze wheat arabinoxylan polymers, thereby increasing the ability of endogenous enzymes to access the nutrients present in wheat. They can also degrade the nutrient-encapsulating effect of indigestible and complex plant cell walls, thereby enhancing the access of endogenous enzymes to their substrates (Bedford, 2000; Leslie et al., 2007; Tahir et al., 2008). Despite the wealth of information available about the beneficial effects of carbohydrase in broilers diets, there is limited understanding of the direct relationship between MC application and energy utilization in broiler diets. Of particular interest is how MC can potentially improve energy utilization in birds fed LCP diets, through increasing N retention and availability of amino acids from dietary ingredients (Yamazaki et al., 2007). Consequently, the aim of this study was to explore the extent to which MC supplementation can affect energy utilization and N balance in broilers fed isoenergetic LCP or standard-CP (SCP) diets.

2. Materials and methods

2.1. Bird management

A total of 32 as-hatched d-old commercial Cobb 500 broilers per run ($n = 64$ in total across 2 runs) were procured from a local supplier (Baiada Pty Ltd, Tamworth, NSW, Australia). The husbandry practices (including temperature, light program, humidity) were undertaken according to Cobb 500 management guidelines (Cobb500, 2018a). Chicks were sexed from feather DNA using a high-resolution melting curve (HRM) analysis (England et al., 2020). Birds were provided ad libitum access to feed and water from d 0 to 28. A 3-phase feeding program was used, including a starter phase from d 0 to 7, a grower phase from d 8 to 17 and a finisher phase from d 18 to 28 according to Cobb 500 nutrient specification (Cobb500, 2018b) with minor modifications to suit the experimental conditions. All birds were fed common wheat-soybean meal-based starter and grower diets (Table 1). On d 18, birds with body weight close to the average were allocated to floor pens (10 birds per pen, 1 pen per dietary treatment), and received their respective experimental diets. On d 21, birds were assigned to 16 calorimetry chambers developed at the University of New England (Swick et al., 2013; Wu et al., 2019), with 2 birds per chamber, one male and one female. Each chamber made of stainless steel (100 cm × 76 cm × 70 cm) was provided with a digital display unit for temperature, humidity and pressure monitoring. Birds were given a 4-d acclimatization period with the chamber lids open. Calorimetric chambers were then closed during a 3-d experimental period from d 25 to 28, and they were briefly open each day for data collection.

Table 1
Ingredients and nutrient of the common starter and grower diets (as-is basis).

Item	Starter diet	Grower diet
Ingredients, %		
Wheat	59.8	52.3
Soybean meal	32.9	27.8
Sorghum	–	10.0
Canola oil	3.13	3.85
Canola ml solvent	–	2.00
Dicalcium phosphate	1.56	1.38
Limestone	1.13	1.03
TiO_2	–	0.500
L-Lys HCl (78.4% Lys)	0.278	0.252
D,L-Met	0.346	0.207
Sodium bicarbonate	0.200	0.200
Salt	0.219	0.171
L-threonine	0.166	0.135
Mineral premix[1]	0.100	0.100
Vitamin premix[2]	0.070	0.070
Choline Cl 70%	0.055	0.058
Multi-carbohydrase[3]	0.005	0.005
Nutrient, %		
AME poultry, MJ/kg	12.8	13.2
CP	23.7	21.9
Ether extract	5.03	6.02
SID amino acids		
Lys	1.28	1.15
Met	0.624	0.470
Met + Cys	0.950	0.774
Thr	0.860	0.770
Calcium	0.880	0.800
Phosphorus avail	0.440	0.400

AME = apparent metabolizable energy; CP = crude protein; SID = standard ileal digestible.
[1] Formulated to supply 23 mg copper, 1.79 mg iodine, 57 mg iron, 171 mg manganese, 0.43 mg selenium and 143 mg zinc per kg finished feed.
[2] Formulated to supply 5,040 mg retinol, 17.5 mg cholecalciferol, 105 mg tocopheryl acetate, 4 mg menadione, 4 mg thiamine, 11 mg riboflavin, 77 mg niacin, 18 mg pantothenate, 7 mg pyridoxine, 0.35 mg biotin, 3.0 mg folate, 0.02 mg cyanocobalamin per kg of finished feed.
[3] Rovabio Advance (xylanase, β-glucanase and arabinofuranosidase).

2.2. Experimental procedures and sampling

The protocol of the present study was approved and conducted in accordance with the Animal Ethics Committee of the University of New England, with authority No. AEC18-072. The study had a 2 × 2 factorial arrangement of treatments, with factors including MC, no or yes, and dietary CP content, LCP or SCP. A total of 4 isoenergetic wheat-based treatment diets were formulated based on Cobb 500 nutrient specification (Cobb500, 2018b) (Tables 2 and 3). Each dietary treatment was replicated 8 times. The standard ileal digestible (SID) AA were calculated according to Adedokun et al. (2008) and Zeitz et al. (2019). Digestible essential AA met or exceeded the requirements for the birds in all dietary treatments. The SCP diets contained on average 21.6% CP, whereas the LCP diets contained on average 17.8% CP (analyzed, Table 3).

The treatments with MC enzymes were supplemented with Rovabio Advance T-Flex in a powder form, which was thermostable (T-Flex) up to 90 °C during feed pelleting (Horrox, 2018). These enzymes are produced by *Talaromyces versatilis* fermentation, and their main enzymatic activities are xylanase, β-glucanase and arabinofuranosidase. They were added to diet at a rate of 50 g/t of formulated feed to provide a minimum of 25,000, 17,200 and 9,250

Table 2
Compositions of dietary treatments (%, as fed basis).

Item	SCP diet		LCP diet	
	–	+	–	+
Wheat	65.0	65.0	67.9	67.9
Soybean meal	21.1	21.1	7.39	7.39
Sorghum	5.00	5.00	14.0	14.0
Canola oil	3.00	3.00	1.88	1.88
Canola ml solvent	2.00	2.00	1.63	1.63
Dicalcium phosphate	1.29	1.29	1.42	1.42
Limestone	1.00	1.00	1.74	1.75
TiO_2	0.500	0.500	0.500	0.500
L-Lys HCl 78.4	0.255	0.255	0.680	0.680
DL-Met	0.203	0.203	0.309	0.309
Sodium bicarbonate	0.200	0.200	0.569	0.569
Salt	0.166	0.166	–	–
Mineral premix[1]	0.100	0.100	0.100	0.100
L-Thr	0.089	0.089	0.274	0.274
Vitamin premix[2]	0.070	0.070	0.070	0.070
Choline chloride	0.048	0.048	0.092	0.092
Multi-carbohydrase[3]	–	0.005	–	0.005
Potassium carbonate			0.399	0.399
L-Arg			0.370	0.370
L-Ile			0.238	0.238
L-Val			0.235	0.235
L-Leu			0.203	0.203
L-His			0.026	0.026
L-Trp			0.018	0.018

SCP = standard crude protein; LCP = low CP.
[1] Formulated to supply 23 mg copper, 1.79 mg iodine, 57 mg iron, 171 mg manganese, 0.43 mg selenium and 143 mg zinc per kg finished feed.
[2] Formulated to supply 5,040 mg retinol, 17.5 mg cholecalciferol, 105 mg tocopheryl acetate, 4 mg menadione, 4 mg thiamine, 11 mg riboflavin, 77 mg niacin, 18 mg pantothenate, 7 mg pyridoxine, 0.35 mg biotin, 3.0 mg folate, 0.02 mg cyanocobalamin per kg of finished feed.
[3] Rovabio Advance (xylanase, β-glucanase and arabinofuranosidase).

Table 3
Main nutrients of dietary treatments (%, as-is basis).

Item	SCP diet		LCP diet	
	–	+	–	+
Analyzed nutrient				
AME poultry, MJ/kg	13.6	14.5	14.2	14.5
CP	21.5	21.7	18.0	17.6
Calculated nutrients				
Ether extract	5.14	5.15	4.24	4.24
SID amino acids				
Arg	1.08	1.08	1.05	1.05
Lys	1.01	1.01	1.01	1.01
Met	0.448	0.448	0.495	0.495
Met + Cys	0.750	0.750	0.750	0.750
Trp	0.207	0.207	0.160	0.160
His	0.430	0.430	0.330	0.330
Leu	1.21	1.21	1.08	1.08
Ile	0.700	0.700	0.700	0.700
Thr	0.650	0.650	0.650	0.650
Val	0.800	0.800	0.800	0.800
Calcium	0.760	0.760	1.033	1.032
Phosphorus avail	0.380	0.380	0.380	0.380
Sodium	0.160	0.160	0.195	0.195
Potassium	0.804	0.804	0.775	0.775
Chloride	0.205	0.205	0.195	0.195

AME = apparent metabolizable energy; CP = crude protein; SID = standard ileal digestible; SCP = standard crude protein; LCP = low CP.

visco units (VU)/g of xylanase, β-glucanase and arabinofuranosidase, respectively, in a complete feed. One visco unit of xylanase and β-glucanase activity is defined as the amount of enzyme, which, at 30 °C and pH 5.5, can hydrolyzes barley β-glucan and wheat arabinoxylan, respectively, to lower the viscous fluidity up to 1 arbitrary unit/min. One visco unit of arabinofuranosidase corresponds to the amount of enzyme that hydrolyzes wheat arabinoxylan to release 1 nmol arabinose/min at 50 °C and pH 4 (Cozannet et al., 2017).

Data for calculating energy partitioning, weight gain (WG), feed intake (FI) and feed conversion ratio (FCR), were collected daily from d 25 to 28. Gaseous exchange (O_2 consumed and CO_2 exhaled) were recorded daily to determine respiratory quotient (RQ) and heat production (HP). The CO_2 exhaled by birds was trapped in 32% KOH connected to each chamber, and the KOH sub-samples were collected at the end of each day of the calorimetric run. On d 28, all birds were euthanized by electrical stunning and cervical dislocation, and digesta was collected from the whole ileum, and pooled per chamber. Samples of tissue from the duodenum (proximal section) and breast muscle (*Pectoralis superficialis*, middle right side) of the male birds were collected for gene expression analysis.

2.3. Sample processing and laboratory analyses

The digesta samples were frozen at −20 °C immediately after collection and then freeze-dried, ground (through a 0.5-mm screen) and stored in sealed plastic containers at room temperature for further analysis. Samples for excreta and feed were analyzed for gross energy (GE) using an adiabatic bomb calorimeter (Parr 6400 automatic isoperibol calorimeter, USA). The TiO_2 concentration in the diets and digesta samples were analyzed following the protocol described by Short et al. (1996). Diet and digesta samples were analyzed for DM by oven-drying at 105 °C until constant weight, GE content by adiabatic oxygen bomb calorimeter (Parr 6400 automatic isoperibol calorimeter, USA) and N content by LECO FP- 2000 automatic nitrogen analyzer (Leco Corporation, St. Joseph, MI). KOH samples were analyzed for CO_2 recovery following the $BaCl_2$ precipitation method described by Annison and White (1961).

Tissue samples were cleaned with chilled PBS, placed into 2-mL Eppendorf tubes containing RNAlater and then stored at −20 °C until RNA extraction. Total RNA extraction was performed with QIAcube HT System (Qiagen, Hilden, Germany) using a RNeasy 96 QIAcube HT Kit, following the manufacturer's protocols. A NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was used to determine the concentration and purity of RNA, and an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany) was used to measure RNA integrity determined by the RNA integrity number (RIN). The RIN values greater than 7.5 were considered high RNA quality (in the current study, 7.5 and 9.8 for duodenal RNA, and 7.5 and 9.6 for muscle RNA). The extracted RNA from each sample was reverse transcribed to complementary DNA (cDNA) using a Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, including gDNA elimination optional step. RotorGene 6000 quantitative real-time PCR (qPCR) machine (Corbett, Sydney, Australia) was employed to convert RNA into cDNA. Sample cDNA was diluted 8 times with nuclease free water, and then stored at −20 °C for qPCR analysis.

The qPCR analysis was performed in duplicate using a SYBR Green kit SensiFAST SYBR No-ROX (Bioline, Sydney, Australia), and a RotorGene 6000 RT-qPCR machine (Corbett Research, Sydney, Australia) was used to perform the amplification. Each reaction contained a volume of 10 μL reaction in total, which included 5 μL of SensiFAST master mix, 2 μL of diluted cDNA template, 400 mmol/L of each primer (reverse and forward) and 2.2 μL of nuclease free water. After PCR reaction, the cycle threshold (Ct) values of target genes were normalized using qBase+ version 3.0 (Biogazelle, Zwijnbeke, Belgium) software against 2 optimized housekeeping genes (TATA-box binding protein [*TBP*] and hydroxymethylbilane synthase [*HMBS*] in the muscle, and hypoxanthine phosphoribosyltransferase

1 [*HPRT*] and succinate dehydrogenase complex flavoprotein subunit A [*SDHA*] in the intestine). Data output obtained were expressed as relative expression of genes to the average geometric value of all samples and were exported to Excel for further analysis. The information of the target and reference genes are listed in Table 4.

The primers (Table 4) used in this study were either obtained from previous studies or designed using the primer tool from NCBI (https://www.ncbi.nlm.nih.gov/). All primers were checked for amplification specificity with their PCR amplicons run on Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Germany) using Agilent DNA 1000 Kit (Agilent Technologies, Inc., Germany) before qPCR analysis.

2.4. Calculation and statistical analyses

Feed AME was calculated by subtracting excreta GE from GE intake divided by FI. The O_2 consumed was directly calculated from the gravimetric change in O_2 cylinder weight before and after the chamber was operating. The volumes (in liters) of gaseous exchange were used to calculate HP (in kcal) using the modified Brouwer (1965) equation as follows:

$$HP = 1.200 \times CO_2 + 3.866 \times O_2.$$

The difference between AME intake and HP resulted in RE, and NE was calculated as RE plus the fasting HP (FHP), with a value of 450 kJ/per metabolic body weight. The metabolic body weight was calculated as body weight raised to the power of 0.70. The N content in diets and excreta was multiplied by 6.25 to obtain CP. The apparent ileal digestibility coefficient (AIDC) was calculated using the following equation:

$$AIDC = 1 - [\text{Digesta nutrient (g/kg DM)}/\text{Digesta } TiO_2 \text{ (g/kg DM)}]/[\text{Diet nutrient (g/kg DM)}/\text{Diet } TiO_2 \text{ (g/kg DM)}].$$

Data were statistically analyzed as a 2 × 2 factorial arrangement of treatments (with run as a covariate) using 2-way ANOVA with Minitab General Linear Model (GLM) procedure (Minitab Inc., State College, PA, USA), after Anderson-Darling testing to assess the normality of the data. Johnson transformation was employed to fit a normal distribution of non-normal data prior to analysis. Pearson method was used for correlation analysis, and Turkey's pairwise comparisons test were used to separate significant different means. The values of $P < 0.05$ were considered significant, and $0.05 < P < 0.10$ were considered as a trend to be significant.

3. Results

3.1. Bird performance, feed energy characteristics and N balance

The results of MC recovery analysis of experimental diets indicated 1,842; 2,745 and 7,412 VU/kg for β-glucanase, xylanase and arabinofuranosidase, respectively in the supplemented LCP diet. The supplemented SCP presented 1,854, 3,227 and 8,713 VU/kg for the recovered β-glucanase, xylanase and arabinofuranosidase, respectively. The mean standard deviations for the recovered enzymes were 108, 220 and 40 VU/kg for β-glucanase, xylanase and arabinofuranosidase, respectively. Enzyme activities were analyzed for endogenous enzymatic activities in the control.

Tables 5 and 6 illustrate bird performance, feed energy characteristics and N balance. A reduction in FI ($P < 0.05$) was seen in birds fed the diets supplemented with MC. Supplemental MC significantly increased feed AME ($P < 0.01$), AMEn ($P < 0.01$), NE ($P < 0.05$), AME/GE ($P < 0.01$), AME-to-CP (AME:CP) ratio ($P < 0.01$); N efficiency (N retained/N intake, $P < 0.05$) and FCR ($P < 0.01$) from d 25 to 28. Improvements ($P < 0.001$) in AME/GE and N efficiency were observed in birds fed the diets with LCP level. Birds fed the LCP diet also presented increased ($P < 0.001$) AME:CP ratio and RQ, as well as a higher ($P < 0.01$) proportion of energy retained as fat per total energy retention (REf/RE), compared to birds fed the SCP diets. On the other hand, birds fed diets containing SCP concentration showed comparatively greater WG, higher FI and lower FCR ($P < 0.001$ for all). The SCP treatment also resulted in reduced AME intake (AMEi)/WG ($P < 0.001$) and NE intake (NEi)/WG ($P < 0.01$) compared with the LCP diet. Ileal GE and CP digestibility, NE/AME and HP were not affected ($P > 0.05$) by either MC supplement or dietary CP level. There were no interactions observed between MC and protein level for any of these measured variables.

Table 7 shows that AME:CP ratio was positively correlated with AME, NE, REf/RE, RQ and FCR ($r = 0.537$, $P < 0.01$; $r = 0.442$, $P < 0.05$; $r = 0.673$, $P < 0.001$; $r = 0.577$, $P < 0.001$ and $r = 0.467$, $P < 0.01$, respectively), and negatively correlated ($P < 0.001$) with N intake, N retention, FI and WG ($r = -0.948$, $r = -0.875$, $r = -0.742$ and $r = -0.715$, respectively). Bird FI had a very strong positive correlation with WG ($r = 0.909$, $P < 0.001$) as expected, and

Table 4
Primers used for gene quantitative RT-PCR.

Gene	Accession No.	T_a, °C	Size, bp	Primer sequence (5′-3′)	Reference
ATP5A1W	XM_429118.5	60	232	F-GGCAATGAAACAGGTGGCAG R-GGGCTCCAGCTTGTCTAAGTGA	Nafari (2019)
COX III	KC847880.1	60	72	F-AGTCACCGTTACATGGGCTCA R-AGAGTTAGTGCGTGGATGGCTT	Nafari (2019)
HMBS	XM_417846.2	60	131	F- GGCTGGGAGAATCGCATAGG R- TCCTGCAGGGCAGATACCAT	Yin et al. (2011)
HPRT	NM_204848.1	63	245	F-ACTGGCTGCTTCTTGTG R-GGTTGGGTTGTGCTGTT	Yang et al. (2013)
ND2	JQ970529.1	60	147	F-AGGCTCCTCCCTAATCACTGC R-CCCATTCAGCCTCCGATTAG	Nafari (2019)
PGC-1A	XM 015285697.2	60	160	AGATCACCGTACAGTCGCAGAC CACGACGCTCTTCAATTGCT	This study
SDHA	NM_001277398.1	60	74	F-ATACGGGAAGGAAGGGGTTG R-TGCTGGGGTGGTAAATGGTG	Nafari (2019)
TBP	NM_205103 D83127	62	147	F-TAGCCCGATGATGCCGTAT R- GTTCCCTGTGTCGCTTGC	Li et al. (2005)

ATP5A1W = ATP synthase subunit alpha; *COX III* = cytochrome c oxidase subunit III; *HMBS* = hydroxymethylbilane synthase; *HPRT* = hypoxanthine phosphoribosyltransferase 1; *ND2* = nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 2; *PGC-1A* = peroxisome proliferator-activated receptor gamma (*PPAR-G*) coactivator 1 alpha; *SDHA* = succinate dehydrogenase complex flavoprotein subunit A; *TBP* = TATA-box binding protein; T_a = annealing temperature; bp = base pair.

Table 5
Effect of multi-carbohydrase and dietary CP on feed energy characteristics and N balance from d 25 to 28.

Item	Main effect means				Pooled SEM	*P*-value			
	MC		CP			MC × CP	MC	CP	Run
	No	Yes	Low	Standard					
Feed energy, MJ/kg DM									
GE	18.60	18.53	18.29	18.84	0.0511	–	–	–	–
AME	13.93[b]	14.46[a]	14.33	14.06	0.1260	NS	<0.01	NS	<0.01
AMEn	13.20[b]	13.73[a]	13.63	13.29	0.1230	NS	<0.01	0.071	<0.01
NE	10.42[b]	10.85[a]	10.70	10.57	0.1170	NS	<0.05	NS	<0.05
AME:CP ratio, MJ/kg:%	0.7114[b]	0.7437[a]	0.8042[a]	0.6509[b]	0.0020	NS	<0.01	<0.001	<0.01
Ileal digestibility coefficient, %									
GE	70.90	72.70	72.70	70.90	1.090	NS	NS	NS	<0.01
CP	80.90	82.30	82.00	81.30	1.500	NS	NS	NS	<0.01
Energy utilization, %									
AME/GE	74.92[b]	78.09[a]	78.35[a]	74.66[b]	0.7000	NS	<0.01	0.001	0.001
NE/AME	74.80	75.00	74.70	75.10	0.3000	NS	NS	NS	NS
Nitrogen balance									
N intake, g/d	3.96[a]	3.75[b]	3.27[b]	4.44[a]	0.1180	NS	<0.05	<0.001	0.001
N retained, g/d	2.635	2.554	2.311[b]	2.878[a]	0.0584	NS	NS	<0.001	<0.05
N efficiency[1], %	67.00[b]	68.77[a]	70.88[a]	64.89[b]	0.7000	NS	<0.05	<0.001	<0.01
Respiratory quotient	1.049	1.044	1.063[a]	1.030[b]	0.0045	NS	NS	<0.001	<0.01

MC = multi-carbohydrase; DM = dry matter; GE = gross energy; AME = apparent metabolizable energy; AMEn = AME corrected for nitrogen; NE = net energy; AME:CP ratio = AME-to-CP ratio; CP = crude protein; N = nitrogen; SEM = standard error of the mean.
[a, b] Means within a row with different superscripts differ significantly ($P < 0.05$, Tukey Pairwise Comparison test).
[1] N efficiency = N retained/N intake.

Table 6
Effect of multi-carbohydrase and dietary CP on energy balance and growth performance from d 25 to 28.

Parameter	Main effect means				Pooled SEM	*P*-value			
	MC		CP			MC × CP	MC	CP	Run
	No	Yes	Low	Standard					
Energy balance, kJ/(kg $BW^{0.70}$•d)									
HP	799.6	795.5	795.2	799.9	3.830	NS	NS	NS	NS
RE	587.8	589.5	569.7	607.6	9.860	NS	NS	NS	NS
AME intake	1,387	1,385	1,365[b]	1,408[a]	10.80	NS	NS	<0.05	NS
NE intake	1,038	1,040	1,020	1,058	9.860	NS	NS	0.056	NS
REf/RE, %	46.45	47.75	49.51[a]	44.69[b]	0.000	NS	NS	<0.01	NS
Growth performance									
FI/d, g (as is)	138.5[a]	129.4[b]	126.0[b]	141.0[a]	2.490	NS	<0.05	<0.001	<0.001
WG, g/d	84.31	84.22	76.37[b]	92.15[a]	2.350	NS	NS	<0.001	<0.001
FCR, g/g (as is)	1.647[a]	1.550[b]	1.660[a]	1.537[b]	0.0233	NS	<0.01	<0.001	<0.01
AMEi/WG, kJ/g	20.79	20.56	21.64[a]	19.70[b]	0.3740	NS	NS	<0.001	<0.001
NEi/WG, kJ/g	15.55	15.42	16.16[a]	14.81[b]	0.2560	NS	NS	<0.01	<0.01

MC = multi-carbohydrase; CP = crude protein; SEM = standard error of the mean; $BW^{0.70}$ = metabolic body weight; HP = heat production; RE = retained energy; AME = apparent metabolizable energy; NE = net energy; REf/RE = proportion of RE as fat per total RE; FI = feed intake; WG = weight gain; FCR = feed conversion ratio; AMEi = AME intake; NEi = NE intake.
[a, b] Means within a row with different superscripts differ significantly ($P < 0.05$, Tukey Pairwise Comparison test).

negatively correlated with feed AME and NE ($r = -0.710$ and $r = -0.547$, respectively). Furthermore, FCR was negatively correlated ($P < 0.001$) with N intake and N retained ($r = -0.591$ and $r = -0.613$, respectively), and positively correlated with N efficiency ($r = 0.405$, $P < 0.05$). The REf/RE was positively correlated with RQ, feed AME and NE ($r = 0.695$, $P < 0.001$; $r = 0.541$, $P < 0.01$ and $r = 0.665$, $P < 0.001$, respectively). In turn, RQ was negatively correlated ($P < 0.05$) with N intake and N retained ($r = -0.453$ and $r = -0.451$ respectively). The NE/AME had a negative correlation with HP and a positive correlation with REf/RE ($r = -0.747$, $P < 0.001$ and $r = 0.543$, $P < 0.01$, respectively).

3.2. Gene expression

The present study investigated mRNA expression of genes related to energy metabolic pathways in the intestinal and skeletal muscle mitochondria (Table 8). An interaction ($P < 0.05$) between MC and dietary CP concentration on intestinal ATP synthase subunit alpha (*ATP5A1W*) mRNA expression was observed, whereby in the absence of MC, *ATP5A1W* expression was significantly lower in birds fed the LCP diet compared to those fed the SCP diet, but in the presence of the enzymes, the difference between LCP and SCP diets disappeared. In addition, there was a tendency for an interaction on *PGC 1A* ($P = 0.080$), indicating that the expression of this gene in the duodenum was upregulated by MC supplementation. There was no significant effect of supplemental MC and dietary CP content or their interaction on duodenal cytochrome c oxidase subunit III (*COX III*) and nicotinamide adenine dinucleotide dehydrogenase subunit 2 (*ND2*) expressions. Muscle *ATP5A1W*, *COX III*, *ND2* and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PGC-1A*) were upregulated ($P < 0.01$, $P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively) in chickens fed the LCP diets, regardless of MC supplementation.

Table 9 illustrates the correlation results between gene expressions, feed characteristics and bird performance. Duodenal *ATP5A1W* expression had a weak negative correlation with REf/RE

Table 7
Correlations between feed characteristics and bird performance from d 25 to 28[1].

Item	AME	NE	GE dc	CP dc	AME/GE	NE/AME	Ni	Nr	Nr/Ni	HP	REf/RE	RQ	FI	WG	FCR
NE	0.931 ***														
GE dc	0.668 ***	0.593 ***													
CP dc	0.555 **	0.452 *	0.877 ***												
AME/GE	0.956 ***	0.870 ***	0.630 ***	0.516 **											
NE/AME	0.205	0.547 ***	0.058	−0.064	0.143										
Ni	−0.506 **	−0.371 *	−0.395 *	−0.363 *	−0.717 ***	0.160									
Nr	−0.397 *	−0.258	−0.369 *	−0.366 *	−0.608 ***	0.211	0.969 ***								
Nr/Ni	0.596 ***	0.491 **	0.383 *	0.282	0.765 ***	−0.049	−0.818 ***	−0.656 ***							
HP	−0.026	−0.298	−0.006	0.060	−0.063	−0.747 ***	0.136	0.164	−0.015						
REf/RE	0.541 **	0.665 ***	0.257	0.137	0.647 ***	0.543 **	−0.514 **	−0.453 *	0.467 **	−0.304					
RQ	0.064	0.156	−0.121	−0.187	0.243	0.269	−0.453 *	−0.451 *	0.277	−0.329 †	0.695 ***				
FI	−0.710 ***	−0.547 ***	−0.626 ***	−0.613 ***	−0.805 ***	0.156	0.872 ***	0.858 ***	−0.692 ***	0.102	−0.337 †	−0.104			
WG	−0.506 **	−0.364 *	−0.570 ***	−0.555 ***	−0.634 ***	0.179	0.865 ***	0.865 ***	−0.656 ***	0.102	−0.302	−0.142	0.909 ***		
FCR	0.094	0.003	0.350 †	0.341 †	0.222	−0.202	−0.591 ***	−0.613 ***	0.405 *	−0.004	0.167	0.137	−0.508 **	−0.810 ***	
AME:CP ratio	0.537 **	0.442 *	0.345 †	0.263	0.760 ***	−0.044	−0.948 ***	−0.875 ***	0.876 ***	−0.116	0.673 ***	0.577 ***	−0.742 ***	−0.715 ***	0.467 **

NE (MJ/kg DM) = net energy; GE dc (%) = ileal digestibility coefficient of gross energy; CP dc (%) = ileal digestibility coefficient of crude protein; AME (MJ/kg DM) = apparent metabolizable energy; AME/GE (%); NE/AME (%); Ni (g/d) = nitrogen intake; Nr (g/d) = N retained; HP (kJ/kg $BW^{0.70}$ per d) = heat production; $BW^{0.70}$ = metabolic body weight; REf/RE (%) = the proportion of energy retained as fat per total energy retained; RQ = respiratory quotient; FI (g, as is) = feed intake; WG (g) = weight gain; FCR = feed conversion ratio; AME:CP ratio (MJ/kg:%) = AME-to-CP ratio.
[1] Probability values are indicated as: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and † = $P < 0.10$ (tendency).

Table 8
Effect of MC and dietary CP on energy related gene expressions.

Parameter		Duodenal genes				Breast muscle genes			
MC	CP	*ATP5A1W*	*COX III*	*ND2*	*PGC-1A*	*ATP5A1W*	*COX III*	*ND2*	*PGC-1A*
No	Low	0.856[b]	1.083	0.9710	0.8140	1.103	1.278	1.197	1.383
No	Standard	1.193[a]	0.9793	1.030	1.193	0.7661	0.7150	0.6500	0.7890
Yes	Low	1.024[ab]	1.103	1.067	1.527	1.170	1.199	1.176	1.298
Yes	Standard	0.9650[ab]	1.023	1.095	1.130	0.9638	0.8560	0.8663	0.9890
Main effects									
MC	No	1.024	1.031	1.000	1.003	0.935	0.996	0.924	1.086
	Yes	0.9950	1.063	1.081	1.328	1.067	1.028	1.021	1.144
CP	Low	0.9401	1.093	1.019	1.170	1.136[a]	1.238[a]	1.186[a]	1.341[a]
	Standard	1.079	1.001	1.063	1.161	0.865[b]	0.786[b]	0.758[b]	0.889[b]
Pooled SEM		0.0401	0.0413	0.0534	0.1100	0.0484	0.0860	0.0689	0.1010
P-value									
MC × CP		<0.05	NS	NS	0.080	NS	NS	NS	NS
MC		NS	NS	NS	NS	NS	NS	NS	NS
CP		NS	NS	NS	NS	<0.01	<0.01	0.001	<0.05
Run		NS	NS	NS	NS	NS	<0.05	<0.05	NS

MC = multi-carbohydrase; CP = crude protein; SEM = standard error of mean; *ATP5A1W* = ATP synthase subunit alpha; *COX III* = cytochrome c oxidase subunit III; *ND2* = nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 2; *PGC-1A* = peroxisome proliferator-activated receptor gamma (*PPAR-G*) coactivator 1 alpha.
[a, b] Means within a row with different superscripts differ significantly ($P < 0.05$, Tukey pairwise comparison test).

($r = -0.377$, $P < 0.05$), coupled with a correlation tendency with FCR ($r = -0.357$, $P = 0.053$). In terms of skeletal muscle genes, *ATP5A1W* mRNA expression was positively correlated with REf/RE, N efficiency, RQ and feed NE, with $r = 0.587$, $P < 0.001$; $r = 0.385$, $P < 0.05$; $r = 0.500$, $P < 0.01$ and $r = 0.430$, $P < 0.05$, respectively. This gene was also negatively correlated with HP ($r = -0.589$, $P < 0.001$). The expression of *COX III* was positively correlated to REf/RE and RQ ($r = 0.411$, $P < 0.05$ and $r = 0.653$, $P < 0.001$, respectively), and negatively correlated with HP, N intake and N retained ($r = -0.603$, $P < 0.01$; $r = -0.446$, $P < 0.05$ and $r = -0.496$, $P < 0.01$, respectively). Similarly, *PGC-1A* expression was negatively correlated with N intake, N retained, HP, FI and WG ($r = -0.486$, $P < 0.01$; $r = -0.530$, $P < 0.01$; $r = -0.485$, $P < 0.01$; $r = -0.440$, $P < 0.05$, and $r = -0.451$, $P < 0.05$), and positively correlated with REf/RE, RQ and FCR ($r = 0.376$, $P < 0.05$; $r = 0.477$, $P < 0.01$ and $r = 0.359$, $P < 0.05$, respectively). There were also positive

Table 9
Correlations between gene expression against feed characteristics and bird performance.[1].

Gene	AME	NE	AME:CP ratio	Ni	Nr	Nr/Ni	HP	REf/RE	RQ	FI	WG	FCR
Duodenal												
ATP5A1W	−0.116	−0.163	−0.263	0.250	0.267	−0.138	0.179	−0.373 *	−0.290	0.152	0.256	−0.357 †
COX III	0.176	0.240	0.225	−0.159	−0.069	0.268	−0.108	0.323 †	0.231	−0.069	−0.093	0.036
ND2	0.375 *	0.439 *	0.072	−0.044	0.037	0.171	−0.147	0.211	−0.038	−0.159	−0.085	−0.074
PGC-1A	0.427 *	0.424 *	0.209	0.308	−0.117	0.194	−0.051	0.275	0.121	−0.266	−0.098	−0.172
Muscle												
ATP5A1W	0.332 †	0.430 *	0.562 ***	−0.490 **	−0.467 **	0.385 *	−0.589 ***	0.587 ***	0.500 **	−0.346 †	−0.294	0.144
COX III	0.027	0.122	0.457 *	−0.446 *	−0.496 **	0.179	−0.603 **	0.411 *	0.653 ***	−0.243	−0.298	0.320 †
ND2	0.083	0.068	0.539 **	−0.471 *	−0.408 *	0.494 **	−0.302	0.277	0.537 **	−0.195	−0.208	0.180
PGC-1A	0.274	0.312 †	0.447 *	−0.486 **	−0.530 **	0.245	−0.485 **	0.376 *	0.477 **	−0.440 *	−0.451 *	0.359 *

ATP5A1W = ATP synthase subunit alpha; *COX III* = cytochrome c oxidase subunit III; *ND2* = nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 2; *PGC-1A* = peroxisome proliferator-activated receptor gamma (*PPAR-G*) coactivator 1 alpha; AME (MJ/kg DM) = apparent metabolizable energy; NE (MJ/kg DM) = net energy; AME:CP ratio (MJ/kg:%) = AME-to-CP ratio; Ni (g/d) = N intake; Nr (g/d) = N retained; Nr/Ni (%) = N intake/N retained; HP (kJ/kg $BW^{0.70}$ per d) = heat production; $BW^{0.70}$ = metabolic body weight; REf/RE (%) = proportion of energy retained as fat per total energy retained; RQ = respiratory quotient; FI (g, as is) = feed intake; WG (g) = weight gain; FCR = feed conversion ratio.
[1] Probability values are indicated as: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and † = $P < 0.10$ (tendency).

correlations between AME:CP ratio against *ATP5A1W*, *COX III*, *ND2* and *PGC-1A*, with $r = 0.562$, $P < 0.001$; $r = 0.457$, $P < 0.05$; $r = 0.539$, $P < 0.01$ and $r = 0.447$, $P < 0.05$, respectively.

4. Discussion

The present study investigated the impact of supplementing MC enzymes in broilers fed isoenergetic diets containing low or standard CP levels on energy utilization and performance. The MC enzymes used in this study differ from other carbohydrase on market by their individual enzymatic activity profile, as MC combines xylanase, β-glucanase and arabinofuranosidase activities. Xylanase and β-glucanase reduce digesta viscosity in cereal-based diets, such as wheat and rye, by partially hydrolyzing the backbone of xylans and arabinoxylans. A complete arabinoxylans depolymerizing action by xylanase is enhanced by the synergistic action of arabinofuranosidase by removing arabinose from the arabinoxylans, thereby facilitating access of xylanase to the xylan backbone (Marquardt, 1997; Lei et al., 2016).

The application of MC in diets increased feed energy (by 3.7% for AME and 4.0% for NE), GE efficiency for AME utilization (AME/GE) and FCR, regardless of the dietary CP level. This is in agreement with previous studies reporting that supplementing broiler diets with MC improves feed AME and FCR (Adeola et al., 2010; Cozannet et al., 2017; Govil et al., 2017). As energy digestibility in birds is compromised by dietary non-starch polysaccharides, especially in their soluble form, the increase in wheat-based diets by MC, regardless of CP content, can potentially indicate the positive impact of these carbohydrase on feed energy improvement.

In addition, a reduction in FI, which resulted in lowered N intake, was noted in the MC-supplemented diets. This was likely due to the comparatively increased feed energy made available for utilization as a result of the enzymes. This heightened energy that could have exceeded the optimal AME to CP ratio is evidenced by an increase of AME:CP ratio by 4.3% in the MC-treated birds compared to the non-enzyme birds. This is in line with Bird (1997) who stated that the benefit of supplemental MC can only be fully realized upon reducing feed nutrient density, taking into account the dietary energy and AA digestibility enhanced by the enzyme addition. This may not only result in a cheaper feed formulation (Bedford, 1997), but also in a reduced AME:CP ratio and body fat deposition. Ravindran (2013b) stated that birds consume feed until their energy requirements are satisfied. Therefore, the excess of energy to CP ratio in the enzyme-supplemented diets observed in this study could have initiated the birds to reach satiety sooner and stopped eating before their N intake was comparable to that of the non-enzyme supplemented birds. This is confirmed by a negative correlation observed between AME:CP ratio and FI, indicating that bird FI reduced with increasing AME:CP ratio. The higher N efficiency observed in the enzyme-fed group could have resulted in lowered N intake, as this was strongly negatively correlated to N efficiency. This suggests that the lower N intake led to lesser N excreted, which resulted in a higher N efficiency.

As expected, N intake and N retained were reduced in birds fed the LCP diets, regardless of MC addition. However, the LCP-fed birds had comparatively high N efficiency and energy metabolizability efficiency (AME/GE), irrespective of MC supplementation. Improved N efficiency in the LCP-fed birds likely indicates that there was suboptimal N intake, and thus less N was excreted, as the majority of the dietary N was utilized. Moreover, poorer growth performance (FI, WG, FCR) was noted in birds fed the LCP diets. This resulted in higher energy cost for growth (AMEi/WG and NEi/WG) observed in this group compared to birds fed the SCP diets. These findings are in agreement with Blaxter (1989) and Bregendahl et al. (2002) who stated that the administration of LCP diets can be associated with a decrease in protein gain, which is followed by the increased energy utilization for growth.

In the present study, the negative correlation between WG and feed energy (AME and NE) and ileal digestibility of energy (CP and GE), coupled with a positive correlation between WG, N intake and N retained were observed. These correlations could suggest that improvement in feed energy and energy digestibility (from protein, carbohydrate and fat) increases energy-to-CP ratio, thereby increasing body fat accretion due to assistive energy provided by the feed. This is evidenced by the REf/RE which was positively correlated to feed energy (AME and NE), AME:CP ratio and energy digestibility. Moreover, FI was strongly correlated to WG as expected; higher FI increases N intake and N retained, providing

proteins indispensable for growth. In addition, the observed negative correlation between FCR and N intake and retained, and thus N efficiency, was also expected. This suggests that protein gain increased when there was more N intake, which, in turn, increased N excretion to a higher proportion than the increase in N retention, thereby lowering N efficiency. The positive correlation between N efficiency and FCR can be linked to the low N content in the LCP diets.

The RQ, which is a gas-exchange ratio between CO_2 and O_2, indicates the degree of nutrient oxidation during mitochondrial energy production (Choct, 2004). In the present study, the higher RQ value in birds fed the LCP diets than those fed the SCP diets, coupled with a positive correlation between RQ and REf/RE, and negative correlations between RQ and N intake and N retained, were observed. These imply that the LCP-fed birds consumed more energy from carbohydrates and fat, which was deposited as body fat. This is in agreement with Matarese (1997) who stated that fat synthesis from carbohydrate increases the RQ value. MacLeod (1997) also stated that broilers fed the LCP diets deposit more body fat because they consume a great proportion of energy from carbohydrates and fats. This author explained that energy from carbohydrate and fat substrates is more efficiently deposited as body fat than an excess of protein would be. Furthermore, the negative correlation between NE/AME and HP, coupled with a positive correlation between NE/AME and REf/RE observed in the present study, could indicate that HP decreased with increasing NE/AME, which augmented with increasing REf/RE. This is in agreement with Noblet (2007) who stated that utilization of AME for NE is highest for fat and starch and lowest for protein (90%, 82% and 62%, respectively). This may imply that appropriate levels of NE/AME and N in the diet should be considered in order to maximize energy efficiency, whilst ensuring that carcass quality is not compromised by excess deposition of fat in the animal.

In this study, the dietary Ca concentration was 10 g/kg in the LCP diets and 8 g/kg in the SCP diets. It was noted that Ca concentration above 8.5 g/kg can negatively affect bird performance (Fallah et al., 2019), but according to the results from Abdollahi et al. (2016) and Akter et al. (2018) there was no significant impact on growth performance (FI, WG and FCR) between broilers fed diets containing 8 and 10 g Ca/kg. However, the results from Fallah et al. (2019) show low WG in birds fed diets with 10 g/kg of Ca from limestone in comparison to those fed 7 g/kg of Ca. However, the difference was marginal (4.3% difference) and there was no statistical difference for FI and FCR between those *2* diets. Moreover, there was no influence of dietary Ca levels on dietary energy response (Abdollahi et al., 2016; Akter et al., 2018). Thus, the different growth performance observed in the present study between the diets with 2 levels of CP is entirely due to the difference in dietary CP content. Moreover, the growth performance data in this study were obtained using 2 birds/replicate over a 3-d period of experimentation, and a large-scale feeding trial is therefore needed for more conclusive results. Furthermore, substantial differences in energy and N characteristics, as well as gene expression were observed between run 1 and 2. These differences resulted in the difference in body weight between the 2 runs (Noblet et al., 2010; Noblet, 2015), whereby the average of initial body weight of birds before starting the experiment (on d 25) was 10% higher in run 1 than run 2 because the birds were obtained from 2 different batches. Due to the nature of the study with constraint availability of the number of calorimetric chambers, it is necessary to perform different runs to achieve the sufficient number of replicates; however, it is recognized that sufficient replicates and animals in a single run is much beneficial to achieve more significant test power for the treatments.

In the study herein, most of duodenal genes correlating with bird performance showed an opposing effect with muscle genes correlated with bird performance. This suggests that those genes may function differently in intestinal and muscle tissues, whereby the expression of intestinal genes upregulated with increased bird performance, whereas muscle gene expressions increased with impaired bird performance. It was mentioned that a differential expression of same genes in different tissues (breast muscle and intestine) could be due to variations in cell response to oxidative stress, as a constant turnover of cells in intestinal epithelium removes oxidized proteins, whereas a slow turnover of muscle cells leads to the accumulation of oxidized proteins (Ojano-Dirain et al., 2007b). Furthermore, differences in gene regulations and inherent metabolism exist between these tissues. Therefore, the gene functions in different tissues in relation to animal performance may be an interesting area to explore.

The *ATP5A1W* or F1F0-ATP synthase or complex V gene encodes proteins catalyzing ATP production from ADP in the presence of a proton gradient and inorganic phosphate (Ojano-Dirain et al., 2007a). An interaction between MC and CP concentration on duodenal *ATP5A1W* expression was observed in the present study. This gene was downregulated when birds were fed the LCP diets in comparison with those fed the SCP diets, but the application of the enzymes eliminated this difference. Upregulation of intestinal *ATP5A1W* in the LCP fed group by the enzymes to the level of the SCP-fed group suggests that supplementing the MC in the LCP diets enhanced nutrient absorption by reducing digesta viscosity, possibly coupled with an increased access of endogenous proteases to substrates. As a result, more N could have been released and absorbed, leading to more N retained, thereby reducing the energy:CP ratio and fat gain. This is evidenced by a weak negative correlation observed between intestinal *ATP5A1W* and REf/RE, coupled with a tendency for negative correlation between this gene and FCR. Therefore, as opposed to muscle *ATP5A1W*, the expression of intestinal *ATP5A1W* increased with improved bird performance (increased lean muscle and reduced FCR). On the other hand, Zhang et al. (2018) observed an increase in *ATP5A1W* mRNA expression in the muscle of broilers with high growth, and Ojano-Dirain et al. (2007b) observed no difference in muscle *ATP5A1W* expression between low and high feed efficient broilers. More studies to investigate the relationship between *ATP5A1W* expression and performance, possibly with different dietary treatments, are warranted for a clear picture regarding the expression of this gene in different tissues.

The mRNA expressions of *ATP5A1W*, *COX III*, *PGC-1A* and *ND2* levels were higher in the breast muscle of birds fed the LCP than SCP diets, irrespective of MC addition. This implies that the LCP birds consumed more energy than their requirement for normal growth and metabolism, and the excess was stored as fat (Leeson and Summers, 2001). In so doing, muscle genes involved in energy production were likely overexpressed to produce more energy, as de novo lipogenesis is an ATP-consuming process (Hardie, 2003). In contrast to the expression of intestinal *ATP5A1W* gene, skeletal muscle *ATP5A1W* expression was positively correlated to RQ, N efficiency, feed NE and REf/RE. This shows that muscle *ATP51W* gene expression was heightened with increased body fat deposition. In addition, the expression of this gene was negatively correlated to HP. This suggests that upregulation of this gene in the muscle was associated with less HP, indicating that the energy substrates oxidized contained less N, as protein oxidation release more heat than fat and carbohydrates (Gous, 2010). This correlation confirms tissue variation in mitochondrial gene expression, whereby increased expression of energy genes in skeletal muscles compromised bird performance, whereas the increased expression of these genes in the intestines improved bird performance. This can be evidenced by a correlation observed between REf/RE and *ATP5A1W* expression, whereby the REf/RE was negatively correlated with

intestinal *ATP5A1W*, and positively correlated with muscle *ATP5A1W*.

The *COX III* gene is a subunit of complex IV responsible for regulating proton pumping and electron transport along the mitochondrial respiratory chain, and is associated with oxidative phosphorylation efficiency. This gene is therefore responsible for the efficiency of mitochondrial energy production. Its lower expression may be associated with oxidative damage caused by high reactive oxygen species (ROS) production, low cell efficiency or aging (Del Vesco et al., 2013; Tarai et al., 2017). It was demonstrated that protein oxidation, coupled with a high ROS production observed in low feed efficient chickens can alter mRNA expression of mitochondrial genes (Ojano-Dirain et al., 2007b). In the present study, *COX III* expression level was higher in the muscles of the LCP-fed birds than the SCP-fed groups. The expression of muscle *COX III* was positively correlated to REf/RE and RQ, suggesting that over-expression of this gene in muscle contributed to energy production needed for fat synthesis. In addition, this gene was negatively correlated with HP, N intake and N retention, denoting that an increase of *COX III* expression in muscle resulted in a lowered muscle accretion. It was previously reported that HP increases with elevated protein accretion and muscle deposition (Close, 1990; MacLeod, 1997).

Avian *PGC-1A* is a gene responsible for stimulating the expression of mitochondrial transcription factor A and nuclear respiratory factor-1 in chicken, that in turn initiate mitochondrial and nuclear genes encoding proteins required for ATP production and fat metabolism (Ojano-Dirain et al., 2007b; Olesen et al., 2010). Upregulation of *avPGC-1A* mRNA expression in skeletal muscles in broilers fed LCP diets was observed in this study. The positive correlations noted between muscle *avPGC-1A* gene expression and REf/RE, N efficiency and FCR, coupled with its negative correlations with, FI, N intake, N retained and WG, show that overexpression of this gene in skeletal muscle reduced bird performance. This suggests that higher expression of muscle *avPGC-1A* is an indicator of slower growth. *ND2* is another gene that controls the production of the mitochondrial ROS and is negatively affected by high dietary fat content and bird aging (Zhang et al., 2016). The results from this study demonstrated that the level of muscle *ND2* mRNA expression was higher in the LCP- than the SCP-fed birds. It was also previously found that a high-fat diet reduces the expression of genes responsible for oxidative phosphorylation and biogenesis in skeletal muscle mitochondria (Sparks et al., 2005). The negative correlation between HP and muscle *ATP5A1W*, *COX III* and *PGC-1A* perhaps occurred because energy substrates contained more carbohydrates and fat than N, which are easily deposited as fat with less HP (MacLeod, 1997). This is also evidenced by the observed positive correlation between the above genes and REf/RE. In addition, a positive correlation observed between the AME:CP ratio and muscle expression of *ATP5A1W*, *COX III*, *ND2* and *PGC-1A* also implies that excess of energy in relation to protein induced overexpression of genes related to energy production in the muscles.

To conclude, the use of MC in isoenergetic diets can increase feed energy and N efficiency, and lowered FCR, regardless of dietary CP content. However, if the released energy exceeds the optimal point of energy-to-CP ratio, FI and Ni will be lower. This is because an excess of energy-to-CP ratio induces birds to reach satiety before their N requirements are met. Therefore, the full benefit potential of supplemental MC can be achieved by using feed lower in energy content. In this case, the energy released by the enzymes would not exceed the energy-to-protein ratio appropriate for chickens, thereby improving bird growth performance and feed efficiency. Further research on optimal AME:CP ratio to achieve this is warranted.

Author contributions

Sosthene Musigwa: conceptualization, data curation, formal analysis, methodology, investigation, drafting manuscript; **Natalie Morgan**: data collection, critically reviewing the manuscript; **Pierre Cozannet**: design, methodology, critically reviewing the manuscript; **Robert Swick**: methodology, feed formulation, critically reviewing the manuscript; **Sarbast K. Kheravii**: methodology, critically reviewing the manuscript; **Shu-Biao Wu**: conceptualization, data curation, investigation, project administration, critically reviewing the manuscript.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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