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DIFFERENTIAL EXPRESSION OF MAJOR AMINO ACID TRANSPORTER GENES IN WEANED RABBITS FED LOW DIETARY CRUDE PROTEIN ENRICHED WITH FREE AMINO ACIDS.

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Abstract:

The mechanism by which transport mediators are expressed remains a complex integrative process which may involve genetic alterations. This study therefore evaluates the effect of altering crude protein levels in diets of rabbits on the mRNA expression of amino acid transporters in a 45-d trial. Twenty four mixed breed rabbits of average body weight of 5.6 Kg were assigned to four dietary groups of 20 % CP, 18% CP, 16 % CP and 14 % CP in a completely randomized design. Diets were supplemented with crystalline amino acid (AA) to achieve equal contents of Lysine, Methionine, Threonine and Tryptophan, and were provided ad libitum. After 45d, rabbits were slaughtered and samples from their jejunum were examined. Compared with the values obtained in the 20 % CP group, the expressions of ASCT, $B^{O+}AT$, Pep T1 and ATB^{0} mRNA in the jejunum were increased by 9%, 11%,14%, 24%; 37%, 39%, 47%, 52% and 16%, 23%, 29%, 34% in the 18%, 16% and 14 % CP groups, respectively. These findings indicate that a 14% CP diet supplemented with crystalline amino acid may supply enough AA to body cells of rabbits depending on the nutritional quality of the diet. The low CP diet also lessens the excretion of nitrogen into the environment and minimizes cost of feeding while enhancing the development of rabbits.

Keywords: mRNA, expression, crude protein, rabbits, jejunum, genetic

Introduction:

It has been documented in Literature that diets low in both crude protein and supplemented amino acids (AA) contents, could reduce feed cost and Nitrogen excretion that act as pollutant (Gallo *et al*., 2018, Liu *et al*., 2019, Recklenward *et al*., 2018). However, the levels of crude protein in diets of weaned animals has been suggested to be one of the major factors that affect growth performance (Speier *et al*., 2018), feed efficiency and gastro intestinal health (Acciaiolio *et al*., 2017) of animals. This is because weaning is associated with reduced food consumption in young mammals (Daniel, 2019), which pre disposes them to reduction in body gains. As such, diets are to be formulated with caution.

Diets that contain high levels of protein have been proposed for weaned animals ration to compensate for stress and losses of nutrients post suckling (Wang *et al*., 2018). An increase in the entry of nutrients (tri-peptide, di-peptide, lysine, arginine, histidine) from optimal dietary crude protein intake into the lumen of the small intestine to the enterocytes can enhance tissue protein synthesis and improve the efficiency of utilization of dietary nutrients, that could selectively modulate the gene expression of amino acid transporter in intestinal cells (Wang *et al*., 2018).

Similarly, nutrients absorption through the small intestine is also mediated by transport protein as they passed through the epithelium of the small intestine into the blood stream via special transporters. These special nutrients transporter, according to Poncet and Taylor (2019), have different gene expression patterns and functions based on the amino acids flux that they sense in the enteric cavity. Thus, modulating those free amino acids across apical and brush border membranes of the small intestine. For example, amino acids are transported into the intestinal epithelial cells as di-peptides by the H^+ dependent peptide transporter 1, Pep T_1. Hence, variety of amino acid transporters act on specific amino acid as substrate in either neutral, basic or acidic system (medium) and expresses them as anionic, cationic, excitatory and neutral amino acids (Opapeju *et al*., 2019) or regulates the intracellular amino acid pool.

Studies on gene expression level of intestinal fatty acid (Xie*et al*, 2018), sodium glucose transporters (Dong *et al*., 2019), excitatory amino acid transporter (Liu *et al*., 2019), Sodium and proton transporter (Lie *et al*., 2017) and activity of digestive enzymes (Deng et al., 2018) have been documented.

Improving the efficiency of protein use and maintaining performance have been reported to be achieved by reducing crude protein content of diet while ensuring the amino acid balance (Gloaguen *et al*., 2018). However, Gallo *et al*., (2018) demonstrated that rabbit's performance may be impaired when dietary crude protein level reduced from 230g/kg to 170g/kg. As such, amino acid deficiency may change the efficiency of Amino acid absorption and transportation (Deng *et al*., 2018). Nutrient transporter gene expression therefore, could be an indicator for growth and absorptive capacity of small intestine.

To meet the need of growth, the small intestine has been reported to undergo dramatic changes in morphology, digestive enzymes activities and nutrient transporters (Dong *et al*., 2019). Intestinal amino acid transporters expression is known to be induced by raised levels of dietary protein. These nutrients is expected to provide a signal for induction of transporter mRNA. This study investigated the effect of reducing dietary crude protein in diets while

supplementing with essential amino acid on the gene expression of amino acid transporters in weaned rabbits.

Materials and methods

Experimental site

The study was conducted at the Rabbitry unit of the Demonstration farm, Department of Animal Health and Production Technology, Kogi State Polytechnic, Itakpe campus which is located in the Guinea savannah of coordinates Latitude 7.638 ^{0} N and Longitude 6.348 ^{0} E. The average annual temperature of Itakpe is 26.64 ^{0}C, 1280 mm average rainfall and 76 %, relative humidity.

Experimental Diets and Management of Experimental Animals

Twenty-four, 25 days old mixed male rabbits were purchased and are randomly allocated to an individual cage for 56 days feeding trial. Experiment started at a 7 day adaptation period when the rabbits were weighed to determine an initial mean body weight (5.6± 0.64). Rabbits were allocated to four (4) different dietary groups with each treatment replicated (6 each per group). Each group were fed diets with different levels of crude protein – 20 % (T1), 18 % (T2), 16 % (T3) and 14 % (T4), respectively as shown in Table 1. The dietary treatments however, met the National Research Council (NRC) nutrient specification for 5 – 8 kg rabbit. Rabbits had free access to feed and drinking water at all times throughout the experimental period.

Table 1- Ingredients and percentage composition of experimental diets.

Ingredients	**T1**	**T2**	**T3**	**T4**
Maize	38.50	38.70	38.40	38.60
Groundnut meal	16.50	16.30	16.60	16.40
Rice offal	32.00	32.00	32.00	32.00
Soybean	5.00	5.00	5.00	5.00
Beniseed	2.00	2.00	2.00	2.00
Bone meal	2.00	2.00	2.00	2.00
Salt	0.50	0.50	0.50	0.50
Fish meal	3.00	3.00	3.00	3.00
Vitamin premix*	0.50	0.50	0.50	0.50
Total	**100.00**	**100.00**	**100.00**	**100.00**
Calculated				
Dry matter %	88.91	88.60	89.00	89.00
Crude protein %	20.00	18.00	16.00	14.00
Crude fibre %	10.05	10.00	09.89	09.76
Ether extract %	06.12	06.11	06.10	06.10
Amino acids				
Isoleucine	04.00	04.00	04.00	04.00
Threonine	05.00	05.00	05.00	05.00
Methionine	02.80	02.80	02.80	02.80
Lysine	03.60	03.60	03.60	03.60

**Vitamin premix* - *Lysine 0.25, Methionine1.00, oyster shell 0.3, L-arginine 0.11, amino acids 1.35, Xylanase 0.19.*

Sample Collection

At the termination of the feeding trial, rabbits were anesthetized with chloroform and slaughtered. 5g of Jejunum tissue samples were carefully excised and immediately frozen in liquid nitrogen and stored at - 80^0C for analysis of mRNA expression.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from 5g of Jejunum stored in liquid nitrogen using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Briefly, the samples were lysed and homogenized in Trizol reagent with the help of homogenizers. 10 mg of tissue was used per one millilitre of Trizol reagent.

After extraction with chloroform, precipitation with isopropanol and washing with 70% ethanol, the extracted RNA was then re suspended in RNase free water. The quality and integrity of RNA were evaluated by using the Nano drop 1000 spectrophotometer (Thermofisher Scientific).

Real time quantitative polymerase chain reaction (PCR) analysis were performed on an ABI – fast Real – time PCY system (Applied Biosystem USA) with a total volume of 10 containing 5 ng of cDNA, 5µl SYBR Green mix, 0.2 µl ROX reference dye (50 x), and 0.2 µl each of the forward and reverse primer (Zuo *et al*., 2018). Primers for the selected genes were designed using oligo 6.0 software (molecular biology CO, USA) and target mRNA and β-*actin* mRNA were amplified with comparable efficiencies. The gene primers y^+LAT 1, ASCT 2, CAT 1, EAAC 1, Pep T1, ATB^{01+}, B^{0+}AT, LAT- 4 and β-actin were designed as sequences from Gen Bank as follows: y^+LAT1 forward, 5'-GCTTCCGAGAGCCAAGAACT- 3' reverse, 5'-TCT TTAACGCCTGGGTCG CT-3'; EAAC 1 forward, 5'-GCTTTCTTCTCCCAGGTCCG-3' reverse, 5'-CTGGGCAATGTGCTTGCTTG -3'; B^{0+}AT, forward, 5'- GAGAGGTTTGGT CTTACTGC-3'; reverse, 5'-GCTATGACCAAGACGGAGCG-3'; LAT 4, forward 5'-TTTGT CTGACCGGCCTTTCC-3', reverse, 5'- GAGAGTCTCCTTGCCTGGTC-3'; ATB^{0+} forward, 5'-TGGTGCATTTTGATTGCCAG-3', reverse, 5'-CTCCCCAGTTCAGGTAGTGG-3'; CAT, forward, 5'- CTCGAACCCCACCAAGGAC T-3', reverse, 5'- GAGGTAGGACGGCACAGA CT-3'; β *actin* forward, 5'-TGGATGCAAGAGATCAGGCT-3', reverse, 5'- ATCCTGCTGG GAGTGGACAA-3'.

Statistical Analysis

All data were subjected to a one way analysis of variance (ANOVA, SAS Version 9.2) and were presented as means $\pm$ standard and error of the means. Differences with P- values of < 0.05 were considered to be statistically significant.

Results and Discussion.

A shown in table 2, there occur a significant change in the mRNA abundance of five amino acid transporters (ASCT2, EAAC1, B^{0+}AT, Pep T_1, ATB^{01+}) while, there was no significant difference ($P > 0.05$) in y^+LAT1, CAT 1 and LAT- 4 mRNA expression in the jejunum of rabbits. The abundance of ASCT2, EAAC1, B^{o+}AT, Pep T1, and ATB^0 mRNA expression

significantly differed among the treatment groups ($P < 0.05$) as they showed similar expression profiles. Compared with the values in the 20 % CP group, the expression of ASCT2, Pep T1, $B^{0+}AT$ and ATB^{o1+} mRNA in the jejunum increased by 9 %, 11 %, 14 % , 24 %; 37 %, 39 %, 47 %, 52 % and 16% , 23 **%,** 29 % **,** 34 %, in 18 %, 16 % and 14 %,respectively. The 14 % **C**P group recorded the highest expression of ASCT2, Pep 1, ATB^{01}amino acids exporters mRNA genes and the lowest expression of EAAC 1.

The increase in mRNA abundance of peptide transporters, identified as Pep T1 in the jejunum of rabbits on 14 % CP group when compared with other groups suggested predominantly expressed nutritional profile in the apical membrane of intestinal epithelial cells of rabbits (Chen *et al.* 2019, Daniel, 2019). The intestinal amino acid transporters might have up regulated the absorption of amino acid/ peptides across intestinal epithelial cells from the lumen into the portal blood circulation and promotes Nitrogen metabolism during both absorptive and post absorptive states (Zuo *et al*, 2018). Amino acids, in the form of peptides transported in the enterocytes of jejunum might have been more effective than free amino acid transporters. As such, jejunum plays a central role in nutrient adsorption (Chen *et al*., 2019). Its absorption capacity is therefore, evaluated by studying nutrients transporter gene expression pattern.

Table 2: Jejunal amino acid transporters mRNA abundance in weaned rabbits

Amino acid transporters	T1	T2	T3	T4	SEM	P-Value
Y^{+} LAT-1	1.00	1.24	1.48	1.40	0.13	0.034
ASCT-2	1.05^{d}	1.25^{c}	1.40^{b}	1.58^{a}	0.16*	0.026
CAT-1	1.92	1.60	1.53	1.53	0.15	0.016
EAAC-1	2.01^{a}	1.90^{b}	1.84^{c}	1.41^{d}	0.05*	0.037
Pep T-1	0.60^{d}	0.85^{c}	0.98^{b}	1.98^{a}	0.04*	0.084
ATB^{01+}	1.01^{d}	1.12^{c}	1.47^{b}	1.49^{a}	0.42*	0.014
$B^{O+}AT$	1.00^{d}	1.06^{c}	1.56^{b}	1.68^{a}	0.17*	0.016
LAT-4	1.02	1.09	1.51	1.69	0.16	0.014

y+ LAT-1: Na^{+} dependent cationic and sodium dependent neutral amino acid transporter 1, CAT-1: Na^{+} dependent cationic amino acid transporter 1, EAAC-1: Excitatory amino acid transporter subunit 1, Pep T1: Solute carrier family 15 member 1, $ATB^{O'+:}$ Na^{+} and Cl^{-} dependent neutral and cationic amino acid transporter, LAT-4: Large neutral amino acids transporter small subunit 4, $B^{O+}AT$: Na^{+} dependent neutral amino acid transporter 1.

The mRNA abundance of $B^{o+}AT$, a Na^{+} independent transporter responsive for transporting cationic and neutral amino acids into jejunum cells in exchange for intracellular neutral amino acids suggested better utilization of low protein (14 %) diets by rabbits. This corroborates the findings of Daniel (2019), who reported that the abundance of peptide and amino acid transporters in the intestine of piglets indicates efficient protein adsorption. In the present study, diets with the lowest crude protein (14 %) increased mRNA abundance of most amino acid transporter which resulted into improved and efficient absorption of peptide and amino acids. On the contrary, Gallo *et al*, (2018) observed that the expression of amino acid transporter genes tended to increase with an increase in the dietary crude protein levels. The reason for variation in observations was established by Dong *et al*, (2019) who opined that modulation of mRNA nutrient sensing genes is not a consequence of the amount of crude protein in diets alone but, the quality of protein in the diet. The similarity in mRNA

expression patterns of y LAT 1, CAT 1 and LAT - 4 indicated their importance in rapid growth as they have high affinity for glutamate and aspartate (Opapeju *et al*, 2019). Possibly, glutamate, an oxidative fuel source that is involved in variety of metabolic sequence in enterocytes might have increased the amount of energy needed to meet the growth of the rabbits and enhanced the endogenous synthesis of arginine via the intestinal-renal axis (Zhang *et al*., 2018). Furthermore, glutamate is required for the synthesis of N-acetyl glucosamine, a common substrate for the synthesis of glycoproteins that are found in the intestinal mucosa (Wu *et al*, 2019). Opapeju *et al*, (2019) also, observed that the use of low crude protein (14 %) amino acid supplemented diets in piglets may decrease the transport capacity of amino acids in the small intestine. The difference in the observed results may be due to variations in sex, breeds or species of livestock studied and differences in Geographical locations. This study used rabbits and rabbits are hindgut fermenters, capable of converting feeds in to volatile fatty acids and utilizing non protein sources. Rabbits practice coprophagy in conjunction with hind gut fermentation in the large intestine which supplies considerable amount of B-vitamins and amino acids that prevent amino acids deficiency and makes available other nutrients (Dong *et al*., 2019). Possibly, rabbits might have recycle their soft faeces to supplement the needed quantity of protein.

Skalli *et al., (2020)* suggested that dietary protein levels may regulate the expression of amino acid transporter genes through a complex regulating system which may affect the energy balance and endocrine system. Thus, amino acids types and hormones plays an important role in the regulation of genes expressions through a complex physiological process that is influenced by many factors (Zhang *et al*, 2018).

Conclusion

This study revealed that a decrease in dietary crude protein (14%) with free amino acids supplementation resulted in a significant increase in the expression of amino acid transporter genes in the jejunum of rabbits. Although, this observation contrasts several reports. However, results from this study have established that the amount of crude protein in diets alone may not determine the efficiency of the diet but the quality of the components of the diet. Further research will be needed to elucidate the underlying mechanism involve in transporter lipoproteins that are involve in nutrient absorption capacity of rabbits.

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