Exploring the Functional and Metabolic Effects of Feeding Garra Fish Meal to Broiler Chickens

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Abstract

The present study evaluated the metabolic and functional effects, beyond nutritive value, of feeding garra meal to broiler chickens. Three hundred twenty Sasso-breed day-old chicks were randomly assigned to four dietary treatments with either 0, 10, 20 or 30% garra meal added on top of a formulated starter and grower basal diets. The experiment lasted for 42 days. There was a gradual increase in feed intake and body weight gain with increasing garra meal addition ($p < 0.05$). Broiler chickens fed 30% garra meal were more efficient in converting feed to body weight and yielded the highest carcass weight ($p < 0.05$). Crude protein and crude fat digestibility coefficients were higher with the garra meal receiving groups with the highest values (74.8 ± 1.6 and 92.3 ± 0.6) recorded with 20% and 30% garra meal additions, respectively ($p < 0.05$). The increase in individual and total esterified carnitine concentrations in dried blood spots demonstrated the elevated metabolic rate with increasing garra meal addition ($p < 0.05$). A better supply of glucogenic substrate to the citric acid cycle was seen with garra meal addition due to the increase of propionylcarnitine to acetylcarnitine ratio ($p < 0.05$) while no effect was observed on ketogenesis as measured through the 3-hydroxybutyrylcarnitine to acetylcarnitine ratio ($p > 0.05$). Yet, it likely showed that part of the higher amino acids (Leucine, Methionine and Citrulline) uptake due to garra meal was used as glucogenic substrate ($p < 0.05$). Histo-morphology showed gradual increases in villus height, crypt depth and their ratio in the proximal parts of the intestine (duodenum and jejunum) with the opposite results observed in the more distal part (ileum) with garra meal addition ($p < 0.05$). It can be concluded that garra meal strongly improved broiler performance when added to a plant-based diet and only few parameters warranted for caution when using more up to 30% garra meal addition. Beyond growth performance, garra meal generated a shift to a more efficient digestion and nutrient metabolism, and induced changes in edible muscle that may aid in solving shortages in the Ethiopian human diet.

Introduction

Fish meal has been a successful ingredient in poultry diets throughout the world. In some regions such as the European Union, its use has been banned together with meat and bone meals after the BSE crisis in 1996 (Eliaszewicz 2004; Thiry et al. 2004). Further, fishmeal has been criticized as a non-sustainable feed resource for aquaculture. Yet, in specific situations, by-catch of small fish from fisheries is still discarded without taking the opportunity to use it as a feed resource. In Ethiopia, an indigenous fish (*Garra spp.*) is commonly considered as an unimportant waste from fisheries. Yet, this small fish can be a valuable source of nutrients (Bayissa et al. 2021a).

Despite of their presence in some Asian and African countries (Getahun and Stiassny 1998), about 60% of the African garra fish species are found in Ethiopia (Dejene et al. 2008; Getahun 2000). The positive impact of this endemic fish (*G. ignestii*, *G. blanfordi*, mainly) on enhancing water transparency, nutrient availability and phytoplankton productivity has been documented by Dejene et al. (2009) in reservoir water bodies in the Tigray region. The higher productivity of such reservoirs together with the absence of predator fish have elevated garra's reproductive capacity, resulting in tons of yield (Dejene et al. 2008,

2009). As long as the by-catch of garra continues and its addition in the human diet is still minimal, the potential of this fish as a resource for animal feeding requires attention.

The present poultry diets in Ethiopia are usually imported and based on cereals (Ebsa et al. 2019). The latter contain anti-nutritional factors such as difficult-to-digest fibre fractions and may have imbalanced amino acid and mineral profiles if not properly formulated (Fickler 2002; Blair 2018). Using local garra “waste” to produce fishmeal could improve the ecological sustainability of poultry diets in Ethiopia, and improve their nutritive value.

Fish meal is indeed known as a highly digestible nutrient source, in particular protein, with a well-balanced amino acid pattern that can support growth of the chickens (Karimi 2006). Despite these interesting nutritional characteristics of fish meal, its addition may need to be limited depending on the accumulation of problematic compounds. Poor conservation may easily lead to putrefactive compounds such as biogenic amines (Auerswald et al. 2006; Feng et al. 2016). In most studied cases, these products are negatively associated with intestinal health (Apajalahti and Vienola 2016). In particular, the addition of garra may exert biogenic amines (e.g. histamine) that can increase intestinal permeability in the consumer chickens. Higher levels of addition can be related with increased histidine decarboxylation effects by L-histidine decarboxylase, hence histamine levels (Holecek 2020). In general, chickens have been reported to have low histamine levels and its concentration can be considered as an indicator of food quality to ensure consumer safety (Mejrhit et al. 2018). To thoughtfully apply garra meal in poultry diets, the optimal addition level needs to be determined, where a good balance is found between performance and gut health. Reports of recent studies indicated that high levels of fish meal induced decreased abundance of intestinal microbiota compositions such as butyrate-producing-strains of the Ruminococcaceae and Lachnospiraceae families, and lactic acid-producing bacteria (*Lactobacillus johnsonii*) when broiler chicks are fed fish meal-based diet (Antonissen et al. 2016).

Also the lipid fraction may trigger opposite effects: on the one hand, the high proportion on long-chain n-3 fatty acids may benefit the health of the chickens and their consumers, but these lipids are also highly susceptible to peroxidation, leading to increased dietary content of oxidative agents (Amaral et al. 2018; Alagawany et al. 2019). As a third example of nutrients that may determine the optimal addition level of garra meal in poultry diets, the concentration and bioavailability of important minerals may need consideration. A large proportion of Ethiopian infants suffer from several micromineral deficiencies, such as iron and selenium (Mekonnen et al. 2005). Preliminary data demonstrated that those minerals are quite high in garra, and one may assume that their bioavailability will be high because they are mainly chelated (e.g. iron in haemoglobin) or covalently bound (e.g. selenium in selenomethionine). Recent works on the Ethiopian garra nutrient composition have reported a higher zinc and iron concentration than in other fish in the same aquatic environment (Bayissa et al. 2021a).

Because small garra fish by-catch is still discarded as a waste in several locations, the use of its fish meal in poultry nutrition could be a sustainable solution to reduce the need for dietary protein source in the sector. We here investigate how fish meal made of garra, a small fish species endemic to Ethiopia,

may affect nutrient metabolism and health parameters beyond overall performance in broiler chickens. Knowledge on the metabolic effects of garra meal can identify additional benefits or disadvantages beyond growth performance that are often neglected in similar poultry trials. Hence, the present study chooses an approach of on-top feeding of garra meal so as to investigate the impact of graded additions of garra meal to broiler diets on broiler performance, oxidative stress (malondialdehyde), inflammatory challenge (histamine), nutrient metabolism, and functional feed aspects (minerals and other compounds).

Materials And Methods

Garra-based poultry feed formulation

The experimental feed formulation and feeding trials were carried out at Mekelle University, Ethiopia. *Garra* (*G. dembecha*) fish were caught from several water bodies and reservoirs of the Tigray region, Northern Ethiopia. Whole fish were uniformly distributed for drying on a triangular shaped entirely mesh-enclosed drier stand (2 m x 12 m x 2.5 m) established on the roof of a 15 m high building and sun-dried for a fixed time period of 5 ± 1 d. The drying environment was with maximum average temperature of 28 ± 1.4 °C and wind speed at 2.72 m/s across the drying periods, December, January and February 2019/20. During the drying period, whole fish were uniformly turned over using a brush-ended clean stick to facilitate drying. Dried garra meal was collected and milled to acceptable smaller size using a grinder machine and made ready as feed substituent. Commercially available formulated broiler starter (d 0-21) and finisher (d 22-42) feeds were purchased from EthioChicken Plc. For both growth phases, a 10, 20, and 30% garra meal addition to the formulated commercial diets were prepared. We deliberately chose for addition of the garra meal instead of substitution with other ingredients to obtain the same nutrient profile. Both scenarios inevitably lead to confounding factors, since we wanted to know the pure effect of garra meal, we opted for addition, in the awareness that this implied different dietary nutrient concentrations. The macronutrient compositions of the garra meal, broiler starter and finisher basal diets were determined by proximate analysis (AOAC 1990) (Table 1).

Similarly, as a starting protocol for the experiment, garra meal, broiler starter and finisher basal diets were further evaluated for amino acid, minerals, and fatty acid contents. Amino acids assay was done using reversed-phase high-performance liquid chromatography analysis at feed & food quality laboratory, Belgium (Table 2). The total ash content was determined following the ISO method (ISO 936, 1998) and the mineral (Ca, P, Mg, K, Na, Br, Cu, Fe, Mn and Zn) concentrations of the garra meal and feed samples were evaluated after dry ashing mineralization according to ISO method (ICP-AES: ISO 11885) as per the procedures described by Gorsuch, (1970) at the Chemistry laboratory, Ghent University (Table 3) and the fatty acid composition at Lanupro, Ghent University (Table 4).

Experimental animal set up and feeding protocol

The experiment involved 320 day-old broiler chickens, randomly divided over 20 indoor floor pens with 16 chickens each (at a density of 0.28 m²/chicken). Each pen was randomly attributed to one of four dietary treatments, *i.e.* 0, 10, 20 and 30% garra meal additions on top of the broiler starter and finisher diets. Broiler chickens (Sasso-C44 breed) were purchased from hatchery supply of EthioChicken Plc.

Pens were considered as experimental units. All pens were designed to be similar in all outlines, with concrete floor having wood shavings as bedding at a depth of 5-10 cm, and built in accordance with the international and institutional research ethics and welfare requirements for experimental animals. Each pen was set with separate feeding and watering troughs, and an electrically controlled warming 200 W bulb lamp hung from the top of each pen. Indoor heating temperature was controlled using wall-fixed digital thermometers and maintained at 30 ± 1.0 °C across the starter periods of the experiment. All equipments were properly cleaned and disinfected using detergents and the entire indoor pens were fumigated with a mix of potassium permanganate and formaldehyde (500 g : 250 ml) 10 d before stocking of the experimental chickens.

All experimental chickens were procedurally vaccinated against Infectious bursal (Gumboro) disease, Newcastle disease and Fowl typhoid as per the manufacturer's recommended vaccination protocols for day-old chickens. The experiment lasted for 42 d and individual chickens were fed on experimental unit bases. Weighed amounts of feed were given on daily basis and feed leftovers were collected, cleaned, air-dried, and then weighed and recorded daily before the next morning feeding, at 08:00am. Feed and clean water were given *ad libitum* and chickens were allowed to stay indoors throughout the experimental period.

Measurement of growth performance indicators

Performance indicating factors were assessed for chickens in each experimental unit. Following fasting for 12 h, chickens were weighed on weekly basis on d 0, 7, 14, 21, 28, 35 and 42 in the morning before feeding starts at 08:00am. The average weekly feed intake (g/wk), body weight gain (g/wk) and feed conversion ratio (FCR, g/g) were evaluated at each sampling time point following standard procedures. Mortality cases were checked and recorded from every replicate of each treatment group (n = 80) on a daily basis and correction factors were made for all dependent parameters across the study period. Mortality was calculated as follows:

$$\text{Mortality (\%)} = (N \div 80) \times 100$$

Where: N stands for the number of dead chicken in every replicate of each treatment group, 80 is the total number of chickens per group.

Evaluation of apparent nutrient digestibility using titanium (IV) oxide as a biomarker

Marker-feed preparation and feeding

Starting from d 22 onwards, titanium (IV) oxide (VWR Chemicals®, Belgium, PN84853, > 99.0% purity) was supplemented into each experimental diet of grower chickens at 0.4% addition. To assure homogenous mixing of the marker, premixing was applied on a small amount of diet (1% of the total diet). Feeding with the marker diet started on the first day of the growth phase (d 22) and continued till d 42 of the experimental period.

Excreta collection and processing

Prior to sampling, all wood shavings were removed and replaced with plastic mats for easier collection of excreta. Excreta sampling from each experimental unit was done for six consecutive days of the last week of the experimental period, d 37-42. Excreta were collected using a blunt-flat scraper from the floor quantitatively for 24 h at 2x/day (06:00 and 18:00), pooled and weighed daily and kept frozen (-20 °C) during the sampling days. Only clean and uncontaminated excreta were collected from several predetermined locations of each pen. Finally, samples were pooled per pen, homogenized with a mixer (1-2 min), and sample of known weight (about 300 g/pen) was taken in plastic bags from each experimental unit. Excreta samples were then freeze-dried to stable weight and kept at -20 °C till analysed for their nutrient and marker concentrations (Schaafstra et al. 2017). Feed and water were given *ad libitum* across the collection period.

Measurement of apparent digestibility coefficients

Freeze-dried excreta samples and the experimental diets, 0, 10, 20 and 30% garra meal formulations, were all analysed for their individual nutrients. The DM, CP and EE contents were evaluated according to AOAC (1990). The CP contents were evaluated using the Kjeldahl method as described by AOAC (2001) and the TiO₂ contents as per the procedures stated by Myers et al. (2004). All samples were evaluated in duplicates at Ghent University, Belgium. Obtained mean values were used for the calculation of apparent digestibility coefficients of individual nutrients using the biomarker and the following formula:

$$\text{Apparent digestibility (\%)} = 100 - \left(100 \times \left(\frac{\% \text{TiO}_2 \text{ in diet}}{\% \text{TiO}_2 \text{ in excreta}} \right) \times \left(\frac{\% \text{nutrient in excreta}}{\% \text{nutrient in diet}} \right) \right)$$

Evaluation of chicken blood biochemical profiles

Blood sample collection and processing

Blood samples (5 mL/chicken) were drawn from wing veins of three randomly selected chickens from each experimental unit at the end of the starter (d 22) and grower phases (d 43). Blood samples were allowed to coagulate at room temperature for 15 min, then centrifuged (1,500 × *g* for 10 min) and serum were collected in Eppendorf tubes and stored at -20 °C until analyses. The same chickens were traced and sampled for their serum in round two blood sampling.

Measurement of biogenic amines and oxidative stress biomarkers

Serum samples (100 μ L) were evaluated for histamine concentration. Following sample preparation and acylation, histamine quantification was performed using Enzyme-Linked Immunosorbent Assay (ELISA) following the manufacturer's kit protocol (Ridascreen[®] Histamine (enzymatic), Art No. R1605) at Ghent University, Belgium. Serum (150 μ L) malondialdehyde concentrations were analysed as thiobarbituric acid reactive substances (TBARS) according to the method of Grotto et al. (2007) at Lanupro, Ghent University.

Acylcarnitine profiling

For the evaluation of shifts in nutrient metabolism, profiling of acylcarnitine concentrations were done. A drop (50 μ L) of blood was taken from each of the whole blood samples collected and placed on circular specimen collection papers (Whatman protein saver cards, 903[™], UK). Each specimen kit was allowed to dry, and later sent to Ghent University Hospital (Laboratory for Clinical Chemistry) for their biochemical assay.

Evaluation of intestinal histo-morphological changes

Intestinal sample collection

At the end of the experimental feeding period, following 12 h fasting, the traced 3 chickens from each experimental unit were sampled for their intestines on d 42. Chickens were weighed and then euthanized humanely using sodium pentobarbital (IP, 28 mg/kg BW) to death. The entire small intestine segment was exposed under aseptic post-mortem examination.

A fixative solution (mixture of glutaraldehyde [3%] and paraformaldehyde [4%] fixative solution in 0.1 mol/L cacodylate buffer [pH 7.4]) was prepared and injected into the intestinal lumen of the middle part of each intestinal segment. The entire small intestine spanning from the gizzard to pancreatic and bile duct (duodenum), from bile duct to Meckel's diverticulum (jejunum), and from diverticulum to the ileo-caecal-colonic junction (ileum) was then excised and placed into separate bottles containing the same fixative solution. Similarly, the cecum part were also separately removed and placed in the same fixative solution until morphologically evaluated.

Histo-morphological examination

Gut tissue samples (2cm long) were collected from the middle part of each intestinal segment, dehydrated under graded alcohols and xylene, and then embedded in paraffin. Serial paraffin sections (5 μ m) were made from samples and kept at 37 °C for 12 h or more. Then after, sample sections were prepared in slides and stained with haematoxylin-eosin stain following a routine protocol. Finally, the mucosal structures were observed under a Nikon phase-contrast microscope and examined using MicroComp integrated digital image-analysis system with the help of Microscope camera. The villous height and crypt depth of 10 well-oriented villi were measured per section. The average value for each tissue was obtained from 3 sections per tissue sample. That is, 10 villi/crypt per section and 3 sections

per sample were measured and the average value used for statistical analysis (Fan et al. 1997). Furthermore, the villus height to crypt depth ratio was also calculated for each value obtained.

Evaluation of intestinal permeability using the everted sac test (*ex vivo*)

The effect of garra meal supplements in the intestinal permeability were evaluated. For this, 20 cm segment of the hind intestine (ileum part) was cut and washed in phosphate buffer saline (PBS) solution along the hollow, weighed, and then kept in an ice water mounted petri dish. The hollow segment was then inverted using a small crochet needle inserted into the lumen. One part of the lumen was ligated while filled with the PBS solution from an elevated container through the other end of the sac and then ligated on either side. The sacs were fully immersed and incubated in a glass beaker filled with PBS solution containing a cobalt marker with a constant oxygenation through aqua-aerators. The glass beakers were kept in an open water bath set up at 39.3 ± 0.8 °C for an hour. Then after, sacs were taken out, rinsed with water, and 10 ml of the intestinal fluid contents were collected with syringe into a tube and stored until analysed. Similar 10 mL sample were collected from the ringers' solution with markers at the start of the experiment for conjoint evaluation. Spectrophotometric reading of cobalt concentration was done at 640 nm (Hamilton 2014).

Data management and analysis

The different data obtained from performance indicator parameters, blood biochemical profiles, and intestinal measurements were entered into a spreadsheet Excel separately and analysed accordingly. Data from experimental diets (four percent levels) were subjected to one-way analysis of variance (ANOVA) and analysed using SPSS software (version 27.0). Every replicate was considered as the experimental unit for all statistical analyses. In either case, results were expressed as treatment means with their pooled standard error of the mean (SEM). A $P < 0.05$ probability value was described to be the level of significance and the notable differences between the treatment groups were determined via Tukey multiple comparisons test.

Results

Growth performance indicators

Mortality throughout the experimental period was low: three dead chickens in the control group and one dead in the 10% garra meal group, all in the first week, with no apparent relationship to the treatments.

There was a gradual increase in feed intake and body weight gain with increasing garra meal addition ($P < 0.001$) (Table 5).

Broiler chickens fed 30% garra meal were more efficient in converting feed to body weight (1.7 ± 0.01 g feed/g gain, $P < 0.001$) across the feeding experiment with the lowest feed conversion ratio observed at the starter phase (1.6 ± 0.02 g feed/g gain). Likewise, the highest carcass weight (1103 ± 36 g, $P < 0.001$)

was recorded at the highest garra meal addition with similar carcass dressing percentages among treatment groups ($P = 0.218$) (Table 5).

Apparent nutrient digestibility

Apparent crude protein digestibility was higher with the garra meal receiving groups with the highest value recorded with 20% garra addition (74.8 ± 1.6 , $P = 0.006$). Similarly, crude fat digestibility was higher with the gradual garra meal additions with the highest digestibility recorded by 30% garra meal addition (92.3 ± 0.6 , $P < 0.001$). No difference in dry matter digestibility was noticed among the treatment groups ($P = 0.068$) (Table 6).

Acylcarnitine profiles

The blood spot concentrations of the short-chain acylcarnitine moieties – free carnitine (C0), acetylcarnitine (C2), propionylcarnitine (C3), butyrylcarnitine (C4), isovalerylcarnitine (C5) and hexanoylcarnitine (C6) – increased gradually with garra meal addition ($P < 0.05$) (Fig. 1). Also the total sum of acylcarnitines increased gradually with garra meal addition. The ratio of bound to free carnitine decreased due to garra meal addition.

There was a gradual increase ($P < 0.05$) in the ratio of propionylcarnitine to acetylcarnitine (C3:C2) with ascending garra meal addition (Fig. 2). The ratio of 3-hydroxybutyrylcarnitine to acetylcarnitine (3OHC4:C2) remained unaffected while the malonylcarnitine to acetylcarnitine ratio (C3DC:C2) was slightly increased with garra meal addition.

Dried blood spot concentrations of some free amino acids (leucine, methionine and citrulline) increased with garra meal addition whereas other amino acids (phenylalanine, tyrosine and ornithine) decreased with garra meal addition (Fig. 3).

Serum histamine and malondialdehyde levels

Chicken serum samples from all treatment groups were found to contain histamine far below the detection limit, 5 mg/mL, and no difference was observed among treatments ($P > 0.05$). However, detectable gradual increase in malondialdehyde levels with garra meal additions were noticed in the starter growth phase, 21 d, of chickens with the highest level showed by 30% garra meal fed groups (9.5 ± 0.8 nmol/mL, $P = 0.054$). No significant effect of garra meal addition was seen at the end of grower phase, 42 d, of chickens ($P = 0.726$) although all values recorded were above the detection limit, 2.5 nmol/mL (Table 7).

Intestinal histo-morphology

Garra meal addition elevated the villus height in the duodenum and jejunum compared with the control group ($P = 0.005$). However, a gradually decreasing villus height of ileum was noted with increasing garra meal addition ($P = 0.005$).

The small intestine crypt depth of the duodenum and jejunum in the garra meal groups were deeper than the control group ($P = 0.003$), while there were no differences between the control and 30% garra meal addition ($P > 0.05$). The crypt depth of ileum was deeper in the control groups than the garra meal supplemented groups ($P < 0.003$), while there was no difference among the 10, 20 and 30% garra meal additions ($P > 0.05$).

The villus : crypt ratio of the duodenum, jejunum and ileum in the chickens with garra meal supplementation was higher than the control group ($P < 0.001$), except for the ileum of 20% garra meal addition being the lowest ratio. The villus : crypt value of the duodenum with 30% garra meal addition was the highest among all other values computed.

The cecal internal endothelial folding/crust heights were higher for the garra meal addition groups than that of the control groups ($P = 0.031$). However, no difference was observed between 20 and 30% garra meal addition groups ($P > 0.05$) (Table 8).

Everted sac test

The everted sac test was used to reflect the absorptive capacity of the intestinal lumen of broiler chickens fed diets with graded additions of garra meal. The cobalt (Co) absorption across the lumen was higher in the 30% garra meal addition than in the group without garra meal ($P = 0.007$) (Fig. 4).

Discussion

The clear gradual increase in performance of the broiler chickens with addition of garra meal was according to expectations, given the high protein and energy content of this fish meal made of garra. It is quite remarkable that adding garra meal on top of an assumedly balanced diet still induced a far better growth performance. Garra meal indeed appears as a balanced diet ingredient with highly digestible nutrients and high palatability.

Less obvious than the increased growth, was the observed increase in feed intake with increasing garra meal, as in most situations, chickens will reduce feed intake with increasing energy density (Mikulec et al. 2004, Karimi 2006, Incharoen et al. 2010). Yet, other factors than just energy density are regulating feed intake. This was not the focus of our study, but we refer to for instance the minimal total discomfort theory (Forbes 2003) that showed that any nutrient or other requirement can affect voluntary feed intake. The increased intake can explain the higher growth rate with increasing garra meal, but the associated higher feed utilisation efficiency emphasizes the balanced supply of digestible nutrients from garra meal.

Data on whole-body composition of garra were recently published by Bayissa et al. (2021a) but we added here the fatty acid profile of garra, demonstrating its interestingly high content of long-chain n-3 fatty acids. These fatty acids have well-known health effects in chickens (Swanson et al. 2012, Al-Zuhairy and Jameel 2014, Ibrahim et al. 2018) and the accumulation of these fatty acids in chicken tissues can affect

human health as well (Baiao and Lara 2005, Al-Zuhairy and Jameel 2014). As tackled further in this discussion, garra meal is also rich in specific functional nutrients such as carnitine and citrulline that may support the animal's metabolism.

Clearly, the maximum for protein deposition was not yet reached with the basal diet without garra meal, because the birds not only grew faster with garra meal addition, but still had the same dressing percentage (and even numerically higher). Also the unaltered malonylcarnitine to acetylcarnitine ratio in the dried bloodspots confirms that fat synthesis remained independent of garra meal addition. Malonyl coenzyme A is indeed a known marker for lipogenesis (Schooneman et al. 2013) and the higher production of malonylcarnitine is a step forward in the synthesis of fats in the citric acid cycle (Yang et al. 2019). It also matches the lower feed conversion ratio due to garra meal addition, which implies that the nutrients and energy from garra meal were more efficiently used than that of the basal diet. An important factor to explain this effect is the combination of highly digestible ingredients with a low fibre content. More garra meal addition led to higher lipid digestibility, even up to the 30% addition, but protein digestibility seemed to reach an optimum at 20% addition, likely because the capacity for protein digestion was reached at that point. A concomitant concern of suboptimal protein digestion is the consequences for gut health, because providing more protein as substrate to the cecal microbiome may lead to damaging fermentation end-products such as biogenic amines (Apajalahti and Vienola 2016, Mejerhit et al. 2018). In the present study, the serum concentrations of histamine, a major biogenic amine, and malondialdehyde, an oxidative stress indicator, could however not show such a negative effect of high garra meal addition. The only measurement that pointed to a possible optimum for garra meal addition, was the increased gut permeability at 30% garra meal addition. Increased gut permeability, as proven *ex vivo* in the present study, could evoke increase in intestinal mucosal paracellular permeability enhancing entry of pathogenic bacteria and/or their toxins into the systemic circulations that could trigger diseases (Chelakkot et al. 2018, Hollander and Kaunitz 2020).

Despite this impact on gut permeability at the higher garra meal addition level, the intestinal development was prominently affected: gradual increases in villus height, crypt depth and their ratio in the proximal parts of the intestine (duodenum and jejunum) typically reflect a higher digestive and absorptive capacity when adding more garra meal. Irrefutably, Incharoen et al. (2010) have found that the villus heights in duodenum and jejunum were lower in chickens fed low-crude protein diets with a lower mitotic number in crypt cells than those on normal dietary crude protein. In fact, the intestinal endothelium, with its villi, is a dynamic environment and often changes to adjust itself in response to intestinal contents, including dietary compositions (Yamauchi 2002, Prakatur et al. 2019). Hence, longer intestinal villi are associated with increased surface areas and capacity for absorption of nutrients (Izadi et al. 2013) and deeper crypts reflect active endothelial cell mitosis beneath each villus for the regeneration of new villi in the lumen (Hamedi et al. 2011). On the other side, lower villus : crypt ratios are associated with fewer absorptive cells and numerous epithelial secretory cells. The latter are involved in excess mucus secretion that can reduce nutrient absorption and lead to increased intestinal coping functions (Hamedi et al. 2011). In the more distal part (ileum), the opposite was observed, likely referring to the fact that higher proximal digestibility led to less digesta in the distal part of the chickens' digestive tract. Ileum is indeed a site

where water and minerals are absorbed while the absorption of proteins, carbohydrates and fat mainly takes place in the jejunum (Svihus et al. 2010, Ariyadi et al. 2019).

The avian caecum is particularly important to process uric acid from protein breakdown (Clench 1999), hence the increase in cecal crust height with more garra meal addition. The larger and numerous cecal folds can further be related to its involvement in the digestion of small protein loads being pushed against the cecal sphincter from the intestinal loads and/or their absorption (Clench 1999, Majeed et al. 2009).

As a result of increased absorptive capacity and increased feed intake, garra meal addition must have induced a higher supply of nutrients in metabolism. This requires a metabolic “machinery” that can handle the elevated demand for nutrient processing. Our group has been using carnitine esters in diverse species to reflect changes in the corresponding coenzyme A moieties in nutrient metabolism (e.g. frogs: Brenes-Soto et al. 2019, cows: Bedada et al. 2021, fish: Bayissa et al. 2021b) but on itself, the carnitine status of an animal can affect metabolic efficiency (Schooneman et al. 2013, Gucciardi et al. 2015, Yang et al. 2019). In the present study, the main acylcarnitines and their sum positively responded to increased garra meal addition, demonstrating the increased metabolic activity due to enhanced nutrient absorption and concomitant growth. The increase of the propionylcarnitine to acetylcarnitine ratio shows a relatively better supply of the often limiting glucogenic substrates to the citric acid cycle (Bayissa et al. 2021b). It is not sure if this was instrumental for improving feed utilisation efficiency because no significant effect was observed on the production of ketone bodies deviating from the citric acid cycle, as measured through the 3-hydroxybutyrylcarnitine to acetylcarnitine ratio. 3-Hydroxybutyrylcarnitine is an indicatory moiety of ketogenesis in the body (Xu et al. 2016). Yet, it likely shows that part of the higher amino acid uptake due to garra meal was used as glucogenic substrate. Methionine is an example of a glucoplastic amino acid that can enter the citric acid cycle through propionyl coenzyme A converting into succinyl coenzyme A (Krajcovicová-Kudláčková et al. 2000), and its free concentration in the dried bloodspots was clearly rising with garra meal addition. Yet, even the concentrations of the pure lipogenic amino acid leucine (Schooneman et al. 2013) went up with garra addition, whereas other free amino acids such as tyrosine and its precursor phenylalanine decreased when more garra meal was added. This suggests that these free amino acids profiles are not just representing metabolic requirements but also simply an influence of the dietary amino acid composition.

At least for citrulline and carnitine, two amino-acid-like substances, their dose-responsive increase in concentration must have been due to their supply through garra meal. Carnitine is not present in plant material, whereas most animals including fish are rich sources of carnitine (Li et al. 2020). There was a marked increase in the free as well as bound carnitine in our study. The reduction in the bound to free carnitine ratio that was seen with higher garra meal addition could be interpreted as a relative drop in metabolic rate, but in this case, this was clearly due to the much higher absorption of carnitine from the garra meal that may even have supported metabolic efficiency. We acknowledge that we cannot claim this effect since other factors may have caused improved efficiency independent of carnitine supply, but

at least studies have demonstrated beneficial effects on carnitine supplementation to plant-based diets for broiler chickens (Janssens et al. 2000, Karadeniz et al. 2008, Hrnčár et al. 2015).

Citrulline cannot be synthesized in birds because they lack the urea cycle – in contrast with mammals – that can generate citrulline from ornithine (Tsuji and Kanazawa 1987). Citrulline can be an interesting functional nutrient given its antioxidant action (Coles 2007), especially in hot environments: heat stress is a strong inducer of oxidative stress (Akbarian et al. 2016). This effect was not studied here but we hypothesize that the better broiler performance in this study when feeding more garra meal was due to nutritional effects (improved supply of digestible nutrients) as well as metabolic modulator effects (e.g. carnitine, citrulline, n-3 fatty acids). The decrease in ornithine with garra meal additions however could possibly reflect dietary dilution effect of garra meal in the experimental diets.

In conclusion, garra meal strongly improved broiler performance when added to a plant-based diet. Only few parameters warranted for caution when using up to 30% garra meal addition, but apart from digestibility and absorptive capacity, also nutrient metabolization efficiency seemed increased. The latter may have been caused by a number of functional components in the garra meal that may warrant further investigation.

Declarations

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Compliance with ethical standards

This study was carried out following the guidelines and approval of the research policy of the Animal Ethics Committee, Mekelle University, Ethiopia. The care of experimental chickens was performed in accordance with the international ethical standards declaration.

Competing interests

The authors declare that they have no any conflict of interest.

Data availability statement

Any further data related to the manuscript will be available upon request.

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Tables

Table 1 Ingredients, energy concentrations and macronutrient composition (dry matter basis, %) of broiler starter and finisher basal feeds

Component	Starter feed	Finisher feed
Ingredients (%)		
Maize	49.5	58.0
Soybean	21.5	15.0
Wheat bran	15.0	14.2
Groundnut expeller	10.0	10.0
Broil start EC ^a	2.0	0.0
Limestone	1.6	1.6
Toxibinder (TOXO-MX) ^b	0.2	0.1
Bactoban (Fysal SP dry) ^c	0.2	0.1
Niger seed meal	-	1.0
Metabolic energy, MJ/kg	11.9	13.6
Macronutrients		
Crude protein	23.8	24.1
Crude fiber	4.7	4.8
Neutral detergent fiber	51.9	54.4
Crude fat	2.8	2.9
Ash	7.4	5.4

^aBroil start EC, mixture of cracked corn and ground roasted soybeans

^bTOXO-MX, activated and purified smectite binding agent

°Fysal Dry SP, dried blend of short chain organic acids

Table 2 Amino acid composition of garra meal, broiler starter and finisher basal feeds

Amino acids (g/kg)	Garra	Starter feed	Finisher feed
Asparagine + Aspartic acid	22.3	12.4	18.6
Threonine	10.8	5.9	7.1
Serine	11.0	6.9	9.3
Glutamine + Glutamic acid	36.3	30.2	35.4
Glycine	30.1	07.3	8.9
Alanine	16.8	8.6	10.0
Valine	11.9	7.5	8.9
Isoleucine	10.4	6.0	7.9
Leucine	17.8	12.4	16.5
Tyrosine	9.0	5.4	7.2
Phenylalanine	9.7	6.9	9.5
Histidine	5.8	4.0	5.6
Lysine	18.2	7.2	10.0
Arginine	18.6	9.4	13.4
Proline	14.1	10.4	11.6
Cysteine	2.8	2.9	3.7
Methionine	7.5	2.7	3.6
Tryptophan	2.1	1.9	2.2
Sum total	255.2	148.0	189.4

Table 3 Mineral composition of garra meal, broiler starter and finisher basal feeds

Minerals	Garra	Starter feed	Finisher feed
Macrominerals (g/kg)			
Calcium	47.4	10.1	3.5
Phosphorus	23.8	5.3	5.0
Magnesium	2.1	2.4	2.1
Potassium	8.6	9.6	8.6
Sodium	3.7	1.5	1.6
Microminerals (mg/kg)			
Copper	6.61	23.3	24.2
Iron	1,160	997	669
Manganese	77.9	107	110
Zinc	259	86.6	79.8

Table 4 Fatty acids composition of garra meal, broiler starter and finisher basal feeds

Fatty acid (g/100g fatty acid)	Garra	Starter feed	Finisher feed
Saturated fatty acids			
C8:0	0.014	0.112	0.091
C10:0	0.021	0.235	0.190
C12:0	0.199	0.098	0.078
C14:0	3.963	0.185	0.187
C15:0	0.847	0.074	0.076
C16:0	28.362	21.528	19.487
C17:0	0.870	0.679	0.500
C18:0	5.627	6.459	6.960
C20:0	0.365	0.146	0.088
C22:0	1.280	0.493	0.090
C24:0	0.201	1.302	1.214
Total	65.04	7.33	7.18
Monounsaturated fatty acids			
C14:1	0.070	0.038	0.042
C16:1	12.487	0.210	0.308
C17:1	1.256	0.037	0.059
c9C18:1	20.875	34.300	34.873
c11C18:1	3.760	1.149	1.077
C20:1	0.765	0.577	0.511
C24:1	0.158	0	0
Total	61.34	8.50	9.14
n-6 polyunsaturated fatty acids			
C18:2n-6	5.301	27.353	29.135
C18:3n-6	0.544	1.029	1.174
C20:2n-6	0.381	0.390	0.301
C20:3n-6	0.595	1.371	1.208
C20:4n-6	0.019	0	0

Total	11.0	7.09	7.91
n-3 polyunsaturated fatty acids			
C18:3n-3	4.130	1.898	2.029
C20:3n-3	0.278	0	0
C20:4n-3	0.361	0.118	0.123
C20:5n-3	3.432	0	0
C22:5n-3	1.429	0	0
C22:6n-3	2.194	0.091	0.094
Total	18.42	0.49	0.56
Sum total	155.82	23.41	24.79

Table 5 Effect of garra meal addition to a formulated broiler diet on feed intake, weight gain, feed conversion ratio, carcass weight and dressing percentage of broiler chickens

Parameter	Garra meal addition (%)				<i>P</i>
	0	10	20	30	
Feed intake (g/wk)					
1-21 d	3,611 ± 143 ^a	4,010 ± 193 ^b	3,944 ± 133 ^b	3,868 ± 88 ^{a,b}	< 0.001
22-42 d	7,211 ± 256 ^a	7,812 ± 138 ^a	7,957 ± 157 ^{a,b}	8,535 ± 119 ^b	< 0.001
1-42 d	5,411 ± 400 ^a	5,911 ± 428 ^b	5,950 ± 439 ^b	6,304 ± 512 ^c	< 0.001
Weight gain (g/wk)					
1-21 d	1,920 ± 36 ^a	2,210 ± 47 ^{a,b}	2,267 ± 21 ^{a,b}	2,384 ± 17 ^b	< 0.001
22-42 d	2,914 ± 47 ^a	3,596 ± 40 ^b	3,939 ± 38 ^b	4,826 ± 28 ^c	< 0.001
1-42 d	2,417 ± 39 ^a	2,903 ± 50 ^b	3,103 ± 51 ^b	3,604 ± 65 ^c	< 0.001
FCR (g/g)					
1-21 d	2.0 ± 0.04 ^b	1.9 ± 0.03 ^{a,b}	1.8 ± 0.03 ^{a,b}	1.6 ± 0.02 ^a	< 0.001
22-42 d	2.6 ± 0.04 ^c	2.2 ± 0.03 ^{a,b}	2.0 ± 0.02 ^b	1.8 ± 0.01 ^a	< 0.001
1-42 d	2.3 ± 0.03 ^c	2.0 ± 0.02 ^b	1.9 ± 0.01 ^{a,b}	1.7 ± 0.01 ^a	< 0.001
CW (g)	834 ± 34 ^a	863 ± 22 ^{a,b}	962 ± 20 ^b	1,103 ± 36 ^c	< 0.001
DP (%)	59 ± 1.5	59 ± 1.6	58 ± 0.5	63 ± 1.2	0.218

^{a,b,c}Values within a row with no common superscripts differ significantly ($P < 0.05$)

Mean values represent mean ± SEM based on 16 chickens per replicate and 5 replicates per treatment

FCR, feed conversion ratio, CW, carcass weight, DP, dressing percentage

Table 6 Apparent digestibility of dry matter, crude protein and crude fat using TiO₂ as a biomarker in broiler chickens fed with different garra meal additions

Digestibility (%)	Garra meal addition (%)				<i>P</i>
	0	10	20	30	
Dry matter	67.7 ± 1.0	63.9 ± 0.9	67.8 ± 0.6	64.7 ± 1.8	0.068
Crude protein	64.3 ± 3.4 ^a	67.4 ± 2.1 ^{a,b}	74.8 ± 1.6 ^b	67.2 ± 2.7 ^{a,b}	0.006
Crude fat	76.1 ± 2.4 ^a	83.9 ± 0.2 ^b	91.6 ± 0.5 ^c	92.3 ± 0.6 ^c	< 0.001

^{a,b,c}Values within a row with no common superscripts differ significantly ($P < 0.05$)

Mean values represent mean ± SEM based on 16 chickens per replicate and 5 replicates per treatment

TiO₂, titanium dioxide

Table 7 Effect of dietary garra meal addition on serum histamine and malondialdehyde biomarkers in broiler chickens at d 21 and 42

Biomarker	Garra meal addition (%)				<i>P</i>
	0	10	20	30	
Histamine (mg/mL)					
1-21 d	0.5 ± 0.001	0.1 ± 0.001	0.1 ± 0.001	0.1 ± 0.002	0.576
22-42 d	0.1 ± 0.001	0.2 ± 0.001	0.2 ± 0.001	0.3 ± 0.004	0.462
MDA (nmol/mL)					
1-21 d	5.8 ± 0.3	7.6 ± 1.0	8.7 ± 1.2	9.5 ± 0.8	0.054
22-42 d	8.3 ± 0.7	9.6 ± 1.0	8.7 ± 0.8	8.9 ± 0.8	0.726

^{a,b,c}Values within a row with no common superscripts differ significantly ($P < 0.05$)

Mean values represent mean ± SEM based on 3 chickens per replicate and 5 replicates per treatment

MDA, malondialdehyde

Table 8 Microscopic villous height, crypt depth, villi to crypt ratio, and cecal crust height of duodenum, jejunum, ileum and cecum segments of broiler chickens fed diets with graded additions of garra meal at d 42

Section	Garra meal addition (%)				<i>P</i>
	0	10	20	30	
Duodenum (μm)					
Villi	682.3 ± 15.1 ^a	729.8 ± 17.3 ^{a,b}	854.1 ± 16.1 ^b	915.9 ± 23.8 ^c	0.005
Crypt	67.9 ± 2.2 ^a	69.5 ± 1.6 ^a	77.4 ± 1.6 ^c	73.2 ± 1.9 ^b	0.003
v:c ratio	10.4 ± 0.2 ^a	10.7 ± 0.2 ^a	11.08 ± 0.2 ^b	12.6 ± 0.2 ^c	< 0.001
Jejunum (μm)					
Villi	724.1 ± 14.9 ^a	756.4 ± 14.6 ^{a,b}	805.2 ± 25.5 ^c	756.0 ± 14.9 ^{a,b}	0.005
Crypt	65.8 ± 1.9 ^a	71.5 ± 1.6 ^{a,b}	76.2 ± 2.6 ^c	66.9 ± 1.2 ^a	0.003
v:c ratio	10.3 ± 0.2 ^a	11.8 ± 0.4 ^b	10.8 ± 0.2 ^a	12.4 ± 0.2 ^c	< 0.001
Ileum (μm)					
Villi	613.9 ± 19.5 ^c	577.4 ± 14.3 ^b	549.7 ± 13.4 ^a	540.1 ± 16.8 ^a	0.005
Crypt	66.9 ± 2.0 ^c	60.4 ± 2.5 ^a	62.2 ± 2.0 ^b	58.4 ± 2.2 ^a	0.003
v:c ratio	9.3 ± 0.2 ^a	10.0 ± 0.3 ^{a,b}	9.0 ± 0.3 ^a	9.6 ± 0.3 ^{a,b}	< 0.001
Cecum (μm)					
crust height	400.7 ± 17.4 ^a	439.9 ± 19.5 ^{a,b}	458.6 ± 9.5 ^b	458.3 ± 12.8 ^b	0.031

^{a,b,c}Values within a row with no common superscripts differ significantly ($P < 0.05$)

Mean values represent mean ± SEM of 30 measurement units based on 3 chickens per replicate and 5 replicates per treatment

v:c ratio, villus height to crypt depth ratio

Figures

Figure 1

Box and whisker plots of acylcarnitine concentrations in dried blood spots of broiler chickens fed with different garra meal additions. C0, free carnitine, C2, acetylcarnitine, C3, propionylcarnitine, C4, butyrylcarnitine, C5, isovalerylcarnitine, C6, hexanoylcarnitine, acyl:free, ratio of acylated against free

carnitine, Sumcarn, sum of all carnitine esters. Mean values are based on 3 birds per replicate and 5 replicates per treatment

Figure 2

Box and whisker plots of concentration ratios of targeted acylcarnitines from dried blood spots of broiler chickens fed diets with graded additions of garra meal. C3/C2, propionylcarnitine to acetylcarnitine ratio, 3OHC4/C2, 3-hydroxybutyrylcarnitine to acetylcarnitine ratio, C3DC/C2, malonylcarnitine to acetylcarnitine ratio. Mean values are based on 3 birds per replicate and 5 replicates per treatment

Figure 3

Box and whisker plots of free amino acid concentrations in dried blood spots of broiler chickens fed diets with graded additions of garra meal. Leu, leucine, Met, methionine, Fen, phenylalanine, Tyr, tyrosine, Orn, ornithine, Cit, citrulline. Mean values are based on 3 birds per replicate and 5 replicates per treatment

Figure 4

Ex vivo mean cobalt ions absorbed across the intestinal lumen using the everted sac test from chickens fed diets with graded additions of garra meal. Absorbance differences of cobalt concentrations were measured at 640 nm spectrophotometry. Mean values are based on 3 birds per replicate and 5 replicates per treatment