Number 4
2020
Volume 53
53 (4) 161–208
ISSN 1337-9984 (Print)
ISSN 1338-0095 (Online)

Slovak Journal of Animal Science

NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

SLOVAK JOURNAL OF ANIMAL SCIENCE

Formerly
Journal of Farm Animal Science

AIMS AND SCOPE

Slovak Journal of Animal Science (ISSN 1337-9984, E-ISSN 1338-0095) is an international peer-reviewed scientific journal published by the National Agricultural and Food Centre – Research Institute for Animal Production Nitra.

SJAS publishes original scientific papers, reviews and short communications on the topics of biotechnology, husbandry and nutrition of livestock animals, quality of animal products as well as animal environment and behaviour.

Slovak Journal of Animal Science (SJAS) is published quarterly.

Online version of the journal (E-ISSN 1338-0095) is available at http://www.sjas.ojs.sk

Annual subscription rates (print journal): 65 € collecting in the editorial office directly,
75 € Slovak Republic, 85 € Czech Republic, 95 € Europe, 105 € ROW.

Slovak Journal of Animal Science is published under the authorization and direction of the National Agricultural and Food Centre - Research Institute for Animal Production Nitra, Slovak Republic.

Editorial office, orders, subscription and distribution: National Agricultural and Food Centre – Research Institute for Animal Production Nitra, Hlohovecká 2, 951 41 Lužianky, Slovak Republic
IČO: 42337402, Phone: +421 376 546 249, e-mail: sjas@nppc.sk
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Filed at the Ministry of Culture of the Slovak Republic: EV 3659/09.

Slovak Journal of Animal Science, 53, 2020 (4)

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NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

Slovak J. Anim. Sci., 53, 2020 (4): 161–167

ISSN 1337-9984
E-ISSN 1338-0095

SEASONAL VARIATIONS IN TESTICULAR MEASUREMENTS, FRESH SPERM QUALITY AND POST-THAW SPERM MOTILITY IN GURCU GOAT BUCKS

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ABSTRACT

This study was aimed to determine seasonal variations in testicular measurements and semen characteristics of four Gurcu goat bucks. Data were collected over a whole year. Body weight and morphometric testicular parameters were measured once a month. Semen samples were collected weekly through an artificial vagina, then diluted in a skim milk extender and frozen. Semen volume, sperm concentration, the total number of spermatozoa and the mass activity were evaluated in each extended semen sample. In contrast, sperm progressive motility was assessed prior to the cryopreservation process. In autumn, the testis was larger than in other seasons ($P < 0.01$). Ejaculate volume increased in winter ($P < 0.01$), while the total number of spermatozoa increased during the winter even the seasonal effect was not significant ($P = 0.24$). Post-thaw motility was highest for the semen collected in autumn. In conclusion, although seasonal variation in the characteristics of fresh semen was limited, it may be appropriate to collect semen in autumn, when sperm doses should be cryopreserved for the genetic preservation of this breed.

Key words: Gurcu goats; freezability; seasonal variation; semen characteristics; testis

INTRODUCTION

Gurcu goats are locally raised in the North-Eastern Anatolia region especially around Ardahan-Çıldır City, Turkey. These goats are critical Turkish genetic resources that are threatened by an extinction. They are a dairy-type local breed producing approximately 200-250 liters of milk for a lactation period of 150-180 days (Yalcin, 1990). Recently, the Gurcu breed has been officially recognized. However, the number of goats of this breed is in the hundreds and decreasing over time. There is currently only one herd that breeds Gurcu goats. Generally, preservation of the Gurcu goat is essential for local adaptability and genetic diversity of Turkish goats. Cryopreservation of sperm has been a preferred method for the genetic preservation of goats in Turkey (Sezgin *et al.*, 2010; Kulaksız *et al.*, 2016; Kuru *et al.*, 2017; 2018).

Because fewer males than females are kept, therefore, an individual male makes a more significant contribution to the next generation. Hence, it is crucial to assess their reproductive fitness. Characterizing both testis morphology and semen traits are essential for determining fertility rates and ensuring the continuity of breeds (Tekin, 1994). Gordon (1999) reported that goat buck semen has a relatively small amount of seminal

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Received: July 5, 2020
Accepted: September 28, 2020

fluid (0.5 – 1.5 mL), a high density of spermatozoa (~4 × 10^9 cells per mL) with a high rate of motility (70 % – 90 %). Semen quality and quantity, as indicators of reproductive efficiency, are influenced by factors such as breed, age, season, frequency of ejaculation, techniques of breeding and even by variation among individual goats within the same herd (Greyling and Grobbelaar, 1983; Webb *et al.*, 2004). Among these factors, the season has important influences on semen quality. Previous studies have focused on seasonal variation in testicular measurements and the characteristics of fresh semen of goats (Ahmad and Noakes, 1996; Kamal *et al.*, 2005; Zamiri and Heidari, 2006; Talebi *et al.*, 2009).

The varieties of goat breeds are another critical factor affecting the success of cryopreservation. For instance, semen from native or cultured goat breeds may have different sensitivity against cryopreservation (Kulaksız *et al.*, 2013). However, there are several studies that investigated the effect of a season on frozen-thawed goat semen (Tuli and Holtz, 1995; Wang *et al.*, 2015). Therefore, this study was aimed at examining the effect of a season on testicular measurements and characteristics of fresh and frozen-thawed semen of Gurcu bucks.

MATERIAL AND METHODS

Four male bucks of the Gurcu goat breed were used in this study. They were 12 months old, when the study commenced, and weighed 40 – 45 kg. The bucks were housed at the Education Research and Practice Farm, Faculty of Veterinary Medicine, Kafkas University, Turkey at 40° 34' 33" N, 43° 02' 35" E at an altitude of 1751 m. The bucks were kept under natural photoperiod. They were fed 1 kg of hay and 0.9 kg concentrate mix, containing 12 % protein per animal daily, and had free access to vitamin / mineral block and freshwater all the time. A general management schedule for de-worming, disease prevention, and hoof trimming was followed throughout.

The seasons were classified as autumn (September – October – November), winter (December – January – February), spring (March – April – May), and summer (June – July – August). These seasons were determined by considering the descriptions of the mating period that Kuru *et al.* (2017) reported in their study conducted under the natural conditions of Kars province, Turkey.

Body weight (BW) and testicular measurements (TM) were recorded once a month throughout the study. The scrotal circumference was measured with a flexible metric tape (A Neogen Company, MI, USA). Testes width, testes length and scrotal thickness were determined by using a digital caliper (Insize Co., Ltd., GA, USA). The volume of each testis was measured volumetrically using the Archimedes principles of water displacement in a measuring cylinder (Demirci, 2002). Before the start of the research, all bucks were trained for semen collection using an artificial vagina. Each time semen was collected, the same female goat (in heat or not) was used as a phantom. Semen was always collected at the same time (09:00 – 10:00) and by the same person. Semen was obtained from each buck once a week for an entire one-year period.

A total of 113 ejaculates were evaluated. The volume of ejaculated semen was recorded immediately after collection into a graduated collection vial. Sperm progressive motility was subjectively evaluated under a phase contrast-supplied microscope equipped with a heating stage at 37 °C and magnification of 400× after dilution (1:10) with skimmed milk extender. Sperm concentration was determined using a hemocytometer after diluting semen (1:400) with Hayem solution (Tekin, 1994). Before freezing, the semen was diluted with a skim milk-based egg yolk extender (Kulaksız and Daşkın, 2010). The composition of the skim milk-based solution was 10 g of skim milk powder and 0.9 g of glucose dissolved in a water to a final volume of 100 mL, to which 10 % (v/v) egg yolk, 5 % (v/v) glycerol, 500 IU of penicillin and 500 µg of streptomycin sulphate per mL were added. The extended semen samples were stored at 5 °C. Diluted semen was loaded into 0.25 mL straws (IMV. Technologies; L'Aigle, France) in a concentration of approximately 10^8 spermatozoa / straw. Plastic straws were sealed with a polyvinyl alcohol powder. The straws containing the semen were then placed into a refrigerator at 5 °C and allowed to equilibrate for 2 h before being frozen. After equilibration, the straws were frozen horizontally on a rack about 4 cm above a liquid nitrogen (LN_2) level in an insulated container. The nitrogen vapour reduced the temperature within

the straws to -120 °C in approximately 15 min. Then, the frozen straws were plunged into LN_2 and were stored for a month before thawing.

Two straws from each buck were thawed in a water bath at (37 °C) for 1 min and sperm progressive motility was subjectively examined for each frozen-thawed semen sample. Precisely, a 3-µL aliquot of each sample was placed on a warm (37 °C) slide and covered with a coverslip. Four or five different fields were recorded using a phase-contrast microscope (Nikon Eclipse E400, Nikon Corp., Japan) at 400× magnification and results were expressed as the percentage of progressive motile sperm for each sample. Throughout the experiment, two trained technicians (as a double-blind manner) evaluated all the samples and results were expressed in percentages as the mean value of their observations (Kulaksız and Daşkın, 2010).

Statistical analysis

The SPSS software program (SPSS 20.0, Chicago, IL, USA) was used to analyze the data. Distributions of the data were evaluated by the Shapiro–Wilk test. Repeated measured analysis of variance (ANOVA) was used to determine the significance of effects and the Bonferroni test was used for comparing means of live weight, scrotum and testicular measurements, as well as pre- and post-thaw semen characteristics (semen volume, mass activity, progressive sperm motility, sperm concentration, total spermatozoa and post-thaw progressive sperm motility) by seasons. If "n" was not equal in comparison variables, one-way ANOVA and Dunnett T3 (if n equals, Tukey's (honestly significant difference (HSD) test) tests were used for multiple comparisons of data. The results were presented as mean ± standard error. $P < 0.05$ was considered as statistically significant in evaluating the results.

RESULTS

The bucks ranged in weight between 47 and 55 kg. Bodyweight increased in autumn and winter seasons compared to spring and summer season ($P < 0.01$; Table 1). Except for scrotal skin thickness, the testicular characteristics also changed seasonally ($P < 0.01$). Scrotal circumference, right and left testicular length, right and left testicular width and testicular volume were generally highest in autumn and least in spring ($P < 0.01$; Table 1).

The quantity and intensity of the native spermatological characteristics of the Gurcu bucks were significantly affected by a season ($P < 0.001$) (Table 2). The ejaculate volume was higher during the winter season than in other seasons ($P < 0.001$). The effect of a season, on the concentration of spermatozoa was found to be statistically significant ($P < 0.001$). Semen with the highest spermatozoa concentration was obtained in the spring and summer seasons ($P < 0.001$). Progressive spermatozoa motility was significantly affected by a season ($P < 0.001$). The rates of progressive

Table 1. Seasonal variation in body weight and testicular measurements of Gurcu goats

Parameters	Spring	Summer	Autumn	Winter	P-value
Body weight (kg)	47.07 ± 1.66^{a}	50.27 ± 0.81^{a}	55.20 ± 0.87^{b}	54.60 ± 1.03^{b}	<0.001
Scrotal circumference (cm)	23.10 ± 0.52^{a}	25.40 ± 0.35^{b}	26.77 ± 0.25^{c}	25.73 ± 0.24^{b}	<0.001
Testes length (cm) right	9.37 ± 0.21^{a}	10.64 ± 0.25^{b}	11.68 ± 0.26^{c}	10.21 ± 0.25^{b}	<0.001
Testes length (cm) left	9.67 ± 0.20^{a}	10.97 ± 0.25^{b}	11.94 ± 0.22^{c}	10.51 ± 0.20^{b}	<0.001
Testes width (cm) right	5.02 ± 0.10^{a}	5.53 ± 0.13^{b}	6.11 ± 0.09^{c}	5.47 ± 0.09^{b}	<0.001
Testes width (cm) left	5.03 ± 0.12^{a}	5.61 ± 0.15^{b}	6.11 ± 0.10^{c}	5.45 ± 0.11^{ab}	<0.001
Testicular volume (mL)	280.33 ± 6.41^{a}	342.67 ± 8.89^{b}	380.33 ± 9.12^{c}	317.33 ± 7.64^{b}	<0.001
Scrotal skin thickness (cm)	0.66 ± 0.04	0.66 ± 0.03	0.65 ± 0.03	0.71 ± 0.03	0.269

Spring: March – April – May, Summer: June – July – August, Autumn: September – October – November, Winter: December – January – February. Different subscripts in the same row (a-c) indicate significance difference ($P < 0.001$). The Data are presented as mean ± standard error (SE).

Table 2. Seasonal variation in semen characteristics of Gurcu goats

Season	Semen volume (mL)	Mass activity	Progressive sperm motility (%)	Sperm concentration (x 10^9 mL)	Total Spermatozoa (x 10^9 mL)	Post-thaw progressive sperm motility (%)
Autumn	1.07 ± 0.07[a]	4.83 ± 0.08	78.04 ± 1.32[a]	2.50 ± 0.17[a]	2.49 ± 0.27	36.50 ± 3.05[a]
Winter	1.39 ± 0.06[b]	4.68 ± 0.08	81.70 ± 1.35[a]	2.48 ± 0.80[a]	3.34 ± 0.28	23.89 ± 3.11[b]
Spring	0.84 ± 0.06[ac]	4.83 ± 0.09	81.25 ± 1.45[a]	3.94 ± 0.22[b]	3.11 ± 0.37	20.96 ± 2.77[b]
Summer	0.66 ± 0.06[c]	4.45 ± 0.26	66.25 ± 4.00[b]	3.92 ± 0.29[b]	2.70 ± 0.37	15.45 ± 5.58[b]
P-value	< 0.001	0.176	< 0.001	< 0.001	0.244	0.021

Autumn (n = 23 for fresh semen): September – October – November, Winter (n = 41 for fresh semen): December – January – February, Spring (n = 29 for fresh semen): March – April – May, Summer (n = 20 for fresh semen): June – July – August. Different subscripts in the same row (a-c) indicate significance difference ($P < 0.05$). The Data are presented as mean ± standard error (SE).

spermatozoa motility decreased in summer but did not change dramatically in other seasons. The examination of post-thawing sperm progressive motility revealed that the best motility was reached in the autumn season ($P = 0.021$).

DISCUSSION

No information or scientific study is available about the reproductive characteristics of Gurcu goat bucks in Turkey, particularly regarding the seasonal variation in semen quality and quantity. The present study demonstrates novel and relevant data regarding the reproductive aspects of Gurcu bucks.

This study showed that the testicular measurement was significantly affected by the season and testicular measurement values (scrotum circumference, testis length, and testis width) of the Gurcu bucks were higher in autumn than in spring, summer or winter. These results are consistent with the findings of Webb *et al.* (2004), Kamal *et al.* (2005), Barkawi *et al.* (2006) and Chentouf *et al.* (2011), who reported seasonal variations in the testis measurements. Souri and Mirmahmoudi (2014) found the highest values of the Markhoz bucks' scrotum circumference (35.2 cm), testis length (14.7 cm) and testis width (6.1 cm) in autumn. These values were higher than the values in the breed used in the present study. Thus, the results obtained in the present study were lower than those shown in the literature, and the factors attributing to this fact were: the breed, age, weight, care and nutrition of the goats used in the study, the measurements periods, the person making the measurements and the measurement technique.

This study showed that the ejaculate volume was significantly affected by the season. The ejaculate volume of the Gurcu bucks was higher in winter than in spring, summer or autumn. These results were consistent with the findings of Greyling and Grobbelar (1983), Ahmad and Noakes (1996) and Zamiri and Heidari (2006), who reported monthly and seasonal variations in the amount of semen. Chentouf *et al.* (2011) reported that Morocco domestic bucks had the highest (0.92 mL) and lowest (0.44 mL) semen volumes during the summer and winter months, respectively, indicating that seasonal differences were significant. Roca *et al.* (1992) and Barkawi *et al.* (2006) reported the seasonal variation in the amounts of semen in the Murciana-Granadina and Zaraibi bucks, respectively. They have also indicated that the highest sperm count was obtained in the autumn season. In this regard, the findings of the present study were different from those of Chentouf *et al.* (2011), Roca *et al.* (1992) and Barkawi *et al.* (2006). This difference might be due to different goat breeds used, geographical location, climatic conditions and care-feeding conditions of the region where the study was conducted.

Spermatozoa motility of the Gurcu bucks was significantly influenced by seasons. Spermatozoa motility was highest in winter (81.70 %) and lowest in summer (66.25 %). Some researchers reported

that changes in semen motility were significantly affected by months and seasons (Barkawi *et al.*, 2006; Kridli *et al.*, 2007; Talebi *et al.*, 2009; Wang *et al.*, 2015), while other researchers did not observe this (Chentouf *et al.*, 2011; Dorado *et al.*, 2010). Besides, the fresh spermatozoa motility in this study was higher than the year-round motility values detected by some researchers (Kamal *et al.*, 2005; Talebi *et al.*, 2009; Chentouf *et al.*, 2011; Qureshi *et al.*, 2013; Wang *et al.*, 2015). The reasons for the differences between the motility values obtained in this study and those obtained in other published studies included the breed, age, evaluator and methods of the evaluation. However, the motility values in this study were similar to those reported by Roca *et al.* (1992), Dorada *et al.* (2010) and Farshad *et al.* (2012).

In the present study, ejaculates with the higher spermatozoa concentration were collected in the spring season. In Korean domestic bucks (Choe *et al.*, 2006) and in Saanen and Nubian bucks (Kamal *et al.* 2004), no difference was found between the seasons in terms of spermatozoa concentration. In this regard, the findings of the present study differed from those of Choe *et al.* (2006) and Kamal *et al.* (2004). Furthermore, the spermatozoa concentration of the Gurcu buck semen observed in the present study was found to be either higher (Talebi *et al.*, 2009; Souri and Mirmahmoudi, 2014; Qureshi *et al.*, 2013; Wang *et al.*, 2015), lower (Ahmad and Noakes, 1996; Karagiannidis *et al.* 2000; Barkawi *et al.*, 2006; Al-Ghalban *et al.*, 2004), or similar (Roca *et al.*, 1992; Kamal *et al.*, 2005; Kridli *et al.*, 2007; Chenteouf *et al.*, 2011) to the values reported in the literature. The reasons for the difference in the spermatozoa concentrations in the present study and other studies included the breed, age, care method, nutritional conditions, geographical location of the region where the research was conducted, climatic conditions, the evaluator and the methods used.

Studies examining the seasonal variation in freezability of the spermatozoa are limited. Tuli and Holtz (1995) found that the freezability of Boer buck semen was influenced by a season. They detected the best post-thawing spermatozoa motility and viability percentages during winter. In this regard, the present study differs from the study conducted by Tuli and Holtz (1995). Wang *et al.* (2015) have been reported that the best seasons for the freezing of Xinong Saanen buck semen were the summer and autumn seasons. The results of this study are consistent with the findings of Wang *et al.* (2015).

CONCLUSION

Although many studies have been conducted to examine the semen characteristics in different goat breeds, studies on the semen characteristics of regional / domestic genotypes are limited. This novel study determined the sperm characteristics of Gurcu goat bucks and paved the way to establish an infrastructure for future studies about artificial insemination in Gurcu goats. Although the Gurcu bucks have continued sperm production throughout the year, getting regular semen ejaculate from the some Gurcu bucks throughout the year is not possible; also the sustainability of such studies is challenging. It would be more appropriate in Gurcu bucks to carry out the sperm intake process in a short time interval during the breeding and nonbreeding seasons rather than spreading this process all year round. Although seasonal variations in fresh spermatological characteristics were limited, it may be appropriate to prefer the autumn season in this breed, when the semen usually is cryopreserved. Taking into account the native and post-thawing spermatological characteristics of the Gurcu bucks, determined in this study, the success rate in future artificial insemination studies on the Gurcu bucks might be increased.

ACKNOWLEDGMENTS

This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK, Project No: TOVAG 112 O 193).

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NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

Slovak J. Anim. Sci., 53, 2020 (4): 168–183

ISSN 1337-9984
E-ISSN 1338-0095

EFFECTS OF DIETARY INCLUSION OF *ZYMOMONAS MOBILIS* TREATED SAWDUST ON HAEMATOLOGY, SERUM BIOCHEMISTRY, CARCASS CHARACTERISTICS AND SENSORY EVALUATION OF MEAT OF BROILER CHICKENS

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ABSTRACT

This study was conducted to investigate the effects of including *Zymomonas mobilis*-treated sawdust (ZTSD) as unconventional feed in broiler chicken diets for 56 days by observing the haematology, serum metabolites, carcass characteristics and sensory evaluation. A total of 375, 1-day old broiler chicks were randomly assigned into 5 groups, each with 5 replicates of 15 birds each in a Completely Randomized Design for the study. Five diets containing untreated and treated sawdust were formulated to replace wheat offal at 0, 50 and 100 % at starter and finisher phases. At the starting phase, the broiler chickens, fed 100 % untreated sawdust-based diet (UTSD), had highest ($p < 0.05$) values for packed cell volume (PCV), red blood cell (RBC) and lymphocytes. Whereas the birds, fed 100 % *Zymomonas mobilis*-treated based diet (ZTSD), had the highest value for white blood cell (WBC). The finishing broiler chickens, fed 50 % UTSD, had the highest values for total protein (TP), albumin, globulin and uric acid, while the birds on 50 % ZTSD had the least values for TP and albumin. The birds, fed 50 % ZTSD, had highest ($p < 0.05$) value for liver, while the highest value of the whole gastrointestinal tract was observed at the group with 100 % ZTSD. The control group had the highest value for the overall acceptability of the meat. In conclusion, the inclusion of ZTSD improved PCV, Hb, RBC, TP and blood glucose. ZTSD, given at 50 %, increased the breast, thigh, drumstick and back of the broiler chickens. The application 100 % UTSD and 100 % ZTSD increased the sensory attributes of the meat.

Key words: haematology; serum biochemistry; carcass characteristics; sensory evaluation; sawdust; *Zymomonas mobilis*

INTRODUCTION

The utilization of wheat offal as a major dietary fibre source in most parts of poultry producing areas in many countries has escalated its price, thereby necessitating a search for a cheaper and locally available alternative (Lamidi *et al.*, 2008). Therefore, animal nutritionists are currently focusing on cheap but suitable alternative feedstuffs especially crop residues and industrial by-products, to sustain livestock industry (Alhassan, 1985). The evaluation of these unconventional feed resources besides other strategies would reduce pressure on the demand for conventional feed resources thereby ensuring attainment of feed security for poultry (Fajimi *et al.*, 1993).

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Received: May 5, 2020
Accepted: July 31, 2020

Sawdust is a lignocellulosic material that is burnt away annually in industrial sites resulting in pollution thereby aggravating the existing environmental hazards. Millions of tons of these lignocellulosic materials, which are wasted every year are found around industrial sites such as sugar mills and sawmills (Pigden and Bender, 1975). Although, there is scarce information concerning the utilization of sawdust by chickens (Oke and Oke, 2001). Ibrahim (2003) attempted a study on exploring the potentials of sawdust as a livestock feed and reported encouraging results. Moreover, Anigbogu *et al.* (2008) revealed that sawdust, ammoniated sawdust and biodegraded sawdust have been used in animal feeding. Gohl (1981) reported the use of sawdust in livestock feed in an attempt to solve the problem of disposing of the by-product. The major limitation in the use of sawdust as feed (which constitute about 62.10 % of crude fibre-lignocellulosic plant material) is the crystalline nature of the cellulose and recalcitrance of lignin (Chesson *et al.*, 1983).

Efforts to improve the bioconversion of cellulosic material as feed have been made during the last decades. The chemical and physical methods of treatment have improved to some extent the availability of nutrients in feeds, but are not yet acceptable to the farmers (Igba-Shan and Miller, 1983).

Moreover, microbial technology using efficient microorganisms and Innovation Solid-State Fermentation (SSF) technology (Anigbogu *et al.*, 2009) may be appropriate for the biological conversion of sawdust (lignocellulosic waste) to valuable feed, making enzymatic hydrolysis more accessible in the rumen (Lewis *et al.*, 1996). Oke and Oke (2007) reported that up to 80 $g.kg^{-1}$ sawdust from *Daniella ogea* tree can be included in broiler chicken diets without any detrimental effect on their weight gain and reduced cost of production. The authors suggested biological and chemical treatment of this lignocellulose waste in order to improve its digestibility. *Zymomonas mobilis* is a bacterium belonging to the Zymomonas genus that is known for its bio-ethanol production efficiency (Seo *et al.*, 2005, Gunasekaran and Chandra, 1999), with activities that surpass yeast in some aspects. It is generally found in African palm wine and Mexican pulque. It is a rod-shaped gram-negative bacterium. It is 2 – 6 µm long and 1 – 1.4 µm wide but can vary significantly (Yanase *et al.*, 2005; Cazetta *et al.*, 2007). Its ability to efficiently ferment carbohydrates using the Entner-Doudoroff pathway makes it an attractive option for life-enzyme for animal feed (Onyejekwe, 2010). *Zymomonas mobilis* has shorter fermentation time (300 – 400 %) than yeast with higher ethanol yield (92 – 94 % versus 88 – 90 % for yeast). It can convert sugar mixtures to ethanol with 90 – 95 % efficiency as reported in the University of Energy Efficiency and Renewable Energy report, United States of America (Ichita, 2006).

However, blood functions in the transportation of nutrients, gases and metabolic waste products around the body of livestock (Zhou *et al.*, 1999), it can be used to assess the clinical and nutritional health status of the animals (Olorode and Longe, 2000). The effects of feedstuffs on the haematological factors of the poultry birds can be used in deciding whether such feedstuffs should be included or not in poultry nutrition (Mmereole, 2008). Mitruka and Rawnsley (1977) reported that high packed cell volume and haemoglobin contents are linked with high feed conversion efficiency while a high percentage of lymphocytes is associated with the ability of the poultry birds to perform well under very stressful conditions (Mmereole, 2008). The chemistry of serum or analysis of serum metabolites such as cholesterol, urea, etc., in the blood system, is for the purposes of detecting organ diseases in domestic animals and the amount of available proteins in the diets (Iyayi and Tewe, 1998). The serum biochemical constituents are positively correlated with the quality of the diet (Brown and Clime, 1972; Adeyemi *et al.*, 2000). Kaneko (1997) reported that the serum protein profile and the absolute values of individual fractions are an excellent basis for a tentative diagnosis.

Therefore, this study was carried out to determine the effects of untreated or treated sawdust on haematology, serum metabolites, carcass characteristics and sensory evaluation of the meat of broiler chickens.

MATERIAL AND METHODS

Research Station and tested ingredients

The study was carried out at the poultry unit, Directorate of University Farms, Federal University of Agriculture, Abeokuta, Nigeria. The sawdust was

collected from different sawmills in Abeokuta, Ogun State, Nigeria, bagged and stored in pallets. The sawdust was biologically treated by the traditional setting according to Anigbogu *et al.* (2009) using 50 kg sawdust placed into fermentation vat (Volume = 200 litres) with 100 litres of water added to 5 kg of previously fermented dough containing *Zymomonas mobilis,* which acted as starter inoculums. The sample was homogeneously mixed and kept to be fermented for a period of 20 days at room temperature in the range of 23.1 °C to 24.6 °C, after which the fermented product was sun-dried, analysed and stored as life-enzyme (sawdust degraded *Z. mobilis* microbes) for the experimental study.

Management of birds and experimental diets

Three hundred and seventy-five (375) 1-day old unsexed marshal broiler chicks were obtained from Obasanjo Farms Nigeria Limited, Nigeria. They

Table 1. Percentage composition of experimental broiler chicken diets (DM- Basis)

Ingredients	Starter diets					Finisher diets				
		-*Z. mobilis*	-*Z. mobilis*	+*Z. mobilis*	*Z. mobilis*		-*Z. mobilis*	-*Z. mobilis*	+*Z. mobilis*	+*Z. mobilis*
	0 %	50 %	100 %	50 %	100 %	0 %	50 %	100 %	50 %	100 %
Maize	53.60	53.60	53.60	53.60	53.60	54.60	54.60	54.60	54.60	54.60
Soybean meal	29.50	29.50	29.50	29.50	29.50	23.50	23.50	23.50	23.50	23.50
Fish meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Groundnut cake	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Wheat offal	5.00	2.50	0.00	2.50	0.00	10.00	5.00	0.00	5.00	0.00
Sawdust	0.00	2.50	5.00	2.50	5.00	0.00	5.00	10.00	5.00	10.00
Bone meal	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Limestone	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Broiler premix[ab]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Lysine	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Methionine[c]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Toxin binder	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Salt	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
TOTAL	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Calculated analysis:										
Metabolizable energy (MJ/Kg)	12.00	11.88	11.76	11.95	11.91	11.92	11.68	11.44	11.83	11.74
Crude Protein (%)	23.38	23.01	22.64	23.14	22.89	21.68	20.94	20.20	21.19	20.70
Crude Fibre (%)	3.62	4.97	6.32	4.69	5.77	3.67	6.37	9.07	5.82	7.97
Ether Extract (%)	3.83	3.75	3.68	3.86	3.90	3.83	3.69	3.54	3.91	3.98
Ash (%)	2.60	2.82	3.03	2.65	2.70	2.26	2.68	3.10	2.36	2.46
Nitrogen Free Extract (%)	50.57	49.45	48.33	49.65	49.33	52.56	50.32	48.09	50.72	48.89
Calcium (%)	1.48	1.48	1.48	1.48	1.48	1.47	1.47	1.46	1.47	1.46
Phosphorus (%)	0.57	0.59	0.61	0.59	0.61	0.54	0.57	0.61	0.57	0.61

[a]Starter Vitamin-Mineral Premix: (Rotinol) based on 2.5 kg/ton (Thiamine, 2000 mg, riboflavin, 7000 mg, pyridoxine, 5000 mg, cyanocobalamine, 1700 mg, niacin, 30,000 mg, D-panthotenate, 10,000 mg, folic acid, 800 mg, biotin, 2000 mg, Retinyl acetate, 12,000 iu., cholecalciferol, 2,400,000 iu., tocopherol acetate, 35,000 iu., menadione, 4,000 mg, ascorbic acid, 60,000 mg, manganese, nill, iron, 70,200 mg, zinc, nill, copper, nill, cobalt, 200 mg, iodine, 400 mg, selenium, 80 mg, choline chloride, 500,000 mg.

[b]Finisher Vitamin-Mineral Premix: (Rotinol) based on 2.5 kg/ton (Thiamine, 1000 mg, riboflavin, 6000 mg, pyridoxine, 5000 mg, cyanocobalamine, 25 mg, niacin, 60,000 mg., D-panthotenate, 20,000 mg, folic acid, 200 mg, D-biotin, 8 mg, Retinyl acetate, 40 mg, cholecalciferol, 500mg, tocopherol acetate, 40,000 mg., menadione, 800 mg, ascorbic acid, 60,000 mg, manganese, nill, iron, 80,000 mg, zinc, nill, copper, nill, cobalt, 80 mg, iodine, 400 mg, selenium, 40 mg, choline chloride, 80,000 mg.

[c]Methionine Hydroxyl Analog (MHA): (Novus International Inc.St. Charles, MO), feed supplement providing 84% Methionine activity.

were weighed and randomly allotted to five dietary treatments. A total of 75 birds were used per treatment and the treatments were replicated 5 times with 15 birds each. The chicks were brooded for 2 weeks. All routine vaccinations and necessary medication were administered to the birds. Feed and water were supplied to the broiler chickens *ad libitum*. The birds were raised for eight weeks (0 – 4 weeks for the starter phase and 4 – 8 weeks for the finisher phase). The test diets were actually formulated to include untreated and *Z. mobilis* - treated sawdust at varying levels of 0, 50 and 100 % replacing wheat offal weight for weight basis.

The composition of the experimental broiler chicken diet is shown in Table 1.

Chemical analysis

The proximate composition of crude protein, crude fibre, ether extract and ash of the samples of untreated and treated sawdust was determined according to the standard procedures of AOAC (2015). The fibre fraction such as neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) was determined by the methods of Van Soest *et al.* (1991). Calcium and phosphorus of the test ingredients were determined by the methods of Grueling (1966). Gross energy of the samples was determined using a Gallenkamp Ballistic bomb calorimeter (Cam Metric Ltd., Cambridge, UK).

Data collection

Blood collection and Analysis

Blood samples were collected individually from 75 broiler chickens (3 birds per replicate) via the wing veins using sterilized syringe at the end of the starting and finishing phases of the feeding trials. About 2.5 ml of the blood sample were collected from each bird into vials containing ethylene diamine tetra-acetic acid (EDTA) as an anticoagulant for the determination of haematological parameters. However, another set was collected into heparinised tubes for serum biochemistry measurement. Haemoglobin concentration was estimated using the cyanmethaemoglobin method (Cannan, 1958), packed cell volume (PCV), red blood cell (RBC) and white blood cell (WBC) count of the blood samples were determined in Wintrobe haematocrit tube according to the method of Schalm *et al.* (1975). Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Volume (MCV) and Mean Corpuscular Haemoglobin Concentration (MCHC) were calculated as described by Jain (1986).

Carcass and organ weight determination

At the end of 8 weeks, seventy-five broiler chickens (3 birds per replicate) were selected at random and starved for about 18 hours to empty their crops for each experiment. They were slaughtered by cervical dislocation, allowed to bleed, scalded in warm water and defeathered. They were thereafter taken to the laboratory where other measurements like the dressed weight, the weight of the cut parts and organs were taken with a sensitive electronic scale within 1 hour after slaughtering. The weight of the cut-up parts and organs was expressed as a percentage of live weight according to "Modified Kosher" method as described by Abe *et al.*, (1996), while the dressing percentage was calculated as follows:

$$\text{Dressing \%} = \frac{\text{Eviscerated weight}}{\text{Liveweight}} \times \frac{100}{1}$$

Sensory evaluation

The sensory evaluation of cooked samples of broiler chicken breast minced meat from three birds per replicate was carried out by twenty panellists. Parameters that were evaluated by the panellists include colour, juiciness, flavour, tenderness and overall acceptability. Each meat sample was coded and presented one after the other to each member of the panel. Each member rinsed his or her mouth with water after assessing each meat sample to avoid carry-over effect. The panellists awarded scores using a nine (9) point hedonic scale of (i) Dislike extremely (ii) Dislike very much (iii) Dislike moderately (iv) Dislike slightly (v) Intermediate (vi) Like slightly (vii) Like moderately (viii) Like very much (ix) and Like extremely (Ogunwole *et al.*, 2013).

Experimental design and Statistical analysis

The experimental design used for this study was a completely randomized design (CRD). All data collected were subjected to one-way analysis of variance (ANOVA) as outlined by Daniel (1995) with the aid of SAS (2001) and the significant means separated by Duncan's multiple range test at 5 % level of significance (Steel and Torrie, 1980).

RESULTS

The result of the proximate composition of the untreated and treated sawdust is shown in Table 2. The biodegradation of the sawdust with *Zymomonas mobilis* led to increased ($p < 0.05$) values of crude protein, ether extract, nitrogen free extract, gross energy and metabolizable energy. There was a significant reduction ($p < 0.05$) in the values of crude fibre, fibre fraction and ash of the sawdust. The crude fibre, neutral detergent fibre, acid detergent fibre and acid detergent lignin had 21.37 %, 35.32 %, 58.92 % and 189.36 % reduction, respectively after degradation.

Table 2. Proximate analysis of untreated and treated sawdust* (SD) (DM-basis)

Components (%)	Untreated SD	Treated SD	t-test (*P*-value)
Dry matter	89.51[a]	86.50[b]	21.69 (0.0001)
Moisture	10.49[b]	13.50[a]	-21.69 (0.0001)
Crude protein	2.14[b]	7.13[a]	-35.95 (0.0001)
Crude fibre	62.48[a]	51.50[b]	79.10 (0.0001)
Ether extract	0.60[b]	5.00[a]	-31.70 (0.0001)
Nitrogen free extract	15.82[b]	20.87[a]	-36.38 (0.0001)
Ash	8.47[a]	2.00[b]	46.61 (0.0001)
Neutral detergent fibre	89.31[a]	66.00[b]	167.93 (0.0001)
Acid detergent fibre	76.76[a]	48.00[b]	207.20 (0.0001)
Acid detergent lignin	40.51[a]	14.00[b]	191.00 (0.0001)
Calcium ($g.kg^{-1}$ DM)	0.05	0.04	1.23 (0.2879)
Phosphorus ($g.kg^{-1}$ DM)	0.85	0.83	0.144 (0.8924)
**Metabolizable energy ($MJ.kg^{-1}$)	2.90[b]	5.92[a]	-21.76 (0.0001)

Means on the same row having different superscripts are significantly different ($P < 0.05$).
*Average of three determinations.
**Metabolizable energy values were calculated using the method.
37 x % CP + 81 x % EE + 35.5 x % NFE for poultry birds (Fisher and Boorman, 1986).

The haematological and serum metabolites of starting broiler chickens fed diets containing untreated and *Zymomonas mobilis*-treated sawdust are shown in Table 3. The dietary treatments influenced ($p < 0.05$) the packed cell volume (PCV), red blood cell (RBC), white blood cell (WBC), heterophil, lymphocytes, eosinophils, monocytes and basophils. The broiler chickens fed 100 % untreated sawdust-based diet (UTSD) had the highest ($p < 0.05$) values for packed cell volume, red blood cell and lymphocytes while the birds fed 100 % *Zymomonas mobilis*-treated sawdust-based diet (ZTSD) had the highest value for white blood cell and birds on 50 % ZTSD had the highest value for heterophil. However, the birds fed 100 % ZTSD had the lowest values for PCV, RBC, WBC and lymphocytes while the least value for heterophil was recorded in 100 % UTSD. The dietary treatments significantly ($p < 0.05$) influenced the total protein, albumin, globulin, glucose, cholesterol, uric acid, creatinine, AST and ALT. The birds fed 50 % ZTSD had highest ($p < 0.05$) values for total protein, albumin and globulin but the lowest values for these serum metabolites were obtained in the control group and 100 % ZTSD.

Haematological and serum metabolites of finishing broiler chickens fed sawdust-based diets are shown in Table 4. The PCV, haemoglobin, RBC and WBC were influenced ($p < 0.05$) by the experimental diets. The birds fed 50 % UTSD had the highest value for PCV while the least value was recorded in 100 % ZTSD. The highest values recorded in birds fed 50 % UTSD for haemoglobin and RBC were similar ($p > 0.05$) to those of the birds fed the control diet. The MCH, MCHC and MCV were affected ($p < 0.05$) by the dietary treatments. The highest value for MCV recorded in birds fed 50 % ZTSD was similar ($p > 0.05$) to the values obtained for birds on the

Table 3. Haematological parameters and serum metabolites of starting broiler chickens (0 – 4 weeks) fed diets containing untreated and treated sawdust

Parameters	Dietary treatments					
	1 Control diet	2 50 % UTSD	3 100 % UTSD	4 50 % ZTSD	5 100 % ZTSD	SEM
Haematological parameters:						
Packed cell volume (%)	27.50^{c}	30.50^{b}	32.00^{a}	26.00^{d}	26.50^{cd}	0.65
Haemoglobin (g.dl^{-1})	8.90	10.00	10.50	8.50	8.70	0.31
Red blood cell (x 10^{12}.L^{-1})	2.25^{ab}	2.70^{ab}	2.88^{a}	2.15^{b}	2.50^{ab}	0.11
Mean Corpuscular Haemoglobin (pg)	40.19	37.06	37.35	39.56	35.88	1.61
Mean Corpuscular Haemoglobin in Concentration (g.dl^{-1})	32.41	32.86	32.80	32.77	32.85	0.86
Mean Corpuscular Volume (fl)	123.07	112.96	113.66	121.54	108.67	3.66
White blood cell (x 10^{9}.L^{-1})	19.50^{b}	13.90^{d}	12.05^{e}	16.20^{c}	21.15^{a}	0.91
Heterophil (%)	31.00^{cd}	33.50^{bc}	29.00^{d}	37.00^{a}	36.00^{ab}	0.86
Lymphocytes (%)	65.50^{b}	63.50^{b}	70.00^{a}	61.50^{b}	62.50^{b}	0.95
Eosinophil (%)	0.50^{b}	1.00^{a}	0.00^{b}	0.00^{b}	0.50^{b}	0.12
Monocytes (%)	1.50^{a}	2.00^{a}	0.50^{b}	0.50^{b}	0.50^{b}	0.18
Basophils (%)	1.50^{a}	0.00^{c}	0.50^{bc}	1.00^{ab}	0.50^{bc}	0.17
Serum metabolites:						
Total protein (g.dl^{-1})	3.50^{b}	4.20^{a}	3.40^{b}	4.50^{a}	2.55^{c}	0.19
Albumin (g.dl^{-1})	1.30^{b}	2.10^{a}	2.00^{a}	2.20^{a}	1.35^{b}	0.11
Globulin (g.dl^{-1})	2.20^{a}	2.10^{a}	1.40^{b}	2.30^{a}	1.20^{b}	0.13
Glucose (mg.dl^{-1})	118.50^{d}	112.00^{e}	127.50^{a}	125.50^{b}	123.50^{c}	1.49
Cholesterol (mg.dl^{-1})	90.00^{c}	88.50^{c}	97.00^{b}	103.00^{a}	90.50^{c}	1.48
Uric Acid (mg.dl^{-1})	4.20^{c}	5.75^{a}	4. 20^{c}	5.35^{b}	3.95^{d}	0.19
Creatinine (mg.dl^{-1})	0.65^{b}	0.50^{c}	0.75^{b}	0.70^{b}	0.95^{a}	0.04
Aspartate Amino-Transferase (µKat.L^{-1})	1.11	0.80	0.92	0.87	0.80	0.05
Alanine Amino-Transferase (µKat.L^{-1})	0.36	0.35	0.45	0.42	0.37	0.04

Means on the same row having different superscripts are significantly different ($P < 0.05$); SEM: Standard Error of Mean; n = 5.

control diet and 100 % ZTSD while the least value was recorded in broilers fed 50 % UTSD. The birds fed the control diet had the highest value for WBC, however, the least value was observed in birds fed 50 % ZTSD. The highest value for heterophil was recorded in 50 % UTSD with a similar value in 100 % UTSD while the lowest value obtained in 50 % ZTSD was similar ($p > 0.05$) to the value in control group and 100 % ZTSD. The finishing broiler chickens fed 50 % UTSD had the highest values ($p < 0.05$) for total protein, albumin, globulin and uric acid while birds on 50 % ZTSD had the least values for total protein and albumin. The AST and ALT were significantly ($p < 0.05$) affected by the dietary treatments. The highest value of AST was obtained in 100 % ZTSD while the lowest value was recorded in the control diet. Moreover, the birds fed 50 % UTSD had the highest ($p < 0.05$) value for ALT while the lowest value was recorded in 100 % UTSD.

The carcass characteristics of broiler chickens fed sawdust-based diets are shown in Table 5.

The dietary treatments influenced ($p < 0.05$) the dressed weight and eviscerated weight. The broiler chickens fed 50 % ZTSD had the highest value while the least value was observed in birds fed 100 % UTSD. The birds on other diets had similar ($p > 0.05$) values with the control group. The birds fed 50 % UTSD had highest ($p < 0.05$) value of eviscerated weight which had similar ($p > 0.05$) values with birds on the control diet and 100 % ZTSD. The highest value ($p < 0.05$) of breast recorded in the birds fed control diet was similar ($p > 0.05$) to birds fed 100 % UTSD while the lowest value was observed in birds fed 50 % UTSD. The highest

Table 4. Haematological parameters and serum metabolites of finishing broiler chickens (4 – 8 weeks) fed diets containing untreated and treated sawdust

Parameters	Dietary treatments					
	1 Control diet	2 50 % UTSD	3 100 % UTSD	4 50 % ZTSD	5 100 % ZTSD	SEM
Haematological parameters:						
Packed cell volume (%)	39.00^{b}	53.00^{a}	37.00^{b}	34.00^{c}	28.00^{d}	2.23
Haemoglobin (g.dl^{-1})	12.50^{a}	11.90^{a}	12.40^{a}	11.00^{b}	9.50^{c}	0.31
Red blood cell (x 10^{12}.L^{-1})	3.30^{ab}	3.50^{a}	3.00^{bc}	2.60^{cd}	2.30^{d}	0.13
Mean Corpuscular Haemoglobin (pg)	37.91^{ab}	34.01^{b}	42.14^{a}	42.30^{a}	41.29^{a}	1.12
Mean Corpuscular Haemoglobin in Concentration (g.dl^{-1})	32.09^{a}	22.47^{b}	33.57^{a}	32.40^{a}	33.96^{a}	1.22
Mean Corpuscular Volume (fl)	31.25^{b}	44.53^{a}	29.83^{b}	30.98^{b}	29.55^{b}	1.58
White blood cell (x 10^{9}.L^{-1})	23.10^{a}	17.00^{c}	15.60^{d}	12.90^{e}	18.20^{b}	0.91
Heterophil (%)	31.00^{bc}	35.00^{a}	32.00^{ab}	28.00^{c}	31.00^{bc}	0.70
Lymphocytes (%)	65.00^{b}	60.00^{c}	67.00^{ab}	70.00^{a}	68.00^{ab}	1.05
Eosinophil (%)	0.00^{b}	1.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.12
Monocytes (%)	3.00^{a}	2.00^{ab}	1.00^{bc}	1.00^{bc}	0.00^{c}	0.30
Basophils (%)	1.00^{ab}	2.00^{a}	0.00^{b}	1.00^{ab}	1.00^{ab}	0.21
Serum metabolites:						
Total protein (g.dl^{-1})	3.10^{c}	6.30^{a}	4.60^{b}	2.10^{e}	2.70^{d}	0.41
Albumin (g.dl^{-1})	1.80^{b}	2.90^{a}	1.50^{bc}	1.00^{c}	1.80^{b}	0.18
Globulin (g.dl^{-1})	1.30^{c}	3.40^{a}	3.10^{b}	1.10^{d}	0.90^{e}	0.29
Glucose (mg.dl^{-1})	120.00^{c}	122.00^{b}	112.00^{e}	115.00^{d}	133.00^{a}	1.94
Cholesterol (mg.dl^{-1})	87.00^{b}	76.00^{e}	84.00^{c}	82.00^{d}	90.00^{a}	1.29
Uric Acid (mg.dl^{-1})	3.20^{b}	5.00^{a}	4.80^{a}	3.00^{b}	2.80^{b}	0.28
Creatinine (mg.dl^{-1})	0.10^{c}	0.40^{ab}	0.40^{ab}	0.30^{b}	0.50^{a}	0.04
Aspartate Amino-Transferase (µKat.L^{-1})	0.77	0.85	0.83	0.82	1.07	0.05
Alanine Amino-Transferase (µKat.L^{-1})	0.41	0.48	0.24	0.32	0.39	0.04

Means on the same row having different superscripts are significantly different ($P < 0.05$); SEM: Standard Error of Mean; n = 5.

value of thigh recorded in the birds fed control diet were similar ($p > 0.05$) to the values of birds fed 50 % UTSD, 100 % UTSD and 50 % ZTSD except in birds fed 100 % ZTSD which had the least value. The back had similar ($p > 0.05$) values across the dietary treatments except in 100 % ZTSD which had the least value. The organ weight was affected ($p < 0.05$) by the dietary treatments. The highest value of the whole gastrointestinal tract was observed in 100 % ZTSD while the lowest value was recorded in 50 % UTSD.

The sensory evaluation of meats from broiler chickens fed sawdust-based diets is shown in Table 6. The dietary treatments significantly ($p < 0.05$) influenced the colour, juiciness, flavour, tenderness and overall acceptability. The highest value recorded in the control diet for colour was statistically ($p > 0.05$) similar to the values obtained in 100 % UTSD and 50 % ZTSD but the lowest value was recorded in 50 % UTSD. The control diet had the highest value for overall acceptability which was similar ($p > 0.05$) to the values obtained in 100 % UTSD and 50 % ZTSD but the lowest value was recorded in 50 % UTSD.

DISCUSSION

The proximate composition of the experimental diets met the nutrient requirements of the starting and finishing broiler chickens in the tropics as stated by Olomu (1995).

Table 5. Carcass characteristics of broiler chickens fed diets containing untreated and treated sawdust

Parameters	Dietary treatments 1 Control diet	2 50 % UTSD	3 100 % UTSD	4 50 % ZTSD	5 100 % ZTSD	SEM
Live weight (g)	1850.00	1900.00	1900.00	1900.00	1850.00	17.75
Dressed weight (g)	1720.00^{b}	1740.00^{b}	1500.00^{c}	1850.00^{a}	1680.00^{b}	33.07
Eviscerated weight (g)	1320.00^{ab}	1360.00^{a}	1280.00^{b}	1250.00^{b}	1300.00^{ab}	12.73
Dressing percentage (%)	71.35	71.79	67.47	65.77	70.33	0.97
Cut parts (% of LW)						
Head (%)	3.24^{b}	3.16^{b}	3.16^{b}	3.16^{b}	4.32^{a}	0.13
Breast (%)	21.62^{a}	18.95^{c}	21.05^{ab}	20.00^{b}	19.46^{c}	0.30
Thigh (%)	10.81^{a}	10.53^{a}	10.53^{a}	10.53^{a}	9.73^{b}	0.10
Drumstick (%)	10.81	10.53	10.53	10.53	11.89	0.24
Wing (%)	8.65^{c}	9.47^{b}	8.42^{d}	9.47^{b}	16.22^{a}	0.78
Back (%)	16.22^{a}	16.84^{a}	16.84^{a}	15.79^{a}	9.73^{b}	0.74
Neck (%)	3.24^{b}	3.16^{b}	3.16^{b}	4.21^{a}	4.32^{a}	0.17
Shank (%)	5.41^{a}	5.26^{a}	4.21^{b}	5.26^{a}	5.41^{a}	0.15
Organ weight (% of LW)						
Heart (%)	0.39^{c}	0.42^{b}	0.59^{a}	0.53^{ab}	0.59^{a}	0.03
Spleen (%)	0.13^{b}	0.16^{b}	0.19^{b}	0.29^{a}	0.19^{b}	0.02
Liver (%)	1.89^{d}	2.11^{c}	2.37^{b}	2.63^{a}	2.16^{c}	0.07
Kidneys (%)	0.22^{b}	0.21^{b}	0.21^{b}	0.21^{b}	0.32^{a}	0.01
Gizzard (%)	2.16^{c}	2.11^{c}	4.21^{a}	3.16^{b}	2.16^{c}	0.22
Whole GIT (%)	12.97^{c}	11.71^{e}	13.68^{b}	12.63^{d}	14.05^{a}	0.22

Means on the same row having different superscripts are significantly different ($P < 0.05$); SEM: Standard Error of Mean; LW: Live weight; GIT: Gastro-intestinal tract; n = 5.

Table 6. Sensory evaluation of meat from broiler chickens fed diets containing untreated and treated sawdust

Parameters	Dietary treatments 1 Control diet	2 50 % UTSD	3 100 % UTSD	4 50 % ZTSD	5 100 % ZTSD	SEM
Colour	6.65^{a}	5.75^{c}	6.60^{a}	6.60^{a}	6.25^{b}	0.09
Juiciness	6.05^{b}	5.35^{c}	6.35^{a}	5.95^{b}	6.12^{ab}	0.10
Flavour	5.75^{a}	5.15^{b}	6.10^{a}	5.70^{a}	5.85^{a}	0.10
Tenderness	6.20^{c}	5.95^{d}	6.45^{b}	6.65^{a}	6.40^{b}	0.07
Overall acceptability	6.50^{a}	6.00^{b}	6.40^{a}	6.40^{a}	6.15^{b}	0.05

^{abc}Means on the same row having different superscripts are significantly different ($P < 0.05$); SEM: Standard Error of Mean; n = 5.

Proximate composition of *Zymomonas mobilis*-fermented sawdust

The crude protein of *Zymomonas mobilis*-fermented sawdust of 7.13 % was greater than the value of 5.56 % reported by Anigbogu and Anosike (2010) for *Zymomonas mobilis*-degraded sawdust but lower than the value reported for wheat offal by Aduku (1993). The crude fibre of 51.50 % obtained in the study was lower than the value (58.85 %) reported by Anigbogu and Anosike (2010). However, the value of ether extract of 5.00 % was greater than 0.16 % reported by the same authors. The difference might

be due to the species of trees, length of storage of the timber, sawdust at the sawmills and the milling pattern (Oke and Oke, 2007). There was an improvement in the gross energy of the fermented sawdust. This might be due to the hydrolysis of the crude fibre into disaccharides and monosaccharide which resulted in the availability and utilization of liberated energy (Faniyi, 2006; Adedire *et al.*, 2012).

Haematological and serum metabolites of starting broiler chickens

At the starter phase, the dietary treatment influenced the haematological parameters with the exception of haemoglobin, mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC). The blood variables most often influenced by dietary treatments were identified as red blood cell (RBC), packed cell volume (PCV), plasma protein, glucose and clotting time (Aletor, 1989; Aletor and Egberongbe, 1992). The PCV, haemoglobin (Hb) and RBC values were within the normal ranges (22.00 – 35.00 %), (7.00 – 13.00 g.dl^{-1}) and (1.58 – 3.28 x 10^{12} L) reported by Jain (1986) and Bounous *et al.* (2000). High PCV, Hb and RBC improved oxygen-carrying capacity of the cells, which result in better availability of nutrients (Oleforuh-Okoleh *et al.*, 2015). Although there were no differences among dietary treatments for MCH, MCV and MCHC, their values were within the normal ranges (33.00 – 47.00 pg), (26.00 – 35.00 g.dl^{-1}) and (90.00 – 140.00 fl) cited by Jain (1993) and Benerjee (2004). This may indicate similar haemoglobin content. This observation agreed with the findings of Fasuyi and Aletor (2005). They reported no significant differences in MCH, MCV and Hb when cassava leaf protein concentrate replaced fish meal in broiler diets. The white blood cell values were within the normal values for broiler chickens (1.20 – 3.00 x 104 µl) reported by Jain (1993). The WBC, heterophil, eosinophil, basophils, monocytes and lymphocyte indicate the immunity potential of the chickens. The sawdust-based diets influenced the serum metabolites of the broiler chickens. The values of total protein were within the normal range for *Gallus gallus* species that is 2.5 to 4.5 g.dl^{-1} as cited by Thrall (2007). The values of albumin obtained in the control group and 100 % ZTSD are similar but are lower than the normal range (2.00 – 3.50 g.dl^{-1}) reported by Anon (1980) and Jain (1986). The birds on 100 % UTSD and 50 % ZTSD had values of globulin which were lower than the normal range (2.00 – 3.50 g.dl^{-1}) reported by Marieb and Hoehn (2007). However, the values were within the normal range (0.5 to 1.8 g.dl^{-1}) as cited by Thrall (2007). The total protein, albumin and globulin values obtained in the study attest to the nutritional adequacy of untreated and treated sawdust in replacing wheat offal in meeting the protein needs of the broiler chickens.

Moreover, the values of glucose (112.00 – – 127.50 mg.dl^{-1}) obtained in this study were lower than the normal range (200 – 500 mg.dl^{-1}) reported by Café *et al.* (2012). The values of cholesterol (88.50 – 103.00 mg.dl^{-1}) observed in the study were within the values (58.00 – 128.00 mg.dl^{-1}) reported by Zomrawi *et al.* (2012) for broiler chickens fed ginger root powder at levels 0.5, 1 and 1.5 % respectively. The birds fed 100 % ZTSD had the lowest value of uric acid compared to other diets. Babatunde and Pond (1987) observed that blood urea concentration is inversely related to protein quality, therefore, the lowest value of total protein observed in 100 % ZTSD may be due to the inferior protein quality and/or nutrition of the sawdust.

Haematological and serum metabolites of finishing broiler chickens

The dietary treatments influenced the haematological parameters of the finishing broiler chickens. The birds fed the control diets, 50 % UTSD and 100 % UTSD had higher values of PCV compared with the normal range (22.00 – 35.00 %) by Bounous *et al.* (2000) while the values of PCV of birds on 50 % ZTSD and 100 % ZTSD were within the normal range. This is an indication that the fibrous feedstuffs ensure the good health status of the birds because low PCV values indicate anaemia. The haemoglobin values were within the normal range (7.00 – 13.00 g.dl^{-1}) reported by Jain (1993). This might indicate that the replacement of wheat offal with untreated and treated sawdust in the broiler chicken diets was nutritionally adequate in providing a sound plane of nutrition. Lindsay (1977) reported that haemoglobin concentration decreased in livestock on low protein intake, parasite infection or liver damage. However, the PCV and Hb are correlated with the nutritional

status of the livestock which directly relates to the nutritional balance of the diet fed to the livestock (Church *et al.* 1984, Babatunde *et al.* 1987). This further indicated that all the broiler chickens had higher tendency to resist respiratory stress because Hb which is carried by the RBC is the oxygen-carrying pigment as earlier reported by Muhammad and Oloyede (2009). The red blood cell values obtained in the study were within the normal range (2.54 – 3.30 x 10^6 mm^{-3}) reported by Aletor and Egberongbe (1992). It had been reported by Ugwuene (2011) that reduced RBC indicates a reduction in the level of oxygen that would be carried to the tissues as well as the level of carbon dioxide returned to the lungs. The values of MCH observed in the study were within the normal range (33.00 – 47.00 pg) reported by Jain (1993). The values of MCHC in birds fed 50 % UTSD were lower than the normal range (26.00 – 35.00 $g.dl^{-1}$) for broiler chickens (Jain 1993). However, all the values obtained for MCV were lower than the normal range (90.00 – 140.00 fl) reported by Jain (1993). MCV is an important trait which is responsible for the cell size of erythrocytes and it is an essential factor in determining the ability of poultry birds to withstand prolonged oxygen starvation (Mitruka and Rawnsley, 1977). The values of WBC were within the normal range of 9.20 – 31.00 x 10^6 mm^{-3} reported in the literature (Riddell, 2011; Mitruka and Rawnsley, 1977; Banks, 1974) for healthy Nigerian local chickens. The white blood cells play an essential role in disease resistance, especially in the production of antibodies and the process of phagocytosis. The lymphocytes were the most numerous and frequent white blood cell type followed by heterophils, eosinophils and the monocytes (Afolabi *et al.*, 2011). The same trend was observed by Bounous *et al.* (2000) and described the lymphocytes as the most numerous WBC in chickens and turkeys. However, the result of the study did not agree with their reports because the values obtained for eosinophil were lower than the values of monocytes. The lymphocytes and monocytes, which are agranulocytes of WBC, were within the normal range from 47.2 to 85.0 % and 0.06 to 5.0 % respectively for a healthy chicken (Riddell, 2011; Mitruka and Rawnsley, 1977). However, the birds on 100 % ZTSD had zero value for monocytes which was lower than the value reported in the literature. Banks (1974) reported 6 % monocytes for domestic chickens and Islam *et al.* (2004) reported 3.42 +/- 0.50 %) monocytes for local chicken of Bangladesh. Moreover, lymphocytes are involved in antibody production, as they are reactive cells in inflammation and delayed hypersensitivity (Banks, 1974). Small lymphocytes may be responsible for the development of clones of plasma cells while monocytes are phagocytic cells. The high lymphocytes and heterophil count in this study are consistent with the findings of Afolabi *et al.* (2010) who also observed high lymphocytes and heterophils in chickens. This is in contrary to the reports of Oyewale (1987) who observed higher white blood cell count and lower lymphocyte counts in Nigerian fowls. The heterophils and the eosinophils that are granulocytes of WBC were within normal range from 10 to 53.6 % and 0.00 to 15 % respectively for a healthy chicken (Riddell, 2011; Pampori and Iqbal, 2007; Mitruka and Rawnsley, 1977). The eosinophils function in phagocytosis while the basophils are responsible for the elaboration of histamines and heparin in circulating blood (Afolabi, *et al.*, 2011). Moreover, the dietary treatments influenced the serum metabolites of the finishing broiler chickens. The values of total protein obtained in birds fed 50 % ZTSD (2.10 $g.dl^{-1}$) and 100% ZTSD (2.70 $g.dl^{-1}$) were lower than the normal range (3.00 – 5.00 $g.dl^{-1}$) reported by Obikaonu *et al.* (2012) but the higher value (6.30 $g.dl^{-1}$) was recorded in 50 % UTSD was within the normal range (5.00 – 8.00 $g.dl^{-1}$) reported by Anon (1980). Reddy and Salunkhe (1984) reported decreased total protein which was attributed to inhibition of protein utilization in broiler chickens. The value of albumin (1.00 $g.dl^{-1}$) recorded in 50 % ZTSD was lower than the normal range (2.10 – 3.45 $g.dl^{-1}$) reported by American Metabolic Testing Laboratories (2001). However, the values of globulin obtained in the control group, 50 % ZTSD and 100 % ZTSD (0.90 – 1.30 $g.dl^{-1}$) were lower than the normal range (2.00 – 3.50 $g.dl^{-1}$) reported by Marieb and Hoehn (2007). Globulin carries essential metals through the bloodstream to the various parts of the body of farm animals. It helps to fight infections in the body of animals. Therefore, high globulin levels are often pronounced in birds with serious infections because of abnormally increased production of antibodies.

The values of globulin observed in the study revealed that the inclusion of treated sawdust in the broiler diet did not precipitate any severe effects on the health status of the birds. Serum urea can be used as a test of protein break down, renal function, hydration status and liver failure (Agboola *et al.,* 2013). The concentration of uric acid also depends on a diet especially those with high protein content. However, the values of uric acid in this study were lower than the normal range (7.00 – 21.00 mg.dl^{-1}) reported by American Metabolic Testing Laboratories, (2001). The values of uric acid obtained in 50 % ZTSD and 100 % ZTSD were similar to the value in the control group. This probably suggested that there were better digestion, utilization and absorption of protein from the treated sawdust used which invariably improved protein utilization. However, a high concentration of urea may be toxic to both the liver and kidney while low levels could be due to low protein intake or severe liver failure (Oyebimpe, 2012). It had been reported by Baron (1973) that increased concentration of creatinine is associated with renal impairment. The values of glucose in this study were within the normal range (65.00 – 140.00 mg.dl^{-1}) reported by American Metabolic Testing Laboratories (2001). The cholesterol values were within the values (76.30 – 115.57 mg.dl^{-1}) reported by Onyimonyi *et al.* (2012) who fed dried garlic powder to broiler chickens. However, the values were lower than (100.30 – 108.21 mg.dl^{-1}) reported by Aderemi (2004), (93.33 – 116.67 mg.dl^{-1}) by Nworgu (2004) and (143.10 – 163.00 mg.dl^{-1}) reported by Nworgu *et al.* (2007). This will restore the confidence of consumers who earlier had reduced or stopped their consumption of chicken due to cholesterol scare. Also, this will protect the consumers from the negative effect of cholesterol which includes obesity, heart attack and stroke (Onyimonyi *et al.*, 2012). Ekpenyong and Biobaku (1986) reported that the levels of SAST and SALT are normally low in blood but they become high when the plane of nutrition is low or when there is an occurrence of liver damage by toxic substances. The values of aspartate aminotransferase (AST) were comparable to the values reported by Sobayo *et al.* (2013) when they fed graded levels of Garcinia kola (Bitter kola used as phytobiotic in broiler chicken diets. Moreover, the values of alanine aminotransferase (ALT) were within the values reported by the same authors.

Carcass characteristics of broiler chickens fed diets containing untreated and treated sawdust

There was no difference in the live weight of the broiler chickens fed the experimental diets. This was in concert with the observation of Odeh *et al.* (2016) who reported no differences between treatments for the live weight. However, there were differences in dressed weight and eviscerated weight. This was contrary to the findings of Abdulraheem *et al.* (2006) who observed no statistical differences between treatment groups when rice bran was used to replace maize in broiler chicken diets. Birds on 50 % ZTSD had superior higher value of dressed weight compared with a control diet. The higher dressed carcass weight (1850.00 g) of broiler chickens fed 50 % ZTSD may be considered to be a direct consequence of the better body weight and FCR of the broiler chickens in this treatment. Although birds fed the control diet with a dressed weight of 1720.00 g and 100 % ZTSD with a value of 1680.00g did not have the highest live weight per finisher broiler chicken, they manifested remarkable dressed weight as a percentage of live weight indicating that all diets supported a proportional cumulative weight gain. However, it implies that the dressed weight of broiler chickens was not directly proportional to the weight gain or performance traits. Also, high weight gain value may not imply a concomitant increase in the dressed weight value expressed as a percentage of live weight (Fasuyi and Aletor, 2005). The dressing percentage was not influenced by dietary treatments. This may suggest that untreated and treated sawdust can be utilized to replace wheat offal in broiler diets. The values of dressing percentage in the study were lower than the values (74.15 – 86.29 %) reported by Odeh *et al.* (2016). The dietary treatments influenced the cut-up parts of the broiler chicken except for the drumstick. The birds fed 100 % ZTSD had the lowest value of thigh and back compared with birds fed other diets. It may imply that the replacement of wheat offal by treated sawdust may not fully support the growth of some body parts of the broiler chickens. This observation agrees with the reports of Fasuyi and Aletor (2005).

The birds fed 100 % UTSD had the highest value of gizzard which might be due to the extra muscular activity required to process high fibre content of the feed. The increased weight of liver of the birds fed 50 % ZTSD and 100 % UTSD might be due to the role of this organ in eliminating metabolic waste and toxin from farm animals. Also, Onyeyilli *et al.* (1998) reported that the liver was a primary organ of biotransformation in farm animals. The values of the whole gastrointestinal tract did not follow any trend, but birds fed 100 % UTSD and 100 % ZTSD had highest values while the least value was obtained in birds on 50 % UTSD. Abdelsamie *et al.* (1983) reported that higher fibre contents at similar feed intakes enhanced relative weight and length of the gastrointestinal tracts of broiler chickens. Longe and Ogedengbe (1989) reported that the gravity of feeding dietary fibre on growth response is a function of the source and concentration of the fibre source.

Sensory evaluation of meat from broiler chickens fed untreated or treated sawdust

The replacement of wheat offal with untreated and treated sawdust had an influence on the sensory attributes of the broiler chicken meat. The values obtained in 100 % UTSD, 50 % ZTSD and 100 % ZTSD were similar to the control group. This implied that the taste of panellists could not differentiate the meat samples. The sawdust-based diets improved the juiciness and tenderness of the meat. Therefore, *Z. mobilis*-treated sawdust promoted overall acceptability of meat from broiler chickens without any deleterious influence on the meat quality. Breidenstein and Carpenter (1983) reported that colour, flavour, juiciness and tenderness are the essential parameters of the eating quality of meat. Also, Pippen *et al.* (1969) reported that the components responsible for flavour are from the lean portion and dissolved in the fat during cooking. However, Awosanya *et al.* (1990) observed that the only factor which was responsible for consumers' overall acceptability of rabbit meat is the age at which the animal is slaughtered. Therefore, the younger the age of the livestock, the more acceptable is its meat. Moreover, juiciness is important in the tenderness of meat because it provides lubrication to the consumers, and enhances mouthfeel (Owens *et al.*, 2004). Tenderness had been reported as a major quality determinant and probably the most essential sensory characteristic of meat (Deatherage, 1963). Tenderness score followed a similar trend as juiciness and flavour. Ouali (1990) and Smulders *et al.* (1991) reported that meat tenderization is a multifactorial process which depends on a number of biological and environmental factors. The utilization of treated sawdust increased the degree of tenderness, as assessed by the taste of panellists. This agreed with the findings of Omojola and Adesehinwa (2007) who reported that exogenous enzyme increased the degree of tenderness of breast meat from broiler chickens. However, the result for texture, colour and overall acceptability did not agree with the reports of the same authors who observed that these parameters were not affected by the inclusion of enzyme in the broiler chicken diets.

The dietary treatments influenced the sensory parameters.

CONCLUSION

Dietary inclusion of 100 % *Zymomonas mobilis*-treated sawdust replacing wheat offal for broiler chicks is not recommended at the starter phase since it potentiates increased white blood cell counts of the chicks. This may be reflective of the imbalance defence mechanism of the starting chicks to the introduced bacteria. As a result of the established gut ecology of the older birds, *Zymomonas mobilis* treatment of the sawdust posed no negative effects on the health status of the finishing broilers. Dietary inclusion of 50 % *Zymomonas mobilis*-treated sawdust significantly improved carcass yield in terms of breast, thigh, drumstick and back weights. Hence, dietary inclusion of *Zymomonas mobilis*-treated sawdust is only recommended at the finisher phase.

ACKNOWLEDGEMENTS

We are grateful for financial support of the World Bank African Centre of Excellence in Agricultural Development and Sustainable Environment, Federal University of Agriculture, Abeokuta, Nigeria.

Professor Anigbogu, N. M., Michael Okpara University of Agriculture, Umudike, Nigeria for the provision of *Zymomonas mobilis* suspension and

technical assistance.

Mrs. Alade, Felicia Omolara, ASUU-UNAAB Cooperative Multipurpose Society Limited for financial support.

Mr. Obadina, James Folorunso, Ogun State Technical College, Abeokuta, Nigeria for technical assistance.

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NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

Slovak J. Anim. Sci., 53, 2020 (4): 184–191

ISSN 1337-9984
E-ISSN 1338-0095

EFFECT OF FIBER CONTENT ON ABSORPTION AND DISTRIBUTION OF NITROGEN IN GROWING PIGS

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ABSTRACT

The experiment was conducted to evaluate the effect of added fiber into diets on absorption and distribution of nitrogen at different levels of dietary nitrogen. Four diets were designed with a combination of two concentrations of fiber: high (4.46 %; HF) and low (3.25 %; LF) level of fiber, and two levels of dietary nitrogen: high (18.8 %; HP) and low (14.0 %; LP) dietary nitrogen. Significant effects of fiber intake on increased dry matter intake were found only in one case. The nitrogen intake was not affected by the fiber content. Changes in the proportion of excreted nitrogen in urine and faeces were proven. Changing the nitrogen content of the feed has a more significant effect ($P < 0.03$) than changing the fiber content ($P < 0.05$). Very high coefficient of determination between nitrogen absorption and nitrogen intake calculated for the diets with low fiber content was $R^2 = 0.91$ and for diets with high fiber $R^2 = 0.97$. By comparing the differences in nitrogen digestibility expressed in $g.d^{-1}$ nitrogen, significant deviation was found only in the HFHP group (+6.37 $g.d^{-1}$ = +19.9 %; $P = 0.03$) among the groups with different fiber content. In retention, we found a positive change comparing the groups LFHP vs. HFHP, +6.22 $g.d^{-1}$ = +25 % ($P = 0.05$). It is the same pair of diets in which we found also a significant difference in dry matter intake (+15 %). Absorption and retention of nitrogen, expressed as a percentage of N intakes, did not decrease in any of the experimental groups irrespective of nitrogen and fiber content in the diet. These data suggest that fiber added into a diet with higher content of CP increased nitrogen in faeces, reduced nitrogen in urine, positively affected the overall balance of N and had only weak effect on its absorption.

Key words: crude fiber; nitrogen excretion; pigs

INTRODUCTION

Fiber from various sources is a common part of monogastric animal feed. Diets with optimum amount of by-products from the food industry are an effective way to improve animal health and mitigate environmental impacts. Potential value is the opportunity to use crude fiber concentrates as 'functional' feed additives to improve young pigs' growth and welfare (Jarrett and Ashworth, 2018). Dried beet pulps, a product of processing sugar, contain crude fiber, which is useful for growing pigs. Lizardo and Aumaître (2001) introduced that the inclusion of beet pulps into a diet improved growth performance immediately after weaning and carcass composition at slaughter. Increased fiber levels promote bacteria fermentation, which is responsible for the shift of urine excretion of N in faeces (Nahm, 2003; Aarnink and Verstegen, 2007). Physical properties of fiber such as viscosity and solubility affect digestion, satiety, and transit time (Williams *et al*., 2001; Montagne *et al*., 2003). Fiber is actually a possible medium for reducing releases of nitrogen production and for improving pig intestinal health and welfare (Longland *et al*., 1994; Basset-Mens and Van der Werf, 2005). Similarly, other studies have been done to investigate the relationships between feeding and nitrogen excretion (Fernandez

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Received: August 6, 2020
Accepted: November 9, 2020

et al., 1999; Le Goff *et al*., 2003; Galassi *et al*., 2007) with particular reference to ammonia emissions.

For example, inclusion of dietary fiber can alter the gut microbiota in ways that could reduce the need for antibiotics, while controlled addition of certain fiber types may reduce nitrogen losses into the environment and so reduce the environmental cost of pig production.

The objective of this study was to verify the effect of different levels of dietary fiber on digestibility and distribution of nitrogen.

MATERIAL AND METHODS

To evaluate the effect of fiber in diets on redistribution of nitrogen eight crossbred gilts, progeny of Large White sows and Landrace boars (initial BW 29.9 ± 1.7 kg), were used. The gilts from the experimental herd of Research Institute for Animal Production Nitra were individually housed in balance cages, located in the Laboratory of digestive physiology of monogastric animals. All experimental procedures were reviewed and approved by the Animal Care Committee of the Research Institute for Animal Production Nitra. Pigs were housed individually in an environmentally controlled (21 °C) room in metabolic cages. The pigs were randomly allotted to four dietary treatments according to a 4 x 4 Latin square design.

The diet was based on wheat, corn and soybean meal. Of these, four dietary treatments were prepared and applied in the form of a Latin square with a 2 x 2 factor arrangement. The included diets were as following: Diet 1 (LFLP) with lower fiber content and low protein (CP 13.92 %) supplemented with isoleucine, lysine, methionine, threonine, tryptophan, and valine (NRC, 1998). Diet 2 (LFHP) with higher content of CP 18.38 %: the base of this diet was fortified wheat, corn, more soybean meal and crystalline amino acids (lysine and methionine); Diet 3 (HFLP, CP 14.08 %) contained similar ingredients as Diet 1 with 150 $g.kg^{-1}$ dried beet pulps. Diet 4 (HFHP, CP 19.29 %) contained similar ingredients as Diet 2 with 15 % dried beet pulps. The same energy level in the diet was reached by means of supplementation with rapeseed oil (Table 1). The pigs were fed by two equal doses at 7 a.m. and 5 p.m. for a daily rate of 90 $g.kg^{0.75}$. Water was provided *ad libitum*. Pigs were weighed at the beginning and at the end of each period.

The experimental period began after a 5-day habituation to become accustomed to the cages. The 10-day experimental period consisted of a 6-day adaptation period, within which the animals adapted to the experimental diet, and a 2 x 48 hour collection period (day 7-8 and day 9-10). During the collection period, samples of urine and faeces were collected separately. Urine was collected via catheters, without addition of sulphuric acid, and stored in ice-cooled containers. Urine pH was measured before freezing each day. 10 % aliquot was stored at -20°C. Faeces were collected, pooled, and stored at -20 °C until analysis.

Samples of diets, urine and faces were analyzed for dry matter, total N and fiber. Analyzes were performed in accordance with the standard methods of AOAC (1998). Feed and faecal samples were analyzed for dry matter (DM) after drying at 105 °C for 8 hours. Crude protein (N x 6.25) was determined by the Kjeldahl method using a Kjell-Foss 16200 auto analyzer (method 978.02). The crude fiber contents were determined using The Fibertec™ 2010 fiber analyzer Tecator, Hoganas, Sweden (method 2002.04). Chemical analyses were performed in duplicate.

The data were subjected to one-way ANOVA using Statgraphic Plus (version 3.1., Statistical Graphics Corp., Rockville, MD, USA). Differences in mean values between groups were assessed using Student's t-test of the statistical significance of the difference between the two samples. Estimation of the normal distribution of small samples and of the effect of dietary fiber and nitrogen concentration were evaluated using regression analyses.

RESULTS AND DISCUSSION

The values provided by chemical analysis of nitrogen intake and intake of pulp in the groups were affected by the methodology intent, therefore in these cases it is not possible to evaluate the differences. Inclusion of beet pulps into the diets reflected differently in all experimental groups. The dry matter intake was reduced between groups LFLP vs. HFLP - 0.09 $kg.d^{-1}$ (-7 %) and increased in LFHP vs. HFHP + 0.17 $kg.d^{-1}$ (+12 %). This significant

Table 1. Composition of basal diets and analysed content of nutrients

	Diet[a]			
Ingredient *(g.kg^{-1} diet)*	LFLP	LFHP	HFLP	HFHP
Wheat	300.00	300.00	300.00	280.00
Maize	552.00	426.00	388.00	276.00
Soybean meal	86.90	223.00	88.80	231.00
Corn starch dextrinized	10.00	10.00	10.00	10.00
Dried beet pulp	-	-	150.00	150.00
Rapeseed oil	8.30	9.60	22.60	23.90
L-isoleucine	0.90	-	1.00	-
L-lysine.HCl	5.80	1.70	5.70	1.40
DL-methionine	0.90	-	1.20	-
L-threonine	2.20	0.30	2.40	0.40
L-tryptofan	0.40	-	0.50	-
L-valine	0.90	-	1.10	-
Monocalcium phosphate	14.10	12.70	14.10	12.70
Limestone	11.00	10.70	7.90	7.60
Salt	3.90	3.80	3.50	3.40
Vit.-min. premix[1]	3.00	3.00	3.00	3.00
Analysed nutrient contents	*(g.kg^{-1} air-dry)*			
Dry matter	883.5	886.3	887.5	890.1
Crude protein	139.2	183.8	140.8	192.9
Crude fiber	30.4	34.6	45.6	43.6

*LFLP = reduced fiber and protein diet; LFHP = reduced fiber and high protein diet; HFLP = high protein +15% dried beet pulp diet; HFHP = high protein + 15% dried beet pulp diet.

[1]Supplied per kg of diet: vit. A 9 000 IU; vit. D3 1 500 IU; α – tocopherol 35.0 mg; vit. B1 1.7 mg; vit. B2 6.0 mg; vit. B6 2.5 mg; Ca-panthothenate 15.0 mg; niacin 38.0 mg; vit. K3 2.0 mg; biotin 0.12 mg; cyanocobalamin 0.03 mg; choline 156 mg; Fe 103.0 mg; Zn 116.5 mg; Mn 49.0 mg; Cu 40.0 mg; I 1.2 mg; Co 0.4 mg; Se 0.3 mg.

difference was recorded only in the group with higher levels of nitrogen. In comparison among groups with different nitrogen content in the diet no significant differences were found. In comparison to the same groups in dry matter excretion, the difference was more pronounced, up to + 34.25 g.d^{-1}, +26.0 % (P = 0.014). We found the most excreted dry matter −165.59 g.d^{-1} in the HFHP group (received dry matter 1.29 kg.d^{-1}) In comparison with the group with low fiber content (LFHP vs. HFHP) the difference in the excreted dry matter was more pronounced up to + 34. 25 g.d^{-1}, +26.0 % (P = 0.014).

The results of the nitrogen balance influenced by the addition of pulp are shown in Table 2. Nitrogen balance was affected by the inclusion of beet pulp in all diets, but not all values were statistically significant. Nitrogen uptake was increased by including beet pulp in the HFLP diet compared to HFHP + 32% (P = 0.015), when feed LF was only +18 % (LFLP vs. HFLP). From these values, the benefit of the higher fiber content is the intake of nitrogen from the feed, as in both cases there was approximately the same increase in nitrogen in the feed between the groups.

Changes in the amount of excreted nitrogen through urine and faeces are expressed by their proportion. The largest and significant value was found in the HFLP group by + 48 %, compared to groups with different nitrogen content but the same fiber content (HFLP = 1.3 vs. HFHP = 2.5). The increased amount of total excreted nitrogen (LF + 33 % and HF + 36 %) was adequate to the increased nitrogen intake (+ 35 %, LP vs. HP). The effect of the pulp was not statistically confirmed here at any nitrogen level. However, we found significant differences between the groups in the amount of nitrogen excreted separately in urine and faeces, in the table expressed as the urine / faecal ratio (P < 0.05). Our result

Table 2. Effects of dietary protein and crude fiber level on nitrogen (N) balance

Items	LFLP* SEM	LFHP* SEM	HFLP* SEM	HFHP* SEM
DM intake, *[kg.d^{-1}]*	1.23 ± 0.10^{ab}	1.12 ± 0.08^{a}	1.14 ± 0.1^{ab}	1.29 ± 0.06^{b}
CF intake *[g.d^{-1}]*	42.40 ± 0.41^{a}	46.75 ± 0.50^{a}	58.62 ± 0.66^{b}	62.19 ± 0.34^{b}
N intake *[g.d^{-1}]*	31.07 ± 0.33^{a}	38.30 ± 0.45^{b}	28.96 ± 0.46^{a}	42.33 ± 0.29^{b}
Urinary/Faecal N ratio	1.80 ± 0.11^{a}	3.14 ± 0.12^{c}	1.30 ± 0.11^{b}	2.5 ± 0.13a
Total N excretion *[g.d^{-1}]*	12.65 ± 0.25^{a}	18.86 ± 0.28^{b}	11.83 ± 0.27^{a}	18.60 ± 0.23^{b}
Absorbed N *[g.d^{-1}]*	26.45 ± 0.31^{a}	32.06 ± 0.47^{a}	23.77 ± 0.33^{a}	38.43 ± 0.37^{b}
Retained N *[g.d^{-1}]*	18.43 ± 0.29^{a}	19.18 ± 0.35^{a}	17.12 ± 0.29^{a}	25.40 ± 0.30^{b}
Absorption, % of intake	84.74 ± 0.18^{a}	87.27 ± 0.12^{a}	81.66 ± 0.13^{a}	85.38 ± 0.13^{a}
Retention, % of intake	59.01 ± 0.27^{a}	52.66 ± 0.18^{b}	58.72 ± 0.19^{a}	56.51 ± 0.19^{a}
Retention, % of absorbed	69.41 ± 0.15^{a}	60.56 ± 0.29^{b}	71.86 ± 0.15^{b}	66.10 ± 0.14^{a}

Different letters ($^{a, b}$ and ^{c}) on the same row indicate that treatment means are significantly different at $P < 0.05$.
*LFLP = reduced fiber and protein diet; LFHP = reduced fiber and high protein diet; HFLP = high protein +15% dried beet pulp diet; HFHP = high protein + 15% dried beet pulp diet.

with low protein diets is consistent with the findings of Galassi *et al*. (2007), who obtained similar results in a traditional Italian heavy pig on average weight (150 kg) with a diet with CP 14.0 % and CF 4.1 % compared to the control group with CP 13.5 % and CF 3.2 %.

Absorbed nitrogen calculated as a difference between nitrogen intake and nitrogen in faeces (including endogenous nitrogen) was the difference in mean values. Large differences in mean values in groups with different nitrogen content (LFLP vs. LFHP +5.61 (17 %) and HFLP versus HFHP +14.65 (38 %)) were not significant (Methodical intention). Significant evidence for better functioning of fiber on nitrogen absorption in higher nitrogen diets was found in groups with different fiber content LFHP vs. HFHP +6.37 g.d^{-1} = +19.9 % (P = 0.03). The opposite tendency was observed in the LFLP diet compared to HFLP -2.68 g.d^{-1} = -10.1 % (P = 0.13).

Similar results were observed in nitrogen retention +4 % at low fiber levels (LFLP vs. LFHP) and +33 % (+8.28 g.d^{-1}) at high fiber levels (HFLP vs. HFHP). Nitrogen retention, expressed as a percentage of N intake, was reduced in pigs fed with the high-fiber diets (-4 %) and it was even more affected by the loss of fiber on feed up to -12 %.

Nitrogen excretion was affected by the inclusion of beet pulp into the diet (Figure 1). Switch from urinary to faecal excretion was detected in all animals, while the largest and most significant it

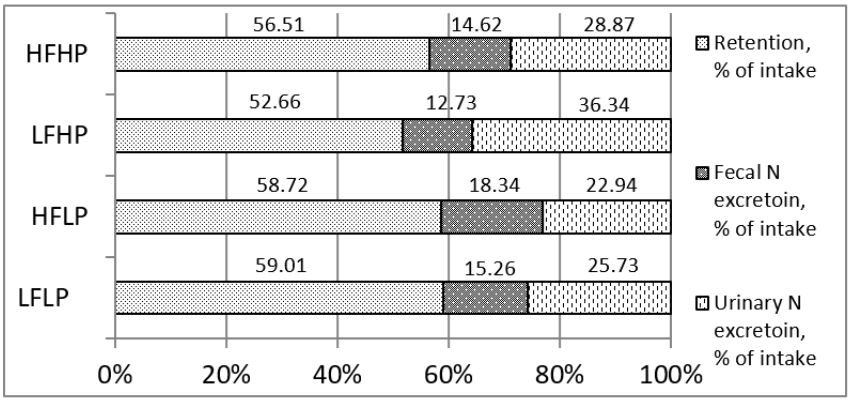

Figure 1. Distribution of nitrogen intake in to faeces and urine (expressed as % of nitrogen intake)

was in the HP diet group ($P < 0.05$). The reduction in urinary nitrogen was -7.48 % (LFHP vs. HFHP) and -2.79 % (LFLP vs. HFLP). Total N retention was detected in the higher nitrogen group of +3.85 % (LFHP vs. HFHP). In the lower nitrogen group (LFLP vs. HFLP) we found almost no change. Faecal N excretion increased by +3.1 % (LFLP vs. HFLP) and +1.89 % (LFHP vs. HFHP).

Similar results were found for Italian pigs fed with CP 14.0 % and CF 4.1 % where 12.3 %, 19.3 % faecal N was confirmed in feed with higher protein levels and increasing fiber (CF 6.7 %). Both groups were compared to CP control 13.5 and CF 3.2 % (Galassi *et al*., 2007).

A different, but not significant, effect was observed in nitrogen uptake and retention (Figure 2). A different distribution was observed in the nitrogen level in the faeces, urine, and retention in the diets with different fiber content and approximately same nitrogen content. The results of absorption and nitrogen retention after inclusion of added fiber at lower nitrogen content (LP) were worse in both cases (-1 to -2 $g.d^{-1}$), but in groups with higher nitrogen content (HP) it was improved at absorption by 20 % (+6. 37 $g.d^{-1}$) and at a retention of 32 % (+6.22 $g.d^{-1}$). Zervas and Zijlstra (2002) found decrease in crude protein content of 18.5 % and the addition of sugar beet pulps reduced N retention by 12.0 % compared to the control group.

In our observation at the concentration 19 % of crude protein in the diets, regression analysis showed that absorbed N (y) was a linear function of

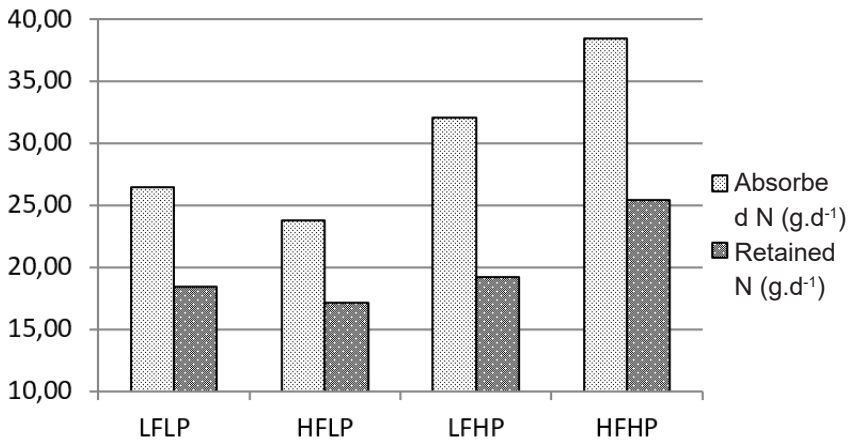

Figure 2. Amount of absorbed and retained nitrogen, according to the fiber and nitrogen content on the diet ($g.d^{-1}$)

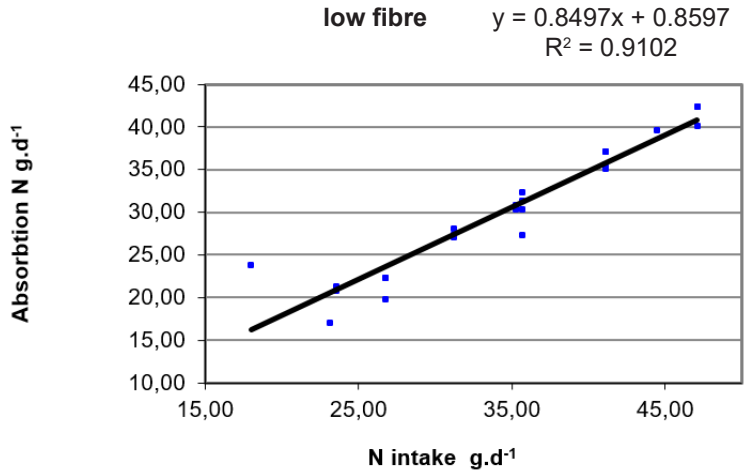

Figure 3. Relationship between nitrogen intake and nitrogen absorption in low fiber diets (LFLP and LFHP)

nitrogen intake (x), as it shown on Figures 3 and 4. The relationship depends on nitrogen content in feed. For low fiber diets it was described by an equation: $y = 0.8497\,x + 0.8597$, $R^2 = 0.9102$. For the diets with high fiber content it was calculated using an equation: $y = 0.9604\,x - 4.3065$, $R^2 = 0.9797$. The slope of the regression equation showed that each gram of nitrogen intake in the LF 849.7 mg and and for HF diets was absorbed up to 960.4 mg N.

The coefficient of determination (R^2) for the regression daily N absorption was predicted from N intake. The values are different according to the low fiber content in diets (LFLP + LFHP) $R^2 = 0.9102$ and (HFLP + HFHPP) $R^2 = 0.9797$. A stronger effect on nitrogen absorption was found in the group with higher fiber content.

From the calculated equations it is possible to estimate the interesting absorption at zero nitrogen intake. In case of low fiber content in feed it is about 0.8 $g.d^{-1}$, at higher levels of fiber it is already negative value (- 4 $g.d^{-1}$). A logical explanation should be the increased microbial colonization of the intestine and the transfer of nitrogen to solid excrement. This nitrogen falls into the category of endogenous production. Endogenous production and nitrogen retention values for different fiber content in feed will be the aim of future investigations. Up to date, we have found that the amount of fiber intake in experimental diets and nitrogen retention are no longer linearly dependent.

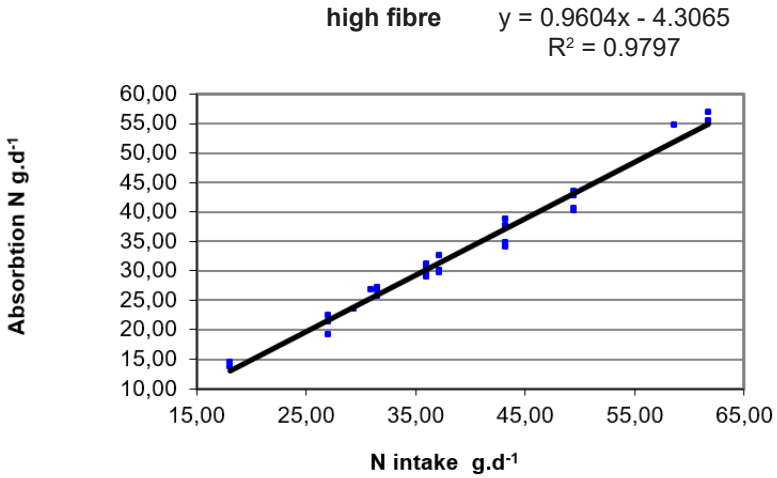

Figure 4. Relationship between nitrogen intake and nitrogen absorption in high fiber diets (HFLP and HFHP)

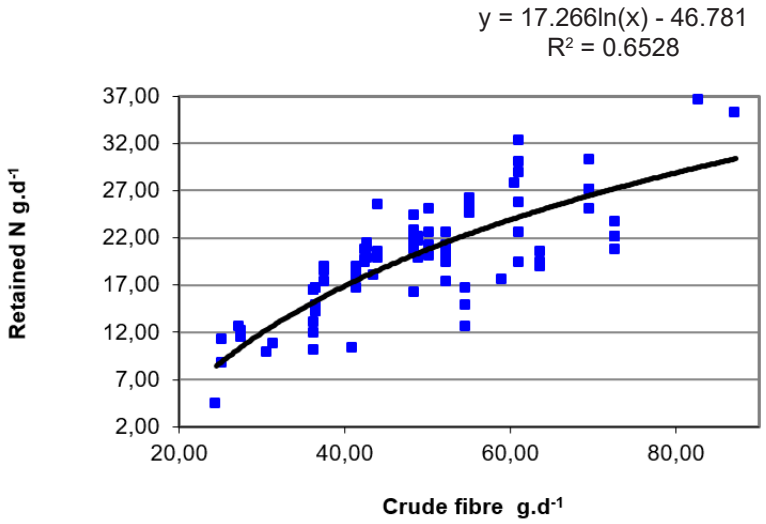

Figure 5. Relationship between crude fiber and nitrogen retention (all groups)

Dependence of nitrogen retention by the fiber content has a logarithmic character of $y = 17.266 \ln(x) - 46.781$, $R^2 = 0.6528$. Figure 5 illustrates the trends in existing data and predictions of future data.

The influence of non-starch polysaccharides from beet pulps on growth performance after weaning, ileal and faecal nutrient digestibility and intestinal enzymes of piglets was investigated in several studies (Ramonet *et al*., 2000; Högberg and Lindberg, 2004). Faecal digestibility of energy and nitrogen was not affected by the presence of 6 – 12 % beet pulps in piglet diets (Lizardo and Aumaître, 2001). These, as well as our present data, suggest that fiber has a greater effect on a diet with a higher content of CP. In both cases it increased the nitrogen in faeces and reduced nitrogen in urine while had no negative effect on the absorption and total amount of nitrogen excreted. According to the logarithmic relationship for retention and fiber content, this theory is rather hypothetic. Specific equation is valid for a specific category of animals and specific quantitative values for fiber content in diets. The opportunity to use crude fiber concentrates as a functional feed additive might be of a potential value to improve young pigs' growth and welfare.

CONCLUSION

A portion of crude fiber is used as an energy source for the pig and a part of the undigested fiber serves as an energy source for microbial populations in the digestive tract. At an optimum amount it does not affect nitrogen absorption but increases microbial population. This population's use of fiber sources high in fermentable carbohydrates can shift nitrogen excretion from urea and other resources to faeces, thereby reducing chances of ammonia emission. From an environmental point of view, nitrogen excreted in the faeces in microbial proteins is more favourable, because it is not rapidly degraded compared with urinary nitrogen. Although these diets do not always maximise pig performance, they provide an effective and economical use of locally grown feedstuffs and hence contribute to sustainable production. The influence of protein and energy nutrition on health and environmental issues in various production systems still require further academic discussion.

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NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA

Slovak J. Anim. Sci., 53, 2020 (4): 192–198

ISSN 1337-9984
E-ISSN 1338-0095

EFFECT OF LAYING AGE AND PLUMAGE COLOUR ON INTERNAL AND EXTERNAL QUALITY CHARACTERISTICS OF NOILER CHICKEN EGGS

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ABSTRACT

This study was conducted to evaluate the effect of laying age and plumage colour on the internal and external quality characteristics of eggs laid by Noiler chicken. Three hundred freshly laid eggs of three plumage colours (brown, barred and black) and age (young and old) were used for this experiment. External and internal quality parameters measured were: egg weight, egg length, egg width, shape index, albumen height, yolk height, yolk width, yolk index, yolk and albumen weight, Haugh unit, shell thickness, shell thickness and shell surface area. The data were subjected to General Linear Model procedure of SAS® (2002) with laying age, plumage colour and their two-way interaction as fixed effects. Significant differences ($P < 0.05$) were observed in albumen height, yolk height and Haugh unit as a result of differences in plumage colour. In addition, the study showed that age had a significant effect on all the parameters considered except egg shape index. The result showed that albumen height, yolk height and Haugh unit decreased with an increase in laying age. In conclusion, it was found that laying age and plumage colour had significant effect on the quality of eggs laid by Noiler chickens.

Key words: Noiler chicken; plumage colour; laying age; external egg quality; internal egg quality

INTRODUCTION

In Nigeria, where the production of animal protein falls far short of meeting the demands of a rapidly growing population (Adene and Oguntade, 2006) and the state of nutrition is characterized by gross inadequate protein intake, poultry is the most common livestock being kept (Amar-Klemesu and Maxwell, 2000). The Nigerian poultry industry in particular has been rapidly expanding in recent years and is, therefore, one of the most important and commercialized subsectors of the Nigerian agriculture (Adene and Oguntade, 2006). The poultry industry serves as a major source of animal protein in form of meat and eggs and has great potential of solving the national problem of inadequacy of animal products. Local chickens are among the many local resources of the poor people, living in the rural areas, which could be harnessed and utilized for poverty alleviation (Njue *et al.*, 2002). The indigenous poultry species, which includes Noiler chicken, makes significant contributions to animal protein availability in Nigeria through cheap poultry products, such as meat and eggs.

Poultry egg remains one of the cheapest, most affordable and acceptable animal product. Eggs possess two yardsticks that make them important as foodstuff; namely, they are rich in nutrient and serve important roles in many food products because of their functional properties (Silversides and Scott, 2001). Egg quality traits including external (egg weight, egg length, egg width, shell quality, shell

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Received: March 2, 2020
Accepted: April 27, 2020

thickness and shell surface area) and internal traits (albumen height, yolk height, yolk width, yolk index, Haugh unit, albumen and yolk parameters) are crucial not only for consumers but also essential for the egg product industry (Song *et al.*, 2000; Wolanski *et al.*, 2007). Egg weight is used to grade eggs into different categories and bigger eggs cause higher price. Also Haugh unit is a measure of overall internal egg quality.

Noiler chicken, a dual purpose breed of chicken with different plumage colours predominantly black, brown and barred, was recently developed by Amo Farm Sieberer Hatchery Limited, Nigeria for smallholder farmers to address the challenges of food and financial insecurities among rural population, especially women. Noiler chicken is bred to survive on low quality feedstuffs to provide good quality meat and eggs, but little or no research work has been done to evaluate the quality of the eggs. Noiler chicken comes in varieties of plumage colour, however, barred, black and brown are the predominant plumage colours. Buss and Guyer (1982) reported that there was some genetic dispersion in eggshell quality characteristics existing between species and between breeds, plumage colour and families within the lines. Many studies showed that hens with coloured feathers lay bigger eggs than hens with white feathers (Halaj and Grofík, 1994; Vits *et al.*, 2005; Halaj and Golian, 2011). The percentage of yolk tends to be larger in larger eggs and egg albumen tends to decrease with egg weight. Iposu *et al.* (1994) and Silversides (1994) reported negative correlations between the egg weight and albumen height as well as between the egg weight and Haugh unit. Padhi *et al.* (2013) reported that the egg weight showed significant difference in external egg characteristics for eggs laid by layers of different age, and the egg weight increases as the age of layers increases. Lee *et al.* (2016) classify age of hens as the major factor that has an effect on the quality of fresh eggs. Silversides *et al.* (2006) reported that the albumen weight is significantly different between different ages of chicken. Increase in the albumen weight with increase in age was reported, and increase in the egg weight at 40 weeks compared to 28 weeks was also reported by Rajkumar *et al.* (2009). As a result of long-term genetic selection, different plumage colours of laying hen vary significantly in the egg shell quality, egg size and production (Curtis *et al.*,1985). Hence, there is a need to assess the effect of laying age and plumage colour on external and internal egg quality. The objective of this study, therefore, was to determine the effect of laying age and plumage colour on external and internal egg quality characteristics of Noiler chickens.

MATERIAL AND METHODS

Experimental Location

The eggs used for this experiment were collected from Livestofarm, Modakeke Osun State, Nigeria and all the necessary measurements were taken at the Department of Animal Sciences, Obafemi Awolowo University, Ile-Ife. The University is located at Log 7031' 18.2" N and Lat 4031' 33.9" E.

Data Collection and Analysis

A totally 300 freshly laid eggs were obtained across three plumage colours (black, brown and barred) at 26 weeks (young age) or 46 weeks (old age) of age from Noiler chicken. Fifty (50) eggs were collected for each plumage colour.

The external egg quality parameters measured were: egg weight, egg width, egg length, shell, shell surface area and percentage of shell thickness, shell weight. The internal egg quality parameters considered were: albumen height, haugh unit, yolk height, yolk index, yolk and albumen weight and yolk width.

Egg weight, yolk and albumen weight and shell weights were measured in grams using KERRO® electronic compact scale (model number BL50001) with a maximum capacity of 5000 g and sensitivity of 0.1 g.

Egg length (EL), egg width (EW), yolk height (YH), albumen height (AH) and yolk width (YW) were measured in centimetres using a Vernier calliper. Shell thickness (ST) in millimetres was measured using a micrometre screw gauge.

Haugh unit was calculated according to Haugh (1937) using the formula below:

$HU = 100 \log (H + 7.57 - 1.7\ W0.37)$

Shape Index = Egg width / Egg length

Yolk index = Yolk height / Yolk width x 100

Shell surface area = $W^{0.66} \times 4.67$

Data were subjected to General Linear Model (GLM) procedure of SAS (2002) with plumage colour, layer's age and their two-way interaction as fixed effects according to the following model:

$Y_{ijk} = \mu + S_i + A_j + (S*A)_{ij} + e_{ijk}$
Y_{ijk} = Trait measured,
μ = Overall means,
S_i = Plumage colour effect (i = 1, 2,3),
A_j = flock Age effect (j = young and old),
$(S*A)_{ij}$ = Interaction between Plumage colour and age,
e_{ijk} = Random error.

When significant differences among means were found, the means were separated using Duncan's Multiple Range and Least Squares Means tests of the same software.

RESULTS AND DISCUSSION

Table 1 shows the effect of plumage colour on internal and external quality characteristics of eggs laid by Noiler chickens. The parameters presented include egg weight, egg length, egg width, albumen height, yolk height, yolk index, yolk and albumen weight, shell weight, shell surface area, percentage shell thickness, shell thickness, egg shape index and Haugh unit.

There were significant differences in the yolk height, albumen height and Haugh unit ($P < 0.05$) as a result of difference in plumage colour, while there were no significant differences in the egg weight, egg length, egg width, yolk index, shell weight, shell thickness and shape index. This result is in agreement with the report of Dahloum *et al.* (2018), who evaluated the effect of plumage colour on egg quality characteristics of indigenous naked-neck chickens in Algeria. They reported that both yolk height and albumen height were influenced by plumage colour. They reported significant effect of plumage colour on all internal egg quality traits. The albumen height as well as yolk height in the present study was greater than those reported by Dahloum *et al.* (2018).

Further, this present result also agrees with the findings of Rayan *et al.* (2013), who reported some reproductive performance parameters and egg quality traits of two commercial layer plumage colour (brown and white variants). The authors reported significant differences in some internal egg qualities (particularly yolk and albumen). In this study, the yolk height of eggs laid by brown hens had significantly higher value compared to those of eggs laid by black and barred hens. Similarly, value of the albumen height of eggs laid by the brown bird was significantly higher compared to eggs

Table 1. Egg quality parameters of different plumage colours of Noiler chicken

Trait	Black ± SD	Barred ± SD	Brown ± SD	SEM	*P*-value
EW (g)	64.43 ± 5.85	63.14 ± 6.92	63.98 ± 6.08	0.500	0.186
EL (cm)	5.840 ± 0.31	5.832 ± 0.32	5.826 ± 0.25	0.024	0.920
EWD (cm)	4.377 ± 0.16	4.330 ± 0.17	4.313 ± 0.44	0.027	0.242
YH (cm)	1.929 ± 0.11^b	1.899 ± 0.16^b	1.967 ± 0.14^a	0.013	0.002
YW (cm)	4.041 ± 0.27	3.997 ± 0.31	4.080 ± 0.24	0.024	0.063
YI	47.700 ± 4.82	47.951 ± 4.34	48.343 ± 4.27	0.444	0.598
AH (cm)	0.821 ± 0.13^b	0.809 ± 0.14^b	0.870 ± 0.13^a	0.012	0.002
YAW (g)	56.164 ± 6.42	54.968 ± 5.31	55.690 ± 5.72	0.476	0.203
SI	75.111 ± 3.89	75.855 ± 5.02	75.700 ± 8.20	0.565	0.618
SW (g)	7.572 ± 0.57	7.522 ± 0.73	7.687 ± 0.79	0.676	0.210
ST (mm)	0.325 ± 0.04	0.323 ± 0.04	0.320 ± 0.04	0.003	0.556
% ST	32.010 ± 4.14	32.590 ± 4.30	32.310 ± 4.37	0.378	0.556
SSA	71.936 ± 4.38	72.928 ± 5.23	72.585 ± 4.55	0.375	0.167
HU	84.45 ± 0.79^b	84.44 ± 0.71^b	84.72 ± 0.63^a	0.067	0.004

EW = Egg weight, EL = Egg length, EWD = Egg width, YH = Yolk height, YW = Yolk width, YI = Yolk index, AH = Albumen height, YAW = Yolk + Albumen weight, SI = Shape index, SW = Shell weight, ST = Shell thickness, SSA = Shell surface area, HU = Haugh Unit, SD = Standard deviation, SEM = Standard error of mean.

laid by black and barred hens. However, there were no significant differences in the egg weight, egg length, egg width, yolk width, yolk and albumen weight, shell weight, shell thickness and shape index.

There were no significant differences in the egg weight, shell thickness and percentage shell thickness among the three plumage colours of Noiler birds. This disagrees with the report of Rayan *et al.* (2013), who reported that egg weight was significantly affected by plumage colour and that there was significant difference in shell thickness due to plumage colour. In their findings, brown plumage-coloured hens laid eggs that had significantly higher shell thickness compared to their white counterparts.

There were significant differences ($P < 0.05$) in the Haugh unit as a result of differences in plumage colour. The brown plumage had the highest value indicating the best in terms of internal quality among the other plumage colours. Consumer's egg preference could be for the brown plumage colour of Noiler chicken because of their superior internal quality.

Table 2 shows the internal and external characteristics of Noiler chicken eggs at different ages (old and young). Parameters evaluated include the mean egg weight, egg length, egg width, yolk height, yolk width, albumen height, yolk and albumen weight, egg shape index, shell weight, shell thickness, shell surface area, percentage shell thickness and Haugh unit.

There were significant differences ($P < 0.05$) in all the internal and external parameters with the exception of egg shape index. Egg weight showed significant ($P < 0.05$) difference between different ages, and the egg weight increased as the age of the birds increases.

In this finding, the albumen weight significantly ($P < 0.05$) increased with the advancement of layer ages. Rossi and Pompei (1995) obtained similar results. Suk and Park (2001) and Rayan *et al.* (2013) observed that the albumen weight increased with advancing age of layers. Shell thickness differed significantly ($P < 0.05$) among the different ages of birds. The shell thickness was higher in young Noiler birds compared to old Noiler birds. This disagrees with the report of Padhi *et al.* (2013), who determined the effect of age on egg quality in chicken and reported no significant differences ($P < 0.05$) among different ages of birds. A probable explanation for thin eggshell in older hens may be lessening of calcium deposition with the passage of time (Bare and Striem, 1998). It has been observed that

Table 2. Effect of laying age on egg quality parameters of Noiler chicken (Young and Old)

Trait	Old ± SD	Young ± SD	SEM	*P*-value
EW (g)	67.516 ± 4.90^a	60.180 ± 5.34^b	0.408	< 0.0001
EL (cm)	5.987 ± 0.25^a	5.679 ± 0.26^b	0.020	< 0.0001
EWD (cm)	4.429 ± 0.15^a	4.251 ± 0.36^b	0.022	< 0.0001
YH (cm)	1.949 ± 0.12^a	1.914 ± 0.16^b	0.010	0.0240
YW (cm)	4.148 ± 0.26^a	3.932 ± 0.24^b	0.020	< 0.0001
YI	47.184 ± 4.22^b	48.812 ± 4.59^a	0.361	0.0015
AH (cm)	0.810 ± 0.14^b	0.857 ± 0.13^a	0.010	0.0020
YAW (g)	58.864 ± 4.72^a	52.350 ± 4.98^b	0.388	< 0.0001
SI	74.973 ± 4.41	76.138 ± 7.18	0.461	0.6184
SW (g)	7.787 ± 0.67^a	7.400 ± 0.69^b	0.055	< 0.0001
ST (mm)	0.302 ± 0.04^b	0.343 ± 0.04^a	0.003	< 0.0001
% ST	34.320 ± 3.89^a	30.286 ± 3.63^b	0.308	< 0.0001
SSA	75.243 ± 3.63^a	69.724 ± 4.06^b	0.306	< 0.0001
HU	84.250 ± 0.72^b	84.827 ± 0.65^a	0.055	< 0.0001

EW = Egg weight, EL = Egg length, EWD = Egg width, YH = Yolk height, YW = Yolk width, YI = Yolk index, AH = Albumen height, YAW = Yolk + Albumen weight, SI = Shape index, SW = Shell weight, ST = Shell thickness, SSA = Shell surface area, HU = Haugh Unit, SD = Standard deviation, SEM = Standard error of mean.

Table 3. Interaction between age and plumage colour on egg parameters of Noiler chicken

Trait	Old Barred ± SD	Young Barred ± SD	Old Black ± SD	Young Black ± SD	Old Brown ± SD	Young Brown ± SD	SEM	*P*-value
EW (g)	68.088 ± 5.10^{a}	58.200 ± 4.23^{c}	68.318 ± 4.11^{a}	60.534 ± 5.34^{b}	66.144 ± 5.88^{a}	61.808 ± 6.08^{b}	0.708	0.0005
EL (cm)	6.067 ± 0.32^{a}	5.597 ± 0.22^{b}	5.982 ± 0.26^{a}	5.699 ± 0.28^{b}	5.913 ± 0.33^{a}	5.741 ± 0.25^{b}	0.035	0.0001
EWD (cm)	4.410 ± 0.19	4.217 ± 0.16	4.465 ± 0.28	4.290 ± 0.33	4.414 ± 0.45	4.247 ± 0.46	0.039	0.9440
YH (cm)	1.957 ± 0.11^{a}	1.839 ± 0.09^{b}	1.913 ± 0.13^{a}	1.945 ± 0.12^{a}	1.977 ± 0.15^{a}	1.957 ± 0.16^{a}	0.019	0.0005
YW (cm)	4.168 ± 0.23^{a}	3.824 ± 0.24^{b}	4.143 ± 0.22^{a}	3.941 ± 0.26^{b}	4.133 ± 0.18^{a}	4.029 ± 0.17^{b}	0.035	0.0033
YI	47.177 ± 4.25	48.224 ± 4.76	46.374 ± 4.56	49.528 ± 4.17	48.002 ± 4.28	48.683 ± 4.33	0.622	0.1047
AH (cm)	0.782 ± 0.12	0.836 ± 0.09	0.796 ± 0.10	0.846 ± 0.11	0.852 ± 0.13	0.889 ± 0.14	0.018	0.8927
YAW (g)	59.276 ± 5.78^{a}	50.660 ± 5.34^{e}	59.652 ± 5.38^{a}	52.676 ± 5.26^{d}	57.666 ± 4.88^{b}	53.714 ± 5.12^{c}	0.673	0.0024
SI	72.737 ± 4.21^{b}	78.972 ± 3.66^{a}	74.822 ± 5.30^{b}	75.399 ± 6.78^{b}	77.359 ± 7.41^{a}	74.041 ± 5.66^{b}	0.8001	<.0001
SW (g)	7.806 ± 0.69	7.238 ± 0.55	7.742 ± 0.61	7.402 ± 0.62	7.814 ± 0.57	7.560 ± 0.48	0.095	0.2388
ST (mm)	0.299 ± 0.02	0.340 ± 0.04	0.304 ± 0.03	0.347 ± 0.04	0.304 ± 0.03	0.341 ± 0.04	0.0053	0.8713
% ST	29.980 ± 3.89	34.040 ± 4.14	30.440 ± 4.10	34.740 ± 4.58	30.440 ± 3.94	34.180 ± 4.45	0.3783	0.8713
SSA	75.676 ± 4.40	68.196 ± 4.56	75.846 ± 5.33	70.010 ± 5.12	74.206 ± 6.16	70.964 ± 4.76	0.5315	0.0004
HU	84.073 ± 0.72	84.815 ± 0.68	84.148 ± 0.71	84.751 ± 0.69	84.526 ± 0.70	84.915 ± 0.73	0.0954	0.1758

EW = Egg weight, EL = Egg length, EWD = Egg width, YH = Yolk height, YW = Yolk width, YI = Yolk index, AH = Albumen height, YAW = Yolk + Albumen weight, SI = Shape index, SW = Shell weight, ST = Shell thickness, SSA = Shell surface area, HU = Haugh Unit, SD = Standard deviation, SEM = Standard error of mean.

the skeletal calcium available for shell calcification decreases with age. Regarding age, a significant ($P < 0.05$) depressing effect was obtained for the values of Haugh units, which decreased as layer age progressed. This agrees with the findings of Verheyen and Decuypere (1991), Yasmeen *et al.* (2008) and Rayan *et al.* (2013), who found that Haugh unit values decreased with increase in the layer age.

Table 3 shows the interaction effect between plumage colour and age on the internal and external egg quality characteristics of Noiler chicken. There were significant differences ($P < 0.05$) in egg weight, egg length, yolk width, yolk and albumen weight and shape index. However, no significant interaction effect on the egg width, albumen height, shell weight, shell thickness and Haugh unit was found.

The result obtained for egg weight indicated that the older black birds had the highest value, while the young barred birds had the least value for egg weight. For egg length, the older barred birds had the highest value, while the young barred birds had the least value. For yolk height and yolk width, there were significant differences as a result of the interaction between laying age and plumage colour. The older brown birds had the highest yolk height followed by older barred birds and older black birds with younger barred birds having the least value for yolk height and yolk width. From physiological point of view, egg weight is positively correlated with progressing age of hen, such phenomena held true also both for yolk or albumen weight as a major egg components. Yolk and albumen weight differs significantly with older black birds having the highest value followed by older barred birds and older brown birds with younger barred birds having the least value. Albumen height and Haugh units are the traits used to evaluate albumen quality, which reduces with age (Liljedahl *et al.*, 1984). There were no significant differences in the albumen height and Haugh unit as a result of the interaction between laying age and plumage colour.

CONCLUSION

External egg parameters including egg weight, egg length, egg width, shell weight, shape index, and shell thickness were not significantly influenced by plumage colour, while internal quality parameters,

such as Haugh units, albumen height and yolk height, were affected by plumage colour with brown plumage-coloured birds having the highest egg internal quality.

Furthermore, laying age had a significant effect on all the internal and external quality parameters considered. The interaction between laying age and plumage colour had significant influence only on the egg weight, egg width, yolk height, yolk width as well as yolk and albumen weight.

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Slovak J. Anim. Sci., 53, 2020 (4): 199–204

ISSN 1337-9984
E-ISSN 1338-0095

EFFECTS OF OXIDASE SUBSTRATES ON THE CHARACTERISTICS OF GOAT SPERMATOZOA MOTILITY: SHORT COMMUNICATION

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ABSTRACT

In this study, the effects of nicotinamide adenine dinucleotide phosphate (NADPH) and phenylalanine on goat sperm motility were investigated. Fresh Shami goat semen was collected and incubated at 37 °C for 45 minutes in Tris-based medium with 1, 2 and 4 mM or without both NADPH and phenylalanine. Sperm motility was assessed using computer assisted sperm analyzer (CASA). Higher velocity values were achieved when 1 and 2 mM of NADPH were added, while the addition of phenylalanine at the same concentrations had reduced the values of all motility parameters, with no significant differences ($P > 0.05$) compared to the control. Addition of the two substrates at a concentration of 4 mM had a clear negative effect ($P < 0.05$) on the values of sperm motility parameters as well as on the percentages of spermatozoa subpopulations, especially for the rapid and the static categories. We concluded that both NADPH and phenylalanine have pronounced effects on goat sperm motility and these effects were dependent on concentration. The supplementation of semen media with NADPH at an appropriate concentration can be useful as a stimulating additive for goat sperm motility.

Key words: hydrogen peroxide; phenylalanine, NADPH, goat, sperm motility

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide (H_2O_2) are chemically reactive molecules resulting from oxygen consumption. In general, ROS family members play an important role in normal sperm function (Ford, 2004), for example, H_2O_2 participates in the regulation of sperm capacitation process by inducing protein tyrosine phosphorylation in human (De Lamirande and Lamothe, 2009), equine (Baumber *et al*., 2000) and bull (Rivlin *et al*., 2004). In contrast, elevated ROS concentrations resulting from an imbalance in the production of ROS and antioxidant systems may adversely affect spermatozoa motility, membrane integrity and IVF outcome (Du Plessis *et al*., 2008).

Spermatozoa may generate ROS through nicotinamide adenine dinucleotide phosphate NADPH-oxidase system located in the plasma membrane (Aitken *et al*., 1992; Rivlin *et al*. 2004). On the other hand, a specific aromatic amino acid oxidase (AAAO) has been identified as the origin of ROS formation from dead sperm (Shannon and Curson, 1982a; 1982b; Upreti *et al*., 1998). We previously showed that the addition of NADPH and phenylalanine (the substrates for NADPH-oxidase and AAAO, respectively), had significantly raised H_2O_2 formation from live and dead bull (Alomar and Donnay, 2006), ram (Alomar *et al*., 2016) and buck sperm (Alomar, 2018). Furthermore, when ram spermatozoa where supplemented with these two substrates, the values of velocity parameters were significantly augmented (Alomar *et al*., 2018).

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Received: July 20, 2020
Accepted: September 28, 2020

Our previous studies clearly indicated the importance of NADPH and phenylalanine in the spermatozoa function of different ruminants' species. However, the influence of NADPH and phenylalanine on goat spermatozoa motility had not been previously studied.

Thus, the main aim of the present study was to determine the effects of the addition of these two specific oxidase substrates on goat sperm motility assessed by CASA technology.

MATERIAL AND METHODS

Semen collection

This study was carried out at Der-Al-Hajar Animal Production Research Station, 33 km south-east of Damascus. Semen was obtained from five sexually experienced Shami bucks, aged between 3 and 4 years. Semen was collected with the aid of an electro-ejaculator (Minitube – Electro Ejaculator, Tiefenbach, Germany). It must be noted that the experiments for this study were approved by the Local Scientific and Ethical Committee of the Atomic Energy Commission of Syria (AECS), Damascus, Syria (permit number 36-Z/M4 – 2019).

Experimental design and medium preparation

Two experiments were conducted with semen from the five bucks and in each experiment a total of 15 ejaculates were used. The ejaculates were mixed in each replicate to isolate the individual effect of males, and each experiment was repeated for three times. Spermatozoa were incubated in tubes in a water bath at a concentration of 25 x 10^6 sperm.ml^{-1} in a final volume of 500 µL of Tris-based medium prepared as a 300 mOsmol.kg^{-1} solution contained the following: 2.44 g Tris (hydroxymethyl) aminomethane, 1.36 g citric acid monohydrate and 1 g glucose in 100 ml of distilled water. The medium components were kept constant at pH 7. The first experiment was designed to examine the effects of three concentrations of NADPH (1, 2 and 4 mM) at 37 °C after 45 minutes of incubation. The second experiment was conducted to examine the effects of three concentrations of phenylalanine (1, 2 and 4 mM), compared to control, on sperm motility at 37 °C after 45 minutes of incubation.

Motility assessment

The motility characteristics of the spermatozoa were assessed by CASA technique using the Hamilton-Thorne motility analyzer (Hamilton Thorne Biosciences, HTM version 12.3, Beverly, USA). For each sperm sample, three viewing fields were selected and counted randomly to assess the data from at least 200-250 sperm cells per sample. The motility characteristics included in the analysis were: the percent motility (MOT %), curvilinear velocity (VCL, µm.s^{-1}), average path velocity (VAP, µm.s^{-1}), straight line velocity (VSL, µm.s^{-1}) and the percent of sperm showing progressive motility (PMOT %: VAP ≥ 75 µm.s^{-1} and STR ≥ 80 %). Spermatozoa subpopulations were defined in four categories by CASA system as following. Rapid (4): fraction of all cells moving with VAP > path velocity (VAP = 25 µm.s^{-1}). Medium (3): fraction of all cells moving with VAP cutoff (5 µm.s^{-1}) < VAP < path velocity (VAP = 25 µm.s^{-1}). Slow (2): fraction of all cells moving with VAP < VAP cutoff (5 µm.s^{-1}) or VSL < VSL cutoff (11 µm.s^{-1}). Static (0-1) fraction of all cells that is not moving at all.

The HTM settings used for goat spermatozoa were negative phase contrast optics at a recording rate of 60 frame/sec, temperature of analysis – 37 °C, light adjustment – 90-110, minimum cell size – 5 pixels, non-motile head size – 5 pixels, non-motile head intensity – 55, low VAP cut off – 21.9 µm.s^{-1}, low VSL cut off – 6 µm.s^{-1}, static size limit – 0.60/8 (min/max), static intensity limit – 0.25/1.50 (min/max), static elongation – 0/95 (min/max).

Statistical analysis

Statistical analysis was conducted using the Minitab program (Minitab Coventry, United Kingdom, Version 13.31, 2000). Data regarding phenylalanine and NADPH effects on spermatozoa were subjected to a factorial analysis of variance for the three concentrations (ANOVA, general linear model procedure, GLM) followed by multiple pairwise comparisons using a post-hoc (Tukey test). The threshold of signification was set at $P < 0.05$.

RESULTS

Table 1 shows the effects of different NADPH concentrations on CASA parameters. Addition of 1 mM

and 2 mM of NADPH had a significant positive effect ($P < 0.05$) on motility parameters MOT %, PMOT %, VAP, VCL and VSL, while a concentration of 4 mM significantly reduced the values of MOT % VAP, VCL and VSL.

Table 2 shows the motility of sperm treated with 0, 1, 2 and 4 mM of phenylalanine during 45 minutes of incubation. Phenylalanine given at concentrations of 1 mM and 2 mM insignificantly ($P > 0.05$) reduced motility values compared to the control. When 4 mM of phenylalanine was added, a significant ($P < 0.05$) decrease in all CASA analyzed parameters was observed. Figures 1 and 2 show the effects of NADPH and phenylalanine on the distribution of goat sperm subpopulations according to the motility. The rapid and static categories were the most affected subpopulations especially when the two substrates were added at a concentration of 4 mM.

Table 1. Effects of NADPH on sperm motility parameters of goat sperm samples

CASA parameter / Treatment	MOT (%)	PMOT (%)	VAP (μm.s^{-1})	VSL (μm.s^{-1})	VCL (μm.s^{-1})
Control	76.22 ± 4.47^{a}	18.66 ± 3.01^{a}	104.33 ± 10.46^{a}	66.11 ± 8.82^{a}	201.00 ± 9.08^{a}
NADPH 1 mM	87.67 ± 5.17^{b}	27.00 ± 2.87^{b}	123.22 ± 9.07^{b}	84.22 ± 8.15^{b}	233.44 ± 15.59^{b}
NADPH 2 mM	84.22 ± 5.67^{b}	25.00 ± 2.73^{b}	116.00 ± 5.5^{b}	78.22 ± 5.24^{b}	218.89 ± 10.56^{b}
NADPH 4 mM	66.88 ± 6.01^{c}	15.89 ± 3.48^{a}	91.78 ± 11.05^{c}	62.11 ± 6.01^{a}	187.33 ± 13.43^{c}

Values with different letters within columns significantly differ (P < 0.05).

Table 2. Effects of phenylalanine on sperm motility parameters of goat sperm samples

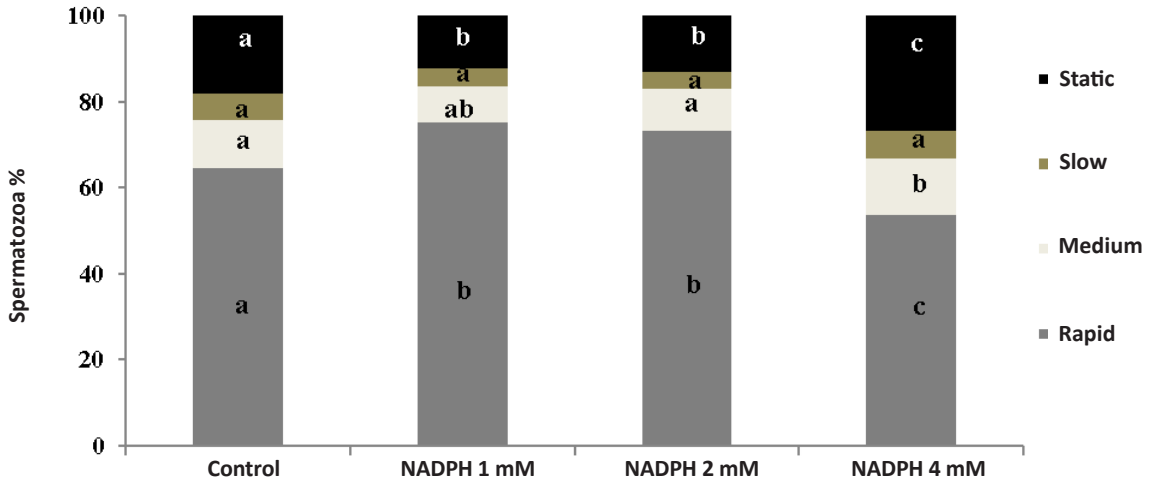
CASA parameter / Treatment	MOT (%)	PMOT (%)	VAP (μm.s^{-1})	VSL (μm.s^{-1})	VCL (μm.s^{-1})
Control	80.44 ± 2.92^{a}	19.56 ± 2.35^{a}	98.11 ± 15.92^{a}	66.22 ± 12.97^{a}	187.66 ± 10.9^{a}
Phenylalanine 1 mM	77.66 ± 5.66^{a}	17.56 ± 5.27^{a}	93.67 ± 19.12^{a}	62.22 ± 16.96^{a}	181.56 ± 16.66^{a}
Phenylalanine 2 mM	75.00 ± 3.08^{a}	16.22 ± 2.08^{a}	92.44 ± 16.83^{a}	61.11 ± 14.38^{a}	179.44 ± 12.3^{a}
Phenylalanine 4 mM	56.67 ± 6.84^{b}	10.00 ± 4.01^{b}	71.22 ± 11.5^{b}	44.56 ± 7.55^{b}	149.44 ± 20.92^{b}

Values with different letters within columns significantly differ (P < 0.05).

DISCUSSION

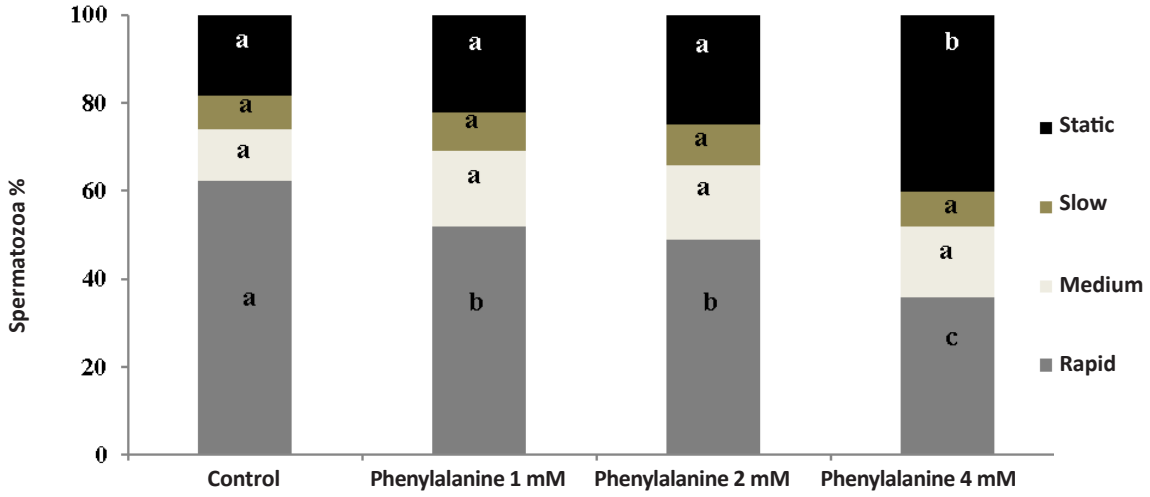
The present data shows for the first time the influences of NADPH and phenylalanine on motility parameters of goat spermatozoa. The effects of these two substrates have been a major issue of our previous studies (Alomar and Donnay, 2006; Alomar *et al*., 2016; 2018; Alomar, 2018), where we have demonstrated their abilities to induce hydrogen peroxide formation from both live and dead ram and bull spermatozoa and also their adverse effects on motility characteristics.

The principal CASA parameters showed an obvious positive effect when spermatozoa were incubated at 1 and 2 mM concentration of NADPH. In agreement with these results, 1 mM of this substrate had positively increased both PMOT % and VAP values of ram spernatozoa (Alomar *et al*., 2018). The controlled amounts of H_2O_2 produced by sperm following NADPH addition could explain these results. It must be noted that NADPH is a source of electrons for ROS generation via a proposed NADPH-oxidase reaction in spermatozoa (Aitken,



Different letters within each subpopulation category significantly differ ($P < 0.05$).

Figure 1. Effects of NADPH on the distribution of motility subpopulations of goat sperm samples



Different letters within each subpopulation category significantly differ ($P < 0.05$).

Figure 2. Effects of phenylalanine on the distribution of motility subpopulations of goat sperm samples

1997). Furthermore, NADPH appears to play a major role in sperm capacitation (Baumber *et al*., 2003; O'Flahertly *et al*., 2006). Stimulation of endogenous superoxide anion and hydrogen peroxide generation by NADPH has resulted in increased capacitation events in buffalo spermatozoa (Roy and Atreja, 2008). According to the previous authors, both O_2^- and H_2O_2 induced tyrosine phosphorylation of 95 kDa protein (p95), which is regulated by a cAMP-dependent PKA pathway. Indeed, sperm changes associated with the capacitation may include an important increase in sperm motility (Yanagimachi, 1970).

Hydrogen peroxide was generated after phenylalanine addition to bull and ram sperm showing AAAO activity in the spermatozoa of these species (Shannon and Curson, 1982b; Upreti *et al*., 1998; Alomar *et al*., 2016). It should be pointed out that the formation of H_2O_2 was reported in fresh, chilled and cryopreserved goat spermatozoa, and when phenylalanine was added to these different spermatozoa types, the H_2O_2 level was significantly raised (Alomar, 2018; 2019). In the present study, the addition of phenylalanine at either concentration reduced the values of motility parameters. In contrast, no adverse effects of phenylalanine on ram sperm motility were noted, when 1 mM of phenylalanine was added and PMOT % and VAP values were significantly raised (Alomar *et al*., 2018). In agreement with our present results, Lapointe and Sirard (1998) showed that phenylalanine had significant negative influence on bull sperms motility. These contradicted results confirm the species-specific pattern of phenylalanine effect on sperm motility, and this could be explained by different abilities of ruminants' spermatozoa to generate hydrogen peroxide formation.

Negative effects on motility values were noted when 4 mM of NADPH and phenylalanine were added. This high concentration of these oxidase substrates may be responsible for a significant increase in H_2O_2 formed by the sperm to a level causing oxidative stress. Oxidative stress is a condition associated with cellular damage and one of the most important factors contributing to the low semen quality (Bucak *et al*., 2010). Exposure of human spermatozoa to NADPH resulted in a dose-dependent generation of reactive oxygen species (ROS), which, at a critical level of intensity, induced lipid peroxidation (LPO), DNA damage and a dramatic decline in sperm motility (Twigg *et al*., 1998). An important inhibition of sperm motility after incubation with ROS was caused by a depletion of ATP and a decrease in the axonemal protein phosphorylation (De Lamirande and Gagnon, 1995).

CONCLUSION

Taken together, the data suggests that NADPH and phenylalanine have pronounced effects on goat spermatozoa motility and these effects are correlated with the concentration used. These results could be related to hydrogen peroxide generation by the spermatozoa. The addition of NADPH to semen media at the appropriate concentration can be useful as a stimulating additive for goat spermatozoa motility.

ACKNOWLEDGMENTS

The author thanks to the Atomic Energy Commission of Syria for their support while conducting the experiments.

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Slovak J. Anim. Sci., 53, 2020 (4): 205–208

ISSN 1337-9984
E-ISSN 1338-0095

USE OF DEAD CELL REMOVAL KIT FOR THE IMPROVEMENT OF RAM SEMEN QUALITY: SHORT COMMUNICATION

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ABSTRACT

The main objective of this preliminary study was to improve the ram semen quality by the removal of dead and apoptotic cells from the ejaculates. For this purpose, ram spermatozoa were incubated with the Dead Cell Removal kit and magnetically sorted using two procedures with different sample loading rate (Deplete and Depletes) by a fully automated cell sorter. Fresh semen samples (control) as well as both sorted fractions (negative and positive) were analysed for motility parameters using CASA and for the proportion of dead cells using flow cytometry. As expected, a significant increase ($P < 0.05$) in the number of dead cells and decrease ($P < 0.05$) in the spermatozoa motility were observed in the positive fractions when sorted by both procedures. However, the viability of negatively sorted spermatozoa was not improved and their motility was insignificantly decreased. In conclusion, although the presented study demonstrates the possible use of a MACS technique for the elimination of ram spermatozoa with compromised membrane, the chosen sorting procedures seem to be insufficient to obtain high purity of spermatozoa with intact membranes. More sensitive depletion programmes should be tested in further studies.

Key words: ram semen; CASA; MACS; dead cell removal; flow cytometry

INTRODUCTION

Generally, the proportion of healthy spermatozoa within the semen affects its fertilizing ability, as the increased number of spermatozoa with damaged membrane or with poor motility obviously is a reason for the decrease in fertility (Januskauskas *et al*. 2003; Dogan *et al*. 2013). Magnetic-activated cell sorting (MACS) has been well established in human assisted reproduction in recent years (Said *et al*., 2006; Oseguera-López *et al*., 2019). This technique is predominantly used to eliminate spermatozoa with externalized phosphatidylserine that is translocated from the inner to the outer plasma membrane during apoptosis (Grunewald *et al*., 2001). Annexin V MicroBead Kit (Miltenyi Biotec, Germany) was used mainly in human (Glander *et al*., 2002; Agarwal *et al*., 2009; Vendrell *et al*., 2014; Bucar *et al*., 2015) or animal (Vasicek *et al*., 2014a; Mrkun *et al*., 2014) studies with the MACS selection of apoptotic spermatozoa.

Additionally, there is another product at the market, named Dead Cell Removal Kit (Miltenyi Biotec, Germany), which according to the producer recognizes a moiety in the plasma membrane of apoptotic or dead cells and can be, thus, used for their removal from the cell suspension using MACS. We have already used this kit together with Annexin V MicroBead Kit in our previous comparable study on rabbit spermatozoa (Vasicek *et al*., 2014b).

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Received: November 14, 2020
Accepted: December 3, 2020

However, in this study, like in the above-mentioned studies, the manual MACS instruments were used for the sorting of low concentrated semen samples. Moreover, any of published papers was focused on the MACS sorting of ram semen.

Thus, in the present preliminary study an automatic magnetic cell sorter was used in order to eliminate spermatozoa with the compromised membrane from the ram semen samples thereby improving their quality.

MATERIAL AND METHODS

Five sexually mature and clinically healthy rams of Native Wallachian (n = 3) and Slovak Dairy (n = 2) sheep breeds aged 2-4 years were used in this preliminary study. They were kept in external conditions in individual stalls at the breeding facility (NPPC, RIAP Nitra, Lužianky, Slovak Republic), fed with hay bale and oats; water and mineral salt were supplied *ad libitum*. Prior to the experiment, semen samples were collected once a week by an electro-ejaculation for the duration of several weeks, as described previously (Baláži *et al*., 2020). The experimental ram sperm samples were immediately after collection transferred to the laboratory in the water bath for the subsequent processing.

Freshly collected semen samples were diluted and analysed by CASA (Sperm VisionTM, MiniTübe, Germany) for concentration (10^9 per mL), total motility (motility > 5 µm.s^{-1}) and progressive motility (motility > 20 µm.s^{-1}) of spermatozoa, as described previously (Baláži *et al*., 2020). The CASA analyses were performed again after MACS sorting in both, negative and positive fractions.

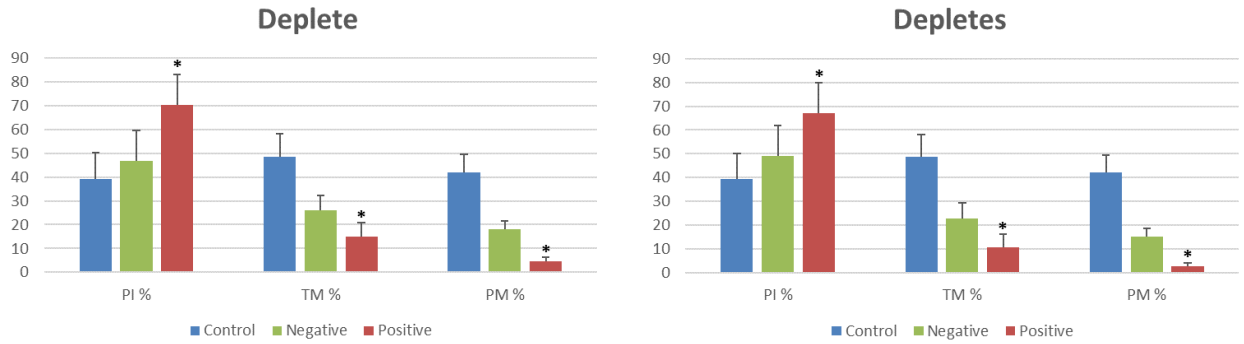
Aliquots of each semen samples (10^8 cells) were diluted in 1 ml of Dead Cell Removal kit (Miltenyi Biotec, Germany) and incubated for 15 min at room temperature according to the producer's manual. After incubation, AutoMACS Pro Separator (Miltenyi Biotec, Germany) was used to remove the dead spermatozoa from the ram semen sample. Since the commercial kit required a buffer with calcium for a proper binding of nanoparticles to the cells, HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, at pH 7.2) was used as a sheath fluid instead of the standard MACS running buffer. This instrument provides several sorting procedures. Two basic depletion programmes (Deplete and Depletes) were used in this preliminary study to sort semen samples from each ram. According to the producer's manual, first depletion procedure with loading rate at 4 mL.min^{-1} should be used in case if recovery of sample is of the highest priority. On the contrary, the second one should be used when a purity of sample is of the highest priority. This programme operates in a sensitive mode with a loading rate at 1 mL.min^{-1}.

Each fresh ram semen sample (control) as well as the negative and positive fractions obtained from MACS sorting were stained with propidium iodide (PI at 50 µg.mL^{-1}; Molecular Probes, USA) in order to determine the proportion of dead cells within the control and MACS-sorted semen samples. Stained samples (at least 10,000 cells) were immediately analysed using a FACS Calibur flow cytometer (BD, San Jose, CA, USA)

Motility parameters and the percentage of dead cells were evaluated using the SigmaPlot software (Systat Software Inc., Germany) with one-way ANOVA (Dunnett's method) and expressed as the mean ± SEM. *P*-values at $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

As far as we know, this is the first study aimed at the MACS removal of apoptotic and dead ram spermatozoa. Ram spermatozoa were sorted by fully automated cell sorter using two depletion procedures that differ in the speed of sample loading. Independently of the used sorting programme, the proportion of dead cells analysed by PI staining increased significantly ($P < 0.05$) in the positive fractions compared to control samples (Figure 1). On the other hand, the number of PI positive cells in the negative fractions did not differ from the control samples. A significant decrease ($P < 0.05$) in the total and progressive motility of spermatozoa was observed in positive fractions. However, the spermatozoa motility in negative fractions was not improved after both MACS sorting procedures and a slight decrease in both parameters, although insignificant in comparison to control samples, was noticed. No differences in the percentage of dead cells between



Deplete – sorting programme with loading rate at 4 mL.min^{-1}; Depletes – sorting programme with loading rate at 1 mL.min^{-1}; PI – percentage of dead cells stained with propidium iodide; TM – percentage of totally motile spermatozoa; PM – percentage of progressively motile spermatozoa; Control – fresh semen samples before sorting; Negative – negatively sorted spermatozoa; Positive – positively sorted spermatozoa; asterisk (*) – statistically significant at $P < 0.05$ in comparison to control sample.

Figure 1. Changes in the motility parameters and proportion of dead spermatozoa after MACS sorting of ram semen samples using two depletion programmes

negative fractions and control samples were noticed also in our previous study (Vasicek *et al*., 2014b), where the effect of Annexin V Microbeads kit and Dead Cell Removal kit on rabbit sperm quality was compared. Moreover, similarly to present study, the motility parameters of negatively sorted spermatozoa also decreased significantly ($P < 0.05$), when compared to controls.

Dead cell removal kit requires a special binding buffer that contains calcium. Thus, the principle of the staining procedure is similar to those for Annexin V Microbeads. In addition, several previous studies (Paasch *et al*., 2003; Delbès *et al*., 2013; Grunewald and Paasch, 2013; Merino-Ruiz *et al*., 2019) referred that this kit contains annexin V microbeads. We can therefore conclude that both kits are very similar in the terms of their function. Moreover, an insignificant loss of progressive motility was also noticed in the negatively sorted human sperm after MACS procedure (Paasch *et al*., 2003), whereas the positively sorted spermatozoa were almost immotile.

CONCLUSION

Present preliminary study indicates the possible use of MACS technique to remove the spermatozoa with compromised membrane from the ram semen samples. However, any of the used automatic cell sorting procedures did not significantly improve the quality of tested semen samples in the terms of their viability and motility parameters. Moreover, the chosen AutoMACS programmes seem not to be effective enough in case of the obtained purity of negatively sorted spermatozoa that could be a reason for these unsatisfying results. Anyway, more sensitive sorting procedures that are provided by the used automatic cell sorter should be tested in further studies.

ACKNOWLEDGEMENTS

The study was financially supported by the Slovak Research and Development Agency (No. APVV-17-0124), by the Scientific Grant Agency (grant no. VEGA 1/0049/19) and Educational Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the Slovak Academy of Sciences (grant no. KEGA 026SPU-4/2018).

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