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ASSESSMENT OF PLASMA BIOCHEMISTRY AND INTESTINAL MICROFLORA IN TRANSYLVANIAN NAKED NECK BREED COMPARED WITH COMMERCIAL BREEDERS'

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Abstract

This study assessed to compare the plasma metabolic status and intestinal microflora of different breeders' genotypes. A total of 110 healthy female breeders (25-week-old) were divided into three groups: Transylvanians Barred Naked Neck (30 birds) and Black Naked Neck (30 birds) vs commercial Ross 308 breeders (50 birds). During a 5-weeks trial, the birds were reared on the floor system in climate-controlled conditions. They were fed a standard commercial laying breeder diet (15.26% crude protein and 11.30 MJ/kg metabolizable energy). Blood and intestinal content samples were collected at 30 weeks for analysis. Results revealed significant differences in plasma protein profile: total protein, albumin, globulin, total bilirubin increased, and uric acid decreased in the Naked Neck varieties vs Ross 308 breeds. Energy profile showed higher glucose and lower HDL-Cholesterol levels in Naked Neck varieties. Plasma mineral profile archive highest calcium and phosphorus values for Naked Neck varieties vs commercial breed, with no significant change in Ca/P ratio. There was no genotype effect on plasma enzyme activities. Cecum microflora was significantly affected by genotype, the Enterobacteriaceae (ENT) and Coliforms population count decreased, while the beneficial population Lactobacillus (LAB) spp. and LAB: ENT ratio increases in the Naked Neck breeds vs commercial breeds.

Key words: *laying breeders, intestinal microflora, Transylvanian Naked Neck, plasma biochemistry.*

INTRODUCTION

It is stated that the local breeds may not compete with the specialised lines in terms of performance indicators, resources and economic efficiency. Still, they could be evaluated as dual-purpose breeds to supply niche markets and can also be used in crossbreeding with commercial breeds (Nolte et al., 2021). The local breed such as Transylvanian Naked Neck has gained attention due to its better adaptability to climatic conditions, efficient valorisation of low feed resources, disease resistance, low mortality, and conserved gene pool (Custură, 2020). Therefore, this local breed could be used for crossbreeding in meat production in alternative rearing systems (semi-intensive or free-range).

Blood biochemical responses are important markers of clinical, physiological, nutritional and health status in chickens (Gheorghe et al., 2017; Filipović et al., 2007; Silva et al., 2007). Biochemical parameter values are affected by genotype, age, sex, season, nutrition, and physiological condition (Toghyani et al., 2010). Previous studies highlighted that the host's genetics influences the microbiota composition in poultry due to the differences between individuals (Guardia et al., 2009), lines (Meng et al., 2014; Zhao et al., 2013; Gabriel et al., 2011) and genotypes (Stanley et al., 2013; Stanley et al., 2012).

The host's genotype could affect the microbial composition directly by the secretions into the gut, modifying the gut motility and epithelial cell surfaces, or indirectly *via* the feed (Gheorghe et al., 2019; Gheorghe et al., 2017; Meng et al., 2014).

The comparison of the biochemical blood profiles and intestinal microflora of Transylvanian Naked Neck breed with different breeders' genotypes has not been reported in the scientific literature. Therefore, this study assessed to compare the plasma metabolic status and intestinal microbial populations of two

Transylvanian Naked Neck varieties with a commercial breeder fed the same standard diet.

MATERIALS AND METHODS

Birds and experimental design

The birds were treated according to the Directive 2010/63/EU for animals used for experimental and scientific purposes (OJEU, 2010). The Ethical Committee of the National Research-Development Institute for Animal Biology and Nutrition (INCDBNA-Balotesti, Romania) approved the trial protocol (no. 366/ 01/2021).

The trial was conducted on a total of 110 healthy female breeders (25-week-old) from two Romanian genotypes: Transylvanians Barred Naked Neck (30 birds; GGTB) and Black Naked Neck (30 birds; GGTN) compared with commercial Ross 308 breeders (50 birds; Ross 308).

The birds were housed during a 5-weeks trial at the INCDBNA-Balotesti research Biobase in climate-controlled conditions and kept on the floor system, fitted with manual feeders and nipple drinkers. A 14-h light photoperiod per day was provided. No veterinary treatment or vaccination protocol was applied during the study period.

During the trial, the birds were fed with a standard commercial laying breeders' diet. The feed (pelleted form) was administered daily in fixed amounts at 07:30, and water was available *ad libitum*. The nutrient composition of standard commercial diet fed to breeders in laying period is shown in Table 1.

Table 1. Chemical composition of laying breeders' diet

Nutrients	% as fed basis
Dry matter	89.29
Crude protein	15.26
Methionine + cysteine	0.64
Lysine	0.70
Crude fibre	5.30
Crude fat	4.09
Ash	9.91
Calcium	2.80
Phosphorus total	0.70
Metabolisable energy (MJ/kg)	11.30

Blood and intestinal content sampling

After a 5-week trial period, a total of 18 birds (6 female/group) were randomly selected for blood and intestinal contents collection. Blood (6 mL/bird) was taken from the wing vein in heparinised tubes in the morning. The blood were centrifuged (3000 rpm for 15 min) at 4ºC, and plasma was stored in 1.5 ml tubes at −20ºC for analysis. Birds were slaughtered by cervical dislocation and dissected. The intestinal content (small intestine and cecum) was sampled in sterile tubes for microbial determinations.

Plasma biochemistry analysis

The plasma profiles protein (total protein, TP; albumin, Alb; total bilirubin, TBil; plasma urea nitrogen, PUN; creatinine, Cre; uric acid, UA), energy (glucose, Glu; C, cholesterol total; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides), mineral (Mg, magnesium; Ca, calcium; IP, inorganic phosphorus), and enzyme (ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase) were assessed by dry chemistry Spotchem EZ SP-4430 analyser and specific reagent strips (Spotchem, Arkray Inc., Japan). Globulins values were calculated as a difference between plasma TP and Alb concentrations; the Alb/Glb ratio, PUN/Cre ratio, and Ca/IP ratio were also calculated. Plasma biochemistry parameters were assessed in duplicate per sample.

Intestinal pH and microbial determinations

The pH of the small intestine and cecum contents was assessed from fresh samples using a portable pH meter (WTW 3310, Germany).

The microbial determinations were done by conventional microbiological techniques using selective agar media. Briefly, one gram of sample was diluted 1:10 in 7 mL Brain Heart Infusion broth (Oxoid Ltd., England) plus 2 mL glycerol and homogenised. Then, 1 mL from dilutions were cultured on selective agar media or determinations of *Enterobacteriaceae* (VRBG agar; Oxoid, CM1082, England), *Enterococcus* spp. (Slanetz-Bartley agar; Oxoid CM0377, England), *Clostridium* spp. (Reinforced Clostridial agar; Oxoid CM0151, England), Coliforms (MacConkey agar; Oxoid CM0007, England), *Staphylococcus* spp. (Mannitol Salt agar, Oxoid CM0085, England), *Salmonella* spp. (Salmonella–Shigella agar; Oxoid CM0099, England), and *Lactobacillus* spp. (MRS agar; Oxoid CM0361, England). The intestinal microbial colonies were counted in duplicate per sample and expressed as log_{10} cfu (colony-forming units)/g of intestinal content.

Statistical analysis

Data were analysed by one-way ANOVA to test the genotype effect (two Romanian Naked Neck varieties *vs* Ross 308 commercial broiler breeders) using the GLM procedure (SPSS v.20, 2011). Each bird sample was considered as the experimental unit for plasma biochemistry and intestinal determinations. The data are given as means and standard error of the mean (SEM). The statistical differences were discussed at $P<0.05$.

RESULTS AND DISCUSSIONS

Plasma biochemistry

The effect of genotype on female breeders' plasma protein and energy profiles is shown in Table 2. Regarding the plasma protein, our study results revealed that the GGTB variety had the highest levels of TP (P=0.004) and Glb (P=0.002), followed by GGTN compared to the Ross 308 commercial breeds. A significant increase in the Alb (P=0.022) and TBil (P=0.031) values were shown on the two varieties of Naked Neck breeds (GGTB and GGTN) compared to the commercial breeds. The lowest UA concentration (P=0.001) was found on the Naked Neck vs commercial breeders. There were no significant changes in the Alb/Glb ratio, PUN, Cre and PUN/Cre ratio as effect on genotype (P>0.05).

The blood TP and Alb values are indicators of dietary protein utilization (Pavlík et al., 2007) and the haemoconcentration level (Kraus et al., 2021). A higher value of TP reflects a better health condition of chickens (Marono et al., 2017), and in layers, this may be due to an estrogenic-induced increase in globulin. The proteins are egg yolk precursors (vitellogenin and lipoproteins), synthesized in the liver, transportted to the ovary *via* plasma, and integrated into the egg cell (Ritchie et al., 1994). Creatinine (Cre), a by-product of phosphorcreatine degradation in skeletal muscles, reflects protein metabolism (Piotrowska et al., 2011). The Cre level is linked with muscle mass and changes by age and physical activities (Szabo et al., 2005).

Comparing the blood parameters of Ross 308 broilers with native Venda chickens, Mabelebele et al. (2017) reported higher TP values in indigenous breed compared to Ross 308 broilers at 42 days and no significant differences at 90 days. These authors also indicated that plasma Cre levels were lower in the indigenous breed than Ross 308 broilers. Other studies (Rehman et al., 2017; Dutta et al., 2013; Peters et al., 2011) reported variations in plasma Cre levels as genetic effects.

Kraus et al. (2021) studied the effects of genotype, housing system, and age on the blood parameters and egg quality of Czech and Slovak native hens and found no significant effect of breed on TP and Alb concentrations at 34, 42, and 50 weeks old.

The UA is the main product of nitrogen catabolism (Lumeij, 1997) and the PUN reflect the ongoing protein metabolism (Kim et al., 2012). Higher PUN concentration and lower UA levels reflect an improvement of protein catabolism (Tao et al., 2021). Similarly, several studies have been reported that the UA level varies as the effect of breeds in poultry (Eleroglu et al., 2015; Isidahomen et al., 2011; Silva et al., 2007) and UA level is higher in female birds due to ovulatory activities (Ibrahim et al., 2012).

The PUN/Cre ratio, as an important marker of renal function, obtained in our study range between normal interval (10-20 mg/dL; Washington & Van Hoosier, 2012) that indicate a good health state.

The results of the plasma energy profile (Table 2) shown that the two varieties of Naked Neck breeds (GGTB and GGTN) had higher values of Glu (P=0.027) and lower levels of HDL-C (P=0.011) than those of the Ross 308 commercial breed. The C level was slightly increased in Ross 308 hens, but no significant genotype effect (P>0.05) was found for C and TG levels.

It is known that glucose (Glu) is the primary metabolite of energy metabolism (Gallenberger et al., 2012). Cholesterol (C) can be synthesised from dietary fats and endogenously within the cells, and increased cholesterol level is a marker of a high risk of cardiovascular disease. Several factors can influence the blood cholesterol level, such as breed, sex, age, and diet composition (Toghyani et al., 2010). Our results partially agree with Abdi-Hachesoo et al. (2011), who reported lower Glu and C concentrations on Iranian indigenous hens compared to Ross 308 hens. The lower C concentration in indigenous hens may be attributed to a higher body activity and higher energy need (Abdi-Hachesoo et al., 2011; Simaraks et al., 2004). Triglycerides (TG) are synthesised in the liver from fatty acids (FAs), proteins, and glucose when the body's requirements are exceeded, and they are accumulated in fatty tissue. At the beginning of laying period, the plasma lipids such as free FAs, TG, and phospholipids increased for the yolk synthesis in oocytes (Moon, 2018). Although the TG plasma level was slightly higher in the two Naked Neck breeds varieties compared to Ross 308 the differences were not significant (P>0.05).

Table 2. Effect of genotype on plasma protein and energy profiles of female breeders

Variables	Genotype			SEM	*P*-value
	Ross 308	GGTB	GGTN		
Protein profile					
TP (g/dL)	4.10^{bc}	4.94^{abc}	4.25^{b}	0.14	*0.004*
Alb (g/dL)	2.10^{b}	2.54^{a}	2.20^{a}	0.08	*0.022*
Glb (g/dL)	2.00^{bc}	2.40^{abc}	2.05^{b}	0.05	*0.002*
Alb/Glb ratio	1.05	1.06	1.07	0.03	*0.876*
TBil (mg/dL)	0.20^{b}	0.32^{a}	0.30^{a}	0.02	*0.031*
PUN (mg/dL)	2.10	2.50	2.20	0.10	*0.071*
Cre (mg/dL)	0.17	0.14	0.13	0.01	*0.481*
PUN/Cre ratio	12.35	17.85	16.92	0.94	*0.984*
UA (mg/dL)	8.14^{a}	6.06^{b}	4.93^{b}	0.43	*0.001*
Energy profile					
Glu (mg/dL)	205^{b}	228^{a}	240^{a}	5.48	*0.027*
C (mg/dL)	112	96.80	91.60	4.67	*0.190*
HDL-C (mg/dL)	57.60^{a}	49.50^{b}	46.72^{b}	1.66	*0.011*
TG (mg/dL)	411	500	509	24.20	*0.183*

GGTB, Transylvanian Barred Naked Neck; GGTN, Transylvanian Black Naked Neck, SEM, standard error of the mean.
TP, total protein; Alb, albumin; Glb, globulin; TBil, total bilirubin; PUN, plasma urea nitrogen; Cre, creatinine; UA, uric acid; Glu, glucose; C, cholesterol total; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides.
^{abc}Means within the row with different superscripts differ significantly (P<0.05).

Regarding the plasma mineral profile (Table 3), the data show that the varieties of Naked Neck breeds (GGTB and GGTN) archive the highest values of Ca (P=0.003) and IP (P=0.028) compared to the values of the Ross 308 commercial breed. The plasma Mg concentration and Ca/IP ratio were not significantly influenced by genotype (P>0.05). Calcium and phosphorus are macro minerals needed for eggshell development in laying breeders. Feeds need to supply adequate quantities of calcium, phosphorus, and their optimum ratio due to the necessity of these minerals for eggshell synthesis and bone turnover and maintaining homeostasis in the body and circulating blood supply (Magnuson, 2015).

Abdi-Hachesoo et al. (2011) found comparable serum Ca values for Ross 308 broiler chickens and native Iranian chickens. Similar to our results, Mabelebele et al. (2017) reported a higher value of serum Ca for indigenous Venda chickens than Ross 308 at 90 days old and a similar value of serum P between these breeds. Suchý et al. (2004) reported a decrease plasma Ca level in meat-type hens compared with egg-type hens and no significant difference in P plasma levels between production types. Decreases in plasma Ca concentration had no significant influence on eggshell quality (Hester et al., 1980). Other studies noticed no link between plasma P concentration and eggshell weight (Pavlík et al., 2009; Boorman & Gunaratne, 2001).

The plasma metabolic enzyme profile of female breeders as the effect of genotype (Table 3) reveals no significant changes in enzyme activities of AST, LDH, ALT, CK, GGT, and ALP in Naked Neck breeds compared with commercial breeder.

The enzymes ALT, ALP, AST and GGT are essential indicators of liver function (Ambrosy et al., 2015). These enzymes may increase due to damaged or diseased cells, indicating the hepatic status. It was noticed that AST and ALT increased in the hyperthyroid subjects and that ALP activity is an indicator of the hen's productivity (Malik and Hodgson, 2002).

Increased plasma activity of the intracellular muscle enzyme CK is linked to an overt muscle injury as response to different pathologies and exposure to environmental stressors (Melesse et al., 2011). Additionally, plasma CK activity rises with age, and it's been suggested that genetic selection for fast- growing broilers causes changes in membrane stability and promotes intracellular enzyme export (Sandercock et al., 2009). Hocking et al. (1998) reported that feed restriction lowers the plasma CK activity in female turkeys. These authors noticed also that changes in plasma CK concentration are correlated with ovarian activity and linked with plasma TG levels.

Table 3. Effect of genotype on plasma mineral and enzyme profiles of female breeders

Variables	Genotype			SEM	*P*-value
	Ross 308	GGTB	GGTN		
Mineral profile					
Mg (mg/dL)	2.14	2.30	2.40	0.06	*0.219*
Ca (mg/dL)	15.28^{b}	17.52^{a}	17.63^{a}	0.43	*0.003*
IP (mg/dL)	2.88^{b}	3.32^{b}	3.80^{a}	0.26	*0.028*
Ca/IP ratio	5.31	5.28	4.64	0.44	*0.170*
Enzyme profile					
AST (UI/L)	256.5	225.8	227.6	21.71	*0.091*
ALT (UI/L)	17.2	15.8	14.4	0.93	*0.497*
LDH (UI/L)	302.4	209.2	301	32.21	*0.104*
CK (UI/L)	1004	825	989	89.84	*0.363*
GGT (UI/L)	45.6	37.4	41.25	1.55	*0.078*
ALP (UI/L)	742	842	672	95.83	*0.327*

GGTB, Transylvanian Barred Naked Neck; GGTN, Transylvanian Black Naked Neck; SEM, standard error of the mean.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; CK, creatine kinase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; Mg, magnesium; Ca, calcium; IP, inorganic phosphorus.
^{ab}Means within the row with different superscripts differ significantly (P<0.05).

Intestinal microflora

As shown in Table 4, the studied genotypes had similar pH values (P>0.05) of the small intestine and cecum. The intestinal pH is one of the major's factors that influence the nutrient metabolism and health status of chickens (Recoules et al., 2017).

The previous study of Mabelebele et al. (2014) compared the gastrointestinal tract (GIT) parameters of Ross 308 broilers with native Venda chickens and reported that native breeds had lower pH value in the small intestine and a similar pH value in the cecum.

The major GIT function is the feed digestion and nutrients absorption of dietary origin in the small intestine or produced by microbial fermentation in the cecum (Apajalahti & Vienola, 2016; Scott et al., 2010). It was stated that intestinal microbiota has a major role in chickens' growth, production, reproduction, welfare, and health status (Ji et al., 2020; Yang et al., 2020). Our results showed that the small intestine microbial populations were not significantly influenced by the genotype (P>0.05), whereas significant changes in the cecum microflora were noticed as an effect of genotype (Table 4). The *Enterobacteriaceae* (P=0.044) and *Coliforms* population counts (P=0.002) decreased, while the *Lactobacillus* spp. (P=0.032) and LAB: ENT ratio (P=0.048) were increased in the naked neck breeds compared to commercial breeds.

Our findings are similar to previous studies (Stanley et al., 2012; Torok et al., 2012; Gabriel et al., 2011) reported that the differences in the microbial composition were higher in cecum content than other intestinal segments efficient and non-efficient chickens. Bikker et al. (2006) stated that the higher *Lactobacilli* counts could lower the Coliform counts, which could be attributed to the microbial colonisation resistance in the intestinal tract. Our results are in line with Bikker et al. (2006), who reported that lactobacilli suppress the activities of pathogenic bacteria (e.g., *Enterobacteriaceae* and Coliforms), improving the intestinal tract environment. It has been shown that *Lactobacillus* and *Lactococcus* spp. have biotechnological value in fermentation and bacteriocin production, with beneficial effects on the host and the reproductive performance (Yang et al., 2020). On the other hand, several studies that compared the composition of faecal microbiota in two lines selected for high or low body weight reported differences in microbiota among the two lines, and also have shown that some microbial species have shown heritability (Meng et al. 2014; Zhao et al. 2013).

Table 4. Effect of genotype on intestinal pH and microbial populations (log_{10} cfu/g) of female breeders

Variables	Genotype			SEM	*P*-value
	Ross 308	GGTB	GGTN		
Small intestine					
pH	6.78	6.65	6.51	0.06	*0.110*
Enterobacteriaceae (ENT)	5.26	4.12	3.63	1.39	*0.288*
Enterococcus spp.	6.05	5.60	5.36	0.62	*0.444*
Clostridium spp.	4.38	3.87	4.38	2.69	*0.973*
Coliforms	3.24	3.10	2.54	2.40	*0.948*
Staphylococcus spp.	3.17	2.77	2.73	2.45	*0.851*
Lactobacillus spp. (LAB)	6.50	6.20	6.15	1.18	*0.431*
LAB: ENT ratio	1.24	1.50	1.69	0.14	*0.504*
Salmonella spp.	absent	absent	absent	-	-
Cecum					
pH	6.40	6.33	6.20	0.04	*0.207*
Enterobacteriaceae (ENT)	6.39^a	5.26^b	4.70^b	0.36	*0.044*
Enterococcus spp.	6.65	6.15	6.24	0.15	*0.277*
Clostridium spp.	6.32	5.80	6.65	0.33	*0.152*
Coliforms	6.25^a	6.10^a	5.80^b	0.18	*0.002*
Staphylococcus spp.	5.50	5.52	6.04	0.22	*0.632*
Lactobacillus spp. (LAB)	8.80^b	9.25^a	9.38^a	0.06	*0.032*
LAB: ENT ratio	1.37^b	1.76^a	1.99^a	0.12	*0.048*
Salmonella spp.	absent	absent	absent	-	-

GGTB, Transylvanian Barred Naked Neck; GGTN, Transylvanian Black Naked Neck; SEM, standard error of the mean.
[ab]Means within the row with different superscripts differ significantly (P<0.05).

CONCLUSIONS

These results revealed significant differences in certain plasma metabolites between Transylvanian Naked Neck and Ross 308 breeders: total protein and its fraction increased, and uric acid decreased; energy profile showed higher glucose and lower HDL-cholesterol levels; mineral profile archive highest calcium and phosphorus values, with no significant change in Ca/P ratio.

Genotype significantly affect cecum microbial populations, the *Enterobacteriaceae* and *Coliforms* population count decreased, while the beneficial population *Lactobacillus* spp. and LAB: ENT ratio increased in the Transylvanian naked neck breeds compared to commercial breed.

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