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Making the most out of energy content in broiler diets using xylanase, emulsifier, and guanidinoacetic acid mixtures

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ABSTRACT

This research endeavour examined several nutritional approaches aimed at optimising the energy content of the broiler diets using mixtures with distinct mechanisms of action in low metabolisable energy diet (LME). Within this context, the impact of various xylanase (Xyl), emulsifier (EM), and guanidinoacetic acid (GAA) mixtures supplemented to LME diets were investigated on the following parameters: growth performance, energy and protein efficiency ratios, oxidative biomarkers, gene expression, and intestinal morphology. Seven hundred one-day-old (Ross 308) male-broilers were assigned to five experimental treatments (28 birds/replicate). The positive control group (PC) fed as breed-recommendations. A dietary change was made to the PC, where the dietary energy content was reduced by 200 kcal/kg feed, served as negative control group (NC). The other experimental diets were as in NC group, fortified with either Xyl+EM, Xyl+GAA or Xyl+EM+GAA. The results demonstrated a negative impact on the NC group's productivity and biometric parameters compared to the PC group ($p < 0.05$). Combinations of Xyl+GAA or Xyl+EM+GAA were as effective as PC in term of growth performance ($p > 0.05$), but they were more efficient in terms of energy efficiency ratio ($p < 0.05$). In contrast to the PC group, The Xyl+EM group had lower protein efficiency ratio ($p < 0.05$). The oxidative biomarkers, gene expression, and intestinal morphology of the NC groups supplemented with various mixtures were better than those of the PC group ($p < 0.05$). In conclusion, the overall benefits in the Xyl+EM+GAA group were notable. It is possible to compensate for LME (-200 kcal/kg feed) by using Xyl+EM+GAA mixture.

Introduction

Feed expenses make up a substantial part of the overall production cost, constituting over 70% of the total costs (Kubiś *et al*., 2020). Feed intake is strongly influenced by the dietary energy content, which is the most expensive component in poultry diets. Therefore, optimising the energy utilisation can have a significant impact on the feed and production cost (Ahiwe *et al*., 2018). The digestive system of broilers, especially the small intestine, is a sophisticated environment where a wide range of enzymatic reactions take place (Ravindran and Reza Abdollahi, 2021). Those enzymatic reactions are highly dependent on the available substates and the antinutritional factors in the feed that hinder or delay the enzymes reactions (Yang *et al*., 2023). In the context of broiler performance, the use of various enzymes can potentially enhance the digestion and absorption of the nutrients at both the intestinal and cellular level (Hashim *et al*., 2023).

Fats or oils are incorporated into the feed due to their superior caloric value compared to other nutrients. However, due to the immature physiological functions and inadequate production of lipase and bile, the ability of newly hatched chick to digest and absorb lipids is limited. Therefore, supplementation of emulsifiers (EM) plays a significant role in maximizing fat digestion. It can help lipase by reducing the size of fat globules and increasing the surface area of for lipase to act on. The latter can improve the digestion and absorption of fats in the intestine (Hosseini *et al*., 2018; Kubiś *et al*., 2020; Kinh *et al*., 2022; Li *et al*., 2022). Recent studies demonstrated that EM supplementation increased the productive performance in low metabolisable energy diets (LME) (Saleh *et al*., 2020) and in diets containing only fat from raw feed ingredients (Ghazalah *et al*., 2021). Additionally, it was reported that EM supplementation improved the antioxidant capacity (Saleh *et al*., 2020; Khalil *et al*., 2024) and intestinal health (Ghazalah *et al*., 2021; Khalil *et al*., 2024).

Xylanase (Xyl) is an enzyme that helps broilers in breaking down the complex sugar (arabinoxylan) present in the plant cell wall. This complex sugar often hinders the digestion of intracellular nutrients like starch and protein. When Xyl interacts with insoluble fibre, it exhibits a prebiotic effect, altering the gut microbiota's composition and increasing the production of short-chain fatty acids. When it acts on soluble fibres, it reduces gut viscosity (Rawash *et al*., 2023). Previous researched confirmed that Xyl supplementation in LME diets had positive effect on the productive performance (Williams *et al*., 2014; Tang *et al*., 2017; Saleh *et al*., 2023), oxidative status (Zhang *et al*., 2018; Pirgozliev *et al*., 2021), growth related genes (Saleh *et al*., 2023), and intestinal health (Liu and Kim, 2017; Hosseini *et al*., 2018; Khalil *et al*., 2024).

It was hypothesized that supplementation of Xyl and EM may have a synergistic effect on both lipid and carbohydrate portions of the diet in LME diets. Results showed that Xyl+EM supplemented group was comparable to either of the individual supplements, indicating that no synergistic effect was noticed (Hosseini *et al*., 2018). However, in another study, Xyl+EM supplementation deepened the crypts significantly compared to the control group ($p< 0.05$) (Kubiś *et al*., 2020). Moreover, in triticale-based diets, supplementation of EM, Xyl and EM+Xyl were studied. Result showed no synergistic effect between Xyl and EM on growth performance ($p > 0.05$) (Wiśniewska *et al*., 2023).

The ultimate end-product of glucose or fatty acid metabolism is ATP. The produced ATPs in the mitochondrial matrix require a shuttling system to transport them to the site of energy utilization, the cytoplasm. This facilitated by the creatine-phosphocreatine shuttle system. Although creatine (Cre) can be de novo synthesized in the animal body, it may be limited in fast growing birds (Khalil *et al*., 2021a, 2021b; Khalil *et al*., 2024). Cre works by increasing the production of ATP, however the efficiency of ATP storage in the form of phosphocreatine (PCre) is highly dependent on the intracellular amount of Cre molecule (Khalil *et al*., 2021b; Majdeddin

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et al., 2023a). Therefore, the dietary fortification of guanidinoacetic acid (GAA), the precursor source of Cre, can boost the intracellular Cre level by overcoming the feedback mechanisms on the first enzymatic step of Cre synthesis, glycine amidinotransferase (Khalil *et al*., 2021a). Many previous studies reported the effectiveness of GAA supplementation in broiler performance (Abudabos *et al*., 2014; Metwally *et al*., 2020; Ceylan *et al*., 2021; Pirgozliev *et al*., 2022), oxidative biomarkers (Khalil *et al*., 2021a; Zhao *et al*., 2021; Khalil *et al*., 2024), growth related genes (Metwally *et al*., 2015; Farshidfar *et al*., 2017; Khalil *et al*., 2024) and intestinal histomorphology (Raei *et al*., 2020; Khalil *et al*., 2024)

This study is a continuation of our prior research, which was carried out under identical experimental conditions. It was hypothesized that using the mixtures of Xyl, EM, and GAA in low metabolisable energy diets (200 kcal/kg) may restore the energy deficit and improve the productive performance of male-broiler-chicken. Also, our study aimed to investigate the effect of combined products on energy and protein efficiency ratio, oxidative biomarkers, protein, and energy related gene expression, and intestinal histomorphology.

Materials and methods

Bird husbandry and experimental design

The handling and care of the birds was approved by the Institu-

Table 1. Ingredient (%) and chemical compositions of starter, grower, and finisher diets[1]

Ingredients	Starter (0-14 Day)		Grower (14-26 Day)		Finisher (26-35 Day)	
	PC	NC	PC	NC	PC	NC
Corn	52.42	51.86	57.23	61.51	62.04	66.4
Soybean meal (46% crude protein)	35	35	31.01	30.75	26.6	26.25
Corn gluten meal (60% crude protein)	3.88	3.7	3.46	3.13	2.52	2.23
Soya oil	3.75	1.93	4.55	0.86	5.34	1.63
Filler (Sand)	-	2.79	-	-	-	-
Dicalcium phosphate (18.2%)	1.39	1.39	1.17	1.16	1.05	1.04
Limestone	1.09	1.09	1.03	1.04	0.93	0.93
Soybeans full fat, toasted	0.85	0.63	-	-	-	-
L-Lysin HCL (78%)	0.32	0.32	0.3	0.31	0.28	0.29
Premix [2]	0.3	0.3	0.3	0.3	0.3	0.3
Salt (NaCl)	0.3	0.3	0.3	0.3	0.3	0.3
DL-Methionine (99%)	0.28	0.28	0.26	0.26	0.24	0.23
Toxin binder	0.15	0.15	0.15	0.15	0.15	0.15
Choline Chloride (60%)	0.1	0.1	0.1	0.09	0.11	0.11
L-Threonine (98.5%)	0.08	0.08	0.06	0.07	0.05	0.05
Sodium bicarbonate	0.07	0.07	0.07	0.07	0.08	0.08
Phytase[3]	0.01	0.01	0.01	0.01	0.01	0.01
Total	100	100	100	100	100	100
Chemical composition (%)						
Dry matter	88.48	88.37	88.55	88.1	88.61	88.16
Crude protein	23.3	23.36	21.26	21.28	19	19
Fat	6.59	4.65	7.33	3.82	8.23	4.7
Metabolisable energy (kcal/kg)	3000	2800	3100	2900	3200	3000
Calcium	0.96	0.96	0.87	0.87	0.79	0.79
Available phosphorous	0.48	0.48	0.43	0.43	0.4	0.4
Potassium	0.97	0.97	0.89	0.89	0.8	0.81
Sodium	0.17	0.17	0.17	0.17	0.17	0.17
Chloride	0.21	0.21	0.21	0.21	0.21	0.22
Dietary electrolyte balance (mEq/kg)	263.42	263.48	240.83	242.33	219.6	220.63
Choline	1700	1700	1600	1600	1550	1550
Total lysine	1.44	1.44	1.3	1.3	1.16	1.16
Total methionine	0.65	0.65	0.6	0.6	0.55	0.54
Total methionine + cysteine	1.03	1.03	0.95	0.95	0.87	0.86
Total threonine	0.98	0.98	0.88	0.88	0.79	0.79
Total valine	1.11	1.11	1.02	1.02	0.91	0.91
Total arginine	1.51	1.51	1.36	1.36	1.21	1.2

[1]PC, positive control; NC, negative control. Xyl+EM: NC supplemented with 0.01% xylanase and 0.025% emulsifier; Xyl+GAA: NC supplemented with 0.01% xylanase and 0.06% guanidinoacetic acid; Xyl+EM+GAA: NC supplemented with 0.01% xylanase, 0.025% emulsifier and 0.06% guanidinoacetic acid.
[2]Vitamins and mineral premix: vitamin A, 12,000,000 IU; vitamin D3, 2,200,000 IU; vitamin E, 10,000 mg; vitamin K3, 2,000 mg; vitamin B1, 1,000 mg; vitamin B2, 5,000 mg; vitamin B6, 1,500 mg; vitamin B12, 10 mg; niacin, 30,000 mg; pantothenic acid, 10000 mg; biotin, 50 mg; folic acid, 1000 mg; copper, 4000 mg; ferrous, 30,000 mg; manganese, 60,000 mg; zinc, 50,000 mg; iodine, 1000 mg; selenium, 100 mg.
[3]Quantum blue (AB-Vista) at 100 g/ton to provide 500 FTU and the following matrix value was considered (calcium, 1000%; phosphorus, 1000%; sodium, 350%; lysine, 170; methionine, 39; methionine + cysteine, 390; threonine, 330; arginine, 130; isoleucine 255; valine, 230).

tional Animal Care and Use Committee at Vet. CU. IACUC (Vet CU 2009 2022465). The study was carried out in a semi-enclosed ventilation system at the Poultry Research Centre, part of the Faculty of Veterinary Medicine at Cairo University in Giza, Egypt. The facility was divided into 25 equal pens, each 2x2 m in size. The birds were housed on a solid concrete floor with wood shavings used as bedding. A total of 700 one-day-old male chicks (Ross 308) were allocated into five experimental groups, each with five replicates (28 birds per replicate). The first group was fed as suggested by the breed manual, served as PC group. The second group was fed low metabolisable energy (LME) (200 kcal/kg), served as NC group. Meanwhile, The third, fourth and fifth groups were fed as NC diet that included 0.01% xylanase(Xyl)(Econase XT; *Trichoderma reesei*, 160,000 BXU/kg; AB Vista, UK) plus either 0.025% emulsifier (EM) (Lysoforte extend lysolecithin, synthetic emulsifier and monoglycerides; Kemin, Belgium) or 0.06% guanidinoacetic acid (GAA) (Creamino, at least 96% GAA; AlzChem Trostberg GmbH, Trostberg, Germany) or a mix of Xyl, EM and GAA, respectively. The birds had free access to food and water. For the first three days, they were subjected to a continuous light programme of 24L:0D, which was subsequently reduced to 20L:4D until they were 35 days old. The temperature was kept at 32°C for the first three days, then steadily dropped by 0.50°C per day until it reached 24°C at the end of the experiment. Throughout the experiment, the humidity fluctuated between 55 and 60%. The regular recommended immunisation schedule against infectious illnesses was administered to all birds.

Diets

Corn-soy-based basal diets were formulated according to the guidelines provided by Aviagen (Aviagen, 2019), except for the reduced energy groups (-200 kcal/kg) Table 1. Starter, grower, and finisher diet were fed to birds aged 0-14, 14-26, and 26-35 days, respectively. A horizontal double ribbon mixed was used to mix all diets. Starter feeds were offered in a crumbled form, meanwhile pelleted feeds (90°C) were offered until the end of the experiment.

Sampling

On day 36, five birds were chosen from each pen in each group to represent the average weight of the pen. Birds were slaughtered by severing the jugular vein. For further investigation, liver, breast muscle and intestinal samples were collected.

Measurements

Growth performance

Birds were weighed at the start of the study, and again after each feeding period to determine their body weight and feed intake (FI). After accounting for mortality, body weight gain (BWG) and feed conversion ratio (FCR) were calculated accordingly. EPEF = (average grams gained/day X % survival rate)/Feed Conversion X 10) is the formula used to calculate the European Production Efficiency Factor (Bawish *et al*., 2023).

Energy and protein efficiency ratio

Energy efficiency ratio (EER) and protein efficiency ratio (PER) were calculated based on total protein intake (TPI) and total metabolisable energy intake (MEI) recorded at the end of each feeding period and cumulatively using the following equations according to (Kamran *et al*., 2011)
MEI = FI x targets ME level of each phase/1000.
EER = WG/100 kcal energy intake.
TPI = FI x target crude protein of each phase/100.
PER = (WG /PI).

Oxidative biomarkers

After homogenising (1 g) of liver tissue in 9 mL of ice-cold PBS, the supernatant was collected and analysed for reduced glutathione (GSH), total glutathione (T-GSH), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) activities. Commercially available kits (Abcam, Cambridge Science Park, UK) were used to measure T-GSH and GSH levels (GSH+GSSG / GSH Assay Kit, No. ab239709), total SOD (Superoxide Dismutase Activity Assay Kit, No. ab65354), and total GSH-Px (No. ab102530) activities according to the manufacturer's instructions.

Gene expression analysis

The collected breast muscle and liver samples were frozen at -80°C for later analysis. The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from different samples, as per the manufacturer's instructions. The cDNA was synthesised with an Omniscript RT reagent kit from Qiagen, and real-time polymerase chain reaction (PCR) was carried out with iQ SYBR Green Supermix (BIO-RAD, USA). The procedures were executed precisely as described by manufacturer's instructions. The mRNA levels of myogenin, myostatin, cystathionine-β-synthase (CBS), and insulin-like growth factor 1 (IGF-1) in the breast muscle as well as adenosine monophosphate-activated protein kinase (AMPK) in liver samples were examined. Sequences of the primers are listed in Table 2. The PCR reactions were run on an Applied Biosystems QuantStudio 5 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) with the following settings: 95°C for 30 s, 40 cycles at 95°C for 5 s and 60°C for 34 s, 95°C for 15 s, and a dissociation stage of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. The levels of expression were normalised to β-actin, and gene expression was calculated as $2^{-\Delta\Delta Ct}$ and expressed as fold change compared to the control group, as described by Livak and Schmittgen (2001). Every single sample was tested three times in all real-time PCRs

Table 2. Nucleotide sequences of specific primers[1]

Gene symbol	Accession number	Orientation	Primer sequence (5′-> 3′)	Amplicon size (bp)
β-Actin	L08165	Forward Reverse	GAGAAATTGTGCGTGACATCA CCTGAACCTCTCATTGCCA	152
AMPK	NM_001039603	Forward Reverse	CGGCAGATAAACAGAAGCACGAG CGATTCAGGATCTTCACTGCAAC	148
CBS	XM_416752.3	Forward Reverse	CTGGGATCTTGAAACCTGGA TCAGGACATCCACCTTCTCC	147
Myogenin	D90157	Forward Reverse	AGCAGCCTCAACCAGCAGGA TCT GCCTGGTCATCGCTCAG	149
Myostatin	NM_001001461	Forward Reverse	TACCCGCTGACAGTGGATTTC GCCTCTGGGATTTGCTTGG	153
IGF-1	AY331392	Forward Reverse	CACCTAAATCTGCACGCT CTTGTGGATGGCATGATCT	140

[1]β-Actin: beta-actin; AMPK: adenosine monophosphate-activated protein kinase; CBS: cystathionine-β-synthase; IGF-1: insulin like growth factor 1

(RT-PCR).

Intestinal histomorphology

Intestinal samples from duodenum, jejunum, and ileum were fixed in neutral buffer formalin (10%) before being processed for hematoxylin and eosin staining (H&E) (Bancroft, 2013). The Leica DM 4B light microscope (Leica, Germany) was used to examine the slides, and the images were captured with Leica DMC 4500 digital camera (Leica, Germany). Image J software (1.45 s, National Institute of health, USA) was used to measure intestinal villus height (from the tip of the villus to the villus-crypt junction) and crypt depth (from the base of crypt up to the villus-crypt junction); the villus height to crypt depth ratio was then calculated (Abdelatty *et al*., 2021).

Statistical analysis

The data collected from productive performance, oxidative biomarkers, gene expression, and intestinal histomorphology were analysed with Minitab Statistical software version 18 and were subjected to one-way ANOVA. Protected Fisher's LSD test at $\alpha < 0.05$ was used to make a comparison between the positive control (PC), which served as a reference group, and the negative control groups. The results shown in tables are means and their pooled standard error of means (SEM).

Results

Growth performance

Results of growth performance are shown in Table 3. Starter phase showed no statistical difference among groups ($p < 0.05$). However, WG and FCR showed a statistical difference among groups at the end of the grower period ($p < 0.05$). In the reduced energy groups, The WG was lower in the NC, Xyl+EM and Xyl+GAA by -6%, -7% and 5% compared to the PC group, respectively ($p < 0.05$), while Xyl+EM+GAA showed no statistical difference compared to the PC group ($p > 0.05$). When compared to PC, FCR increased by 10% and 5% in the NC and Xyl+EM groups, respectively ($P < 0.05$) but it maintained in the Xyl+GAA and Xyl+EM+GAA ($p > 0.05$). In the finisher phase, no statistical difference was noticed among groups ($p > 0.05$). In the overall period, WG decreased by -4% and -5% in the NC and Xyl+EM groups, respectively ($p < 0.05$), whereas WG was maintained in Xyl+GAA and Xyl+EM+GAA when compared to the PC group ($p > 0.05$). Moreover, NC had a 5% increase in FI ($p<0.05$), whereas the Xyl+EM, Xyl+GAA and Xyl+EM+GAA groups were comparable to the PC group ($p > 0.05$). FCR in the NC and Xyl+EM groups increased by 9% and 5% ($p < 0.05$), respectively, but Xyl+GAA and Xyl+EM+GAA showed no statistical difference when compared to the PC group. No statistically difference was noticed in the mortality. However, EPEF showed a tendency to be reduced in NC group ($p = 0.06$).

Energy and protein efficiency ratios

Results concerning energy and protein efficiency ratios are shown in Table 4. During starter phase, The MEI of NC group was comparable to the PC group ($p > 0.05$), whilst the reduced energy groups supplemented with Xyl+EM, Xyl+GAA and Xyl+EM+GAA had lower MEI compared to the PC group ($p < 0.05$). No statistical difference was noticed in EER, TPI and PER ($p > 0.05$). During the grower period, the MEI of NC group was not affected significantly ($p > 0.05$), however the reduced energy groups supplemented with Xyl+EM, Xyl+GAA and Xyl+EM+GAA had significantly lower

Table 3. Effects of xylanase, emulsifier and guanidinoacetic acid mixtures on growth performance[1,2]

		Reduced energy groups (-200 kcal/kg ME)					
Parameters[3]	PC	NC	Xyl+EM	Xyl+GAA	Xyl+EM+GAA	SEM[4]	*p*-Value
			0-14 days				
WG (g)	492.6	491.86	480.2	489.99	492.5	5.59	0.49
FI (g)	575.09	598.32	576.2	586	583.98	6.73	0.15
FCR (g:g)	1.17	1.22	1.2	1.20	1.19	0.02	0.23
Mortality (%)	0.71	0.71	0	0.71	2.86	0.8	0.14
			14-26 days				
WG (g)	1116.2[a]	1045.90[b]	1039.2[b]	1063.70[b]	1099.20[a]	12.42	0.00
FI (g)	1365.55	1411.7	1335.92	1336.52	1344.6	20.46	0.08
FCR (g:g)	1.224[a]	1.351[b]	1.286[b]	1.256[a]	1.223[a]	0.02	0.00
Mortality (%)	0.71	0.71	0	2.14	1.45	0.85	0.47
			26-35 days				
WG (g)	825.5	811.4	782.8	835.1	826.9	28.52	0.72
FI (g)	1388.76	1476.9	1384.49	1352.7	1422.91	32	0.11
FCR (g:g)	1.69	1.83	1.77	1.63	1.72	0.05	0.15
Morality (%)	3.6	2.14	2.86	0	2.96	1.5	0.51
			1-35 days				
WG (g)	2434.30[a]	2349.16[b]	2302.20[b]	2388.79[a]	2418.60[a]	23.9	0.01
FI (g)	3329.40[a]	3486.86[b]	3296.62[a]	3275.22[a]	3351.48[a]	36.35	0.01
FCR (g:g)	1.368[a]	1.485[b]	1.432[b]	1.372[a]	1.386[a]	0.02	0.00
Mortality (%)	5	3.57	2.86	2.86	7.14	1.59	0.30
EPEF	483.98	436.6	446.93	483.37	463.09	12.96	0.06

[1]PC: diets contain standard metabolizable energy; NC: diets with reduced metabolizable energy by 200 kcal; Xyl+EM: NC supplemented with 0.01% xylanase and 0.025% emulsifier; Xyl+GAA: NC supplemented with 0.01% xylanase and 0.06% guanidinoacetic acid; Xyl+EM+GAA: NC supplemented with 0.01% xylanase, 0.025% emulsifier and 0.06% guanidinoacetic acid.

[2]Each mean represents 5 replicates with sample size = 15 birds/pen (75 birds/group).

[3]WG: weight gain; FI: feed intake; FCR: feed conversion ratio; EPEF: European Production Efficiency Factor (average grams gained/day X % survival rate)/Feed Conversion X 10).

[a, b] means that not share a common superscript within a row differ significantly from PC as determined by Fisher's protected LSD ($p < 0.05$).

4SEM: standard error of means.

MEI than the PC group ($p < 0.05$). The EER of the reduced energy groups was comparable to the PC group ($p > 0.05$), whereas Xyl+EM+GAA group showed a greater EER than the PC group ($p < 0.05$). Moreover, the PER of the NC and Xyl+EM was lower than PC group ($p < 0.05$), meanwhile, the reduced energy groups supplemented with Xyl+GAA and Xyl+EM+GAA was comparable to the PC group ($p > 0.05$). In the finisher phase, the MEI of the reduced energy group were not differ significantly compared to the PC group ($p > 0.05$), but the MEI of the Xyl+GAA group was lower than the PC group ($p < 0.05$). In the overall period, The MEI of NC group was not significantly different than the PC group ($p > 0.05$), however, the reduced energy groups supplemented with Xyl+EM, Xyl+GAA and Xyl+EM+GAA had lower MEI than PC group ($p < 0.05$). Moreover, The EER of NC and NC+EM groups was similar to the PC group, however the reduced energy groups supplemented with Xyl+GAA and Xyl+EM+GAA had a higher EER than PC group ($p < 0.05$). The TPI of the reduced energy group supplemented with Xyl+EM, Xyl+GAA and Xyl+EM+GAA were not similar to the PC group ($p > 0.05$). Meanwhile, NC group had higher TPI compared to the PC group ($p < 0.05$). The PER, of the NC and Xyl+EM were lower than PC group significantly ($p < 0.05$), while reduced energy groups supplemented with Xyl+GAA and Xyl+EM+GAA were not significantly different compared to the PC group ($p > 0.05$).

Liver oxidative biomarkers

Results of oxidative biomarkers are shown in Fig. 1. Compared to the PC group, The NC group showed significant reductions in the hepatic GSH, GSH-Px and SOD ($p < 0.05$), however T-GSH remained unaffected ($p > 0.05$). On the other hand, the reduced energy groups supplemented with Xyl+EM, Xyl+GAA and Xyl+EM+GAA improved all oxidative biomarkers compared to the PC group ($p < 0.05$).

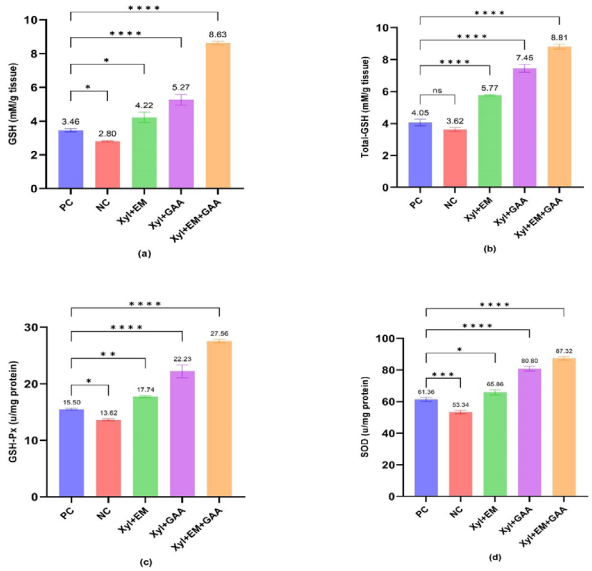

Fig. 1. Levels of (a) GSH: reduced glutathione, (b) T-GSH: total glutathione, (c) GSH-Px: glutathione peroxidase, (d) SOD: superoxide dismutase in the liver from PC, NC, Xyl+EM, Xyl+GAA, and Xyl+EM+GAA groups. Data are expressed as mean ± SE of five birds per treatment group and were analyzed using one-way ANOVA followed by Fisher's protected LSD post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ significance when compared to PC group. ns = not significant).

Relative PCR gene expression

Results of gene expression analysis are shown in Fig. 2. Our results showed that The NC group had a significant increase in AMPK ($p < 0.05$), whilst myogenin, myostatin, IGF-1, and CBS remained an affected compared to the PC group ($p > 0.05$). On the other hand, reduced energy

Table 4. Effects of xylanase, emulsifier and guanidinoacetic acid mixtures on energy and protein efficiency ratios[1,2]

Parameters[3]	PC	Reduced energy groups (-200 kcal/kg ME)				SEM[4]	*p*-Value
		NC	Xyl+EM	Xyl+GAA	Xyl+EM+GAA		
			1-14 days				
Total ME intake (kcal)	1725.28[a]	1675.31[a]	1613.37[b]	1640.81[b]	1635.13[b]	19.18	0.01
EER (gain/100 kcal ME)	28.56	29.3 7	29.77	29.87	30.14	0.37	0.05
Total protein Intake (g/bird)	134	139.41	134.26	136.54	136.07	1.57	0.15
PER (g:g)	3.68	3.53	3.58	3.59	3.62	0.04	0.23
			14-26 days				
Total ME intake (kcal)	4233.20[a]	4093.84[a]	3874.18[b]	3875.92[b]	3899.34[b]	59.76	0.00
EER (gain/100 kcal ME)	26.36[a]	25.58[a]	26.84[a]	27.45[a]	28.22[b]	0.40	0.00
Total protein Intake (g/bird)	290.32	300.4	284.28	284.41	286.13	4.35	0.09
PER (g:g)	3.84[a]	3.49[b]	3.66[b]	3.74[a]	3.85[a]	0.06	0.00
			26-35 days				
Total ME intake (kcal)	4444.03[a]	4430.59[a]	4153.48[a]	4058.09[b]	4268.72[a]	98.88	0.05
EER (gain/100 kcal ME)	18.6	18.33	18.89	20.55	19.39	0.61	0.12
Total protein Intake (g/bird)	263.86	280.6	263.05	257.01	270.35	6.08	0.11
PER (g:g)	3.13	2.89	2.98	3.25	3.06	0.10	0.15
			1-35 days				
Total ME intake (kcal)	10402.50[a]	10199.74[a]	9641.03[b]	9574.81[b]	9803.19[b]	110	0.00
EER (gain/100 kcal ME)	23.41[a]	23.04[a]	23.89[a]	24.95[b]	24.69[b]	0.31	0.00
Total protein Intake (g/bird)	688.18[a]	720.42[b]	681.59[a]	677.96[a]	692.55[a]	7.16	0.00
PER (g:g)	3.54[a]	3.26[b]	3.38[b]	3.52[a]	3.49[a]	0.05	0.00

[1]PC: diets contain standard metabolizable energy; NC: diets with reduced metabolizable energy by 200 kcal; Xyl+EM: NC supplemented with 0.01% xylanase and 0.025% emulsifier; Xyl+GAA: NC supplemented with 0.01% xylanase and 0.06% guanidinoacetic acid; Xyl+EM+GAA: NC supplemented with 0.01% xylanase, 0.025% emulsifier and 0.06% guanidinoacetic acid.

[2]Each mean represents 5 replicates with sample size = 15 birds/pen (75 birds/group).

[3]Total ME intake: feed intake x targets ME level of each phase/1000; EER: energy efficiency ratio (weight gain/100 kcal energy intake; Total protein intake: Feed intake x target crude protein of each phase/100; PER: protein efficiency ratio (weight gain/protein intake).

[a, b] means that not share a common superscript within a row differ significantly from PC as determined by Fisher's protected LSD ($p < 0.05$).

[4]SEM: standard error of means.

groups supplemented with Xyl+EM, Xyl+GAA, and Xyl+EM+GAA showed a significant increase in the aforementioned parameters ($p < 0.05$) compared to the PC group.

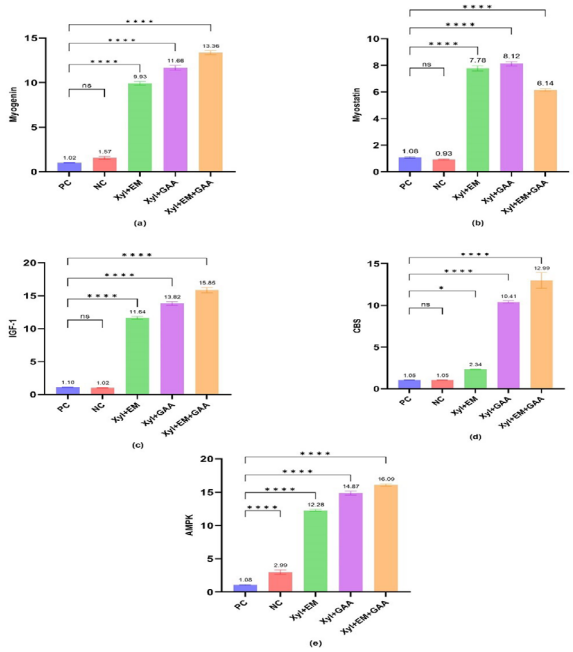

Fig. 2. Relative PCR gene expression of (a) myogenin, (b) myostatin, (c) IGF-1: insulin like growth factor-1, (d) CBS: cystathionine-β-synthase in the breast muscle, and (e) AMPK: adenosine monophosphate-activated protein kinase in the liver samples from PC, NC, Xyl+EM, Xyl+GAA, and Xyl+EM+GAA groups. Data are expressed as mean ± SE of five birds per treatment group and were analyzed using one-way ANOVA followed by Fisher's protected LSD post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ significance when compared to PC group. ns = not significant).

Intestinal histomorphology

Light microscopic examination of different intestinal regions showed normal histological structure in all experimental group without histopathological alterations Fig. 3(a). Goblet cells hyperplasia is observed in ileum segments, particularly in the Xyl+GAA group Fig. 3(b). Results of intestinal histomorphology are shown in Table 5. In the duodenum part, villus height of the NC group showed a significant reduction ($p < 0.05$) in the villus height, meanwhile crypt depth and villus to crypt ratio were comparable to the PC group ($p > 0.05$). On the other hand, the reduced energy groups supplemented with Xyl+EM, Xyl+GAA and Xyl+EM+GAA had greater villus height and crypt depth ($p < 0.05$). While villus to crypt ratio of Xyl+EM group was not statistically different, Xyl+GAA and Xyl+EM+GAA showed significant increases than PC group ($p < 0.05$). Mucus area percentage increased in all reduced energy groups ($p < 0.05$). In the jejunum part, the reduced energy groups supplemented with Xyl+EM, Xyl+GAA and Xyl+EM+GAA showed significant increases in the villus height, crypt depth and villus to crypt ratio ($p < 0.05$), meanwhile the NC group remained unaffected compared to the PC group ($p > 0.05$). The mucus area percentage increased in the energy reduced groups except Xyl+EM+GAA group, which was comparable to the PC group ($p > 0.05$). In the ileum part, villus height and crypt depth were significantly improved in the energy reduced groups supplemented with Xyl+EM, Xyl+GAA and Xyl+EM+GAA ($p < 0.05$), whilst NC group was comparable to the PC group ($p > 0.05$). Villus to crypt ratio of NC, NC+GAA were not statistically different ($p > 0.05$), however Xyl+EM and Xyl+EM+GAA were significantly lower than PC group ($p < 0.05$). Mucus area percentage of NC, Xyl+GAA and Xyl+EM+GAA group increased significantly, whereas Xyl+EM remained unaffected when compared to the PC group ($p > 0.05$).

Discussion

Our prior research investigated the distinct impacts of Xyl, EM, and GAA as a dietary supplement for male-broiler chicken fed LME diets (-200 kcal/kg). At this level of metabolizable energy, it was concluded that Xyl and EM incapable of improving the productive performance, whereas GAA partially restored the productive performance compared to standard energy diet (Khalil *et al.*, 2024). It is generally accepted that reducing the metabolizable energy content of the diet has a detrimental effect on growth performance (Hosseini *et al.*, 2018; Khalil *et al.*, 2024). Previous research reported that the individual supplementation of Xyl and EM resorted the productive performance in LME diets (-100 kcal/kg feed) relative to the high energy group. However, the same research failed to demonstrate a synergistic effect when EM and Xyl were combined (Hos-

Table 5. Effects of xylanase, emulsifier and guanidinoacetic acid mixture on intestinal histomorpholgy[1,2]

		Reduced energy groups (-200 kcal/kg ME)					
Parameters	PC	NC	Xyl+EM	Xyl+GAA	Xyl+EM+GAA	SEM[3]	p- Value
Duodenum							
Villus height (µm)	1789.69[a]	1604.92[b]	2748.69[b]	2910.89[b]	3076.93[b]	44.3	<0.0001
Crypt depth (µm)	203.80[a]	200.17[a]	289.34[b]	259.93[b]	297.44[b]	8.73	<0.0001
Villus: crypt ratio	9.17[a]	8.24[a]	10.24[a]	11.97[b]	10.89[b]	0.39	<0.0001
Mucus (area, %)	2.33[a]	4.33[b]	7.91[b]	8.85[b]	8.11[b]	0.36	<0.0001
Jejunum							
Villus height (µm)	786.93[a]	818.45[a]	1737.66[b]	2569.66[b]	2151.28[b]	46.73	<0.0001
Crypt depth (µm)	118.26[a]	116.9[a]	220.34[b]	249.18[b]	241.99[b]	7.12	<0.0001
Villus: crypt ratio	6.88[a]	7.20[a]	8.43[b]	10.94[b]	9.40[b]	0.36	<0.0001
Mucus (area, %)	8.20[a]	11.07[b]	11.67[b]	11.29[b]	8.33[a]	0.61	<0.0001
Ileum							
Villus height (µm)	767.3[a]	720.94[a]	1246.42[b]	1316.23[b]	1193.45[b]	26.24	<0.0001
Crypt depth (µm)	124.04[a]	126.49[a]	262.91[b]	210.92[b]	226.43[b]	8.24	<0.0001
Villus: crypt ratio	6.62[a]	6.21[a]	4.92[b]	6.93[a]	5.70[b]	0.28	<0.0001
Mucus (area, %)	16.50[a]	19.38[b]	16.79[a]	19.88[b]	18.20[b]	0.56	<0.0001

[1]PC: diets contain standard metabolizable energy; NC: diets with reduced metabolizable energy by 200 kcal; Xyl+EM: NC supplemented with 0.01% xylanase and 0.025% emulsifier; Xyl+GAA: NC supplemented with 0.01% xylanase and 0.06% guanidinoacetic acid; Xyl+EM+GAA: NC supplemented with 0.01% xylanase, 0.025% emulsifier and 0.06% guanidinoacetic acid.

[2]Each mean represents 50 slide section from 5 birds/treatment.

[a, b]means that not share a common superscript within a row differ significantly from PC as determined by Fisher's protected LSD ($p < 0.05$).

[3]SEM: standard error of means.

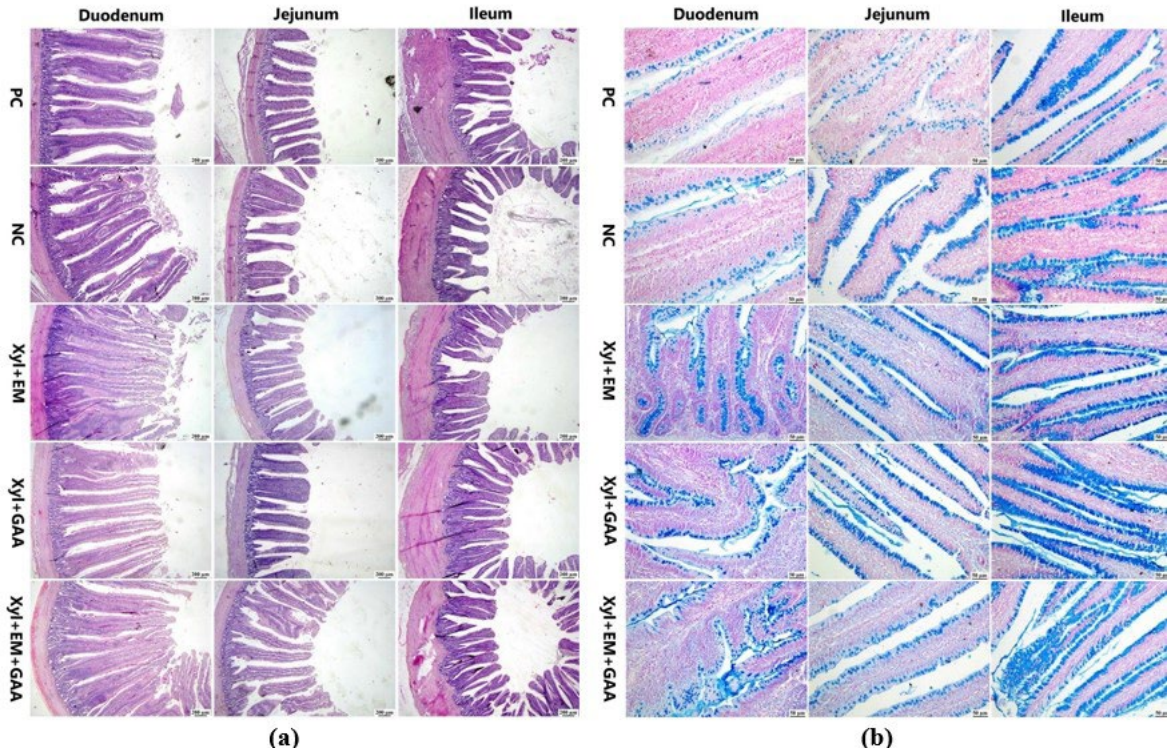

Fig. 3. (a) shows Duodenum, jejunum, and ileum sections of different experimental groups (H&E). Normal structure of different intestinal segments with variation of villus height and crypt depts are shown in different groups. The heights length of villi is observed in the duodenum. Scale bar, 200 µm. (b) Goblet cells stained blue in the lamina epithelialis of different intestinal segments (Alcian blue). Hyperplasia of goblet cells is noticed in ileum segments especially in Xyl+GAA group. Scale bar, 50 µm.

seini *et al*., 2018). Metwally *et al*. (2020) revealed that the growth performance of broiler chicken was significantly enhanced by supplementing a combination of Xyl+EM to LME diets (-150 kcal/kg feed) compared to control group, however this study was lacking of the individual effect at such energy level. In the other hand, EM combined with multienzymes (1,200 U of Xyl, 150 U glucanase, 700 U invertase, 5,000 U protease, 500 U cellulase, 12,000 U amylase, and 60 U mannanase /kg of diet) restored the growth performance of broiler chicken fed LME diet (-100 kcal/kg feed), received same oil level, compared to the PC group (Wickramasuriya *et al*., 2022). Furthermore, Ghazalah *et al*. (2021) reported that growth performance was enhanced when EM was added to LME diets (-100, -150 and -200 kcal/kg in starter, grower, and finisher, respectively) fortified with multienzymes in the basal diets (Protease 40,000 U/g, Xylanase 20,000 U/g and Amylase 2000 U/g). Notably, this improvement occurred despite the absence of added fat in the diet. Therefore, it can be speculated that the Xyl and EM mixture may not have an added value on the growth performance as reported in our study. Yet several factors may attribute to this finding, such as the metabolisable energy level, and the type and amount of fat in the feed (Tenório *et al*., 2022). It seems that using EM together with multienzymes may have a synergistic effect by working together on different substrates level, as reported by (Ghazalah *et al*., 2021; Wickramasuriya *et al*., 2022).

The absorbed glucose and fatty acids will be metabolised intracellularly to generate ATP (Shastak and Pelletier, 2023). The efficiency of energy utilisation is highly dependent on the available enzymatic reactions (Shastak and Pelletier, 2023), oxygen (Khalil *et al*., 2021a), and energy transporters. For example, L-carnitine transports fatty acids to mitochondria (Yousefi *et al*., 2023), whereas Cre transports the produced ATPs from the mitochondrial matrix to the site of energy utilisation in the cytoplasm (Reicher *et al*., 2020). Cre may be limited in fast growing animals due to their higher demand for Cre to supply the fast-growing muscles (Portocarero and Braun, 2021). Recent studies confirmed that dietary GAA greatly improved the energy metabolism by increasing Cre load intracellularly (Majdeddin *et al*., 2023b). In our study the Xyl and GAA mixture compensated for the energy deficit (- 200 kcal/kg feed) resulting in comparable growth performance compared to PC group. Supporting to our finding, Metwally *et al*. (2020) found that Xyl and GAA mixture supplemented to LME diet (-100 kcal/kg feed) overcompensated the energy deficit, resulting in a significant improvement in growth performance when compared to standard metabolisable energy group. It was speculated that further adding of EM to Xyl and GAA mixture may further improve the growth performance at LME diets (-200 kcal/kg feed). In our study, the mixture of Xyl+EM+GAA performed similarly to the PC group, but with a limited advantage over the Xyl+EM mixture in term of weight gain. Therefore, it is possible to speculate that Cre played a key role in modulating the energy metabolism and making the out most of using enzymes and emulsifiers through Cre-Pcre and ATP buffering system. Nevertheless, more research is required to investigate the effect of using multienzymes together with EM and GAA mixture on the growth performance.

It is acknowledged that a favourable state of growth and health is denoted by a high EER and PER (Khalil *et al*., 2024). The former indicates that feed energy is utilised efficiently for growth, whereas the latter indicates that dietary protein is converted into body protein efficiently (Kamran *et al*., 2011). It is well known that broiler eats to fulfil its energy demands as a priority over the growth efficiency (Hosseini *et al*., 2018), therefore it is not surprising that EER in LME diet in our study was comparable to the PC group. Nevertheless, the PER was altered negatively ($p < 0.05$). In accordance with our results, Maliwan *et al*. (2022) reported that EER was not affected by reducing the dietary energy content, meanwhile, the average daily protein intake was reduced as the concentration of dietary energy increased. Our previous study reported that Xyl supplemented to NC group (-200 kcal/kg feed) reduced the total ME intake ($p < 0.05$) and maintained the EER ($p > 0.05$). On the other hand, the PER was lower compared to the PC group ($p < 0.05$). Additionally, the same study showed that EM supplemented to NC group maintained the EER ($p > 0.05$), but PER was lower compared to the PC group ($p < 0.05$). In the contrary, GAA supplemented to NC group significantly increased the EER and maintained the PER compared to the PC group (Khalil *et al*., 2024). Moreover, our previous research showed a pattern of individual effects of Xyl and EM on MEI compared to the PC group. The former demonstrated a decrease in MEI from starter to finisher, with the main effect being on the terminal phase, whereas the latter demonstrated an increase in the MEI from starter to finisher, with the main effect being on the earlier phase (Khalil *et al*., 2024). Therefore, it can be speculated that the effect of Xyl on the ME intake is cumulative owe to its prebiotic effect by building up the fibrolytic microbiota population that produce the short chain fatty acids, meanwhile the effect of EM on ME intake is predominately during the earlier phases owe to the immature digestion of fat. Under current study, Xyl+EM mixture decreased the ME intake steadily from starter to finisher, and cumulatively compared to PC group ($p < 0.05$). Although the cumulative PI was comparable to the PC, the PER was not improved compared to the PC group.

Adding Xyl+GAA and Xyl+EM+GAA mixtures to NC diets in our study decreased the ME intake from starter to finisher, and cumulatively compared to PC group ($p < 0.05$). Additionally, the EER was significantly improved in the aforementioned groups compared to the PC group. Our previous research demonstrated the individual effect of GAA supplementation to the LME diets (-200 kcal/kg feed) on both ME intake and EER.

As a result, it can be concluded that GAA has a significant effect on modulating energy metabolism at cellular level, which may have a significant contribution to restoring the energy deficit when compared to Xyl and EM due to its ATP buffering system indicated by lower MEI and higher EER while maintaining the PER compared to the PC group (Khalil *et al*., 2024). Even though GAA does not provide energy in and of itself, it appears that birds were able to maximise the use of ATP they produce through ATP shuttle and storage system of Cre, achieving a perfect balance between MEI and growth efficiency on LME diets. In our study, both Xyl+GAA and Xyl+EM+GAA groups considerably increased EER ($p < 0.05$), whereas the PER of the two groups was similar to that of the PC group ($p > 0.05$). Further research is warranted to investigate the effect of supplementation of EM+GAA+multienzymes mixture in LME diet on EER and PER.

Beside growth performance, it is crucial to investigate the oxidative status of the birds under LME diets. Redox homeostasis is a critical function for aerobic organisms such as human and animals. Under physiological condition cells maintain redox status by producing oxidants and antioxidants. When this balance is disrupted, oxidative stress can damage the birds' productivity (Bacou *et al*., 2021). In our previous work, LME diet group (-200 kcal/kg feed) showed reductions in GSH and SOD activities compared to the standard energy group. The same study reported an increase of GSH activity by 21 and 93% in LME diets supplemented with EM and GAA, respectively. Additionally, T-GSH increased by 16, 42 and 97% in LME group supplemented with Xyl, EM and GAA, respectively. Only, LME diet supplemented with GAA showed significant increase in GSH-Px and SOD by 47% and 21%, respectively compared to the standard energy group (Khalil *et al*., 2024). In the present study, Xyl+EM increased the GSH activity by 22%, T-GSH by 42%, GSH-Px by 15% and SOD by 7%, which indicate that there was no synergetic effect of Xyl+EM mixture on GSH and T-GSH, however it improved GSH-Px and SOD significantly compared to the PC group. Meanwhile, Xyl+GAA mixture improved GSH by 52%, T-GSH by 84%, GSH-Px by 43% and SOD by 32%, which is nearly matched with the induvial use of GAA alone. Nevertheless, adding Xyl+EM+GAA to the LME improved the GSH activity by 149%, T-GSH by 118%, GSH-Px by 77% and SOD by 87.32%, which clearly showed the synergistic effect of such mixture compared to the PC group. It was reported that Xyl may have antioxidant effect through the degradation of NSP to oligosaccharide and phenolics compounds. Moreover, it was suggested the dietary fibre antioxidant may exist in the insoluble NSP (Bao and Choct, 2010). Moreover, (Saleh *et al*., 2020) revealed that the antioxidant effect of EM may refer to the improvements in fat soluble vitamins, carotenoids, and cell membrane permeability, which increased the availability of α-tocopherol. Additionally, GAA may exert its antioxidant effect through arginine sparing effect and lowering the mitochondrial dependent reactive oxygen species (Khalil *et al*., 2021a). Therefore, it seems that combing the different mode of action of such product had synergistic effect on the overall oxidative biomarkers.

In poultry, several genes are sociated with growth, for example, myogenin is one of the most important gene involved with the muscle fibre formation and muscle growth (Sławińska *et al*., 2013). meanwhile, myostatin is an inhibitor of muscle growth and regulates glucose metabolism by promoting glucose uptake, increasing glycolysis, and inhibiting glycogen formation in the skeletal muscle (Chen *et al*., 2010). IGF-1 is crucial regulator of muscle development and metabolism and was reported to regulate the feeding behaviour in broiler chicken (Fujita *et al*., 2019). CBS, on the other hand, is linked to AMPK via γ subunit of AMPK. This subunit includes four distinct CBS domains, which enable AMPK to detect changes in the AMP/ATP ratio with high sensitivity (Herzig and Shaw, 2018). In our study the NC group showed upregulation of AMPK, significantly, nevertheless the IGF-1, myogenin and myostatin were comparable to the PC group. This finding, however, my indicate the physiological adaptation of the birds fed the LME diet and may highlight genetic improvements in energy metabolism. It is possible that at such LME level, the birds did not lose their physiological function to the point of collapse, as indicated by low mortality rate. Previous research demonstrated the individual effect of Xyl, EM and GAA supplemented to LME diets (-200 kcal/kg). The result showed that the all-aforementioned additives increased the myogenin, myostatin, IGF-1, CBS and AMPK compared to PC group, significantly. However, the key feature of Xyl and EM effects was associated with increase in myogenin and myostatin, whereas GAA was strongly associated with IGF-1, CBS and AMPK (Khalil *et al*., 2024). In our study, all experimental groups showed significant increases in the gene expressions when compared to the PC. It is worth noting that the LME intake and high EER were strongly associated with IGF-1, CBS and AMPK upregulation (Khalil *et al*., 2024). In our study, the EER was significantly increased in Xly+GAA and Xyl+EM+GAA groups that showed higher fold changes in IGF-1, CBS and AMPK compared to Xyl+EM group, which is agreed with (Khalil *et al*., 2024). It is important to point out that AMPK gene expression was investigated in the liver samples, whereas other samples were measured in breast muscle. It is possible that activated AMPK in the liver in response to LME diets altered hepatic lipid homeostasis and energy balance through the immediate and direct modification of metabolic enzymes. For example, fatty acid oxidation is accelerated, fat synthesis is reduced, and gluconeogenesis is increased to boost the concentration of glucose in the body to meet the body's energy needs (Xu *et al*., 2022). The upregulation of IGF-1 in breast muscles may be regulated by blood glucose levels, which in turn enhance glucose uptake and promote growth (Fujita *et al*., 2019). In contrast to myogenin, myostatin has a suppressive effect on growth, however, it might be influenced by the activated AMPK to regulate the energy homeostasis in the breast muscle (Chen *et al*., 2010). Overall, it is reasonable to assume that supplementing GAA has a significant impact on gene expressions, especially those of the IGF-1, AMPK, and CBS genes as reported in (Khalil *et al*., 2024), but adding Xyl or Xyl+EM to GAA has a synergistic effect.

Many previous research demonstrated the effects of Xyl and EM and GAA on the intestinal morphology (Liu and Kim, 2017; Raei *et al*., 2020; Ghazalah *et al*., 2021). Our previous research highlighted the key features of the individual supplement in the different intestinal segments. For instance, Xyl supplementation had the greatest effect on the duodenum segment, GAA had the greatest effect on the jejunum, and Xyl and EM were equally enhanced the Ileum histomorphology compared to the PC group (Khalil *et al*., 2024). Under current study, combing Xyl+EM showed a synergistic effect on all intestinal segments compared to PC group, which was superior to individual supplementation of such additives (Khalil *et al*., 2024). Meanwhile, Xyl+GAA and Xyl+EM+GAA groups showed the greatest synergistic effect compared to the PC group. Nevertheless, when EM was added to Xyl+GAA, our results showed no extra value. The current study, as well as our earlier work, demonstrated changes in the intestinal microbiota in the NC groups supplemented with different mixtures, as indicated by an increase in the mucus area percentage, which may have beneficial effects in the intestinal health via different mode of actions (Khalil *et al*., 2024).

Conclusion

The growth performance as well as the biometric indices of the NC group are negatively affected. Xyl+GAA and Xyl+EM+GAA groups, on the other hand, achieved growth performance that was comparable to the PC group. Additionally, both aforementioned groups outperformed the PC group in the EER. When compared to the PC group, the NC groups fortified with various mixtures improved the oxidative biomarkers, gene expression, and intestinal histomorphology. The overall improvements were notably observed in the Xyl+EM+GAA group. Therefore, it is possible to compensate the LME (-200 kcal/kg feed) by adding Xyl+EM+GAA. The authors recommend further investigating the impact of combining multienzymes with EM and GAA in the LME diet (200 kcal/kg feed) on broiler performance.

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Conflict of interest

The authors declare that they have no conflict of interest.

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