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ABSTRACT

1. The study aimed to compare the responses of broilers to diets supplemented with the same level of guanidinoacetic acid (GAA) but formulated to have different N-corrected apparent metabolisable energy (AMEn) contents. The study involved 1280 day old Ross 308 broilers, in 64 pens, 32 pens males and 32 pens females, (20 birds each) from 0 to 42 days of age.

2. Standard AME levels of 12.55 MJ/kg, 12.97 MJ/kg and 13.18 MJ/kg in starter, grower and finisher diets, respectively, were set for the positive control (PC) feeds. Four dietary treatments were prepared: PC (as above); Negative Control 1 (NC; PC - 0.21 MJ ME /kg as compared to PC); NC 1+ 0.06% GAA; NC2 (PC - 0.42 MJ ME kg as compared to PC + 0.06% GAA). Each diet was offered to 16 pens (8 male + 8 female), following randomisation.

3. Overall, birds fed the NC1 had lower feed intakes (FI) compared to birds fed the PC and NC2+GAA, lower weight gain (WG) compared to all the other diets and lower final body weight than birds fed the GAA diets ($P < 0.05$). There was a diet by sex interaction ($P = 0.039$) as feeding NC+GAA to females improved feed efficiency compared to being fed NC2 and NC1+GAA, but not in males.

4. Birds fed GAA had a higher poultry efficiency factor ($P < 0.001$) than those fed NC1.

5. There were no effects of treatment or sex on litter moisture, footpad score, white striping, wooden breast, AMEn, dry matter and fat retention ($P > 0.05$). However, the diet NC1+GAA had 11.2% higher NR coefficient compared to the NC1 diet ($P = 0.038$).

6. Overall, the results imply that performance depressions induced by a reduction of dietary AMEn in the range of 0.21 to 0.42 MJ/kg can be more than compensated by a supplementing 600 g/t GAA to the feed.

Key words: Guanidinoacetic acid, growth performance, metabolisable energy, broiler

INTRODUCTION

Guanidinoacetic acid (GAA) is formed *de-novo* from L-arginine and L-glycine mainly in the kidney (Wyss and Kaddurah-Daouk, 2000). In a second reaction, GAA is transformed into creatine in the liver via the action of guanidinoacetate N-methyltransferase (Brosnan *et al.,* 2009). Creatine functions in the cell energy metabolism and interacts with the ATP system in the muscle (Wallimann, 2007). Poultry are uricotelic animals, lacking enzymes to synthesise enough arginine *de novo* being required as a precursor for creatine (Campbell, 1995). A significant amount of creatine and phosphocreatine pool is irreversibly converted to creatinine and excreted daily in urine (Brosnan *et al.,* 2009). However, nowadays broilers are fed primarily vegetable-based diets and the supply of exogenous creatine is virtually reduced to zero. Guanidinoacetic acid is seen as an effective alternative source of creatine in broiler diets (Khajali *et al.,* 2020).

Due to its biological functions, commercial application of GAA has consistently been found to improve growth performance (Michiels *et al.,* 2012; Khajali *et al.,* 2020) and protect/ improve meat quality when fed to fast growing broilers (Córdova-Noboa *et al.,* 2018a; Córdova-Noboa *et al.,* 2018b). It has also been suggested that GAA could spare metabolisable energy (ME) by about 50 kcal/kg in commercial broiler feeds (Abudabos *et al.,* 2014; Albiker, 2015; Tabatabaei Yazdi *et al.,* 2017). However, the ME content of feeds for broiler chickens differs between countries or regions, driven mainly by the commercial industrial structure of the chicken market. This situation leads to different economic implications when using GAA with an energy sparing matrix. Therefore, the main objective of the present study was to test the efficacy of GAA when added to wheat-barley-soybean based practical diets, that differ in ME content, on growth performance variables of broiler chickens from 0 to 42 days of age. Dietary ME, nutrient digestibility, leg health and breast meat quality variables were also determined.

MATERIALS AND METHODS

Growth Performance Broiler Experiment

The study was approved by Harper Adams University Research Ethics Committee. One thousand three hundred day-old Ross 308 chicks (half male + half female) were obtained from a commercial hatchery (Cyril Bason Ltd., Craven Arms, UK). On the arrival 1280 day old broilers (excluding any malformed or very small chicks) were divided into 64 pens, 32 pens holding 20 birds of each sex). Each of the pens had a solid floor with an area of 2.1 m^2 that was covered with wood shavings as bedding material. The pens were equipped with a drinker with five nipples and a hopper feeder. The room temperature was maintained according

96 industry standards (Aviagen Ltd., 2014). A standard lighting programme for broilers was used,
97 decreasing the light:dark ratio from 23h:1h from day old to 18h:6h at 7 d of age, which was
98 maintained until the end of the study.
99 Broilers were fed one of four diets, with 16 (8 male + 8 female) replicates per treatments (Table
100 1). Diets were formulated to represent a commercial standard, including enzymes and
101 coccidiostats. Standard apparent metabolisable energy (N corrected, AMEn) levels of 12.55
102 MJ/kg (3000 kcal/kg), 12.97 MJ/kg (3100 kcal/kg), and 13.18 MJ/kg (3150 kcal/kg) in starter
103 (day 0 – 14), grower (day 15 – 25) and finisher diets (day 26 – 42), respectively, were set for
104 the positive control feeds. Nutrient matrix values of GAA were used for feed formulation of the
105 treatment feeds (Table 2). All diets were mixed by Target Feeds Ltd (Whitchurch, UK). Feed
106 raw materials were analysed using NIRs to estimate the amino acid contents, proximate
107 contents, and AMEn for each raw material (AMINONir®, Evonik Operations GmbH, Germany;
108 based on Fontaine *et al.* (2001; 2002)). These values were used in the final feed formulation
109 to assure the desired nutrient levels in the formulation. The trial comprised four dietary
110 treatments (crumbs for starters and pellets for the grower and finisher feeds): Positive control
111 (PC; normal ME); Negative Control 1 (NC1; PC - 0.21 MJ ME (50 kcal) /kg as compared to
112 PC); NC1+ 0.06% GAA; Negative Control 2 (NC2; PC - 0.42 MJ ME kg (100kcal) as compared
113 to PC + 0.06% GAA). The GAA and respective matrix values (Table 2) were provided by
114 Evonik Operations GmbH, Germany. Final feeds were analysed for amino acids (AMINOLab®,
115 Evonik Operations GmbH, Germany) and proximates (Horwitz and AOAC International, 2002).
116 The AMEn of the finished feed was calculated based on the proximate analysis, according to
117 the equation published by (WPSA, 1984). Feed and water were offered for *ad libitum*
118 consumption.
119 Body weights and feed intake (FI) were measured on a per pen basis at day old, 14, 25 and
120 42 days. As the dietary phases changed, the data was recalculated on a per bird day basis.
121 Daily weight gain (WG) and feed conversion ratio (FCR) were calculated. In accordance to
122 commercial standard, FCR was standardised to body weight of dead birds. Mortality was
123 recorded daily. The Production Efficiency Factor (PEF) was calculated as: ((liveability, % x live
124 weight, kg) / (age, days x FCR)) x 100.
125 The litter dry matter was determined at 42 days of age by taking 4 samples from different
126 locations of the floor of each pen and drying them in an oven using standard procedures (Mirza
127 *et al*., 2016). Footpad lesions were scored at 42 days of age on a three-point scale where 0
128 described normal footpads without lesions, and a score of 2 was given for obvious scores on
129 the footpads (Ekstrand *et al.,* 1998). All birds in each pen were assessed. A mean value of the
130 pen for the measurements was used for statistical analysis.
131 The occurrence of white striping (WS) was evaluated at 42 days of age, approximately 3 hours
132 after slaughter. Four breast fillets (two birds from each pen) were taken and used to establish

the different levels of WS. A three point scale was used, where 0 describes breast fillets without any problems (normal), fillets with < 1 mm thin lines (moderate) were considered as moderate (1), and a score of 2 was given to those breasts having > 1 mm white striations covering most of the surface area (severe) (Kuttappan *et al.,* 2012). The wooden breast (WB) scoring was also performed at 42 days of age using the same breast fillets employing a three point scale, normal, moderate (mildly hardened consistency) and severe (markedly hardened consistency) (Sihvo *et al.,* 2017). A mean value of the pen for each of the mentioned measurements was used for statistical analysis.

In-Vivo Determination of Metabolisable Energy of Grower Broiler Diets

At 20 d of age, 2 chickens from each floor pen was selected at random, and transferred to one of 64 raised floor pens (0.360 m^2 floor area) in a controlled environment room. Each pen was equipped with plastic feeders and drinkers. Treatments were randomly allocated to each pen as birds were fed the same diets. To maintain the effect of the floor pen rearing conditions no adaptation period for moving birds to a raised floor pen was allowed. Feed and water were offered for *ad libitum* consumption. The birds selected were kept in the raised floor pens for 96 hrs, and total excreta output was collected twice (every 48 hrs) from the trays beneath. Spilt feed and feathers were removed, and excreta were collected before weighing. Excreta and feed were weighed for the same period. Dietary AMEn was determined as described by (Hill & Anderson, 1958). The coefficients of apparent retention of dietary dry matter (DMR), nitrogen (NR) and fat (FR) retention were determined as described elsewhere (Abdulla *et al.,* 2016).

Laboratory analyses

Dry matter (DM) in feed and excreta samples was determined by drying of samples in a forced draft oven at 105°C to a constant weight (Horwitz and AOAC International, 2002); method 934.01). Crude protein (6.25 × N) in samples was determined by the combustion method (Horwitz and AOAC International, 2002); method 990.03) using a LECO FP-528 N (Leco Corp., St. Joseph, MI). Oil (as ether extract) in diets was extracted with diethyl ether by the ether extraction method (Horwitz and AOAC International, 2002); method 945.16) using a Soxtec system (Foss Ltd., Warrington, UK). The gross energy (GE) value of feed and excreta samples was determined in a bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL) with benzoic acid used as the standard.

Statistical Analyses

The data were analysed by a randomised block ANOVA (GenStat, 18th ed., Lawes Agricultural Trust, VSN International Ltd., Oxford, UK) using 2 × 4 factorial structure to investigate the main treatment factors (sex × diets) and their interaction. In all instances, differences were reported as significant at $P < 0.05$ employing protected LSD test.

RESULTS

The results on bird growth performance are presented in Table 3. Analysis of variance revealed no interactions between dietary treatment and sex except for FCR over the entire production cycle ($P = 0.039$). Feeding NC+GAA to females improved feed efficiency compared to NC1 and NC1+GAA, but not in males (Table 3). Therefore, results are structured in two blocks of the main factors dietary treatment and sex, respectively.

Dietary treatments affected performance significantly in the 0 - 25 and 0 - 42 day periods. In the 0-25 day period body weight ($P = 0.005$) and weight gain ($P = 0.006$), respectively, were affected whereas FI was not, resulting in a significant impact on FCR ($P<0.001$). Body weight and weight gain were lowest with the NC1 feed, while the ME reduced treatments with GAA supplementation (NC1+GAA, NC2+GAA) resulted in higher weights and gain ($P < 0.05$). Consequently, the latter treatments had significantly lower FCR compared to NC1 ($P < 0.05$). Over the entire period of 42 days, FI was significantly impacted by dietary treatment ($P = 0.04$). ME reduction (NC1) decreased FI compared to the PC ($p<0.05$) with the NC2+GAA ($P < 0.05$) showing a similar numerical pattern as in the 0 - 25 day period. Final body weight and weight gain was again lowest in treatment NC1 compared to NC1+GAA and NC2+GAA ($P < 0.05$) while PC broilers had intermediate results. The FCR of males fed PC seemed to be higher than in the remaining three treatments, while for female broilers, NC1 and NC2+GAA showed higher FCR than the PC and NC1+GAA (interaction, $P = 0.039$). Overall liveability did not reveal any treatment effects, while PEF was affected by diet ($P = 0.006$). Productivity index was highest for NC1+GAA and NC2+GAA and lowest for NC1 ($P < 0.05$) while PC was in between.

Except for overall FCR (see interaction above) and liveability, all reported data in Table 3 were significantly affected by sex ($P < 0.001$). Accordingly, FI, body weight, and weight gain was always higher in males than in females and FCR was always lower. Consequently, this resulted in a higher PEF number for males.

Evaluation of foot pad lesion scoring and meat quality abnormalities revealed neither direct nor interactive effects ($P > 0.05$; Table 4). Also, litter moisture content was not affected ($P > 0.05$).

The results on dietary AMEn determination and nutrient retention coefficients are presented in Table 5. There were no differences ($P>0.05$) in AMEn, DMR and FR due to diets or sex. However, compared to diet NC1, NC1+GAA diet had higher NR ($P < 0.05$) but did not differ from PC and NC2+GAA ($P > 0.05$).

DISCUSSION

In order to study the efficiency of GAA on growth performance, dietary energy and nutrient availability, diets were formulated with three different energy contents. The reduction of dietary AME by up to 0.42 MJ/kg was achieved by reducing the inclusion of soybean oil and wheat, whereas at the same time barley was increased. Recalculation of dietary energy based on the proximate analysis of the final diet compositions revealed reasonable agreement although values were not exactly met (Table 1b). Mateos *et al.* (2019) gave cause for concern that differing analytical procedures may partially be responsible for the observed differences. However, in the current trial the feed analyses confirmed the aim to reduce energy stepwise and in each feeding phase. The AMEn reduction in NC2 was always twice as high as in NC1. Averaging the differences across all three feeding phases to PC would confirm 0.21 and 0.42 MJ/kg reduction, thus achieving the dietary objective. Also, the feed mixing protocol with changing proportions of ingredients per se would suggest differences in dietary energy between PC, NC1 and NC2. According to the expectation, diets of NC1+GAA should bring a performance depression of NC1 back to that of PC and for NC2+GAA a similar response was considered possible. In our assay, the determination of AMEn, which was performed only with the grower feed, does not necessarily confirm the expectations for the grower feed. The range of determined AMEn for PC (12.74 MJ/kg) and NC1 (12.46 MJ/kg) were close to the calculated values, which were 12.86 MJ/kg for PC and 12.65 MJ/kg for NC1. Having in mind, that there were no statistically significant treatment effects in determined AMEn (Table 5). Birds fed the NC2+GAA feeds resulted in the lowest value of 12.23 MJ/kg but showed a growth performance like NC1+GAA. Although dietary ME is widely used to describe the available energy concentration in poultry feedstuffs, diets with the same ME are not necessarily used with equal efficiency when fed to poultry (Pirgozliev and Bedford, 2013; Pirgozliev *et al.,* 2015). Indeed, research by Ale Saheb Fosoul *et al.* (2018) found that the growth performance of broilers fed GAA was more sensitive to NE of diet than to AME. Additionally, effects of GAA on NE was more pronounced in diets with lower energy content. Dietary AME is usually calculated as an additive value of the ME of all dietary ingredients. However, different nutrients, i.e.

carbohydrates, proteins and fat are utilised by growing poultry with different efficiency (Groote, 1974) and their ME values may change depending on inclusion rates and interaction within dietary ingredients. The arithmetical assumption doesn't always follow the expected biological effects, thus changed dietary composition, i.e. fat and carbohydrate contents, not always provides the expected changes in available energy (Mateos *et al.,* 2019). For ingredients such as fat sources, where levels of 5 to 10% might be considered suitable and comparable to levels employed in commercial practice, the error may be large (Campbell, 1995).

Dietary treatments significantly affected performance. As expected, reduction of dietary energy by 0.21 MJ AME/kg impaired performance. Surprisingly, overall results indicate that birds did not compensate lower energy concentration by increased feed consumption but the contrary. This is not in line with the concept that broilers always aim to maintain their energy balance by adjusting their feed consumption in case of small changes (Kleyn and Chrystal, 2020). Indeed, Maharjan *et al.* (2020) reported a negative correlation between dietary energy level and feed intake resulting in linear FCR responses. However, increased feed intake did not allow maintaining energy intake, resulting in lower body weight gain, which does not support the view that FI always compensates for dietary energy. Maynard *et al.* (2019), could only partly confirm such response, although lower FI with reduced dietary ME was also not observed. In our case, the overall response was biased by an interaction with sex which became obvious in regard to FCR. Accordingly, females responded with increased FCR with AME reduction, while males showed the opposite resulting in no differences across both sexes (Table 3). However, the relatively high dietary barley inclusion, a cereal rich in fibres, may also explain the FI in the study. The response on FI remains unclear but might relate to the genetic progress of broilers depositing less fat and more meat, affecting energy utilisation against the background that dietary energy levels declined over the last 20 years (Cerrate and Corzo, 2018). Kleyn and Chrystal (2020) also point out that genetic progress and related changes in partition of energy use for fat and protein deposition is an important factor. Moreover, they emphasised that severe dietary energy deficiencies might not be responded by increased but rather decreased feed intake. It remains speculative whether a reduction of 0.21 MJ AME would provoke a severe deficiency. Indeed, AMEn of 12.44 MJ/kg, 12.86 MJ/kg and 13.07 MJ/kg may represent the lower edge of common energy levels. Finally, body weight gain suffered with AME reduction while GAA additions compensated this performance loss even at 0.42 MJ/kg AME reduction.

The PEF standardises technical results, considering FCR, liveability, age and BW and high values are associated with better overall performance. Supplementation of GAA to energy reduced diets maximised this index suggesting that even a reduction of 0.42 MJ/kg AME can be compensated by 600g/t GAA supplementation.

Effects of GAA on the growth performance was likely due to its key role as a creatine precursor and subsequently its ability to increase the muscular creatine and ATP stores (Khajali *et al.,* 2020). Creatine in its phosphorylated form buffers the cell energy metabolism, enhancing the energy delivery to high demanding energy tissues, such as muscles and the heart (Wyss and Kaddurah-Daouk, 2000). The improvements due to GAA were similar in both NC1 and NC2, indicating that 0.06% GAA in diets can compensate not only for a reduction of 0.21 MJ/kg in the diet but also for a reduction of 0.42 MJ/kg ME. In their review, Khajali *et al.* (2020) came up with a regression suggesting that an addition of 600 g GAA/t of feed improves FCR by 2.55% on a relative scale which would mean for the current trial an improvement of 0.043 kg/kg FCR in NC1+GAA over NC1. In fact, the overall effect in this trial was only 0.016 kg/kg. The same paper suggested an improvement of 2.65 % or 74g in body weight. Indeed, average body weights in treatment NC1+GAA and NC2+GAA were 86 g higher than in NC1 and 42 g higher than in PC and, thus, in line with the literature review by Khajali *et al.* (2020). Therefore, GAA was effective as feed additive in this trial.

In the past, interactions of dietary energy and GAA has been investigated by others as well. In experiments where dietary ME was reduced by more than 0.50 MJ/kg compared to the PC, the compensatory effects of supplementary GAA were less pronounced and there were contradictions between reports (Mousavi et al., 2013; Tabatabaei Yazdi et al., 2017; Majdeddin et al., 2018). Most of the reports confirmed that performance drops with a reduction of 0.21 MJ in AMEn but can be more than compensated by the addition of 600 g/t GAA addition (Abudabos et al., 2014; Heger et al., 2014; Dozier and Gehring, 2014; Albiker, 2015; Metwally et al., 2020). Moreover, an economic evaluation revealed that in contrast to the other supplements, addition of GAA to energy reduced feed maximised profitability (Ion et al., 2016). To summarise, our findings with NC1+GAA and NC2+GAA are confirmed by the majority of publications in this context. Despite the obvious variation reported in the literature, it may suggest that supplementation of 600 g GAA/t is equivalent to > 0.21 MJ ME/kg and potentially even 0.42 MJ/kg or more.

Indeed, it was hypothesised by Khajali *et al.* (2020) that especially the improved FCR due to GAA supplementation is the consequence of an improved energy metabolism on cell level. Various publications have reported an increase in muscle creatine content by on average 15% with 600 g/t GAA supplementation which may have a positive impact on energy utilisation (Khajali *et al.,* 2020). A lot of research has been conducted in order to understand the mode of action of creatine in the tissues. Wallimann (2007) reviewed the discovery of the phosphorylated creatine (pCr) shuttle mechanism and describes that creatine is phosphorylated by taking up a phosphorous group from ATP especially in the mitochondria of cells, which then is reduced to ADP. This process is catalysed by creatine kinase. Phosphorylated Cr is then transported outside the mitochondria as an energy-rich compound

and serves as an energy buffer (Wallimann, 2007). Moreover, Wallimann (2007) concluded that increased pCr or rather pCr/ATP ratio would indicate an improved cellular energy status. Research by Michiels *et al.* (2012), Tabatabaei Yazdi *et al.* (2017), and Majdeddin *et al.* (2020) reported significant increases of pCr/ATP in broiler muscle by GAA. Thus, it can be concluded that GAA supplementation improves the energy charge of breast muscle tissue, which accounts for >90% of the body creatine pool (Wyss and Kaddurah-Daouk, 2000). Consequently, synthesis of muscle protein is limited by creatine, which influence feed efficiency. Indeed, the review by Khajali *et al.* (2020) suggested an increased breast meat yield of 1.67% (relative scale) at 600 g/t GAA. Recently, Boney *et al.* (2020) reported even a 2.7% increase of breast meat yield with 600 g/t GAA. So, a reduction of dietary energy might thus be compensated by GAA supplementation. This also suggests that the energy in the PC feeds was used less efficiently due to a lack of creatine reducing the energy charge of breast muscle tissue. Our results, as well as the literature discussed above would support this argumentation. The overall results showed that adding GAA to AMEn reduced diets (-0.21, -0.42 MJ/kg) more than maintained body weight gain. In terms of FCR, we cannot report an impairment with ME reduction (NC1) as especially the male broilers had an opposite response, but the FCR of NC1+GAA and NC2+GAA treatments was not worse than that. This picture together with PEF numbers would indicate that even a reduction of 0.42 MJ ME/kg can be compensated by GAA supplementation at 600 g/t.

It should be noted that the birds may also have been responding to the arginine sparing effect of GAA. Recent research (Johnson et al., 2020) suggests that the modern broiler strains are more responsive to amino acid density than energy, particularly when considering the scale of the ME reduction imposed in this study. Given the analysis of the diets, the NC1 is lower in arginine in the starter than the PC and the ratios of arginine to lysine are marginally lower than ideal in the starters and in some of the growers and finisher diets. The finisher diets with GAA are much higher in arginine than the other two diets, which suggests potential arginine sparing effect of GAA.

The occurrence of WB and WS is linked to fast-growth rates of the birds and their large breast muscles, where an oxidative stress and mitochondrial dysfunction leads to lipidosis, fibrosis, and overall myodegeneration (Petracci *et al.,* 2019). The problem was described as severe in male birds reared to 9 weeks of age but it was less pronounced in birds reared to 6 weeks of age (Petracci *et al.,* 2019). Córdova-Noboa *et al.* (2018a; 2018b) reported no impact in WS of 55 day old broilers fed 0.06% dietary GAA. However, dietary GAA reduced the occurrence in WB in 51 day old birds but not in 55 day old (Córdova-Noboa *et al.,* 2018a). Interestingly, no impact in WB severity was observed in another study (Córdova-Noboa *et al.,* 2018b). However, the live weight of the birds in our study was about 2.8 kg, compared to 4.5 and 5 kilograms live weight in the experiments of Córdova-Noboa *et al.* (2018a; 2018b). The birds

in our study were reared under high welfare conditions in pens of twenty and slaughtered at 42 days of age. There was enough feeding space and water supply and although the growth rate was in line with breeders recommendations, it was much lower than those reported by Córdova-Noboa *et al.* (2018a; 2018b). This may suggest an explanation of the lack of changes in WB and WS in the reported study. However, Maynard et al. (2019) suggested that factors beyond nutrition, growth rate, and age may contribute to the occurrence of these myopathies. In agreement with Çenesiz et al. (2020) it can be concluded that, regardless of dietary energy levels, supplementation of GAA to plant-based diets has the potential to improve growth performance in broilers. The results showed that performance depressions induced by a reduction of dietary AMEn even at 0.42 MJ/kg can be compensated by supplementing 600 g/t GAA to the feed.

Table 1a. Composition and nutritive value of the experimental diets

Dietary phases	Starter Day 0 – 14			Grower Day 15 - 25			Finisher Day 26 - 42		
Dietary treatments[1]	PC	NC1	NC2	PC	NC1	NC2	PC	NC1	NC2
Dietary ingredients (kg/t)									
Wheat	414.87	397.10	379.33	526.70	508.39	490.09	610.38	592.07	573.77
SBM (48 %CP)	273.19	272.43	271.67	208.62	207.87	207.13	155.76	155.02	154.27
Barley	116.75	142.64	168.52	81.09	107.45	133.82	57.24	83.60	109.97
SBM (ff)	90.00	90.00	90.00	80.00	80.00	80.00	80.00	80.00	80.00
Soybean oil	50.25	42.99	35.73	54.65	47.42	40.18	52.02	44.79	37.55
Limestone	17.04	17.05	17.06	15.92	15.93	15.94	14.61	14.57	14.52
Mono-Ca-Phosphate	16.78	16.74	16.70	14.62	14.58	14.54	12.34	12.36	12.37
Sodium bicarbonate	4.92	4.94	4.96	4.89	4.91	4.93	4.92	4.94	4.97
MetAMINO	3.65	3.64	3.63	2.80	2.79	2.78	2.34	2.33	2.32
ThreAMINO	1.44	1.43	1.43	-	-	-	-	-	-
L-Lys-HCl	3.40	3.38	3.37	3.02	3.00	2.99	2.86	2.85	2.83
ValAMINO	1.32	1.31	1.29	1.19	1.18	1.17	1.06	1.05	1.05
NaCl	1.14	1.13	1.11	1.21	1.20	1.18	1.21	1.20	1.18
Maxiban	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60
Choline Cloride	0.54	0.52	0.50	0.59	0.57	0.55	0.54	0.52	0.50
MultiGrain[2]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Premix[3]	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Calculated values									
CP (g/kg)	227	227	227	200	200	200	182	182	182
AMEn (MJ/kg)	12.44	12.24	12.03	12.86	12.65	12.44	13.07	12.86	12.65
Ca (g/kg)	11	11	11	10	10	10	8.5	8.5	8.5
P (g/kg)	7.8	7.8	7.8	7.1	7.1	7.1	6.9	6.9	7.0
av P (g/kg)	5.0	5.0	5.0	4.5	4.5	4.5	4.5	4.5	4.5
Lys (g/kg)	14.2	14.2	14.2	12.0	12.0	12.0	10.5	10.5	10.5
Met+Cys (g/kg)	10.3	10.3	10.3	8.8	8.8	8.8	7.9	7.9	7.9

[1]Guanidinoacetic acid was added as CreAMINO® at 0.6 kg/t to NC ad NC1 in all dietary phases.
[2]RONOZYME® MultiGrain *DSM, Switzerland) is a combination of xylanase, β- glucanase, cellulase and xyloglucanase enzymes.
[3]Provided per kg feed: 2160 µg retinol, 75 µg cholecalciferol; 25 mg α-tocopherol, 1.5 mg menadione, 5 mg riboflavin, 8 mg pantotenic acid, 10 µg cyanocobalamin, 1.5 mg pyridoxine, 1.5 mg thiamine, 0.5 mg folic acid, 30 mg niacin, 60 µg biotin, 0.8 mg I, 10 mg Cu, 80 mg Fe, 0.3 mg Se, 80 mg Mn, 80 mg Zn (Target Feeds Ltd., Whitchurch, UK).

Table 1b. Analyses of the experimental diets

Dietary phases	Starter Day 0 – 14				Grower Day 15 - 25				Finisher Day 26 - 42			
Dietary treatments[1]	PC	NC1	NC1+GAA	NC2	PC	NC1	NC1+GAA	NC2	PC	NC1	NC1+GAA	NC2
CP, g/kg	236	230	231	228	195	195	197	201	183	184	236	230
Lys, g/kg	14.9	14.6	14.6	14.3	11.1	12.8	11.3	12.1	10.4	10.7	14.9	14.6
Met+Cys, g/kg	10.0	10.2	10.4	9.9	7.9	8.4	7.9	8.6	7.6	7.8	10.0	10.2
Thr, g/kg	9.6	9.2	9.3	9.0	7.7	8.1	7.7	7.8	7.1	7.2	9.6	9.2
Arg, g/kg	15.6	14.7	14.9	14.5	12.4	12.3	12.1	12.4	11.1	11.4	15.6	14.7
Val, g/kg	12.3	11.8	11.7	11.6	9.0	8.9	8.9	9.0	8.2	8.4	12.3	11.8
Ile, g/kg	9.7	9.2	9.5	9.1	7.9	7.8	7.8	7.9	7.2	7.3	9.7	9.2
Leu, g/kg	17.3	16.3	16.4	16.1	13.9	13.8	13.8	14.0	12.8	13.0	17.3	16.3
$Gly_{equivalents}$, g/kg[1]	17.7	16.8	17.1	16.6	14.5	14.4	14.4	14.5	13.4	13.5	17.7	16.8
GAA, g/t	<10	<10	521	611	<10	<10	622	701	52	<10	583	638
Recovery, %[2]			90	106			108	122			101	111
AME, MJ/kg[3]	12.59	12.21	12.15	11.78	12.74	12.46	12.44	12.27	12.58	12.44	12.44	12.26
Recovery, %[4]	101	100	99	98	99	98	98	99	96	97	97	97
Difference to PC, MJ/kg		-0.38	-0.44	-0.81		-0.13	-0.15	-0.32		-0.15	-0.15	-0.32

[1] $Gly_{equivalents}$ = Gly + 0.714 * Ser [2] intended concentrations 600 g produced * 0.96 minimum purity [3] based on proximate analysis according to WPSA (1984) AME_N = 15.51 * crude protein, g/kg + 34.31 *ether extract, g/kg + 16.69 * starch, g/kg + 13.01 * sugar, g/kg [4] compared to calculated values

Table 2. Composition and availability of guanidino acetic acid used in the study

Nutrient	Matrix value
Guanidino acetic acid (GAA)	96%
Standardised ileal digestibility	100%
Crude protein	221%
Arginine sparing potential	≥ 77%[1]
AME sparing potential	≥ 349 MJ/ kg (83,333 kcal/kg)[2]

[1] 77% represents least sparing deducted from research (Khajali *et al.,* 2020) [2] 349 MJ (83,333 kca) AME/kg product equals 0.21 MJ (50 kcal) AME/kg compound feed at inclusion of 600 g/ton.

Table 3. Effect of different inclusion levels of guanidinoacetic acid (GAA) on growth performance of broiler chickens.

	Diets				Sex		SEM			Probability		
Treatment groups[1]	NC1	PC	NC1+GAA	NC2+GAA	F	M	Diets	Sex	Interaction	Diets	Sex	Interaction
	Starter period 0 to 14 day old											
Feed intake (g/b/d)	40.9	41.8	42.0	42.6	40.5	43.2	0.43	0.30	0.61	0.065	< 0.001	0.250
Weight gain (g/b/d)	32.0	32.1	32.5	32.3	30.5	33.9	0.24	0.17	0.35	0.406	< 0.001	0.218
Feed conversion ratio[2]	1.284	1.306	1.294	1.322	1.328	1.275	0.0168	0.0119	0.0168	0.138	< 0.001	0.087
Body weight	0.489	0.489	0.496	0.495	0.468	0.517	0.0035	0.0025	0.0050	0.338	< 0.001	0.340
	Grower period 0 to 25 day old											
Feed intake (g/b/d)	69.5	70.8	70.0	71.2	66.3	74.5	0.57	0.40	0.80	0.150	< 0.001	0.433
Weight gain (g/b/d)	47.2^{a}	48.2^{ab}	48.7^{b}	49.1^{b}	45.2	51.3	0.37	0.26	0.53	0.005	< 0.001	0.313
Feed conversion ratio	1.367^{a}	1.361^{ab}	1.335^{c}	1.347^{bc}	1.360	1.345	0.0049	0.0035	0.0070	< 0.001	0.003	0.366
Body weight	1.315^{a}	1.338^{ab}	1.355^{b}	1.365^{b}	1.259	1.428	0.0102	0.0072	0.0144	0.006	< 0.001	0.266
	Overall period 0 to 42 day old											
Feed intake (g/b/d)	108.3^{a}	111.7^{b}	111.2^{ab}	112.7^{b}	103	118.9	0.83	0.59	1.18	0.004	<0.001	0.399
Weight gain (g/b/d)	64.9^{a}	66.8^{b}	67.3^{b}	67.4^{b}	61.8	71.5	0.48	0.34	0.68	0.002	< 0.001	0.471
Feed conversion ratio[3]	1.673	1.673	1.657	1.676	1.672	1.668	0.0063	0.0045	0.0089	0.145	0.559	0.039
Body weight	2.782^{a}	2.826^{ab}	2.868^{b}	2.868^{b}	2.623	3.049	0.0219	0.0155	0.0310	0.022	< 0.001	0.782
Liveability (%)	99.95	99.99	99.95	99.94	99.96	99.95	0.023	0.016	0.032	0.442	0.584	0.279
PEF[4]	394^{a}	405^{ab}	412^{b}	408^{b}	375	434	3.5	2.5	5.0	0.006	< 0.001	0.237

[1] Negative control (NC = PC – 50 kcal AMEn), positive control (PC; normal AMEn with levels of 3000, 3100 and 3150 kcal/kg in starter, grower, finisher diets), NC + GAA (NC + 0.06% GAA), NC2 + GAA (PC – 100 kcal AMEn/kg + 0.06% GAA); GAA was added at 0.6 kg/t

[2] Gram feed intake per gram weight gain

[3] There was diet by sex interaction as feeding NC+GAA to females improved feed efficiency compared to NC and NC1+GAA, but not in males (Female NC 1.687^{a}, Female PC 1.662^{ab}, Female NC+GAA 1.655^{b}, Female NC1+GAA 1.682^{a}, Male NC 1.659^{ab}, Male PC 1.685^{a}, Male NC+GAA 1.659^{ab}, Male NC1+GAA 1.669^{ab}).

[4] European poultry efficiency factor: averaged grams gained per day × survival rate (%) ÷ feed conversion ratio × 10.

Data are means of 16 single sex replicate pens (8 female; 8 male) with 20 birds per pen.

P value describes significance between treatments determined by ANOVA.

Results are statistically significant when $P < 0.05$

Table 4. Effect of dietary treatment on litter moisture, bad footpad score, wooden breast and white strip scores at 42 days of age.

	Diets				Sex		SEM			Probability		
Treatment groups[1]	NC1	PC	NC1+GAA	NC2+GAA	F	M	Diets	Sex	Interaction	Diets	Sex	Interaction
Litter moisture	0.339	0.347	0.340	0.339	0.336	0.346	0.0134	0.0095	0.0189	0.973	0.466	0.873
Bad footpad score	0.006	0.013	0.026	0.016	0.017	0.014	0.0055	0.0039	0.0078	0.100	0.604	0.095
Total wooden breast	0.125	0.062	0.208	0.062	0.146	0.083	0.0565	0.0400	0.0800	0.228	0.274	0.228
Total white striping	0.062	0.062	0.167	0.042	0.115	0.052	0.0381	0.0270	0.0539	0.101	0.107	0.685

[1] Negative control (NC = PC – 50 kcal AMEn), positive control (PC; normal AMEn with levels of 3000, 3100 and 3150 kcal/kg in starter, grower, finisher diets), NC + GAA (NC + 0.06% GAA), NC2 + GAA (PC – 100 kcal AMEn/kg + 0.06% GAA); GAA was added at 0.6 kg/t
Data are means of 16 single sex replicate pens (8 female; 8 male) with 20 birds per pen.
P value describes significance between treatments determined by ANOVA.
Results are statistically significant when $P < 0.05$

Table 5. Effect of dietary treatment on N-corrected apparent metabolisable energy (AMEn), dry matter (DMR), N (NR) and fat (FR) retention coefficients.

	Diets				Sex		SEM			Probability		
Treatment groups[1]	NC1	PC	NC1+GAA	NC2+GAA	F	M	Diets	Sex	Interaction	Diets	Sex	Interaction
AMEn (MJ/kg)	12.52	12.84	12.46	12.23	12.67	12.35	0.147	0.208	0.294	0.142	0.245	0.131
DMR	0.718	0.721	0.734	0.701	0.728	0.709	0.0152	0.0107	0.0215	0.510	0.234	0.128
NR	0.616[a]	0.649[ab]	0.685[b]	0.635[ab]	0.644	0.650	0.0158	0.0112	0.0223	0.038	0.765	0.664
FR	0.829	0.839	0.840	0.755	0.842	0.790	0.0313	0.0221	0.0442	0.204	0.113	0.263

[1] Negative control (NC = PC – 50 kcal AMEn), positive control (PC; normal AMEn with levels of 3000, 3100 and 3150 kcal/kg in starter, grower, finisher diets), NC + GAA (NC + 0.06% GAA), NC2 + GAA (PC – 100 kcal AMEn/kg + 0.06% GAA); GAA was added at 0.6 kg/t
Data are means of 16 single sex replicate pens (8 female; 8 male) with 20 birds per pen.
P value describes significance between treatments determined by ANOVA.
Results are statistically significant when $P < 0.05$

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