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PREFACE

In this edition, Volume 27 No 2, we proudly present articles from animal and veterinary sciences including reproduction; feed technology; animal physiology; and post-harvest from scientist all over the world. The articles published in this edition are:
"Correlation between Semen Quality, Libido, and Testosterone Concentration in Bali Bulls"; "Application of Infrared Thermography as a Determinant of Sub-Clinical Mastitis in Sapera Dairy Goats"; "Effect of Supplementing Ground Leaf of Misai (*Orthosiphon stamineus*) in Diet on Growth Performance of Broiler Chickens"; "Effectivity of Dry and Liquid BS4 Enzymes in Improving Performance of Broiler Chickens Fed Different Nutrient Density Diet"; "Changes in Physiological Condition of Broiler Chickens Sprayed with Water before Transportation" and "Influence of Duck Eggshell Nano-Calcium Fortification on the Chemical Quality of Beef Sausage.

We extent high appreciation to all peer reviewers who make this journal academically high value. Hopefully, these articles would offer any benefit to readers and the end-users of technological innovation, and attract interests from scientists to contribute their papers to the Indonesian Journal of Animal and Veterinary Sciences.

Chief Editor

Bogor, June 2022

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LIST OF CONTENT

Correlation between Semen Quality, Libido, and Testosterone Concentration in Bali Bulls

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ABSTRAK

Iskandar H, Sonjaya H, Arifiantini RI, Hasbi H. 2022. Korelasi antara kualitas semen, libido dan konsentrasi testosteron pada pejantan sapi Bali. JITV 27(2):57-64. DOI: http://dx.doi.org/10.14334/jitv.v27i2.2981.

Indonesia memiliki dua Balai Inseminasi Buatan (BIB) Nasional dan lebih dari 15 Balai Inseminasi Buatan Daerah (BIBD) yang tersebar di beberapa provinsi. Pejantan unggul di BIB dan BIBD, harus memiliki libido yang tinggi dan menghasilkan semen yang berkualitas. Penelitian ini bertujuan untuk mengevaluasi dan menguji korelasi antara libido dengan kualitas semen dan konsentrasi hormon testosteron, serta potensi produksi semen beku dari pejantan sapi Bali di BIBD Sulawesi Selatan. Sepuluh ekor pejantan sapi Bali milik BIBD Sulawesi Selatan digunakan dalam penelitian ini. Koleksi semen dilakukan dua kali setiap minggu dengan evaluasi semen mengikuti protokol BIBD, bersamaan dengan itu dilakukan pengambilan sampel darah dan pengukuran libido dari masing-masing pejantan. Potensi produksi semen beku dihitung dengan mengalikan volume semen, motilitas dan konsentrasi sperma. Hasil penelitian menunjukkan bahwa kualitas semen segar dan konsentrasi hormon testosteron tidak berbeda antara pejantan sapi Bali libido tonggi dan rendah. Libido memiliki korelasi positif dengan volume semen (r= 0,52) dan motilitas sperma (r= 0,62), sedangkan konsentrasi testosteron berkorelasi negatif dengan volume semen (r= -0,65), motilitas sperma. Pejantan sapi Bali libido tinggi dan rendah memiliki potensi produksi semen beku yang baik yaitu berkisar antara 19.755 – 21.640 straw per tahun. Pejantan sapi Bali di BIBD memiliki kualitas semen segar dan konsentrasi hormon testosteron dalam kondisi normal dengan potensi produksi semen beku yang tinggi, meskipun hanya 60% sapi Bali yang memiliki libido tinggi dan 40% memiliki libido rendah.

Kata Kunci: Sapi Bali, Produksi Semen Beku, Libido, Kualitas Semen, Konsentrasi Testosteron

ABSTRACT

Iskandar H, Sonjaya H, Arifiantini RI, Hasbi H. 2022. Correlation between semen quality, libido, and testosterone concentration in Bali bulls. JITV 27(2):57-64. DOI: http://dx.doi.org/10.14334/jitv.v27i2.2981.

Indonesia has two National Artificial Insemination Centers (AIC) and more than 15 Regional Artificial Insemination Centers (RAIC) spread across several provinces. Bulls in the AIC must have a high libido and produce good quality semen. This study examines the correlation between libido with semen quality and testosterone concentration to determine potential frozen semen production from Bali bulls in South Sulawesi RAIC. Ten Bali bull were used in this study. Semen collection was carried out twice a week with semen evaluation following the RAIC protocol. At the same time, blood samples and libido measurements were carried out from each bull. The frozen semen production potential was calculated by multiplying the semen volume, motility, and sperm concentration. The results showed that the quality of fresh semen and testosterone concentrations did not differ between high and low libido of Bali bulls. Libido has a positive correlation with semen volume (r= 0.52) and sperm motility (r= 0.62), while testosterone concentration has a negative correlation with semen volume (r= -0.65), sperm motility (r= -0.60), and libido (r= -0.48). Bulls with high and low libido have good frozen semen production potential, ranging from 19,755 – 21,640 straws per year. Bali bulls in RAIC have fresh semen quality and testosterone concentrations under normal conditions, with high potential for frozen semen production, although only 60% of Bali cattle have high libido and 40% have low libido.

Key Words: Bali Bull, Frozen Semen Productivity, Libido, Semen Quality, Testosterone Concentration

INTRODUCTION

Bali cattle are known to have high environmental adaptability and good reproductive efficiency. Bali cattle are also resistant to low feed conditions, resistant to parasites, with carcasses 52.72-57.6% and good meat quality (Alwiyah et al. 2016; Gunawan et al. 2016; Jakaria et al. 2017). Mating these cattle is mainly done by artificial insemination (AI), using frozen semen produced by the AI centre (AIC). Indonesia has two National AICs, and more than 15 Regional Artificial Insemination Centers (RAIC) spread across several

provinces. Bulls in the AIC must have a high libido and produce high semen quality. Thus, bulls in AIC and RAIC have to be superior, so they can make a good quality frozen semen to support the success of AI in the field and improve the quality of Bali cattle in the area. The bull fertility rate is related to sperm motility (Zubair et al. 2015), libido (Chenoweth 2021), sperm production capacity, and testosterone hormone (Singh et al. 2014).

The problem in the selected Bali bull is their libido. Libido is vital in bull because it will speed up the process of semen collection and also accelerate the process of semen freezing. Libido, or desire to mate, is manifested in the sexual behaviour of bulls and is naturally more active in approaching in estrus females (Abell et al. 2017). With various physiological mechanisms, bull desires mediate libido responses and experiences acquired by bulls (Kowalczyk et al. 2021). Bos taurus has a high libido (Le Danvic et al. 2015), while Bos indicus have a lower libido (Manegassi et al. 2021) compared to the Crossbreed (Mukhopadhyay et al. 2010). Bali cattle (*Bos javanicus*) are native Indonesian breeds with a lower libido than Maduras cattle (Susilawati 2011). For collecting semen purposes in AIC and RAIC, they use a live bull or a dummy. Therefore, the bull libido in AIC is essential, and libido is known to be influenced by the testosterone hormone (Perumal et al. 2020).

Testosterone is the primary androgen required for spermatogenesis in the testes and is responsible for maintaining secondary sexual characteristics and libido (Senger 2012). Testosterone is produced by Leydig cells (Kowalczyk et al. 2021) under the influence of luteinising hormone (LH) from the anterior pituitary (Wang et al. 2021). The testosterone concentration in Bos taurus aged 6 - 7 years is 35.16 ng/mL (Baharun et al. 2021), and in *Bos indicus*, 14.86 ng/mL (Littlejohn et al. 2017). Information on testosterone concentrations in native and local cattle has been reported by Gholib et al. (2020) in Aceh cattle (4.39 ng/mL), Kuantan cattle 2.82±1.99 ng/mL (Anwar & Jiyanto 2019), Ongole crossbreeds 6.14 ng/mL (Widyaningrum et al. 2015) and Bali cattle ranging from 4.57-4.79 ng/mL (Syarifuddin et al. 2017). Male fertility can be determined by analysing the testosterone hormone (Singh et al. 2014).

The relationship between testosterone with sperm quality and sperm motility has not been proven (Rajak et al. 2014a; Rajak et al. 2014b). Bali cattle producing frozen semen's productivity is essential and closely related to the value of semen volume, sperm motility, and sperm concentration (Nugraha et al. 2022). Information regarding the correlation of semen quality, libido, and testosterone concentration in Bali bulls is limited. Bulls in RAIC varied in libido and semen quality. Some bulls show a high libido with medium semen quality; on the other hand, some bulls exhibit low libido but have excellent semen quality. There are also bulls with low libido and low semen quality. Libido and semen quality are essential in RAIC. Therefore, this study aims to evaluate semen quality, libido, and testosterone concentration and examine the relationship between these three variables in Bali bulls' reproductive performance.

MATERIALS AND METHODS

Ethical approval

The Animal Ethics Commission, Hasanuddin University, has approved this research number 302/UN4.6.4.5.31/PP36/2021.

Study period and location

This study was conducted from October 2020 to July 2021. Semen and blood samples were obtained from RAIC South Sulawesi. Sperm motility, volume, and concentration were evaluated at the South Sulawesi RAIC. Blood sample plasma preparation was performed at the South Sulawesi RAIC. The testosterone concentration was measured using the enzyme-linked immunosorbent assay (ELISA), performed according to the manufacturer's protocol at the Primate Research Center, IPB University.

Research animal

This study used ten Bali bulls aged 5-10 years belonging to South Sulawesi RAIC. All bulls were reared following the Standard Operating Procedures (SOP) of RAIC. The RAIC kept Bulls in 2.5x2 m cages equipped with feed and drink containers. Feeding in fresh forage 10% of body weight and concentrate as much as 2 kg per day given twice a day, in the morning and evening, drinking water provides ad libitum.

Semen collection and evaluation

Fresh semen was collected twice a week using an artificial vagina by the RAIC bull master. Semen collection is carried out between 06.00-10.00 AM. The collected semen was immediately brought to the laboratory for macroscopic and microscopic evaluation, referring to Arifiantini (2012). The data displayed is only semen volume, sperm motility, and sperm concentration. These three variables are the data needed to calculate the number of frozen semen produced by each bull.

Semen volume was measured visually on a semen collection tube with mL units. The sperm motility assessment by mixing 10 µL of semen with 40 µL of saline solution. After homogenising, 5 µL was taken, dripped onto the object glass, and covered with a cover glass. The evaluation was done using a binocular microscope (Olympus CX31 RTSF). Calculation of sperm concentration using a photometer SDM6 (Minitub, Germany).

Libido assessment

This study evaluates bull libido during semen collection and records the reaction time of the bull. The mounting enthusiasm was scored during mounts and service. Mounting enthusiasm was a score from -2 to + 2 (Table 1).

Table 1. Mounting enthusiasm of Bali bulls at South Sulawesi RAIC

Score	Criterion	Libido
-2	Bull does not mount	Low
-1	Bull mounts by sliding	Low
0	Bull mounting between sliding and jumping	Moderate
+1	Bull mounts by jumping	High
+2	Bull jumps with great enthusiasm	High

Modification from Hoflack et al. (2006)

Testosterone concentration assessment

Approximately 3-5 mL of blood samples were collected using a 5 mL vacutainer blood collection containing EDTA (three fingers, USA) through the vena jugularis region of the bulls. The blood was centrifuged at 3000 pm at room temperature for 10 minutes. Blood plasma was collected, put into a microtube, and stored at -20°C until analysis. Testosterone analysis was carried out using the Bovine testosterone ELISA kit method (Signalway antibody, #EK0019). Blood plasma was diluted in a ratio of 1:4 using distillate water. Standard solutions with concentrations ranging from 0.2 to 16 ng/mL. Samples and standard solutions were transferred (25 µL each) into ELISA microplate wells (Duplo performed), then added with conjugate enzymes (except blanks) and covered with cling film.

The mixture was homogenised using a vortex for 10 seconds and incubated at room temperature for 60 minutes. Afterwards, the microplate wells were washed 3-4 times with 300 µL of washing solution each, added 200 µL of the substrate, and incubated for 15 minutes (room temperature). The reaction stopped by adding 100 µL of stop solution to each well. The absorbance was read using an ELISA reader at 450 nm (Dasrul et al. 2020; Hafizuddin et al. 2020).

Data analysis

Independent-Sample T Test was applied to the differences between treatments ($P<0.05$), which were considered statistically significant. The data on fresh semen quality were analysed descriptively. The Pearson correlation test analysed the correlation between semen quality, libido, and testosterone concentration. Data were analysed using SPSS version 20 software.

RESULTS AND DISCUSSION

The average semen volume of bulls with high libido was 6.83±0.44 mL and bulls with low libido was 6.71±0.22 mL. The average sperm motility of bulls with high libido was 72.49±0.93 and bulls with low libido was 73.74±1.42. The sperm concentration of bulls with high libido was 1365.90±76.65 and bulls with low libido was 1247.70±69.28 (Table 2). This study showed only three variables of semen quality associated with frozen semen production. Semen volume, sperm motility, and sperm concentration were not significantly different between bulls with high and low libido ($P>0.05$). The high and low percentage of motility sperm in semen is affected by age, individuals, season, and temperature. Semen volume, sperm motility, and sperm concentration are considered essential indicators of sperm quality and fertility. Ismaya (2014) reported that temperatures influence the motility of sperm: cold temperatures will inhibit motility, while hot temperatures will increase motility. Increasing the sperm concentration could improve the chances of conception by increasing the number of normal sperm until the required threshold for conception is reached (Morrell et al. 2018). Murphy et al. (2013) reported that a higher concentration of fresh bull semen might cause an increase in oxidative stress. The semen volume in cattle, according to Ax et al. (2000), is 7-10 mL per ejaculate, with sperm concentration ranging from $1000x10^6$ to $1500x10^6$ sperm/mL. Individual influences on the fresh semen quality of Bali bulls have been reported by Indriastuti et al. (2020). Moreover, the sperm motility of the Bali bull at South Sulawesi RAIC is below sperm motility at Baturiti RAIC (Indriastuti et al. 2020). However, it still meets the quality requirements of fresh semen state of the Minister

Table 2. The quality of the fresh semen samples and testosterone hormone concentration from Bali bulls at South Sulawesi RAIC

Libido	Variable			
	Semen volume (mL)	Sperm Motility (%)	Sperm concentration (× 106 mL)	Testosterone hormone concentration
High	6.83±0.44[a]	72.49±0.93[a]	1365.90±76.65[a]	4.87±1.27[a]
Low	6.71±0.22[a]	73.74±1.42[a]	1247.70±69.28[a]	2.99±0.33[a]

Same superscript letters following numbers in the same column indicate non-significant difference (P< 0.05)

Table 3. Correlation between semen quality, libido, and testosterone hormone concentration of Bali bulls

Variable	Semen volume	Sperm motility	Sperm concentration	Libido	Testosterone hormone concentration
Semen volume	1				
Sperm motility	0.107	1			
Sperm concentration	-0.012	0.194	1		
Libido	0.525*	0.629*	0.109	1	
Testosterone hormone concentration	-0.659*	-0.602*	-0.026	-0.485	1

*Shows a significant relationship between pairs of variables (P<0.05)

of Agriculture of Republic of Indonesia Number 10/Permentan/PK.210/3/2016 in 2016). The Indonesian National Standard for frozen bovine semen is as the SNI number: 4869.1:2017 (BSN 2017) also states that sperm motility of fresh semen to be processed into frozen semen must have sperm motility >70%. Semen quality is influenced by several factors such as age (Hapsari et al. 2018), season and temperature (Soren et al. 2016), semen collection interval (Sankhi et al. 2019), and individual variations (Indriastuti et al. 2020), and genetics (Mohammed & Ahmed 2017).

In this study, Bali bulls at south Sulawesi RAIC show that 60% have a high libido, while 40% show a low libido. The libido of Bali bulls at South Sulawesi RAIC differs due to environmental conditions, nutrition, age, and experience of bulls. Factors influencing libido and mating ability include the clump, number of estrus females, the environment (Hastono & Praharani 2014), and bull age (He et al. 2014). Libido is also influenced by genetic and management factors, environmental conditions, and bull age. The libido of the Brahman cross and Friesian Holstein cattle is higher than the Red Chittagong breed (Islam et al. 2018). Older bulls are more likely to have physical or pathological conditions that reduce libido (Masoumi et al. 2011).

Testosterone hormone concentrations of Bali bulls with high and low libido showed not significant different (Table 2), the blood collection was carried out simultaneously. The bull's reaction to riding the female is naturally related to the presence of stimulation from the female. The testosterone concentration in this study was within normal ranges. Therefore, semen quality was also categorised as good. Bull libido level is affected by testosterone concentration. Testosterone concentrations and semen quality vary throughout the year (Chacur et al. 2013). Furthermore, testosterone concentrations are higher during early animal growth (Gulia et al. 2010) and accelerated physical and testicular growth (Chacur et al. 2018). Testosterone concentration in Holstein bulls aged 8-9 months is 0.49 ng/mL (Gholami et al. 2010); in Aceh bulls, cattle aged 4-5 years are 5.14-13.06 ng/mL (Dasrul et al. 2020). Simmental bull 8-10 years old were 13.39 to 23.27 ng/mL (Baharun et al. 2021). Low libido can be caused by low testosterone levels (Rajak et al. 2014a; Rajak et al. 2014b).

The correlation between semen volume, sperm motility, sperm concentration, and testosterone hormone concentration in Bali bulls is presented in Table 3. In this study, sperm motility has a low positive correlation with semen volume and a low negative correlation with sperm concentration. Bull's libido positively correlates with semen volume, sperm motility, and low correlation with sperm concentration. However, semen volume with testosterone concentration had a strong negative relationship (-0.65). During semen collection, the bull master performs several teases. Teasing stimulates the secretion of seminal plasma, mostly from vesicular glands, which consists of 75% of semen volume (Garner & Hafez 2016).

Table 4. Libido and productivity of Bali bulls at South Sulawesi RAIC ordered from highest to the lowest production

Libido	Number of motile sperm per ejaculate ($x10^6$)*	Number of straws per ejaculate (pieces)**	The potential of frozen semen production annually (pieces)***
High	6762.66	270.50	21640.00
Low	6173.56	246.94	19755.20

*The result of multiplying semen volume x sperm concentration x % sperm motility; ** Represents the number of straws produced in one ejaculate, obtained by dividing the number of motile sperm in one ejaculate by 25 $x10^6$ (insemination dose of frozen bull semen); *** Represents the total straw production for one year, assuming one year is 40 weeks of collection, twice a week (40 x 2 = 80 ejaculates/year); The calculation is multiplying 80 by the number of straw production per ejaculate

Furthermore, teasing also stimulates the secretion of the bulbourethral gland, which secretes an alkaline fluid, cleaning the urogenital tract of the bull. As we all know, urine and semen come out in the same channel; therefore, the alkaline fluid secreted by the bulbourethral gland cleans the urogenital tract, previously passed by urine. This condition is proved by good sperm motility. Mahmood et al. (2014) reported that the age of the bull had a significant correlation with semen volume (r= 0.93) negative correlation with sperm concentration (r= -0.87). All bulls in this study are at the productive age from five to ten years. Availability of sufficient testosterone will cause an increase in testosterone accumulation. As a result, the area activates the metabolism brain and regulates libido to be active (Indrayanto 2021). Andersson (1992) reported a significant correlation between testosterone concentration and fertility in Ayrshire bulls, followed by high sperm motility and sperm concentration. On the other hand, Chacur et al. (2018) reported an increase in serum testosterone levels, testes' growth and bodyweight of young Brahman bulls at 12 and 14 months.

This result exhibit a high negative correlation between semen volume and testosterone concentration. In contrast, Dasrul et al. (2020) reported a relationship between testosterone and semen volume in Aceh bull. In small ruminants such as Bligon goats and Anglo-Nubian x Etawah Grade, Rachmawati et al. (2014) and Hafizuddin et al. (2020) reported similar to Dasrul et al. (2020). One of the testosterone hormone functions is to regulate the work of the accessory glands, which produce seminal plasma (Senger 2012). Testosterone concentration is related to semen quality (Malik et al. 2018), which is plasma testosterone concentrations remain high during active semen production

The testosterone concentration negatively correlates with sperm motility (-0.60). According to Ansari et al. (2007), testosterone did not correlate with motility and sperm concentration in buffalo. Moreover, Souza et al. (2011) state that mass movement and sperm motility had low relationship with testosterone concentration in Simental bull aged 33-41 months. In this study, testosterone and sperm concentrations were negatively very low correlated (-0.02). This condition was also reported by Rajak et al. (2014) that testosterone concentration was not related to sperm concentration of crosses bulls. Testosterone plays a role in spermatogenesis, especially at the stage of spermiogenesis. Furthermore, sperm concentration is determined by mitosis at the time of spermatocytogenesis. Spermatocytogenesis is controlled by Follicle Stimulating Hormone (Senger 2012).

Testosterone concentration and libido had a moderately negative correlation (-0.48). This moderate negative relationship means that the higher the testosterone concentration, the lower the libido. Previous studies have reported that testosterone concentrations are not associated with libido (Schallenberger et al. 1991; Sekasiddhi & Buban 1997), semen quality (Souza et al. 2011) and only have a low association with mass movement and sperm motility (Santos et al. 2004; Souza et al. 2011). Other studies reported that the libido of bulls is significantly related to semen quality and fertilization rate (Singh et al. 2019; Kowalczyk et al. 2021). The testosterone concentration varies within each species, individual bull, age, season, and environment (Rajak et al. 2014b). Bulls' age may also influence this result, the range of bulls' age in this study was 5 to 10 years old, and each had a different testosterone concentration between bull with high and low libido.

This study shows that libido has a moderate relationship with semen volume (0.52) and a strong relationship with sperm motility (0.62). Several studies reported that libido scores were strongly associated with semen volume, sperm motility, and sperm concentration in Brahman, Friesian Holstein, Red Chittagong bulls (Islam et al. 2018), and Sahiwal bulls (Singh et al. 2015). This study showed that bulls with high libido react faster to mount and ejaculation than bulls with low libido. During teasing and courtship, the accessory glands will secrete a seminal plasma.

Semen volume, sperm motility, and sperm concentration, if each variable is seen separately, cannot describe the potential of each bull. The multiplication of these three variables will explain the productivity of

each bull in producing frozen semen (Table 4). Table 4 shows that bulls with high and low libido at South Sulawesi RAIC are superior because they have a high frozen semen production, 19 to 26 thousand straws yearly. Semen production of native or local cattle in the Roadmap for Self-Sufficiency of Indonesian superior bulls (Ditjennak 2018), a minimum of 7500 straws per year. Table 4 shows bulls with high and low libido had good semen quality and high productivity in frozen semen production. Knowledge in evaluating bull libido and frozen semen productivity is fundamental to calculating the ideal population structure in each RAIC. The RAIC could determine their frozen semen production target per year to figure out the bulls needed to reach it.

CONCLUSION

This study concludes that Bali bulls in RAIC with high and low libido has no effect on semen quality and testosterone hormone testosterone. However, there is a positively correlates with semen volume and sperm motility, while testosterone hormone concentration negatively correlates with semen volume, sperm concentration, and libido

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Application of Infrared Thermography as a Determinant of Sub-Clinical Mastitis in Sapera Dairy Goats

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ABSTRAK

Pamungkas FA, Purwanto BP, Manalu W, Yani A, Sianturi RG. Penggunaan termografi inframerah sebagai penentu mastitis sub-klinis pada kambing perah Sapera. JITV 27(2):65-73. DOI: http://dx.doi.org/10.14334/jitv.v27i2.3059.

Penelitian ini mengevaluasi potensi penggunaan hasil pengindraan termografi inframerah (IRT) dibandingkan dengan somatic sell count (SCC) dan tes reagen mastitis dari IPB University (IPB-1) pada deteksi infeksi mastitis sub-klinis kambing perah Sapera. Sebanyak 8 ekor kambing perah sapera dengan rataan berat badan 35-40 kg dievaluasi selama masa laktasi. Parameter yang diobservasi termasuk produksi susu, karakteristik psikokimia, penginderaan SCC, IPB-1 dan IRT putting. Data yang diperoleh kemudian dianalisis mengikuti prosedur MIXED dan CORR dari SAS. Hasil penelitian menunjukan bahwa karakteristik psikokimia susu (lemak; non-fat solid; laktosa; protein; titik beku; pH), SCC dan IRT berbeda ($p<0,05$), khususnya hasil mastitis antara normal dan +3. Rataan produksi susu dengan normal hingga skore +2 mastitis selama laktasi sebesar 1,281±253 ml/hari, sementara pada hasil +3 mastitis adalah sebesar 957±250 ml/hari. Korelasi ditemukan pada ambing kanan dan kiri ini merupakan bukti penting yang menunjukan bahwa mengkombinasikan hasil evaluasi parameter dengan metode IRT dengan SCC dan IPB1-SCC (r=0.70-0.74), IPB1-IRT (r=0.70-0.71), dan SCC-IRT (r=0.62-0.65). Hal ini merupakan bukti penting yang menunjukan bahwa mengkombinasikan parameter hasil tes IRT dengan SCC dan IPB-1 dapat bermanfaat untuk skrining mastitis subklinis pada kambing perah.

Kata Kunci: Kambing, Inframerah, Mastitis, Sel Somatik, Termografi

ABSTRACT

Pamungkas FA, Purwanto BP, Manalu W, Yani A, Sianturi RG. Application on infrared thermography as a determinant of sub-clinical mastitis in Sapera dairy goats. JITV 27(2):65-73. DOI: http://dx.doi.org/10.14334/jitv.v27i2.3059.

Application of infrared thermography (IRT) sensing results versus somatic cell count (SCC) and mastitis test reagent from Bogor Agricultural University (IPB-1) was evaluated in this study for infection detection in dairy goats with subclinical mastitis. Eight Sapera dairy goats with a 35-40 kg live weight were evaluated throughout their lactation. The parameters observed including milk production, physicochemical characteristics, SCC, IPB1, and IRT sensing in the udder. The collected data were analysed using MIXED and CORR procedures from SAS. Results showed that the physicochemical characteristic of milk (fat, non-fat solids, lactose, protein, freezing point, pH), SCC and IRT were significantly different ($P<0.05$), especially the test results for mastitis between normal and +3. The average production of goat milk with a normal until +2 mastitis test score during lactation was 1.281±253 ml/day, while a mastitis test score of +3 was 957±250 ml/day. A positive correlation was found in both the left and right udder of IPB1-SCC (r=0.70-0.74), IPB1-IRT (r=0.70-0.71), and SCC-IRT (r=0.62-0.65). This is substantial evidence that combining IRT results with SCC and IPB1 parameters can be valuable for screening subclinical mastitis in dairy goats.

Key Words: Goat, Infrared, Mastitis, Somatic Cells, Thermography

INTRODUCTION

Mastitis is a term used to describe inflammation of the mammary gland's parenchymal tissue, characterised by physical, chemical, and microbiological changes in the milk produced (Radostits et al 2007). In terms of livestock production, mastitis results in a decrease in milk production, changes in milk quality, an increase in medical costs, labour, and the number of livestock released (Saleh and Faye 2011). Mastitis is divided into clinical, subclinical, and chronic mastitis. Clinical mastitis is derived from the presence of inflammatory signs (such as redness, heat, swelling, pain, and loss of udder function); subclinical mastitis is characterised by

changes in milk composition without signs of severe inflammation of the udder. At the same time, chronic mastitis is characterised by an inflammatory process that can continue from one lactation to the next (Awale et al 2012).

Clinical mastitis causes economic losses due to decreased or discarded milk production. However, subclinical mastitis causes a more significant loss because the continued presence of microorganisms in the mucosa during one or several lactation periods causes a progressive loss of epithelial secretion, thereby reducing milk production (Srivastava et al 2015). Due to this impact, many studies have been directed toward diagnosing and detecting mastitis, including the California Mastitis Test (CMT), Somatic Cell Count (SCC), and enzymatic analysis (Viguier et al 2009). So far, milk somatic cell count has been used as an indicator of udder health. It has been included in selection indexes of different countries to reduce the susceptibility to mastitis (Weigel & Shook, 2018). Somatic cells are epithelial cells that come out of the lining of the mammary glands to secrete milk. Their number will increase due to the presence of pathogens as indicators of infection in milk (Madouasse et al 2012). SCC values lower than 1×10^5 cells mL^{-1} of milk indicates good milk quality, whereas milk infected with bacteria can cause an increase in SCC to above 1×10^6 cells mL^{-1} (Bytyqi et al 2010). However, this technique is subjective, time-consuming and labour-intensive, and less accurate for the detection of early signs of disease and has not been scientifically validated for the detection of clinical mastitis, especially in automated milking systems (Hovinen et al 2008) so that a detection method is needed without having to do milking but can detect early signs of mastitis before the physical, chemical, and microbiological changes in the milk produced.

Infrared thermography (IRT) is a non-invasive sensing method used to measure heat transfer and blood flow changes by detecting changes in body temperature (Nääs et al 2014). Kunc et al (2007) reported that the udder affected by mastitis had a high temperature even before clinical symptoms. Recently, there has been speculation regarding the application of infrared thermography (IRT) to obtain udder surface temperature and use it as a tool for mastitis diagnosis (Berry *et al.* 2003). Based on this background, this study aimed to examine the use of non-invasive and non-milking IRT methods in the early detection of mastitis in dairy goats.

MATERIALS AND METHODS

This research was conducted at the Indonesian Research Institute for Animal Production (IRIAP), located at an altitude of 450 to 500 m above sea level with the potential for rainfall between 3500 to 4000 mm $year^{-1}$. The air temperature in the cage ranged from 20.81-31.59 °C with a relative humidity of 47.19-99.82 % and a wind speed of 1.18-2.02 m/s. The use of experimental animals has obtained approval from Komisi Kesejahteraan Hewan Balitbangtan (KKHB), Ministry of Agriculture of the Republic of Indonesia, with registration number: Balitbangtan/Balitnak/ Rm/04/2019.

Animals

Eight Sapera dairy goats with a 35-40 kg live weight were evaluated throughout their lactation, kept in individual cages measuring 1.6 × 1.0 m^2. The feed given was in the form of commercial concentrate elephant grass silage, each 1400 g/day; the reference for the feeding amount was based on the results of pre-research conducted on the Sapera dairy goats. Feed was given twice a day, namely at 07.00 and 15.00 WIB. Provision of drinking water is made ad libitum by using a drinking bucket provided in each cage. Milking was done manually in the morning at 08.30 WIB.

Experimental design

Goats are kept during lactation (about six months). Individual milk production was recorded every day, while milk samples from each goat for observation were taken every week for two months of lactation. Milk samples were obtained immediately after taking the IRT sensing results. The parameters observed in this study included measurements of physicochemical characteristics, the number of somatic milk cells in each half udder, mastitis score using reagent from Bogor Agricultural University (IPB-1), and the result of IRT sensing of Sapera dairy goats on the udder.

Physicochemical characteristics of milk

Samples for somatic cell count were obtained from each nipple separately and cooled immediately, then transported in a box equipped with ice for immediate analysis. Samples were stored at -20 °C until further testing. Analysis of milk composition included fat content, solids non-fat (SNF), specific gravity, lactose, salts, protein, add water, freezing point, and acidity (pH) using MCC lactoscan (Milkotronic Ltd., Bulgaria).

Somatic cell count

Calculation of the number of somatic cells (SCC) using the Breed method was carried out by taking 0.01 ml of milk sample (using a Breed pipette), which was then distributed over a 1 cm^2 area (on a fat-free object glass). The preparations were awaited to dry, then fixed

over the flame. The milk fat was dissolved by immersing the object-glass in alcohol ether for two minutes and shaking it. The preparations were then stained with methylene blue Löffler for 1-2 minutes. The trials were then rinsed with water and immersed in 96% alcohol to remove the residual dye. After drying, the number of SCC ml^{-1} cells could be counted with a microscope with 1000 x magnification and observed for 30 fields of view. The formula calculates SSC:

$$\frac{SCC}{ml} = mycroscopy\ factor\ x\ a$$

Where microscope factor is 10,000/μr^2 equal to 393,174 (microscopy model type MC300); a is means of somatic cells from 30 fields of view; and r is radius area of view (mm).

Mastitis test score

Mastitis test was carried out using an IPB-1 reagent (Faculty of Veterinary Medicine, IPB University). 2 ml of milk sample was mixed with 2 ml of IPB-1 reagent and homogenised for 15-30 seconds. The interpretation of the results obtained was grouped into five scores; namely, N: average or no reaction, T: trace or slightly slimy, tended to disappear with stirring, +1: dirty or thickened but did not form a gel, +2: gelled, moved as mass during mixing, +3: the gel forms a convex surface and adheres to the bottom of the holding cup. Scores of +2 or +3 are indicative of mastitis, according to the directions of Shearer and Harris (2003).

Infrared Thermography

IRT sensing results using an infrared camera brand FLIR A320 (FLIR Systems Co. Ltd., St Leonards, NSW, Australia) with an emissivity coefficient equal to 0.98. The results of the IRT sensing of each animal were taken on the udder (right and left udder). Thermal images of the udder were taken at a distance of 1 m from the skin of the udder. Udder temperature was analyzed by ThermaCAM Researcher Professional 2.10. ThermaCAM Researcher Professional is a robust real-time digital storage, measurement, and analysis software. Extremely versatile, researcher digitally stores and retrieves static and real-time infrared images, live IR digital video sequences, dynamic high-speed events and data directly from the FLIR IR camera allowing in-depth and precise analysis of thermal events.

Statistical analysis

Collected physicochemical properties, milk somatic cell count, mastitis test, and IRT sensing results were analysed using the MIXED and CORR procedure from SAS (V. 9.1; SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Average milk quality, number of somatic cells, and results of IRT sensing on the right and left udders of the Sapera goat for each mastitis test score are presented in Table 1. In contrast, the description of the results of IRT sensing on the goat udder is illustrated in Figure 1. Several analysed parameters showed no difference in specific gravity between mastitis test scores ($P>0.05$). In contrast, other parameters, namely fat, SNF, lactose, protein, freezing point, pH, SCC, and IRT, were significantly different ($P<0.05$), especially between normal condition mastitis test scores with +3. This is due to damage to udder tissue infected, mastitis-causing an increase in SCC and changes in milk composition. Vural et al (2016) reported that infection due to mastitis would be transmitted from blood to milk so that there is an increase in the amount of protein, enzymes, and some milk minerals that cause changes in milk composition. Furthermore, efforts to balance the osmotic effect of milk due to infection require the role of mineral and lactose levels, which result in a decrease in mineral and lactose levels (Abdelgawad et al 2016). Several previous studies have also reported a reduction in milk lactose (Sharma et al 2014) and freezing point (Akdag et al 2017), which can be used as indicators of subclinical mastitis Further, Caboni et al (2017) reported that milk with high SCC had higher fat and protein content with a lower freezing point when compared to milk with low SCC.

Milk fat content of uninfected goats (4.41±1.28%) decreased compared to goats infected with mastitis category +3 (2.04±0.84%). Similar results were also reported by Kifaro et al (2009) on dairy goats. This is because the milk infected with mastitis has a very high increase in lipase enzyme activity resulting in the breakdown of milk fat and the release of free fatty acids that cause an unpleasant taste in milk (Uallah et al 2005). Likewise, the protein level of uninfected goats' milk (4.10±0.15%) decreased compared to goats infected with mastitis in the +3 category (3.89±0.14%). The same results were also reported by Khan and Khan (2006) in dairy goats. In milk infected with mastitis, there will be an increase in the proteolytic enzyme activity (plasmin), which causes extensive damage to milk proteins in the udder.

SNF levels also showed that goat's milk with a mastitis test score of +3 (8.24±0.29%) was lower than the normal mastitis test score (8.65±0.32 %), a decrease in milk SNF in infected goat udders. Dependent on damage to udder tissue by invading pathogens leading to the reduction in the synthetic activity of the mammary glands (Ben Chedly et al 2010). Similarly,

Table 1. Average quality milk of Sapera dairy goat for each mastitis test score

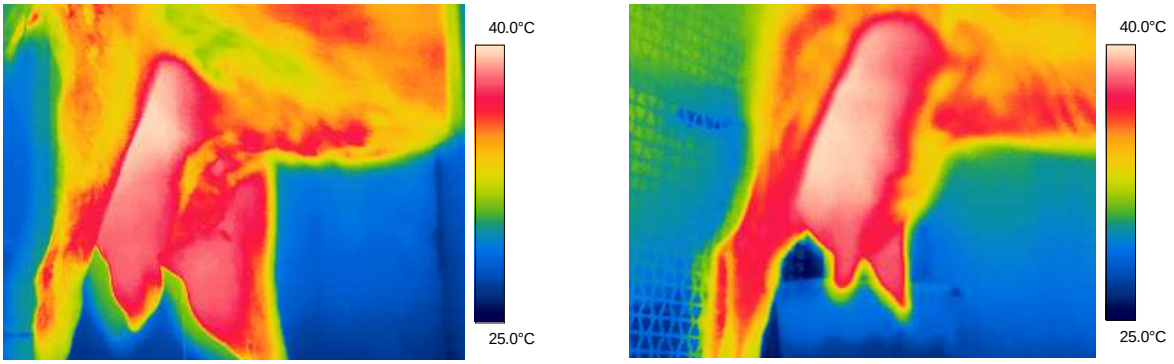
Parameter	Normal		Trace		+1		+2		+3	
	Udder									
	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left
Fat (%)	4.41±1.28^{a}	4.26±0.80^{ab}	3.34±1.03^{ab}	4.32±1.58^{ab}	3.80±1.72^{ab}	3.29±0.72^{ab}	3.06±1.05^{bc}	3.19±0.82^{abc}	3.28±0.48^{ab}	2.04±0.84^{c}
SNF (%)	8.65±0.32^{ab}	8.88±0.20^{a}	8.77±0.29^{a}	8.48±0.34^{abcd}	8.58±0.28^{abc}	8.56±0.44^{abc}	8.67±0.48^{ab}	8.09±1.05^{d}	8.17±0.23^{cd}	8.24±0.29^{bcd}
Specific gravity (g/ml)	1.03±0.000^{a}	1.03±0.000^{a}	1.03±0.003^{a}	1.03±0.000^{a}	1.03±0.002^{a}	1.03±0.002^{a}	1.03±0.004^{a}	1.03±0.004^{a}	1.03±0.002^{a}	1.03±0.000^{a}
Lactose (%)	3.97±0.14^{ab}	4.00±0.09^{a}	3.94±0.13^{ab}	3.82±0.15^{bcd}	3.86±0.12^{abc}	3.85±0.20^{abc}	3.82±0.13^{bcd}	3.81±0.15^{bcd}	3.67±0.10^{d}	3.70±0.13^{cd}
Salts (%)	0.64±0.02^{ab}	0.66±0.01^{a}	0.65±0.02^{a}	0.62±0.03^{abc}	0.63±0.02^{abc}	0.62±0.03^{abc}	0.63±0.04^{abc}	0.60±0.07^{c}	0.60±0.02^{c}	0.60±0.02^{bc}
Protein (%)	4.10±0.15^{ab}	4.22±0.10^{a}	4.16±0.14^{a}	4.02±0.17^{abcd}	4.07±0.13^{abc}	4.06±0.22^{abc}	4.10±0.23^{ab}	3.84±0.50^{d}	3.86±0.11^{cd}	3.89±0.14^{bcd}
Add water (%)	7.65±4.01^{bc}	6.11±1.71^{c}	6.49±2.96^{c}	9.65±3.72^{abc}	8.37±2.94^{bc}	7.52±4.83^{bc}	8.41±4.18^{bc}	13.84±12.37^{a}	12.29±2.58^{ab}	11.66±3.24^{ab}
Freezing point (°C)	-0.48±0.02^{bcd}	-0.48±0.01^{d}	-0.48±0.01^{d}	-0.46±0.01^{abcd}	-0.47±0.01^{bcd}	-0.48±0.02^{bcd}	-0.48±0.02^{cd}	-0.44±0.06^{a}	-0.45±0.01^{ab}	-0.45±0.01^{abc}
pH	6.92±0.04^{ab}	6.92±0.06^{a}	6.90±0.04^{abc}	6.90±0.09^{abc}	6.86±0.13^{abcd}	6.80±0.07^{bcde}	6.78±0.08^{de}	6.80±0.08^{cde}	6.75±0.03^{de}	6.74±0.03^{e}
SCC (x103)	321±72^{b}	259±70^{b}	356±70^{b}	278±73^{b}	477±121^{b}	422±119^{b}	749±119^{b}	604±131^{b}	2690±1384^{a}	2120±938^{a}
IRT (°C)	37.24±0.18^{b}	37.32±0.28^{b}	37.27±0.57^{b}	37.44±0.30^{b}	37.42±0.37^{b}	37.37±0.39^{b}	38.12±0.83^{a}	38.43±0.34^{a}	38.34±0.28^{a}	38.62±0.34^{a}

Different superscripts on the same line for each mastitis test score showed significant differences (P<0.05). SNF= Solid non-fat, SCC= somatic cell count, IRT= infrared thermography

Table 2. Correlation coefficient between IRT sensing results and milk quality, mastitis test scores, and the number of somatic cells in udder

Variable	IPB1	Production	Fat	SNF	Specific gravity	Lactose	Salt	Protein	Add water	Freezing point	pH	SCC
Production	-0.67											
Fat	0.40	-0.53										
SNF	-0.44	0.34	-0.40									
Specific gravity	-0.11	0.29	-0.35	0.63								
Lactose	-0.53	0.39	-0.52	0.84	0.39							
Salt	-0.46	0.36	-0.49	0.96	0.63	0.84						
Protein	-0.45	0.35	-0.44	0.99	0.63	0.86	0.97					
Add water	0.41	-0.22	0.16	-0.97	-0.52	-0.79	-0.90	-0.96				
Freezing point	0.38	-0.24	0.20	-0.98	-0.57	-0.79	-0.91	-0.96	1.00			
pH	-0.67	0.56	-0.55	0.31	0.22	0.31	0.35	0.33	-0.20	-0.20		
SCC	0.72	-0.62	0.25	-0.31	-0.11	-0.38	-0.31	-0.32	0.29	0.28	-0.54	
IRT	0.71	-0.32	0.22	-0.28	-0.15	-0.34	-0.29	-0.30	0.33	0.24	-0.49	0.64

*P<0.05, **P<0.01, ns= no significant. SNF= Solid non fat, SCC= somatic cell count, IRT= infrared thermography



(A) (B)

Figure 1. The description of IRT sensing results on goat udders with a normal milk mastitis test score (A) and a mastitis test score of +3 (B)

lactose levels in goat's milk with a normal milk mastitis test score (3.97±0.14%) decreased if the goat was infected with mastitis with a mastitis test score of +3 (3.70±0.13%). This is possible because lactose is synthesised in udder gland cells from glucose and galactose. Still, during inflammation, the secretory activity of cells is reduced due to damage to epithelial cells by leukocytes (Coulona et al 2002). The degree of acidity (pH) was lower in infected goat's milk with a mastitis test score of +3 (6.74±0.03%) than in goat's milk under normal conditions (6.92±0.04%), where these changes are associated with increased permeability of epithelial cells in the udder resulting in the transfer of components such as citrate and bicarbonate from blood to milk, causing an increase in pH. However, Chen et al (2010) reported no significant difference in the pH of goat's milk between normal and mastitis-infected milk, although higher levels of SCC in milk, if not induced by an apparent intramammary infection, would not cause changes in milk pH.

Results showed that the mastitis test scores using an IPB-1 reagent increased inline with the SCC scores and IRT sensing on the udder. A normal milk mastitis test score results of IRT sensing on goat udders is 37.24±0.18 °C and the SCC value is 321 ± 72 x10^3 cells mL^{-1}) increased if the goat are suggestive of intramammary infection (mastitis test score of +3) with IRT sensing on goat udders is 38.62±0.34 °C and the SCC value is 2,120 ± 938 x10^3 cells mL^{-1}. An increase in SCC is a significant milk primary marker for detecting and diagnosing mastitis (Viguier et al 2009). Further Rainard et al (2018), SCC is a highly sensitive biomarker of mammary gland inflammation in which variations in SCC depend on leukocyte recruitment from blood to milk in response to inflammatory reactions elicited in udder tissue bacterial intrusion into the mammary gland. Under normal conditions, the amount of SCC in goat's milk varied between 210-1,120 x10^3 cells mL^{-1} (Leitner et al 2004). This happened because the mastitis test score was +3; the condition of the goat was in the subclinical mastitis phase and had just experienced an acute inflammatory process. The inflammatory process due to mastitis begins through dilation of blood vessels, increasing blood flow to the infected udder, and increasing udder temperature. However, oedema reduces blood flow when it enters the chronic stage, lowering the temperature (Jones & Plassmann, 2002). Furthermore, studies involving endotoxin infusion into the mammary gland resulted in an increase in udder temperature of 1.5–2.0 °C within 24 hours after infusion (Hovinen et al 2008).

Milk production of individual Sapera goats during lactation is shown in Figure 2. As many as eight Sapera goats were used, it turned out that four of them showed a normal mastitis test score of +2 and four more with a mastitis test score of +3. The graph shows that the milk production of goats with a mastitis test score of +3 is lower than that of goats with a normal mastitis test score of +2. The average milk production of goats with a normal mastitis test score up to +2 for six months of lactation was 1,281±253 ml/day, while for goats with a mastitis test score of +3, it was 957±250 ml/day. According to Le Roux et al (2003), mastitis infection causes damage to the secretory epithelium of the mammary gland and affects the components of milk and milk production. The decrease in milk production due to mastitis in this study was also strengthened by the increase in the SCC value of milk. Sharma et al (2011) reported that high SCC content in milk is a significant indicator of infection in the mammary glands, resulting in decreased milk production.

Data on milk components (fat, SNF, lactose, salt, and protein), IPB-1 mastitis score, SSC, and IRT results were analysed by Pearson correlation ($p<0.05$ and $p<0.01$) to show the strength of the correlation between the various components of milk, presented in Tables 2 and 3. A fairly high and very significant positive correlation ($P<0.01$) was found in both the right udder and left udder between levels of SNF-lactose (r=0.79-0.89), SNF-salt (r=0.95- 0.97), SNF-protein (r=0.99), lactose-salt (r=0.81-0.87), lactose-protein (r=0.81-0.91), salt-protein (r=0.96-0.98), add water-freezing point

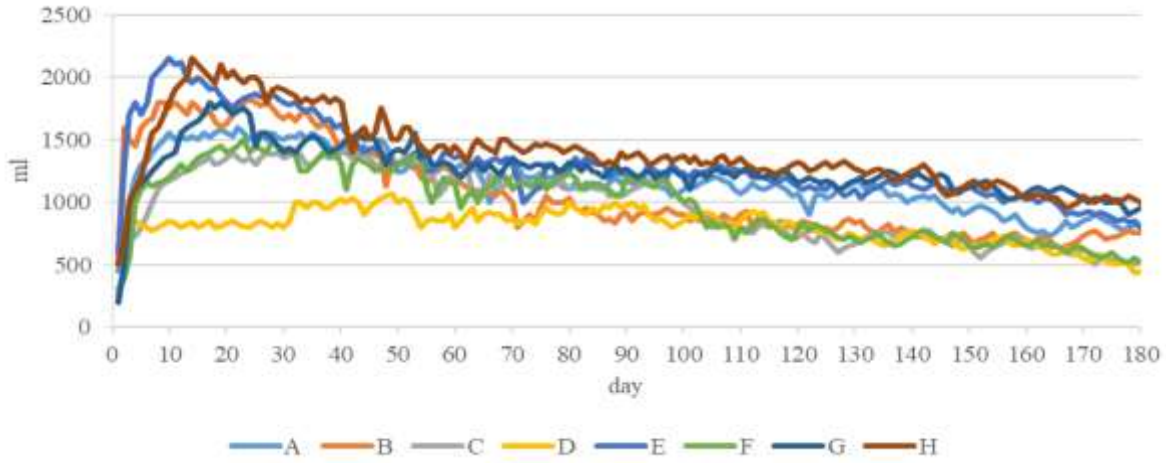


Figure 2. Graph of individual milk production (A-H) of Sapera goats during lactation. Goats A, B, C, and D obtained a normal mastitis test score of up to +2, while goats E, F, G, and H obtained a mastitis test score of +3

(r=1.00), IPB1-SCC (r=0.70-0.74), IPB1-IRT (r=0.70-0 .71), and SCC-IRT (r=0.62-0.65). As the main source of carbohydrates, Lactose controls milk volume by maintaining osmolarity through the rate of lactose synthesis in epithelial cells in the mammary glands (Zhao and Keating 2007). It can also affect the synthesis and secretion of other milk components (Shahbazkia et al 2010). Somatic cell count (SCC) is one of the most important indicators of milk quality. In contrast, SCC is an index of udder health that correlates with the level of infection in the mammary gland, whether subclinical, chronic, or acute mastitis (Kalaydzhiev et al 2021). The increase in milk SCC in response to udder infection causes a decrease in milk quality which is characterised by changes in the chemical composition of milk by decreasing casein, lactose, calcium, and increasing sodium, chloride, and serum protein (Pitkälä et al 2004).

Thermal condition of the surface of each organ reflects metabolic processes that are influenced by fluctuations in the amount and rate of blood supply, in which organs that experience hyperthermia and redness due to accelerated blood flow are used as early signs of inflammation (McCafferty et al 2011). The data at this time revealed that the results of IRT and SCC showed high scores in line with the increase in IPB1 scores. Udder with a mastitis test score of +1 or more had higher udder temperature and SCC than cattle in healthy conditions (Redetzky et al 2005). Therefore, this can explain the positive correlation obtained in this study between IRT, SCC, and IPB1. These results are similar to those previously reported in ruminants (Samara et al 2014). This is substantial evidence that combining IRT results with SCC and IPB1 parameters can be helpful in screening for subclinical mastitis in dairy goats.

A relatively high and very significant negative correlation was found between SNF-add water, SNF-freezing point, lactose-add water, lactose-freezing point, salt-add water, salt-freezing point, protein-add water, and protein-freezing point. These results are similar to those reported in cattle (Dehinenet and Mekonnen 2013). According to Kurwijila (2006), an increase in added water can decrease the specific gravity and increase the freezing point of milk. Still, it affects the physicochemical quality of milk.

CONCLUSION

IRT sensing results showed that the temperature of goats with subclinical mastitis was 38.34-38.62 °C. Average milk production during lactation in goats with a mastitis test score of +3 (957±250 ml/day) was lower than in goats with a normal mastitis test score of +2 (1,281±253 ml/day). The correlation between the IRT right/left udder results with the mastitis test score, and the number of somatic cells of 0.62-0.71 indicates that the IRT sensing results can be a diagnostic method in screening for subclinical mastitis in dairy goats.

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Effect of Supplementing Ground Leaf of Misai (*Orthosiphon stamineus*) in Diet on Growth Performance of Broiler Chickens

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ABSTRAK

Malahubban M, Zakry FAA. 2022. Kesan penambahan serbuk daun misai kucing (*Orthosiphon stamineus*) dalam makanan ke atas prestasi pertumbuhan ayam pedaging. JITV 27(2):74-83. DOI: http://dx.doi.org/10.14334/jitv.v27i2.2982.

Penggunaan herbal untuk pakan ternak merupakan salah satu cara dalam mengatasi kerugian penggunaan bahan kimia buatan yang berlebihan. Penelitian ini mengevaluasi respon ayam pedaging yang diberi pakan tambahan serbuk daun misai kucing (*Orthosiphon stamineus*). Dalam penelitian ini digunakan 160 ekor ayam broiler jantan umur satu hari yang diberi air *ad libitum* dan pakan sampai 20 hari. Perlakuan diberikan pada broiler jantan umur 21 hari. Data dikumpulkan dan dievaluasi setelah dilakukan terminasi ayam broiler jantan umur 42 hari. Terlihat bahwa suplementasi serbuk daun *O. stamineus* dengan konsentrasi 8 g/kg pada pakan ayam pedaging menghasilkan performa pertumbuhan yang sebanding dengan suplementasi tetrasiklin dan vitamin E. Di samping itu pakan mengandung 8 g/kg *O. stamineus* memiliki efek penurunan enzim darah. Pada ayam pedaging yang menerima suplementasi tetrasiklin, bagaimanapun, terlihat adannya aktivitas enzim serum yang signifikan. Hasil penelitian juga menunjukkan bahwa konsentrasi 8 g/kg serbuk daun *O. stamineus* dalam pakan setara dengan 200 mg/kg suplementasi vitamin E. Oleh karena itu, serbuk daun *O. stamineus* dapat mendorong produksi ayam broiler organik, yang aman, sebagai suplemen pakan yang berkelanjutan.

Kata Kunci: Ayam Pedaging, Pakan Tambahan, Serbuk Daun, *Orthosiphon stamineus*

ABSTRACT

Malahubban M, Zakry FAA. 2022. Effect of supplementing ground leaf of misai (*Orthosiphon stamineus*) in diet on growth performance of broiler chickens. JITV 27(2):74-83. DOI: http://dx.doi.org/10.14334/jitv.v27i2.2982.

The use of herbs in animal nutrition is one of the important approaches in overcoming the disadvantages of excessive use of artificial chemicals in animal nutrition. The present study was done to evaluate response of broilers feeding on a diet supplemented with the ground leaf of misai (*Orthosiphon stamineus*). The birds in this, study were 160 one-day-old male broiler chickens, given ad libitum water and feed for up to 20 days. Treatments were given to 21-day-old male broiler chickens. Data was collected and evaluated after slaughtering 42-day- male broiler chickens. It was shown that supplementing broiler diets with *O. stamineus* powdered leaf at a rate of 8 g/kg resulted in growth performance comparable to tetracycline and Vitamin E supplementation. It was also shown that supplementing the diet with 8 g/kg *O. stamineus* had a blood enzyme-lowering effect. In broilers receiving tetracycline supplementation, however, significant serum enzyme activity was observed. Results also showed that 8 g/kg of ground *O. stamineus* leaf in the diet was equivalent to 200 mg/kg Vitamin E supplementation. Therefore, *O. stamineus* leaf powder can promote organic, safe, and sustainable broiler chicken production, and as diet supplement.

Key Words: Broiler, Diet Supplementation, Ground Leaf, *Orthosiphon stamineus*

INTRODUCTION

The use of antibiotics in raising broilers has been limited by the European Union's withdrawal of glycopeptides avoparcin and bacitracin, the macrolides spiramycin and tylosin streptogramin virginiamycin as prophylactic antibiotics and growth promoters between 1995 and 1999 (Muaz et al. 2018). Medicinal herbs have the potential to alternate the use of synthetic antibiotics and antioxidative growth promoters in broiler production. Medicinal herbs are rich in phenolic compounds, flavonoids and aromatic compounds that exhibit natural antibiotic and antioxidative potential to improve the growth performance of livestock by enhancing the immune status of the animals (Kırkpınar et al. 2011; Kiczorowska et al. 2016; Wang et al. 2019; Bai et al. 2019). In this regard, one promising medicinal plant is *Orthosiphon stamineus*, which exhibits a wide range of biological properties, including antibacterial and antioxidant traits (Malahubban et al. 2013a) .

The use of appropriate additives in the diet to prevent lipid oxidation provides major benefits to

animals and consumers. Lipid oxidation, for example, can produce pathological alterations in the mucosal membrane of the gastrointestinal tract, impede enzyme action, and raise cholesterol and peroxide levels in the serum, all of which can contribute to atherosclerosis. Furthermore, lipid oxidation can result in the formation of malondialdehyde, a powerful mutagen and carcinogen (Vandemoortele et al. 2021). According to Khajali & Wideman (2016), broiler chickens are sensitive to lipid peroxidation and the formation of reactive oxygen species due to an excessive accumulation of fat in their bodies, primarily polyunsaturated fatty acids reactive oxygen species (ROS). Then, in broiler chickens, ROS production was linked to the development of pulmonary hypertension syndrome (PHS).

Furthermore, pathogenic bacteria such as *E. coli*, *Salmonella* sp., *Clostridium* sp., and *Campylobacter* sp. can cause disease in chickens. These pathogenic bacteria compete for nutrition with the host microorganisms in the small intestine. Due to the deconjugating effects of bile acids, colonisation may also impair fat and fat-soluble vitamin digestion. Rinttilä & Apajalahti (2013) found that competition lowered growth performance and increased illness incidence.

In the present study, *O. stamineus* ground leaf was added as a supplement to the diet of broiler chickens evaluated for its antibacterial and antioxidant potential, with tetracycline and Vitamin E serving as the respective positive controls. The benefits of the diet additive were assessed in terms of growth performance, carcass characteristics, serum biochemistry and lipid peroxidation in serum and liver of broiler chickens. Moreover, the effect of *O. stamineus* ground leaf supplementation in the diet was investigated on the population growth of *Escherichia coli* and *Lactobacillus* spp of the intestine in broilers.

MATERIALS AND METHODS

Preparation of *Orthosiphon stamineus* used as animal feed additive

Fresh *Orthosiphon stamineus* (OS) samples were collected from the Herbal Farm at Universiti Putra Malaysia. Gene Bank Centre, Faculty of Agriculture, Universiti Putra Malaysia, verified the plant sample. Under nursery settings, four-five nodes stem cuttings were obtained from mature OS plants and planted in black polythene bags containing a mixture of soil, sand, and peat moss (2:1:1). Ten weeks after planting, the first crop OS plant was harvested. OS shoots were trimmed to roughly 30 cm from the tip. Fresh OS leaves were harvested and oven-dried for 72 hours at 60°C. Using a Willey mill (Thomas® Willey cutting mill model 4) and a one mm screen, the dried leaves were pulverised into powder and kept at 4 °C until needed.were

Birds and experimental design

A local hatchery provided 160 one-day-old male broiler chickens (Cobb 500). The broilers were fed a commercial broiler starting meal (0–20 days) and provided unlimited access to water. The broilers were weighed and reassigned to four different feeding regimens at the end of week three in order to attain comparable average weights for each treatment. Each treatment comprised five replications, each containing eight broilers. Broilers were randomly assigned to 20 cages, each measuring 122 cm (length) x 91 cm (width) x 50 cm (height) and equipped with round feeders and round drinkers, with the temperature and humidity set to ambient (28°C) (60 to 89%, respectively).

Dietary treatments

The basal diet should be made without antimicrobials, anticoccidial medicines, or feed enzymes, according to the National Research Council's suggestion (NRC 1994; Applegate & Angel 2014). The feed was provided ad libitum and refilled at 08:30 and 17:30 every day, with the leftovers gathered in the raising cages. AOAC International techniques were used to analyze dietary and nutrition-related chemical composition (George & Latimer 2019).

The broilers were fed the following diets from the 21st to the 42nd day of the experiment: 1) Diet C (Control, basal diet); 2) Diet VE (Positive control) (Basal diet+200 mg/kg Vitamin E); 3) Diet T20 (Positive control) (Basal diet+20 mg/kg Tetracycline); and 4) Diet OS8 (Basal diet+8 g/kg OS). The rate of supplementation at 8 g/kg of OS was the best rate in promoting the growth and carcass characteristics of broiler chickens as reported in the earlier work (Malahubban et al. 2013b). The compositions of the above dietary treatments are as shown in Table 1.

Sunzen Corporation Sdn. Bhd. (Malaysia) supplied tetracycline. Vitamin E in the form of d-1-α-tocopherol acetate was provided by Lutavit E 50 (BASF, Germany). Tetracycline is used as a positive control for antibacterial activity, while Vitamin E served as a positive control for its antioxidant activity.

Parameter measurement

Birds were weighed individually at weekly intervals. Daily observation was conducted for survival and mortality. Total feed intake was recorded per cage

at weekly intervals. Feed intake and feed conversion ratio were modified for mortality.

On day 42, ten broilers from each treatment group were selected at random and individually weighed to the closest gram unit before being slaughtered by severing the carotid artery and jugular veins (Department of Standard Malaysia 2004). Each bird was bathed in hot water for 20 seconds and manually de-feathered for 30 seconds after 5 minutes of bleeding. Manual removal of the feet, skull, and viscera followed.

The dressed bird was opened up, and the liver, kidney, heart, gizzard, proventriculus and intestine were removed and weighed. Carcass samples and liver were stored immediately in a deep freezer at minus 80ºC until required for further analysis. The alimentary tracts of five of the ten slaughtered birds in each treatment group were promptly dissected. The ceca contents and the ileal (about 30 cm long segment of the lower ileum measured from the Meckels diverticulum) were collected and put into plastic 50 mL Falcon tubes. Prior to the evaluation of the intestinal population of bacteria, the collected contents were maintained on ice. The collected ceca contents were processed within 60 to 90 min of collection. All samples were weighed after being diluted 1:10 with normal saline solution. Ten serial dilutions of ceca content were made in normal saline,

Table 1. Ingredients in the dietary treatments and nutritional analysis (adopted from earlier work of Masnindah Malahubban et al. 2013b)

Ingredients	Dietary Treatments[1]			
	C	VE	T20	OS8
Corn	61.0	61.0	61.0	60.2
Soy Bean Meal (SBM) (44%)	25.0	25.0	25.0	25.0
Fish Meal	6.41	6.41	6.41	6.41
Palm Oil	5.00	5.00	5.00	5.00
Limestone	1.26	1.26	1.26	1.26
Salt	0.28	0.28	0.28	0.28
Dicalcium Phosphate (DCP)	0.10	0.10	0.10	0.10
Mineral Mix[a]	0.25	0.25	0.25	0.25
Vitamin Mix[b]	0.25	0.25	0.25	0.25
L-Lysine	0.20	0.20	0.20	0.20
DL-Methionine	0.15	0.15	0.15	0.15
Choline chloride	0.10	0.10	0.10	0.10
Orthosiphon stamineus	-	-	-	0.80
Tetracycline	-	-	(20)[c]	-
Vitamin E	-	(200)[d]	-	-
Calculated analysis (%)				
Metabolize Energy (ME) Kcal/kg	3211	3211	3211	3211
Crude Protein, %	20.00	20.00	20.00	20.00
Crude Fibre, %	4.35	4.35	4.35	4.40
Crude Fat, %	3.21	3.21	3.21	3.21
Calcium, %	0.99	0.99	0.99	0.99
Available P, %	0.33	0.33	0.33	0.33

[a]Premix was given per kg of diet: Mg = 56 mg, Fe= 20 mg, C = 10 mg, Zn= 50 mg, C = 125 mg, I= 0.8 mg. [b]Premix was given the following per kg of diet: Vitamin A= 50.000 MIU; Vitamin D_3= 10.000 MIU; Vitamin = 75.000 MIU; Vitamin K= 20.000 g; Vitamin B1= 10.000 g; Vitamin B2= 30.000 g; Vitamin B6= 20.000 g; Vitamin B12= 0.100 g; Calcium D-Panthothenate= 60.000 g; Nicotinic acid= 200.000 g; Folic acid= 5.000 g; Biotin= 235.000 mg. [c]Tetracycline (20 mg/kg dry matter intake). [d]d- 1-α tocopherol acetate (200 mg/kg dry matter intake). [1]Dietary treatments: C= Control; VE= 200 mg/kg d-1-α tocopherol acetate (positive control); T20= 20 mg/kg Tetracycline (positive control); OS8= 8g/kg *O. stamineus*

and a 100-µL portion of 10^{-7} to 10^{-9} diluted aliquot plated on fresh agar plates.

The total count of facultative anaerobic bacteria was determined on nutrient Agar (NA) (OXOID, U.K.) after incubation for 24 hour at 37ºC (Kırkpınar et al. 2011). The nutrient agar medium could provide nutrients for most cultivable bacteria, including facultative anaerobic bacteria that can live without oxygen and not inhibited when oxygen is available. *Escherichia coli* count was determined from the green metallic surface sheen of colonies following incubation on Eosin–methylene blue (EMB) agar medium (OXOID, U.K.) under the same conditions. *Lactobacillus* count was determined from white colonies appearing on Rogosa Agar (RA)(OXOID, U.K.) after plates incubated in an anaerobic jar at 37ºC for 48-72 hour. Results were expressed as $\log_{10}$ of colony-forming units (CFU) per gram of ileal digesta. Blood samples (4.0 mL) from birds were collected from the wing vein using sterile gauge 23 needles and syringe. Blood samples were taken in a standard vacutainer and centrifuged at 3000 g for 10 minutes to separate them. For the analysis of serum glucose, cholesterol, triglycerides, albumin, total protein, sodium (Na), potassium (K), chlorine (Cl), urea, aspartate transaminase (AST), alkaline transaminase (ALT), and alkaline phosphatase, serum samples were kept at minus 20°C (ALP). Using an auto-analyzer, to analyse certain commercial kits (Roche Diagnostica, Basal, Switzerland) (HITACHI 902, Automatic Auto-analyser). By subtracting serum albumin from serum total protein levels, serum globulin was determined. Lipid oxidation was assessed based on malondialdehyde (MDA) for blood serum and liver. Malondialdehyde, the end-product of lipid peroxidation by reactive oxygen species, was evaluated using the TBARS assay kit (Oxiselect^{TM}, CellBiolabs, U.S.A.).

Statistical Analysis

SPSS software was used to conduct statistical data analysis (IBM SPSS version 21). Differences between means for all parameters were determined using a one-way analysis of variance (ANOVA). Multiple comparisons of means conducted using Duncan's test to show differences among treatments. Differences were considered significant at the 0.05 level.

RESULTS AND DISCUSSION

Result

Broiler Weight Performance

Table 2 shows the weight gain and live weight of broilers fed various diets. No improvement in live weight of broilers fed various experimental diets over the control basal diet was observed from day-21 to day-28. Significant live weight variation was observed only from day-35 to day-42 (final observation day). On day-35, even though broilers fed basal diet treated with OS08 had no significant difference with control diet (C) (P>0.05), the effect of OS08 treatment on live weight of broiler was similar to VE treatment and nearly identical with T20 treatment.

Table 2. Live weight and weight gain of broilers fed different diets for three weeks

Performance	Dietary Treatments			
	C	VE	T20	OS8
Live Weight (g)				
Initial (day 21)	767.3±15.3	784.4 ± 13.6	763.3 ± 22.4	756.6 ± 22.0
Week 4 (day 28)	1217.3 ±22.7	1256.5 ±38.6	1269.8 ±25.3	1230.1 ± 20.2
Week 5 (day 35)	1618.2 ±40.3^{b}	1714.9 ±33.9^{ab}	1776.2 ±35.6^{a}	1717.7 ±38.9^{ab}
Final (day 42)	2164.2 ±35.5^{b}	2305.8 ±55.2^{a}	2379.5 ±41.5^{a}	2296.8 ±47.5^{a}
Cumulative Weight gain (g)	1384.2 ±42.4^{b}	1477.1 ±61.4^{ab}	1569.7 ±57.5^{a}	1491.1 ±57.7^{ab}
Week 4	450.0 ±23.9	472.1 ±38.1	506.5 ±37.9	473.6 ±28.1
Week 5	400.9 ±32.1	454.4 ±39.0	507.1 ±39.2	489.0 ±34.4
Week 6	547.4 ±49.5	591.6 ±43.8	598.8 ±53.3	584.6 ±57.8

Treatment means ± standard errors are presented. Treatment values with the same superscript letters are not significantly different at P>0.05. C= Control; VE = 200 mg/kg d-1-α tocopherol acetate (positive control); T20= 20 mg/kg Tetracycline (positive control); OS8= 8g/kg *O. stamineus*

Table 3. Feed intake, FCR, and mortality of three-week-old broilers fed various diets

Performance	Dietary Treatments			
	C	VE	T20	OS8
Cumulative Feed intake (g)	3117.4±181.4	2998.2±67.7	3264.0±150.5	3112.0±134.5
IWeek 4	840.4±35.3	884.4 ±49.4	878.0 ±79.1	882.0 ±46.6
Week 5	894.6±56.1	907.4 ±41.9	1030.0±69.1	968.0±84.6
Week 6	1382.4±160.3	1206.4±71.4	1356.0±87.7	1262.0±95.4
Cumulative FCR (g)	2.21±0.12	1.97±0.04	2.01±0.09	1.99±0.08
Week 4	1.87±0.07	1.87±0.10	1.73±0.16	1.86±0.09
Week 5	2.23±0.14	2.00±0.09	2.03±0.14	1.98±0.17
Week 6	2.53±0.29	2.04±0.12	2.26±0.15	2.15±0.16
Mortality (%)	1.88	1.25	1.25	1.25

Treatment means ± standard errors are presented. No significant differences between treatments were found (P>0.05). No significant different on mortality rate (X^2 = 0.949, P<0.05. C= Control; VE = 200 mg/kg d-1-α tocopherol acetate (positive control); T20= 20 mg/kg Tetracycline (positive control); OS8= 8g/kg *O. stamineus*

On day-42, birds in OS08 diets showed a significant (P<0.05) increase in the live weight (2296.8±47.5 g) as compared to control basal diet (2164.2±35.5 g), and similar to positive control diets, T20 at 2379.5±41.5 g and VE at 2305.8±55.2 g.

Cumulative weight gain of broilers fed OS08 diet had no significant difference with control diet (P>0.05). However, broilers on the OS08 diet had similar weight gain with broilers on VE diet at 1491.1±57.5 g and 1477.1±61.4 g. Moreover, broilers fed on the OS08 diet challenge cumulative weight gain of broilers on the T20 diet at 1569.7±57.5 g.

Feed intake, feed conversion ratio and mortality

No significant differences in feed intake and feed conversion ratio observed in broilers fed different diets in the present study, as shown in Table 3.

Mortality rate was also low, ranging from 1.25 to 1.88% and no significant difference was detected among treatments (X2= 0.949, P<0.05). On average, broilers fed OS8 recorded slightly lower cumulative feed intake than the control diet at 3112.0±134.5 g and 3117.4±181.4 g, respectively. However, broilers fed on T20 recorded higher cumulative feed intake than broilers fed OS8 and lower cumulative feed intake compared to broilers on the VE diet.

Carcass and Organ Characteristics

Table 4 shows the percentage of carcass yield and relative organ weight of broilers fed different dietary treatments for 42 days.

No significant differences (P>0.05) found on relative percentage weight of carcass, kidney, heart, gizzard, proventriculus and small intestine of broilers fed in all different dietary treatments. However, significant differences found in the abdominal fat and liver weight of treated broilers. Broilers fed diet OS8 showed significantly reduced abdominal fat (P<0.05) at 1.34±0.10% as compared to the control (1.96±0.22%) and diet T20 (2.51±0.26%).

Broilers fed diet T20 increased their abdominal fat significantly (P<0.05) at 2.51±0.26% over control basal diet at 1.96±0.22%. The present study also showed that broilers fed diet OS8 increased their relative liver weight (2.92±0.18%) as compared to the control (2.34±0.09%), diet T20 (2.26±0.09%), and diet VE (2.25±0.11%).

Blood Characteristics

Serum biochemical parameters and experimental outcomes presented in Table 5. In general, serum cholesterol, glucose, triglycerides, potassium (K), aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were found to vary significantly between the experimental treatments (P<0.05). No significant differences found on serum total protein, albumin, globulin, sodium (Na), chlorine (Cl), and urea. In the present study, significantly lower cholesterol recorded in broilers fed diet OS8 (2.90±0.19 mmol/L) as compared to those fed the control diet (3.49±0.22 mmol/L), diet T20 (3.50±0.17 mmol/L), and diet VE (3.52±0.09 mmol/L).

Result of the present study also indicated that diet OS8 lowered serum glucose significantly at 4.18±0.36

Table 4. Effect of experimental diets on carcass yield and relative organ/tissue weights of broilers on day-42

Relative weight (%)	Dietary Treatments[1]			
	C	VE	T20	OS8
Carcass	70.89±2.07	72.41±1.82	73.51±1.65	72.15±1.39
Abdominal Fat	1.96 ±0.22^{b}	1.51±0.11^{bc}	2.51±0.26^{a}	1.34 ±0.10^{c}
Kidney	0.20±0.02	0.21±0.02	0.21±0.01	0.23±0.01
Liver	2.34±0.09^{b}	2.25±0.11^{b}	2.26±0.09^{b}	2.92±0.18^{a}
Heart	0.60±0.02	0.59±0.02	0.57±0.03	0.64±0.01
Gizzard	3.09±0.17	2.86±0.07	2.89±0.22	3.14±0.14
Proventriculus	0.96±0.05	0.88±0.04	0.92±0.03	0.87±0.04
Small intestine	3.99±0.22	4.46±0.21	4.37±0.23	4.40±0.30

Treatment means ± standard errors are presented. Treatment values with the same superscript letters are not significantly different at P>0.05. [1]Dietary treatments: C = Control; VE = 200 mg/kg d-1-α tocopherol acetate (positive control); T20 = 20 mg/kg Tetracycline (positive control); OS8 = 8g/kg *O. stamineus.*

Table 5. Serum biochemical parameters and experimental outcomes of broilers on day-42

Serum Parameters	Dietary Treatments[1]			
	C	VE	T20	OS8
Cholesterol (mmol/L)	3.49±0.22^{a}	3.52±0.09 ^{a}	3.50±0.17^{a}	2.90±0.19^{b}
Glucose (mmol/L)	8.18± 0.24^{a}	6.94± 0.51^{a}	4.81± 0.60^{b}	4.18± 0.36^{b}
Triglycerides (mmol/L)	1.03± 0.19^{a}	0.60± 0.02^{b}	0.98± 0.07^{a}	0.76± 0.04^{ab}
Total protein (g/L)	28.78±0.88	29.48±1.79	31.46±1.82	29.12±1.75
Albumin (g/L)	18.73±1.05	21.82±1.77	19.85±2.14	19.31±1.30
Globulin(g/L)	10.05±1.25	7.66±1.54	11.61±1.53	9.81±1.37
Na (mmol/L)	114.20± 6.90	127.30± 8.40	126.00± 8.90	110.90± 6.60
K (mmol/L)	15.20± 1.30^{b}	22.90± 1.40^{a}	17.00± 2.20^{b}	11.20± 1.10^{b}
Cl (mmol/L)	79.80± 2.40	75.60± 4.40	74.60± 3.70	69.30± 3.00
Urea (mmol/L)	0.33± 0.04	0.24± 0.02	0.24± 0.03	0.28± 0.04
AST (U/L)	254.13± 8.43^{b}	248.49± 8.52^{b}	287.98± 6.71^{a}	213.16± 3.23^{c}
ALT (U/L)	5.52±1.01^{a}	3.97±0.96^{ab}	4.25±0.50^{ab}	2.13±0.12^{b}
ALP (U/L)	990.90± 252.68^{b}	1081.70±225.97^{b}	3682.20±38.84^{a}	874.30±187.28^{b}

Treatment means ± standard errors are presented. Treatment values with the same superscript letters are not significantly different at P>0.05. [1]Dietary treatments: C = Control; VE = 200 mg/kg d-1-α tocopherol acetate (positive control); T20 = 20 mg/kg Tetracycline (positive control); OS8 = 8g/kg *O. stamineus*

mmol/L than the control, and diet VE at 8.18±0.24 mmol/L 6.94±0.51 mmol/L, respectively. The results indicated that diet OS8 was similar to diet T20 4.81±0.60 mmol/L of serum glucose. Broilers fed had significantly lower ALP at 874.30±187.28 U/L than birds fed on diet T20 at 3682.20±38.84 U/L. diet OS8 had equivalent serum triglycerides levels (0.76±0.04 mmol/L) with birds on diet VE (0.60±0.02 mmol/L). However, birds fed on diet VE had significantly lower than control, and T20 diets. Broilers fed diet OS8 showed lowered serum potassium significantly at 11.20±1.10 mmol/L, similar to broilers on control and T20 diets, compared to broilers on diet VE at 22.90±1.40 mmol/L.

Broilers fed diet OS8 had significantly lowered AST levels at 213.16±3.23 U/L as compared to those on diet C (254.13±8.43 U/L), diet VE (248.49±8.52 U/L), and diet T20 (287.98±6.71 U/L). As in serum AST enzyme activity, broilers fed diet OS8 showed significantly lower ALT at 2.13±0.12 U/L than the control at

5.52±1.01 U/L. All in all, broilers fed diet OS8 exhibited a serum enzyme-lowering effect. From the present study also found that birds provided diet OS8 had significantly lower ALP at 874.30 ± 187.28 U/L than birds fed on diet T20 at 3682.20 ± 38.84 U/L.

Lipid peroxidation in serum and liver

Occurrence of lipid peroxidation in serum and liver broilers fed different dietary treatments after 42 days presented in Table 6. Broilers fed diet OS8 (0.20±0.033 nmol/mL) showed significant lower serum lipid peroxidation rates as compared to broilers on the control basal diet (0.31±0.043 nmol/mL), and had significantly similar with broilers on diet VE (0.15±0.027 nmol/mL) and diet T20 (0.29±0.032 nmol/mL). In the liver, lipid peroxidation was also lowered significantly in broilers fed diet OS8 (1.64±0.09 nmol/g) as compared to those on the control (2.55±0.15 nmol/g), and diet T20 (2.29±0.16 nmol/g) but was similar with broilers fed diet VE (1.47±0.13 nmol/g).

Intestinal Microbial Population

The intestinal microbial population of broilers fed different dietary treatments over 42 days shown in Table 7. No significant differences (P>0.05) found in an intestinal population of *Lactobacillus* and *Escherichia coli* in broilers subjected to different dietary treatments in the present study. However, broilers fed diet OS8 had significantly lower (P<0.05) facultative anaerobe populations at 6.19±0.15 log CFU/g as compared to broilers fed the control diet (6.70±0.07 log CFU/g). It was also found that population of facultative anaerobe in broilers provided OS8 was similar to broilers on T20 (6.14±0.17 log CFU/g).

Discussion

OS8 supplemented in broiler chicken diet showed an increase in the live weight compared to the control basal diet. The improvement in the live weight of broiler chicken after fed on OS8 could be due to antioxidant and antimicrobial properties constituted in the *O. stamineus* ground leaf, as Malahubban et al. (2013a) reported. Herbs' chemical composition regulates the intestinal microflora then stimulating the digestion process and eventually increasing weight gain and feed utilization (Elkatcha et al. 2016). However, theeffect of phytochemical composition may not stand alone because it could vary significantly due to variety, location, and climate (Xiao et al. 2012). It may also affect broad aspects of physiology, being positive interaction with the biochemistry of the body of broiler chicken (Mbikay 2012). Therefore, the following data could provide helpful growth-promoting indication and broiler chicken status after fed *O. stamineus* ground leaf supplemented in the diet.

No significant differences in feed intake and feed conversion ratio were observed in broilers fed different diets in the present study (Table 2). They reflected similar results where broilers were fed oregano and garlic essential oils (Kırkpınar et al. 2011). The mortality rate was considerably low in the present study being in the range of 1.25 to 1.88%, comparable to rates experienced in commercially produced broilers where 2 to 7% mortality is common (Idan et al. 2020; Yerpes et al. 2020).

The observed reduction of abdominal fat deposition and the increment of liver weight was consistent with reports from Malahubban et al. (2013b) studies. Similarfindings have also been reported in broilers fed green tea extract (Mohammadpour et al. 2021) or turmeric extract (Utami et al. 2020). Other herbal supplements have, nevertheless, been less successful in this respect. For example, Taufik & Maruddin (2019) reported that broilers fed garlic supplement did not alter abdominal fat deposition. The excessive gain in liver weight of broilers fed diet OS8 demonstrated vigorous hepatic use. A similar incidence was found in rats fed rosemary extract with higher hepatic metabolism and better liver mass (Wang et al. 2019).

In terms of serum biochemical parameters, dietary treatments in the present study affected mainly serum cholesterol, glucose, triglycerides, potassium, and the enzymes aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP). Broilers fed the OS supplement confirmed reduced AST and ALT rates, suggesting that a normal liver

Table 6. Lipid peroxidation in serum and liver of broilers on day-42

Parameters	Dietary Treatments			
	C	VE	T20	OS8
Serum (nmol/mL)	0.31 ± 0.043^{a}	0.15 ± 0.027^{c}	0.29 ± 0.032^{ab}	0.20 ± 0.033^{bc}
Liver (nmol/g)	2.55 ± 0.15^{a}	1.47 ± 0.13^{b}	2.29 ± 0.16^{a}	1.64 ± 0.09^{b}

Treatment means ± standard errors are presented. Treatment values with the same superscript letters are not significantly different at P>0.05. C= Control; VE= 200 mg/kg d-1-α tocopherol acetate (positive control); T20= 20 mg/kg Tetracycline (positive control); OS8= 8g/kg *O. stamineus*

Table 7. Intestinal bacteria population (log CFU/g of intestinal contents) of broiler chickens fed different diets (Readings on Day-42)

Bacteria	Dietary Treatments			
	C	VE	T20	OS8
Facultative Anaerobic	6.70±0.07[a]	6.56±0.17[ab]	6.14±0.17[b]	6.19±0.15[b]
Lactobacillus	4.96±0.52	5.92±0.36	5.46±0.04	5.38±0.07
Escherichia coli	4.11±0.26	3.79±0.33	3.55±0.19	3.75±0.21

Treatment means ± standard errors are presented. Treatment means with different superscript letters within the same column differ significantly (P<0.05). C= Control; VE= 200 mg/kg d-1-α tocopherol acetate (positive control); T20= 20 mg/kg Tetracycline (positive control); OS8= 8g/kg *O. stamineus*

characteristic, this was similar to that reported by Utami et al. (2020) on the effect of turmeric extract in the diet of broiler chickens. In contrast, the increase in AST and ALP activities could indicate injury or damage to the liver as presently indicated in broiler chicken following tetracycline supplement.

Gut microbial population plays an essential role in broiler weight gain. OS supplementation might also affect the population of gut microbes in broilers due to its antimicrobial and antioxidant properties, as mentioned earlier, and as similarly reported in previous studies (Giannenas et al. 2018; Guo et al. 2019). Microflora in gut contributes significantly to overall health performance of the host (Diether & Willing 2019) by influencing the development of the gut system. When infections attach to the mucosa, for example, gut integrity and function are severely harmed, and the immune system is put to the test (Aguzey et al. 2020). Chickens raised in a pathogen-free environment grow 15% quicker than those raised in typical conditions where germs and viruses are present (Akpan et al. 2019). Therefore, the microbial population is one of the crucial indicators of the broiler's health status.

Furthermore, it is widely known that gut microbiota represents a nutritional "burden" in fast-growing broiler chicken (Ravindran & Abdollahi 2021), because activemicroflora components may have a higher energy demand for maintenance and worse nutrient utilization efficiency (Yadav & Jha 2019). With its antimicrobialproperties (Malahubban et al. 2013a), OS could improve digestibility of the feed offered to the animals and regulate and limit growth and colonization of a variety of pathogenic and non-pathogenic species in the gastrointestinal tract (Teng & Kim 2018). Increasing the presence of pathogenic bacteria in broiler guts may result in poor growth and a high feed conversion ratio. While the dietary treatment with OS in the present study did not affect the population of *Lactobacillus* and *Escherichia coli*, the inclusion, however, suppressed the population of the facultative anaerobes that might include pathogenic species such as *Salmonella enterica* (Pham et al. 2022), and this was comparable with tetracycline. The significant effect of OS on total facultative anaerobes might be collective and not selective by reducing the number of intestinal microbial communities rather than the number of specific individuals and characteristics of bacteria. The antibacterial properties of *O. stamineus* could be associated with phytochemical compounds, as demonstrated in the previous phytochemical screening (Malahubban et al. 2013a), which revealed alkaloids, tannins, etc. saponins and steroids in the methanol extract. Antibacterial action of phytochemicals is mediated by a variety of mechanisms. Tannins, for example, work by depriving essential proteins like enzymes of iron, hydrogen bonding, or non-specific interactions (Loo et al. 2020). *Bacteroides fragilis*, *Clostridium perfringens*, and *Enterobacter cloacae* are among the bacteria that are inhibited by tannic acid (Loo et al. 2020). Therefore, the inclusion of 8 g/kg OS in the diet can replace synthetic antibiotic tetracycline or its equivalents.

CONCLUSION

Dietary supplementation of *Orthosiphon stamineus* leaf powder at a rate of 8 g/kg in the feed was comparable to tetracycline and Vitamin E supplementation to promote the growth of broilers. *O. stamineus* leaf powder also reduced abdominal fat deposition and cholesterol levels in the blood serum of the birds. From the present study it is also found that 8 g/kg *O. stamineus* supplementation in diet promoted lowering blood serum enzymes, suggesting metabolic stability. Therefore, *O. stamineus* leaf powder can replace conventional antibacterial and antioxidant compounds as broiler diet supplements in organic and sustainable poultry production.

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Effectivity of Dry and Liquid BS4 Enzymes in Improving Performance of Broiler Chickens Fed Different Nutrient Density Diet

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ABSTRAK

Sinurat AP, Haryati T, Pratiwi N, Purwadaria T. 2022. Efektifitas enzim BS4 kering dan basah dalam peningkatan proforma ayam broiler yang diberi pakan kepadatan pakan yang berbeda. JITV 27(2):84-92. DOI: http://dx.doi.org/10.14334/jitv.v27i2.3051.

Suplementasi enzim ke dalam pakan sudah banyak dilakukan untuk meningkatkan kecernaan gizi pakan dan performa unggas. Suatu enzim (BS4) untuk imbuhan pakan sudah dihasilkan dengan membiakkan *Eupenicilium javanicum.* Efektivitas enzim ini perlu diuji karena banyak faktor yang mempengaruhi efektivitas penambahan enzim ke dalam pakan. Suatu penelitian dilakukan untuk mengetahui efektivitas penambahan enzim BS4 ke dalam pakan terhadap performa ayam broiler. Sebanyak 300 ekor ayam broiler umur sehari dibagi ke dalam 30 kandang dan dipelihara hingga umur 35 hari. Ayam diberi makan ransum percobaan yang merupakan faktorial dari 2 (ransum standard dan ransum kepadatan gizi rendah) X 3 (tanpa enzim, ditambah enzim BS4 cair, ditambah BS4 tepung) dengan 5 ulangan. Pengamatan dilakukan terhadap performa (konsumsi pakan, bobot badan, FCR dan daya hidup) pada periode starter (1-21 h) dan periode selama penelitian (1-35 h). Pada akhir penelitian dilakukan pengukuran persentase karkas, bobot lemak abdomen, rempela dan hati. Hasil menunjukkan bahwa performa ayam 1-35 h tidak dipengaruhi oleh interaksi antara kepadatan gizi dengan penambahan enzim. Kepadatan gizi juga tidak nyata mempengaruhi performa ayam broiler. Penambahan enzim nyata menurunkan konsumsi pakan dan memperbaiki FCR dibandingkan dengan tanpa penambahan enzim. Penambahan enzim BS4 bentuk cair maupun padat menyebabkan penurunan 3,6% konsumsi pakan. Enzim BS4 cair memperbaiki FCR 6,4%, sedangkan BS4 padat memperbaiki FCR 8,9%, namun tidak ada perbedaan yang nyata antara enzim cair dan padat. Perlakuan kepadatan gizi, penambahan enzim maupun interaksi kedua faktor tersebut tidak nyata mempengaruhi persentase karkas, bobot relatif lemak abdomen, hati dan rempela ayam broiler.

Kata Kunci: Performa Ayam Broiler, Enzim BS4, Cair, Kepadatan Nutrisi, Padat

ABSTRACT

Sinurat AP, Haryati T, Pratiwi N, Purwadaria T. 2022. The effectivity of dry and liquid BS4 enzymes in improving performance of broiler chickens fed different nutrient density diet. JITV 27(2):84-92. DOI: http://dx.doi.org/10.14334/jitv.v27i2.3051.

Supplementation of enzymes in feed is now commonly practiced to increase the nutrient availability of feed and the performance of poultry. A new enzyme called BS4 was produced by cultivating *Eupenicilium javanicum*. It is necessary to test the efficacy of this enzyme since the effectiveness of enzyme supplementation depends on many factors. An experiment was conducted to study the effect of dietary BS4 enzyme supplementation in improving the performance of broiler chickens. A number of 300 broilers DOC was distributed into 30 pens and reared until 35 d. Six experimental diets i.e., factorial of 2 (Standard diet, and low nutrient density diet) X 3 (Control, BS4 liquid enzyme, and BS4 powder enzyme) were formulated with 5 replications. The performance (feed intake, body weight, FCR, and survival rates) were observed during the starter (1-21 d) and whole (1-35 d) periods. At the end of the trial, measurements were also made on the carcass yield, abdominal fat, liver, and gizzard weights. Results showed that performances of broilers from 1-35 d were not significantly affected by interaction between nutrient density and enzyme supplement. The nutrient density also did not affect performances of broilers. However, dietary enzyme supplementation significantly reduced feed intake and improved FCR of broilers as compared to the control. Supplementation of BS4 in liquid or powder form, reduced feed intake by 3.6%. Supplementation of liquid and powder BS4 enzymes improved FCR by 6.4% and 8.9%, respectively, but no different effect between liquid and powder BS4 enzymes on performance of broilers. Nutrient density, enzyme supplementation, and interactions between the two factors did not significantly influence carcass yield, abdominal fat, liver, and gizzard relative weights of broilers.

Key Words: Broiler Performances, BS4 Enzyme, Liquid, Nutrient Density, Powder

INTRODUCTION

Enzymes are composed of proteins that act as biological catalysts to accelerate biochemical reactions. Enzymes are now commonly used in poultry feed as a supplement to enhance nutrient digestibility of feed, especially those containing feed ingredients with high anti-nutritive factors such as non-starch polysaccharides (NSP). Many enzymes are used in poultry feed production nowadays, either as single- or multi-

enzymes. Supplementing enzymes into feed is expected to improve performance, reduce feed cost and minimize hazardous effect on the environment due to poultry production (Costa et al. 2008; Alagawany et al. 2018). However, not all enzymes supplement showed a similar degree of improvement on chicken performance. There are many factors affecting enzymes supplementation on chickens performance such as the match between enzymes and the presence of antinutrient factors in feed or type of diet, types of enzymes (Saleh et al. 2018), age of the bird, and feed processing (Amerah et al. 2011; Mbukwane et al. 2022). Therefore, it is imperative to study the effectiveness of enzymes in every environmental condition.

The use of enzymes in feed formulation could be applied in two methods. The first method is called "on top" application, i.e., supplementing into the feed without considering the improvement of the available nutrient due to enzyme (Allouche et al. 2015). In this method, the feed was formulated according to the standard nutrient requirement of the birds and added the enzyme as recommended by the producer on top of the formula. Feed price will be more expensive in this method, but it is expected to be compensated by the improvement in the performance of the birds, especially in improving feed conversion ratio due to enzymes supplementation. The second method is by supplementing the enzyme with a diet formulated to contain lower nutrient contents than the standard as shown by some researchers (Yegani & Korver 2013; Saleh et al. 2018). The reduction is expected to be compensated by the enzyme supplemented. With this method, the feed price will be cheaper than the standard formulae and expecting to achieve similar performance to the standard formula. The second method was used in this experiment to study the effectivity of BS4 enzymes on the performance of broiler chickens.

A new multi-enzyme called BS4 has been produced by cultivating *Eupenicillium javanicum* in a solid substrate fermentation. The BS4 contains β-mannanase, cellulase, β-mannosidase, and α-galactosidase (Sinurat et al. 2014). Studies on the effectivity of the BS4 enzyme on performance of laying hens, growing ducks, and native chickens have been carried out. Reports showed that supplementation of BS4 enzyme in the diet significantly improved the FCR of growing ducks (Purba & Sinurat 2018), and FCR of growing local chickens (Sinurat et al. 2017). All these studies used the BS4 enzyme in liquid form.

Most commercial enzymes are provided in dry powder form, although some are in liquid form. Liquid enzymes have a high-water content which allows the enzymes active all the time and may interact between enzymes that reducing the activity in the case of multi enzymes, especially when the protease is present. High water contents provide more chance of microbial growth when stored at ambient temperatures. Powdered enzymes are produced by removing the water content or immobilization process which automatically inhibits the enzyme to be active. It is easier to handle for storage, and more resistant to various environmental changes and transportation conditions as compared to liquid enzymes (Homaei et al. 2013). Drying is needed to produce the powder which might reduce its activities during the process and may affect its effectiveness when used as a feed supplement. Therefore, a study was designed to evaluate effectivity of BS4 enzyme when given in liquid or powder form.

MATERIALS AND METHODS

This experiment was carried out at the Indonesian Research Institute for Animal Production - Ciawi Bogor – Indonesia. All procedures regarding the use of live animals were carried out according to approval by The Animal Welfare Committee at the Indonesian Agency for Agricultural Research and Development. The approval number for this experiment is Balitbangtan/Balitnak /A/02/2016.

The liquid enzymes were produced by cultivating *Eupenicilium javanicum* by solid-state fermentation process as described by Haryati et al. (2019). The enzyme was immobilized by the absorption method to produce powder enzymes (Haryati et al. 2010). In brief, concentrated liquid BS4 enzymes were added to wheat pollard powder (mesh 60) with a 1:1 (v/w) ratio and mixed thoroughly. The mixture was then dried in an oven at 37ºC for 2 x 24 hours. Activity of both liquid and powder enzymes was determined by saccharification method as described by Haryati et al. (2019). One unit of saccharification activity is defined as the amount of enzyme in ml or g DM that liberates 1 μmol of glucose from palm kernel cake per minute under assay conditions. The amount of enzyme supplemented into the feed have similar activities, i.e., 30 saccharification Unit/kg feed or equal to l500 ml BS4 liquid enzymes/ton feed, or 2000 g BS4 powder enzymes/ton feed.

Three hundred unsexed broiler DOC were purchased from a commercial hatchery. The chicks were reared on pens covered with rice hulls as litter. Each pen-sized 284 x 146 cm (l x w) was filled with ten chicks, and considered as one experimental unit or replication. Every five pens, were fed with one of the six dietary treatments from 1 to 35 days old.

Two diets, i.e., a standard diet and a low-nutrient density diet were formulated. Standard diet was formulated to fulfill nutrient requirement of broiler chickens, and low nutrient density diet contained a lower protein and ME with a similar ME: protein ratios, i.e., 12.9 and 14.3 kcal ME/g crude protein for starter and grower diet, respectively. The standard starter contained 22.5% crude protein and 2900 kcal ME/kg,

Table 1. Ingredients and nutrient composition of starter and grower diets of broiler chicken

Ingredients, %	Starter diet (1-21 day)		Grower diet (22-35 day)	
	Standard	Low Density	Standard	Low Density
Maize	52.64	53.83	59.79	56.83
Meat and bone meal	6.00	4.00	6.00	6.00
Soy bean meal	29.65	30.00	26.90	24.08
Rice bran	7.81	8.67	2.55	10.00
Crude palm oil	2.00	1.00	3.00	1.31
Limestone	0.16	0.20	0.15	0.19
DL-Methionine	0.42	0.40	0.38	0.37
L-Threonine	0.09	0.08	0.05	0.06
L-Lysine	0.36	0.34	0.24	0.27
Dicalcium phosphate	0.30	0.90	0.36	0.31
Premixes (vitamins, mineralsand additives)	0.58	0.58	0.58	0.58
Total	100	100	100	100
Calculated nutrient composition				
Dry matter, %	88.00	88.00	88.00	88.1
Crude fiber, %	4.00	4.00	3.42	3.42
Metabolisable energy (ME), kcal/kg	2900	2815	3000	2910
Crude protein (CP), %	22.50	21.80	21.00	20.37
ME: CP ratio, kcal/g	12.90	12.90	14.30	14.30
Calcium, %	0.90	0.90	0.90	0.90
Available Phosphorous, %	0.50	0.50	0.50	0.50
Lysine, %	1.450	1.397	1.255	1.229
Methionine + Cystine, %	1.040	1.000	0.967	0.940
Dig. Lysine, %	1.270	1.232	1.100	1.067
Dig. Methionine + Cystine, %	0.940	0.912	0.870	0.844

and the standard grower contained 21.0% crude protein and 3000 kcal ME/kg. Low-nutrient density contained 21.8% crude protein and 2815 kcal ME/kg for starter diet, 20.37% crude protein, and 2910 kcal ME/kg for grower diet. The starter diets were fed from 1 to 21 days old, followed by the grower diets from 22 to 35 days old. Ingredients and nutrient composition of the diets are presented in Table 1. Each diet was supplemented with either no enzyme, BS4 liquid enzyme, or BS4 powder enzyme.

Performances of the chickens (body weight, feed intake, feed conversion ratios, and mortalities) were observed during starter period (1-21 days) and whole period of the trial (1-35 days). At the end of the feeding trial, one male and one female chicken from each pen were taken randomly and slaughtered to measure carcass percentage, abdominal fat, gizzard, and liver weight. The performance data were subject to analyses of variance in 2x3 factorial design with five replicates, while data on carcass yield, abdominal fat, gizzard, and liver weights were analyzed in 2x2x3 factorial design with five replications. Further analyses were carried out with the Duncan test to determine the difference between treatments when the ANOVA was significant at $P<0.05$.

RESULTS AND DISCUSSION

Results

Growth performance

Performances of broilers during starter and whole period are presented in Table 2 and Table 3. Feed intake was significantly affected by interaction ($P<0.01$) between dietary nutrient levels and enzyme

supplementation. Broilers fed with low nutrient diet consumed significantly less feed when supplemented with BS4 enzymes. The highest feed intake was found in broilers fed with no enzyme supplementation (1335 g/bird) and significantly different from those diets supplemented with BS4 powder enzymes (1194 g/bird). Feed intake of broilers on diet supplemented with liquid BS4 enzyme (1313 g/bird) was lower than those fed on diet without enzyme and higher than those fed on diet supplemented with powder BS4 enzyme, although the difference was not significant (P>0.05). However, enzyme supplementation did not significantly (P>0.05) affect feed intake of broilers when fed with the standard diet.

The treatments (nutrient levels, enzyme supplementation, and their interactions) during starter period did not affect body weight at 21 days old significantly (P>0.05). The heaviest broiler weight was found on broilers fed with the low nutrient diet without enzyme supplementation (937 g). The lighter weight was found on broilers fed low nutrient diet and supplemented with BS4 powder enzyme.

The treatments also did not affect FCR (P>0.05) during starter period. The lowest FCR (1.381) was achieved by broilers fed standard diet supplemented with BS4 liquid enzymes. On the other hand, broilers fed with the low diet supplemented with BS4 liquid enzyme performed the highest FCR value (1.442). Average body weight of broilers fed with low nutrient diet (902 g/bird) was similar to those fed with standard diet (904 g/bird), but FCR was better on broilers fed with standard diet (1.408) than broilers fed with low nutrient diet (1.424). Average body weight of broilers fed the non-supplemented diet (922 g/bird) was slightly heavier than broilers fed with the liquid supplemented diet (893 g/bird), and BS4 powder enzymes (894 g/bird), although the difference between treatments was not significant. There was no different effect between liquid and powder BS4 enzyme supplementations on body weight at 21 days old. However, average FCR of broilers fed the diet without enzyme was inferior (1.424) to those fed the diet supplemented with BS4 enzyme in liquid (1.412) or powder forms (1.403).

Survival rates of broilers during starter period were also not significantly (P>0.05) affected by nutrient levels, enzyme supplementation, and their interactions. Average survival rate of broilers fed with low nutrient levels (97%) was slightly lower than broilers fed with the standard diet (99%). Survival rates were similar on birds fed without enzyme supplement (98%) with broilers fed enzyme supplement in liquid (98%) or powder forms (99%). Nonetheless, the survival rates in all treatments were still high.

Effect of treatments on performances of broilers during the whole period (1 to 35 days old) is presented in Table 3. Interaction between nutrient level and enzyme supplement did not significantly (P>0.05) influence feed intake, body weight, FCR, and survival rates of broilers during the period. Therefore, performance data presented to describe the main effect only.

Feed intake of broilers from 1 to 35 days was not significantly (P>0.05) affected by the nutrient density. Average feed intake of broilers fed the low diet was slightly higher (3214 g/bird) than the standard (3163 g/bird). However, feed intake of broilers from 1 to 35 d was significantly (P<0.05) affected by dietary enzyme supplementation. Broilers fed the diet without BS4 enzymes consumed (3275 g/bird) more feed than broilers fed the diet with BS4 enzymes. There was no significant difference in feed intake of broilers fed the diet with the liquid (3156 g/bird) and the powder (3135 g/bird) BS4 enzymes.

Broilers' body weight at 35 d of age was not significantly (P>0.05) affected by nutrient density, enzyme supplement nor by interactions between the two factors. Average body weight of broilers at 35 days fed with low nutrient density was slightly heavier (1966 g/bird) than those fed the standard diet (1940 g/bird). Average body weight of broilers fed the diet without enzyme supplement was slightly lower (1909 g/bird) than those fed diet supplemented with liquid (1958 g/bird) or powder (1992 g/bird) BS4 enzyme.

FCR of broilers from 1 to 35 d was not significantly (P>0.05) influenced by the nutrient density. The average FCR of broilers fed the low diet was similar (1,639) to those fed standard diet (1.636). However, enzyme supplementation significantly (P<0.05) influenced the FCR. Average FCR of broilers from 1 to 35 days was higher (1.719) or less efficient than broilers fed the diet supplemented with enzymes. FCR of broilers fed the diet supplemented with BS4 liquid enzyme (1.615) was slightly higher than those supplemented with BS4 powder enzyme (1.579) although the difference was not significant (P>0.05).

Survival rates of the broilers from 1 to 35 d were not significantly (P>0.05) influenced by interaction between nutrient density and enzyme supplementation, nutrient density nor by enzyme supplementation. Average survival rate was between 96 – 100% and 93 – 97% during starter, and the whole period, respectively.

Carcass yield and organ weight

Effect of treatments on carcass percentage, abdominal fat levels, gizzard weihgt, and liver of broilers at 35 d of age are presented in Table 4. The nutrient density, enzyme supplementation, sex, or interactions between the two factors did not significantly (p>0.05) affect carcass percentage, abdominal fat level, gizzard weight, and liver weight of male and female broilers. Only live bodyweight of the broilers was significantly (p<0.01) affected by sex.

Table 2. Performance of broilers fed with different nutrient density and enzyme supplement during starter period (1-21 day)

Nutrient level	Type of Enzyme	BW DOC g/bird	Feed intake, g/bird	BW 21 d, g/bird	FCR	Survival rate, %
Low Nutrients	None	50.8±2.5	1335±59[a]	937±91	1.436±45	96.0±5.5
	Liquid	51.9± 0.5	1313±90[ab]	912±79	1.442±0.065	96.0±5.5
	Powder	50.1±5.2	1194±33[b]	857±23	1.394±0.042	100.0±0.0
Standard Nutrients	None	50.1±2.5	1298±58[ab]	907±30	1.432±0.084	100.0±0.0
	Liquid	48.9±4.2	1206±81[ab]	874±56	1.381±0.063	100.0±0.0
	Powder	48.9±5.7	1312±74[a]	931±45	1.411±0.087	98.0±4.5
Significance (P):						
Nutrient Level (N)		0.27	0.73	0.93	0.63	0.15
Type of Enzyme (E)		0.83	0.09	0.47	0.72	0.78
N x E		0.79	<0.01	0.08	0.60	0.13

BW= body weight, DOC= day old chick, FCR= food conversion rate. Means in the same column with different superscript differ significantly (P<0.05)

Table 3. Performance of broilers fed with different nutrient density and BS4 enzyme supplement during experimental period (1-35 day)

Treatments	Feed intake 1-35 day, g/bird)	Body weight 35 day, g/bird	FCR	Survival rate, %
Effect of Nutrient Density:				
Low	3214 ±128	1966 ±107.0	1.639 ±0.097	94.0 ±7.4
Standard	3163 ±150	1940 ±89.7	1.636 ±0.110	95.3 ±7.4
Effect of enzymes supplementation:				
None	3275 ±87[a]	1909 ±85.0	1.719 ±0.117[a]	97.0 ±6.7
Liquid	3156 ±169[b]	1958 ±84.4	1.615 ±0.065[b]	93.0 ±6.7
Powder	3135 ±117[b]	1992 ±110.2	1.579 ±0.096[b]	94.0 ±8.4
Significance (P):				
Nutrient Density (N)	0.27	0.49	0.93	0.63
Enzyme (E)	0.04	0.23	0.03	0.48
N x E	0.11	0.44	0.97	0.48

FCR= food conversion rate. Means in the same column and factor with different superscript differ significantly (P<0.05)

Average body weight of males (2297 g/bird) was heavier than female broilers (1987 g/bird). The nutrient density and the enzyme supplementation did not significantly (P>0.05) affect body weight, carcass yield, abdominal fat, gizzard, and liver weight.

Discussion

Feeding broilers with a lower nutrient density than the standard recommendation will impair the performance of the broilers. Supplementing enzymes into the low nutrient density diet is expected to restore the performance. However, the result of the present study did not show a significant difference between the standard and the low nutrient density diets. Delezie et al. (2010) reported that broilers fed with a lower nutrient density than the standard diet with a similar ME protein ratio depressed feed intake, body weight gains, and impaired FCR when ME was reduced too drastic (300 kcal/kg). However, when the difference was not too drastic (150 kcal/kg), the performances were not significantly affected by nutrient density.

Abdollahi et al. (2018) also reported that there was only a slight reduction in performance of broilers when

Table 4. Carcass and some organs weight of broilers fed with different nutrient levels and enzyme supplementation

Treatments	Live body weight	Carcass. g/100 g BW	Abdominal fat. g/100 g BW	Gizard. g/100 g BW	Liver g/100 g BW
Nutrient density (N)					
Standard	2107±234	66.7±2.676	1.03±0.276	1.82±0.25	2.32±0.37
Low	2177±243	67.9±2.231	0.98±0.278	1.76±0.28	2.25±0.37
Enzymes (E)					
None	2130±220	67.8±2.414	1.07±0.273	1.75±0.23	2.37±0.42
Liquid	2170±261	66.9±2.241	1.00±0.230	1.86±0.23	2.28±0.41
Powder	2125±245	67.3±2.890	0.95±0.292	1.76±0.32	2.21±0.25
Effect of Sex (S)					
Female	1987±144[a]	67.2±2.615	1.04±0.256	1.82±0.25	2.31±0.32
Male	2297±214[b]	67.5±2.448	0.96±0.294	1.76±0.28	2.26±0.41
Significance (P)					
Nutrient density (N)	0.14	0.08	0.45	0.34	0.44
Enzyme (E)	0.69	0.54	0.41	0.33	0.38
Sex (S)	<0.01	0.69	0.28	0.34	0.59
N x E	0.92	0.84	0.92	0.42	0.15
N x S	0.86	0.94	0.780	0.31	0.96
E x S	0.22	0.90	0.42	0.94	0.16
N x E x S	0.11	0.39	0.34	0.06	0.60

BW= body weight. Means in the same column and factor with different superscript differ significantly (P<0.05)

ME of the diet was reduced by 100 kcal/kg, but the performance reduction was significantly higher when the ME of the diet was 200 kcal/kg lower than the standard diet. Alqhtani et al. (2022) showed that the feed intake and bodyweight gain of broilers were not significantly affected by diet density when the difference of the ME was less than 100 kcal/kg. The ME difference between the standard and the low diet in the present experiment was only 100 kcal/kg. These may be the reasons why there was no difference in performance of broilers fed diet with different densities.

This study showed an interaction effect between enzyme supplementation and nutrient density on feed intake during the starter period but not during the whole period, which indicated that the supplementation effectively reduced feed intake when fed low nutrient density diet during starter period. However, Abudabos (2012) and Alqhtani et al. (2022) showed no interaction between nutrient density and enzyme supplementation on feed intake of broilers during the starter period. No explanation could be described at this stage for this discrepancy.

Beneficial effect of enzyme supplementation in broiler feed is normally expressed in improvement in feed efficiency utilization or reduction of feed conversion ratio (FCR). In general, nutrient digestibility of the diet was low in the early stage of life and increases with increasing age for chicks (Batal & Parsons 2002). This may be related to development of digestive tract and organs related to digestion process, and it is expected that effect of enzyme supplementation on feed efficiency is higher when given in starter period than during grower or whole (starter and grower) period. However, different results were found in the present study and other studies (Abudabos 2012; Alqhtani et al. 2022). The present study showed average improvement in FCR was 6.7%, and 7.1% during starter period (1-21 d), and whole period (1-35 d), respectively. Abudabos (2012) showed 1.3% and 6.0% FCR improvement during starter (1-22 d) and whole period (1-42 d), respectively. Alqhtani et al. (2022) reported 1.6% and 2.8% FCR improvement during starter (1-22 d) and the whole period (1-42 d), respectively. Perhaps, more studies are required to explain this phenomenon.

Supplementation of BS4 enzyme did not influence body weight during the starter and the whole period but significantly reduced feed intake and improved FCR of broilers during whole experimental period, regardless of the nutrient density. Feed intakes were reduced by 119 g/bird (or 3.6% of the standard), and 140 g/bird (or 4.3% of the standard) when BS4 liquid- and powder

enzymes were supplemented, respectively. FCR was 6.4% and 8.9% better than the standard as effect of BS4 liquid or powder supplement, respectively. These result indicated that the BS4 enzyme increased availability of nutrients in the diet, hence less feed was required to perform similar bodyweight as standard. It has been reported that BS4 enzymes mainly consist of carbohydrates such as β-mannanase, cellulase, β-mannosidase, and αgalactosidase (Sinurat et al. 2014). However, the enzymes are capable to increase metabolizable energy (digestibility of carbohydrates), protein, and amino acids of feed ingredients or feed (Pasaribu et al. 2009; Sinurat et al. 2013; 2014; 2015).

Some variations in the poultry performance improvement due to supplementation of BS4 enzymes in the feed have been reported. Supplementation of BS4 enzymes in commercial laying hen's feed increased by 3.2% egg production and improved by 5.8% FCR. There was no improvement in egg production, but FCR was improved by 4.3% in laying native chickens (Sinurat et al. 2019). Bodyweight gain was increased by 7.2% and the FCR by 6.8% in growing ducks (Purba & Sinurat 2018). Bodyweight gain was not affected, but FCR improved by 2.2% in growing native chickens (Sinurat et al. 2017).

A meta-analysis was conducted based on 21published reports to study effect of multi-carbohydrases containing α-galactosidase + xylanase or α-galactosidase+β-glucanase on improving performance of broilers' (Llamas-Moya et al. 2021). This study concluded that multi-carbohydrases have a significant effect on improvement of broilers performance. Body weight gain increased by 56 g/bird, and FCR reduced by 0.042 or 2.4% better than performance of broilers without enzyme supplements. Result of the present study showed that body weight increased by 49, and 83 g/bird when supplemented with BS4 enzymes in liquid, and powder, respectively. The results also showed an improvement in FCR 0.104 (or 6.4%) and 0.14 (or 8.9%) with BS4 enzymes supplementation in liquid, and powder form, respectively. These results indicated effectivity of BS4 enzymes in liquid or powder form to improve the performance of broilers is better than the average effectivity of multi-carbohydrases reported.

Reports on effect of powder and liquid enzymes are scant. The present study showed a slightly better improvement of powder than the liquid BS4 enzymes in improving broiler's performance, but the difference was not significant. Sinurat et al. (2019) also found similar results when BS4 enzymes were supplemented into the diet of native chicken layers. The present study showed that the effectivity of enzymes did not depend on the form but their enzyme activities. Formation process of powder enzyme was immobilization of liquid BS4 on wheat pollard with adsorption method. In this method, the enzyme is not firmly bound to the matrix. Although immobilization process may affect enzyme activity, effect on performance of broilers was not shown since liquid and powder BS4 enzymes added were similar, i.e., 30 saccharification Units/kg feed.

There were some studies on effect of nutrient density on carcass yield and internal organs of broilers with inconsistent results. The present study showed similar results as reported by Kamran et al. (2008); Zhai et al. (2013); and Kim et al. (2016), which showed that carcass yield, relative weights of abdominal fat, and liver were not affected by nutrient density. On the other hand, Li et al. (2010) showed that broilers fed with a lower nutrient density produced lower abdominal fat levels.

Effect of dietary enzyme supplementation on carcass and internal organ characteristics of broiler chickens has been reported by many authors. Most of the studies showed no significant effect of enzyme supplementation on carcass and organs characteristic of broilers. Dietary enzyme supplementation, especially the carbohydrases are expected to increase AME of the diet and may imbalance ME: protein intake ratios if digestibility of protein is not increased. Diets with a higher ME: protein ratio than the standard is well known to increase abdominal fat deposition in broilers (Nahashon et al. 2005; Hada et al. 2013; Fouad & El-Senousey, 2014). This phenomenon did not occur in the present study. The ME: protein ratio imbalances might not occur since the BS4 enzyme improved ME, protein, and amino acids digestibility of the feed (Pasaribu et al. 2009; Sinurat et al. 2014). Emadinia et al. (2014) and Mohammadigheisar et al. (2021) also reported similar results.

Enzymes supplementation helps digest feed in the digestive tract of chickens by biochemical reactions. Activity of digestive organs may be less as compared to those fed without enzyme supplementation. Therefore, smaller size of organs related to the digestion process is expected in chickens fed dietary enzymes. Some reports support this hypothesis, but not all. Agboola et al. (2013) showed no significant effect of dietary enzyme supplementation on carcass and liver weight but reduced gizzard size of broilers. Some researchers reported that adding enzymes to diets did not affect relative weight of liver and gizzard of broiler chickens (Hassanein 2011; Emadinia et al. 2014; Mohammadigheisar et al. 2021). The present study also showed that BS4 enzyme supplementation did not significantly influence relative weight of liver and gizzard of broilers.

CONCLUSION

Based on the results, it is concluded that nutrient density did not affect performances (feed intake, body weight, FCR, and survival rates), carcass yield, abdominal fat-, gizzard-, and liver- relative weights of broilers. Supplementation of BS4 enzymes in broilers'

diet reduced feed intake and improved FCR. Effectivity of liquid and powder BS4 enzymes in improving performance of broiler chickens was similar.

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Changes in Physiological Condition of Broiler Chickens Sprayed with Water before Transportation

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ABSTRAK

Sara U, Raharja DP, Sonjaya H, Azhar M. 2022. Perubahan kondisi psikologi ayam broiler yang disemprot dengan air sebelum transportasi. JITV 27(2):93-99. DOI:http//:dx.doi.org/10.14334/jitv.v27i2.2996.

Pengangkutan menuju RPU (rumah potong unggas) merupakan rangkaian proses yang dapat menyebabkan tingginya tingkat stres pada ayam ras pedaging. Stres panas akan terus meningkat apabila jarak tempuh antara kandang dan rumah potong unggas sangat jauh yang menyebabkan perjalanan akan semakin lama. Salah satu solusi untuk mengatasi stres panas akibat pengangkutan adalah melakukan penyiraman beberapa menit sebelum perjalanan dimulai agar ayam ras pedaging tetap mampu mempertahankan kondisi homeostatisnya. Penelitian ini bertujuan untuk untuk mengetahui pengaruh metode penyiraman sebelum pengangkutan dengan jarak yang berbeda terhadap status hematologis, hormonal, serta kualitas daging ayam ras pedaging. Penelitian ini disusun berdasarkan Rancangan Acak Kelompok (RAK) pola faktorial. Sebanyak 54 ekor ayam ras pedaging strain Cobb umur 35 hari dibagi menjadi 2 perlakuan yakni; tanpa penyiraman (P0) dan penyiraman (P1). Ayam pada masing-masing perlakuan diangkut menuju rumah potong unggas dengan 2 jarak tempuh yang berbeda yakni; 30km (J1) dan 60km (J2), serta 1 perlakuan kontrol (tanpa pengangkutan) (J0). Penyiraman dilakukan sesaat sebelum proses pengangkutan. Hasil penelitian menunjukkan bahwa jarak tempuh pengangkutan meningkatkan nilai kekuningan (b*) daging bagian dada, dan menurunkan nilai kecerahan (L*), serta meningkatkan pH daging bagian paha ayam ras pedaging ($P<0.05$). Nilai hematokrit, kadar hemoglobin, dan konsentrasi hormon triiodotironin (T_3), komponen warna daging lainnya, serta pH daging bagian dada tidak mengalami perubahan signifikan, baik ditinjau dari penyiraman, jarak tempuh, maupun interaksi keduanya ($P>0.05$). Respon ayam ras pedaging terhadap penyiraman sebelum pengangkutan terlihat dari perubahan tingkat penyusutan berat badan. Selain itu, pengangkutan dengan jarak tempuh yang berbeda berpengaruh terhadap sifat fisik daging ayam ras pedaging

Kata Kunci: Jarak Tempuh, Kualitas Daging, Profil Fisiologis, Stres Pengangkutan, Penyiraman

ABSTRACT

Sara U, Raharja DP, Sonjaya H, Azhar M. 2022. Changes in physiological condition of broiler chickens sprayed with water before transportation. JITV 27(2):93-99. DOI:http//:dx.doi.org/10.14334/jitv.v27i2.2996.

Transportation to the slaughterhouse is a series of processes that can cause high levels of stress in broilers. Heat stress will increase if the distance between the farm and the slaughterhouse is far. One of the solutions to overcome heat stress due to transportation is to do watering a few minutes before the transportation so that the broilers are still able to maintain their homeostatic conditions. This study aims to determine the effect of watering methods before transportation with different distances on the haematological, hormonal, and quality status of broiler chickens. This study was arranged based on a factorial randomized block design (RAK). A total of 54 broilers of the Cobb strain aged 35 days were divided into 2 treatments, namely; without watering (P0) and watering (P1). Chickens in each treatment were transported to the poultry slaughterhouse with 2 different distances; 30km (J1) and 60km (J2), as well as 1 control treatment (without transport) (J0). Watering is done just before the transportation process. The results showed that the transportation distance increased the yellowness value (b*) of the breast meat, decreased the brightness value (L*), and increased the pH of the broiler thigh meat ($P<0.05$). Hematocrit values, hemoglobin levels, and concentrations of the hormone triiodothyronine (T3), other meat color components, as well as the pH of the breast meat, did not change significantly, both in terms of watering, distance traveled, and the interaction between the two ($P>0.05$). The solution of water spraying before transportation on different distances could not restore the hematology and hormonal status, as well as the meat quality of the broiler. However, the decline in meat quality was shown in transported broilers.

Key Words: Distances, Meat Quality, Physiological Profile, Transportation Stress, Water Spraying

INTRODUCTION

Transportation is one of the processes in a series of pre-slaughter management on broilers. During transport, broilers are very susceptible to stress caused by several factors: shock, vibration, vehicle speed changing, ambient temperature, noise, high density, dehydration, and lack of food (Zheng et al. 2020). Changes in environmental temperature are a potential stressor during transportation, especially in tropical climates. Thermal stress causes changes in metabolism or physiological processes, which finally decrease meat quality (Xing et al. 2019).

Under stressful conditions, broilers will try to reduce metabolism in their body by declining the concentration of triiodothyronine (T3). Still, it happens temporarily because homeostasis processes in the body require a lot of energy. In this condition, metabolism will increase, even exceeding the normal level. Triiodothyronine does not play a role in this process anymore, but corticosterone. High metabolism requires glucose and oxygen in an amount more. Further, it will cause changes in haematological status and produce undesirable changes in meat quality.

Changes in haematological status will affect the body balance of broilers. Explained that chickens' well-being is supported by the balance of the physiological condition of blood, due to the critical roles of blood in absorbing and transporting nutrients, metabolic waste, hormones, and enzymes. Mainly, stress is caused by high ambient temperatures may change haematological profiles in broilers. Previous research was conducted by Hassan & Reddy (2012) found an increase of hematocrit value on 42-d-old broilers which were experiencing heat stress for 2 hours, however, haemoglobin concentration did not change. A previous study by Bergoug et al. (2013) found that there was no effect of transportation on the hematocrit value of 1-day-old chicks. A study conducted by Ulupi et al. (2018) found no change in the hematocrit value and haemoglobin concentration in broilers both before and after the transportation.

Meat quality decreased due to disruption of physiological processes during stress is a substantial loss. Several studies have proved that heat stress during transport changes meat quality, both in terms of colour, and pH of meat (Aleme & Bekele 2021; Zhang et al. 2012). Indeed, changes in meat color and pH refer to the formation of PSE meat (pale, soft, exudative), or DFD meat (dark, firm, dry), which indicates a decline in meat quality.

The solutions for reducing stress during transportation are the primary interest. The keys can be applied by supplementation vitamins, minerals, or changing environmental temperature by using a cooler on a truck. In this research, the solution was the application of water spraying just before transportation. Additionally, this inexpensive solution with an easy method can be applied by farmers and distributors of broilers. Some previous researchers who used water spraying before transportation could minimise extreme loss of meat quality of broilers (Jiang et al. 2015; Tamzil et al. 2018). However, no study that focused on physiological conditions, and especially haematological status due to the implementation of water spraying before transportation, and its relationship with meat quality. Thus, this study aimed to investigate the effect of water spraying before transport of different distances to the status of broiler's haematological, hormonal, and meat quality.

MATERIALS AND METHODS

This research was carried out at the Poultry Production Laboratory, Livestock Physiology Laboratory, and Animal Products Technology Laboratory, Faculty of Animal Husbandry, Hasanuddin University, as well as Pathology Laboratory, Makassar Health Laboratory Center.

Materials

The materials used include; 54 Cobb strain broilers aged 35 days, disposable syringe volume 3 ml, non-additive vacuum tube, tube containing EDTA K3 volume 3 ml, wax, 70% alcohol, hydrochloric acid (HCL), aquades, label paper, plastic clip, cotton swab, microcapillary hematocrit, and ELFA Triiodothyronine kit. The tools used include: pickup trucks, chicken baskets, electric scales, clinical thermometers, maximum-minimum thermometers, pipettes, microtubes, tube and microtube racks, knives, scissors, tweezers, and surgical boards, haemoglobinometer Sahli, general purpose centrifuge, specialty centrifuge, microhematocrit reading instrument, colorimeter, and pH meter.

Methods

The method used in this research includes research design, watering process, conditions in the transportation process, data collection and data analysis.

Research design

This study was arranged based on a 2x3 factorial randomized block design (RBD) with 3 transport groups (replicates), and each consisted of 3 sub replications, so that there were 54 experimental units. Two factors used in this study are; the first factor (I) is

watering, which P0 is No Watering and P1 is Watering. The second factor (II) is the distance traveled with J0 is 0 km, J1 is 30 km, and J2 is 60 km.

Watering process

The watering process based on the method of Jiang et al. (2015) and Wang et al. (2016) with some modifications. Chickens are watered using 4 sprinklers which are sprayed on both the right and left sides of the pick-up truck. The sprinkler used Nozzle Kit 8, with settings in *shower mode*. The water temperature is 27^{o}C and at an ambient temperature of ±30°C. Watering occurred for 30 seconds after the chickens are loaded onto a pick-up truck before the transport process.

Transportation process

The vehicle used to transport broilers from the cage to the poultry slaughterhouse is a pick-up type car. The baskets used are made of latticed plastic (0.78 x 0.58 x 0.28 m). Each basket can accommodate about 9-10 chickens. The minimum and maximum temperatures of the environment around the chickens are measured during the journey. Chickens are divided into 2 groups, namely; transportation distances of 30 km and 60 km. When arriving at the slaughterhouse, the chickens are weighed and rested for a short time (10-15 minutes) before slaughtering (Vieira et al. 2013). The series of handling processes before up to the time of the slaughtering process for broilers can be seen in Figure 1.

Data collection

The process of taking blood samples in broilers is carried out after transportation. Blood samples were taken from each chicken from each experimental unit through the brachial vein using a syringe. Blood is accommodated in two different tubes, namely; vacuum tube containing EDTA K3 anticoagulant, and non-additive vacuum tube. The blood sample in the EDTA K3 vacuum tube was used to test the parameters of the hematocrit value and hemoglobin levels, while the blood sample in the non-additive vacuum tube was centrifuged to obtain a serum sample which was used to test the Triiodothyronine hormone concentration parameter. After postmortem for 15 minutes, all the pectoralis major muscles (chest muscles) and gastrocnemius muscles (thigh muscles) were sampled to determine meat quality. Muscle samples were stored in plastic clips for later use for testing the color and pH parameters of the meat. Measuring meat color using a Minolta CR400 A colorimeter consisting of; L* (brightness), a* (redness) and b* (yellowish) (CIELAB color system) on the surface of the chest and thigh muscles.

Data analysis

The data obtained were analyzed using the Analysis of Variance (ANOVA) to test the diversity of the data and if there was a significant effect, continued with Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

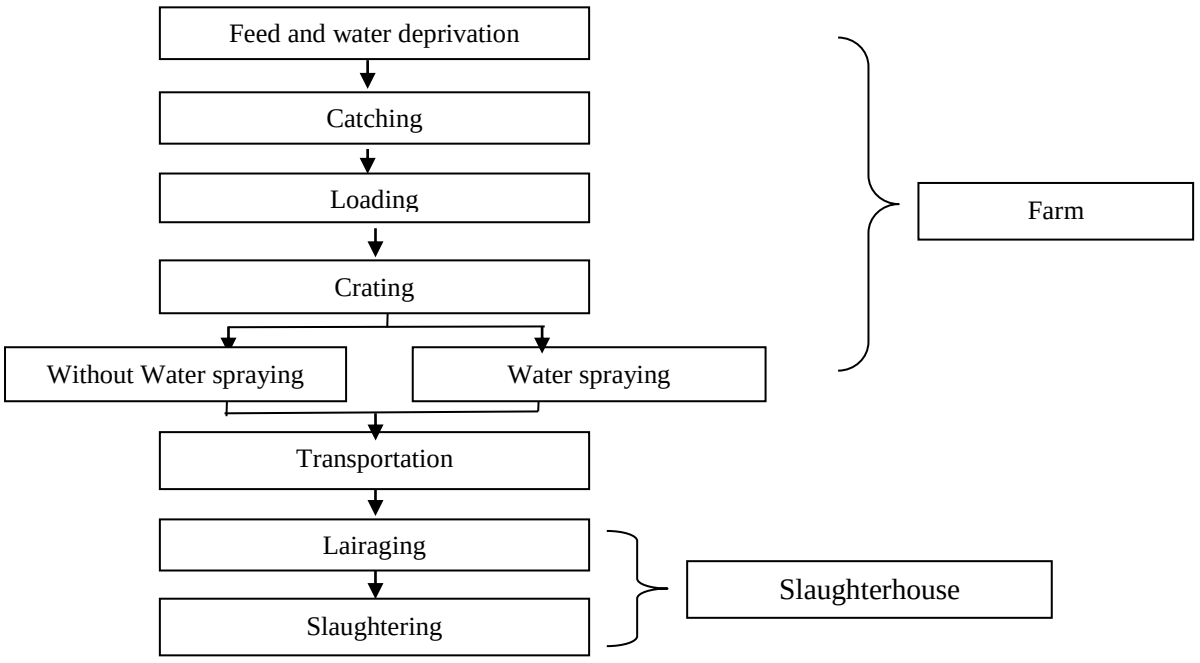

Figure 1. Flow chart of broilers handling sequence

RESULTS AND DISCUSSION

The haematological and hormonal status of broiler watered before transportation at different distances is shown in Table 1. Hematocrit values, hemoglobin concentration, and triiodothyronine levels did not change significantly, because of water spraying, distance, or their interaction (P>0.05).

There was no significant effect of water spraying, distances, and their interaction on the hematocrit values of broilers. These results indicated that both water spraying and different distances had not changed the number of blood cells in broilers. These results were obtained in a previous study by Adeyemi et al. (2015) who reported that feed deprivation time did not affect hematocrit values of broilers. Another study by Tamzil et al (2018) which applied water spraying before transportation, did not find any change in the hematocrit value. Although hematocrit values in this study did not differ, these values were lower than the normal standard. Broiler's normal hematocrit values range from 30 to 50% (Tamzil et al. 2013). Several factors affected low hematocrit values; erythropoiesis decline, increasing water consumption, hemodilution, and others (Tamzil et al. 2014). Besides, water spraying before transportation applied in this study also did not affect hematocrit values. A previous study by Yadav et al (2016) who investigated misting and wallowing in buffaloes found that under heat stress conditions, untreated buffalo would experience hemodilution more quickly, so the hematocrit value was lower than watered or wallowed buffalo.

Haemoglobin concentration was also not different in this study. Ulupi et al. (2018) reported that there was no change in the blood haemoglobin concentration of broilers after transportation. In this research, the stress of distances could not cause excessive product of glucocorticoid, thus water and sodium chloride reabsorption in kidney and caecum were still in normal condition, and hemodilution had not happened. Furthermore, stress during transportation was also unable to reduce the production of erythrocytes, thus erythropoietin could not stimulate erythropoiesis. The water spraying method applied in this research did not alter hemoglobin concentration. These results indicated that water sprayed broilers or not were still able to maintain haemoglobin concentration in their body. The study conducted on Murrah buffalo showed no change in haemoglobin concentration, both with water spraying or not (Yadav et al. 2016).

The triiodothyronine levels which were not different in this study were consistent with the finding of Hussnain et al. (2020) on broilers who experienced transportation and feed deprivation. Recent research on buffalo also showed that the cooling method or different seasons did not change triiodothyronine levels (Kumar et al. 2019; (Yadav et al. 2016). However, another study found a decrease in triiodothyronine concentrations after chickens underwent 2 and 4 hours transportation and feed restriction (Azis et al. 2012; Zheng et al. 2020). Change in triiodothyronine levels on transported broilers Changes in triiodothyronine levels was affected by deiodinase type III activity. Deiodinase type III was an enzyme that catalysed the deiodinase of thyroid hormones and had an essential role in controlling the bioavailability of hormones (Ciavardelli et al. 2014). Triiodothyronine levels in this research indicated that stress-caused transportation had not been able to increase the activity of deiodinase type III in broilers.

Table 1. Hematocrit values, haemoglobin concentrations, and triiodothyronine levels of broilers were watered before transportation at different distances

Parameters	Water Spraying	Distances			Mean
		J0	J1	J2	
Hematocrit Values (%)	P0	24.39±5.74	25.11±1.83	28.55±2.26	26.01±3.75
	P1	26.05±2.05	27.22±2.99	22.67±6.43	25.31±4.22
Mean		25.22±3.96	26.16±2.50	25.61±5.38	
Hemoglobin Concentration (gdL-1)	P0	8.29±1.82	7.86±1.44	8.58±1.12	8.24±1.33
	P1	8.82±0.60	8.41±0.99	7.89±1.67	8.37±1.09
Mean		8.56±1.25	8.14±1.15	8.24±1.33	
Levels Triiodothyronine (T3) (ng/mL)	P0	1.23±0.31	0.52±0.31	0.39±0.13	0.71±0.45
	P1	1.93±0.69	1.11±1.14	0.63±0.47	1.22±0.86
Mean		1.58±0.60	0.81±0.76	0.51 ± 0.31	

[a-b] Means within a column with superscripts are significantly different (P<0.05); P0= Without water spraying, P1= Water spraying; J0= 0 km (without transportation), J1= Transportation distance of 30 km, J2= Transportation distance of 60 km

Table 2. Meat color and pH values of broiler watered before transportation at different distances.

Parameters	Water Spraying		Distances			Mean
			J0	J1	J2	
				Breast		
	P0	*L**	46.76±2.08	47.36±0.80	43.47±2.52	45.86±2.47
		*a**	3.51±1.55	3.01±1.43	3.30±0.60	3.27±1.11
Meat Color		*b**	2.41±0.53	4.26±0.56	2.96±0.89	3.21±1.01
	P1	*L**	43.55±5.14	46.58±1.57	48.24±0.37	46.12±3.38
		*a**	3.81±1.22	2.02±1.03	3.26±0.69	3.03±1.18
		*b**	2.24±0.90	3.21±0.47	3.75±1.16	3.07±1.01
		*L**	45.15±3.92	46.97±1.97	45.85±3.06	
Mean		*a**	3.66±1.26[a]	2.51±1.24[b]	3.28±0.57[ab]	
		*b**	2.33±0.67[a]	3.74±0.74[b]	3.35±1.02[b]	
				Thigh		
	P0	*L**	52.10±2.93	49.43±1.26	48.02±1.59	49.85±2.53
		*a**	5.70±1.07	4.64±0.78	5.95±0.61	5.43±0.94
		*b**	3.52±0.43	4.50±0.79	3.08±1.29	3.70±1.00
	P1	*L**	51.29±1.53	49.37±1.34	49.43±1.35	50.03±1.54
		*a**	6.03±1.51	4.45±1.70	5.50±1.86	5.32±1.62
		*b**	3.52±0.07	3.15±0.54	3.74±0.74	3.47±0.53
		*L**	51.69±2.13[a]	49.40±1.16[b]	48.72±1.53[b]	
Mean		*a**	5.86±1.18	4.54±1.19	5.72±1.26	
		*b**	3.53±0.27	3.82±0.96	3.41±1.01	
				Breast		
Meat pH	P0		6.63±0.06	6.70±0.25	6.85±0.03	6.73±0.16
	P1		6.67±0.23	6.72±0.13	6.72±0.93	6.70±0.14
Mean			6.65±0.15	6.71±0.18	6.79±0.94	
				Thigh		
	P0		6.77±0.03	6.99±0.12	6.97±0.11	6.91±0.12
	P1		6.76±0.25	6.89±0.12	6.79±0.08	6.82±0.16
Mean			6.77±0.16[a]	6.94±0.12[b]	6.88±0.11[ab]	

[a-b] Means within a column with superscripts are significantly different (P<0.05); P0 = Without water spraying, P1= Water spraying; J0= 0 km (without transportation), J1= Transportation distance of 30 km, J2= Transportation distance of 60 km

Table 2 shows the meat quality of broiler watered before transportation at different distances. Significant differences were seen in the b* values (yellowness) of breast meat, and L* values (lightness) of thigh meat on transported broiler (P<0.05). b* values of breast meat showed an increase, whereas L* values of thigh meat decreased on transported broiler. However, water spraying treatment and interaction between water spraying and distances did not affect significantly color values. Moreover, the other color values had no significant effect, both in terms of water spraying, distances, and the interaction of these two factors (P>0.05). The significant difference in pH values was shown in thigh meat. pH values of thigh meat were increased after transportation (P<0.05). But, no significant effect in pH values of breast meat could be observed in this experiment (P>0.05).

Previous studies that found an increase in b* values on breast meat had also been observed by Tang et al. (2013) and Hu et al. (2020). In addition, L* values

decreased after transportation was an early indication of the formation of DFD (dark, firm, dry) meat. These results corroborated the findings of Brossi et al. (2018), who reported a decrease in L* values in broilers under Acute Heat Stress (AHS) conditions. Decreasing of L* values due to glycogen depletion, as a result, intracellular water increased in broiler meats. Hence, the meat's surface was too dry, and dense, and the ability of meats to absorb the light, was so high. Eventually, the meats look dark (Mir et al. 2016). The application of water spraying in this experiment did not show a significant difference in all meat colours, breast, and thigh meat. These results indicated that broilers could maintain meat quality though not completely.

In contradiction to these results, previous studies reported a decrease in pH value, either on breast or thigh meats (Tang et al. 2013; Zhang et al. 2012). Furthermore, there was no significant effect of all treatments on the pH values of chest meats. These findings were also obtained by Yalçin & Güler (2012), both on long or short distances. Additionally, Kim et al. (2017) found that heat stress change the pH values of thigh meat of broilers. There was very little evidence clarifying an increase in pH values of meat after transportation, but the results of this study were in line with a decrease in L* values (lightness) of thigh meats described previously. The increase of glycogen utilisation is because of energy requirements during stress (Yalçin & Güler, 2012). Commonly, lactic acid which was a by-product of glycogen had a role in decreasing pH values of 7.0 to 5.7 in the rigour mortise phase, yet the poor glycogen levels in meats due to stress resulted from an increase in pH values of meat. High meat pH caused a lower denaturation of myoglobin, and aerobic metabolism increased on the surface of the meat. Furthermore, meat's WHC (water holding capacity) increased, thus its shape became too dense, dark, and dry because the water was suspended in the meats (Mir et al. 2017; Wideman et al. 2016).

CONCLUSION

Transported & watered broiler had no difference in haematological status compared to no transported broilers. This indicates that watering could maintain the haematological status of broilers. However, transportation could not maintain the meat quality, because there are some changes in the color of the thigh and breast meat that lead to the pale, soft, and exudative condition.

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Influence of Duck Eggshell Nano-Calcium Fortification on the Chemical Quality of Beef Sausage

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ABSTRAK

Prayitno AH, Rukmi DL, Widiyawati A, Prasetyo B. 2022. Pengaruh fortifikasi nano kalsium kerabang telur itik terhadap kualitas kimia sosis sapi. JITV 27(2):100-106. DOI:http://dx.doi.org/10.14334/jitv.v27i2.3009.

Kerabang telur itik merupakan salah satu *bio-waste* dari industri peternakan unggas dan rumah tangga yang dibuang ke tempat sampah. Kerabang telur itik mengandung kalsium tinggi yang dapat diaplikasikan sebagai alternatif sumber kalsium harian tubuh. Kalsium kerabang telur itik berstruktur nano dapat digunakan sebagai bahan tambahan pangan dalam pengolahan sosis sapi. Tujuan penelitian ini adalah untuk mengetahui pengaruh fortifikasi nano kalsium kerabang telur itik terhadap kualitas kimia sosis sapi. Materi penelitian ini terdiri atas daging sapi, tepung tapioka, isolat protein soya, minyak sawit, garam, lada, bawang putih, bawang merah, bawang bombay, ketumbar, pala, gula, *frankfurter*, sodium tripolifosfat, monosodium glutamat, es, dan kerabang telur itik berstruktur nano. Perlakuan fortifikasi nano kalsium kerabang telur itik yaitu 0; 0,15; 0,3; 0,45; dan 0,6% dari total adonan. Parameter yang diuji yaitu kadar air, protein, lemak, karbohidrat, serat, abu, gula, kalsium, natrium, dan energi. Setiap perlakuan terdiri dari 5 replikasi. Data hasil uji kualitas kimia dianalisis dengan analisis variansi pola searah dan jika terdapat perbedaan yang signifikan (P<0,01) diuji lanjut dengan uji *Duncan's New Multiple Range Test*. Hasil penelitian menunjukan bahwa fortifikasi nano kalsium kerebang telur itik yang berbeda berpengaruh sangat nyata (P<0,01) terhadap kadar protein, lemak, abu, gula, kalsium, dan natrium, tetapi tidak mempengaruhi kadar air, karbohidrat, serat, dan energi sosis sapi. Forifikasi nano kalsium kerabang telur itik sampai level 0,6% dapat meningkatkan kadar protein, abu, dan kalsium tetapi menurunkan kadar lemak, gula, dan natrium sosis sapi.

Kata Kunci: Sosis Sapi, Kualitas Kimia, Kerabang Telur Itik, Fortifikasi, Nano Kalsium

ABSTRACT

Prayitno AH, Rukmi DL, Widiyawati A, Prasetyo B. 2022. Influence of duck eggshell nano-calcium fortification on the chemical quality of beef sausage. JITV 27(2):100-106. DOI:http://dx.doi.org/10.14334/jitv.v27i2.3009.

Duck eggshells are one of bio-wastes from poultry industry and household that have been disposed. Duck eggshells contain high calcium which can be applied as an alternative source of daily calcium for the body. Nanostructured duck eggshell calcium can be used as a food additive in beef sausage processing. This study was conducted to determine the chemical quality of beef sausage fortified by duck eggshell nano-calcium. The materials include beef, soy protein isolate, palm oil, garlic, salt, pepper, shallot, onion, tapioca, monosodium glutamate, sodium tripolyphosphate, nutmeg, coriander, frankfurter, sugar, duck eggshell nano-calcium, ice, and nano-structured duck eggshell. Treatments for fortification of duck eggshell nano-calcium were 0; 0.15; 0.3; 0.45; and 0.6% of the total dough. Parameters tested were moisture, protein, fat, carbohydrate, fiber, ash, sugar, calcium, sodium, and energy of the sausage. Each treatment consisted of 5 replications. Data collected was analyzed by analysis of variance using completely randomized design and if there was significant different (P<0.01) then further tested by the Duncan's New Multiple Range Test. Results showed that the fortification of duck eggshell nano-calcium had a highly significant effect (P<0.01) on protein, fat, ash, sugar, calcium, and sodium, but did not affect moisture, carbohydrate, fiber, and energy of beef sausage. Fortification of duck eggshell nano-calcium up to 0.6% increased protein, ash, dan calcium but decreased fat, sugar, and sodium of beef sausage.

Key Words: Beef Sausage, Chemical Quality, Duck Eggshell, Fortification, Nano-calcium

INTRODUCTION

Duck eggshell is one of bio-wastes from duck farming which is abundant with high calcium content (Prayitno et al. 2022) and a lot of it is produced from household waste. Utilization of bio-waste from duck eggshells can be used as a source of dietary calcium. Duck eggshells contain calcium carbonate as much as 94-97% (Nurlaela et al. 2014), while eggshells in the form of flour contain calcium around 50.75% (Prayitno et al. 2016). The properties and added value of duck eggshells can be enhanced by the application of nanotechnology to produce eggshell nanoparticles. High energy ball milling (HEM) is a nanotechnology that can

be applied to convert particles from eggshells to nano-sized ones. The application of HEM technology can change duck eggshell particles from 13,229 nm to 347 nm (Prasetyo & Prayitno 2020) with a calcium content of 54.36-59.27% (Prayitno et al. 2020).

The performance of nano-sized particles is better due to an increase in surface area (Habte et al. 2019). Shabnam et al. (2020) stated that nano-sized minerals addition to food have a better and faster absorption rate in stomach than micro-sized minerals. Nano-calcium oxide (NCaO) is one type of metal oxide that is widely applied to food products such as meat products. Nano-calcium oxide can be applied as an antibacterial (Roy et al. 2013), food additives (Suryanto et al. 2014; Prasetyo & Prayitno 2021), catalyst (Gopalappa et al. 2012), a drug delivery system (Balaganesh et al. 2018) that can increase absorption (Jampilek et al. 2019) so that it can be absorbed almost 100% by the body (Suptijah et al. 2012).

The International Osteoporosis Foundation (IOF) reports that prevalence of osteoporosis in women in Indonesia at the age of 50-70 years and over 70 years is 23% and 53% (Pusdatin 2020). Men after 55 years of age have a higher risk of osteoporosis than women (Jahari & Prihatini 2014). Premenopausal women have a risk of developing osteoporosis with a higher risk of 21.7% than men with a risk of osteoporosis of 14.8% (Mansoben et al. 2021). Low intake of calcium into the body can cause the risk of osteoporosis. Nurmaliza et al. (2021) stated that one of the risk factors that cause osteoporosis in women is calcium intake. Nano calcium in duck eggshells can be used as an excellent food additive compared to other sources of calcium as a mineral that is functional and has a positive impact on health.

One of the macro minerals that the body needs to meet bone health is calcium. The body's calcium is usually used to be met from spinach, broccoli, soybeans, milk, and processed products. Fulfillment of body calcium can be met in another way, is by consuming calcium-fortified foods. Micro-sized calcium is only absorbed by the body about 50% which can lead to deficiency (Prayitno et al. 2021). Eggshell nano-calcium as a natural source of calcium has been developed as a fortification material for functional food products. Sausage is the most consumed processed meat products in Indonesia. Sausage is one of the processed meat products which is processed with or without addition of other food additives that are inserted into casing with or without cooked (SNI 2015).

Foods fortified with eggshell calcium can be used as a functional food to reduce the risk of osteoporosis, especially in the elderly (Arnold et al. 2021). Calcium-fortified foods can increase calcium intake (Palacios et al. 2021) one of which is sausage products. Effect of eggshell nano-calcium fortification on beef sausage has been investigated on sensory quality (Prasetyo & Prayitno 2021) and physical quality (Prayitno et al. 2022). Sausage is a processed meat product that is relatively low in calcium (Engeloug et al. 2017; Huang et al. 2021). People of all ages who are starting to worry about their bone health have prompted the food industry to respond by fortifying calcium in foods (Cormick et al. 2021). Supporting research on duck eggshells nano-calcium fortification on the chemical quality of beef sausage has not been investigated. This research was designed to develop new healthier sausages fortified with nano-calcium and study its effect on the chemical quality of sausages taking into beneficial to the calcium intake. Chemical quality of sausages is one of the important variables in determining the quality of sausage products that are objectively tested. Therefore, this study aimed to determine the chemical quality of beef sausage fortified with duck eggshell nano-calcium.

MATERIALS AND METHODS

Materials

The materials used in this research include beef, tapioca flour, soy protein isolate, palm oil, garlic, pepper, salt, shallot, onion, monosodium glutamate, coriander, nutmeg, sugar, frankfurter, sodium tripolyphosphate, ice, nano-structured duck eggshell, and collagen casing.

Methods

This research started from the preparation of duck eggshell nano-calcium, preparation of formulations and ingredients, sausage processing, chemical quality test, and statistical analysis.

Preparation of duck eggshell nano-calcium

Duck eggshell nano-calcium were made using high-energy ball milling (Prasetyo & Prayitno 2020). Duck eggshells were soaked in hot water for 10 minutes, dirt and eggshell membranes were cleaned, dried at 105□C for 12 hours, and mashed. Eggshell flour was calcined at 1,000□C for 2 hours and further processed using HEM for 60 minutes to produce a duck eggshell nano-calcium powder.

Preparation of formulations and ingredients

Beef sausage formulation, ingredients, and beef sausage processing were made according to Prasetyo & Prayitno (2021). The formulation and ingredients of

beef sausage fortified with duck eggshell nano-calcium was presented in Table 1.

Preparation and sausage processing

Treatmentin in this research was according to Prasetyo & Prayitno (2021), with level of duck eggshell nano-calcium fortification as follow: 0; 0.15; 0.3; 0.45; and 0.6% of the total dough. The beef was cleaned of connective tissue, cut into small pieces, then ground. The ground beef and oil were mixed, and salt, sodium tripolyphosphate, duck eggshell nano-calcium, and ice were added. All spices were ground, soy protein isolate, tapioca, oil, and ice were mixed until smooth. Sausage dough was inserted into the collagen casings. The raw sausage was then boiled for 45 minutes at a temperature of 60-70°C and cooled at room temperature, and then a chemical quality test was carried out.

Table 1. The formulation and ingredients of beef sausage fortified with duck eggshell nano-calcium

No.	Ingredients	Percentage (%)
1.	Beef	50
2.	Tapioca flour	16.5
3.	Soy protein isolate	2.5
4.	Palm oil	10.5
5.	Salt	1.2
6.	Sodium tripolyphosphate	0.5
7.	Monosodium glutamate	1
8.	Pepper	0.2
9.	Garlic	1.2
10.	Shallot	2
11.	Onion	2
12.	Coriander	0.2
13.	Nutmeg	0.2
14.	Sugar	0.5
15.	Frankfurter	1
16.	Ice	10.5

Chemical quality test

The fortified sausage was analyzed for chemical quality including moisture, protein, fat, carbohydrate, fiber, ash, sugar, calcium, sodium, and energy (AOAC 2019).

Statistical analysis

Chemical quality data was analyzed by analysis of variance using completely randomized design and if there was significantly different ($P<0.01$) then further tested by the Duncan's New Multiple Range Test (Riadi 2014).

RESULTS AND DISCUSSION

Moisture

Results showed that the fortification did not significantly affect ($P>0.01$) moisture content of beef sausage. The moisture content ranged from 41.42-41.70%. This result was lower than the results of Cunningham et al. (2015) the moisture ranged between 56.8-67%. El-Nashi et al. (2015) reported beef sausage has a moisture content around 50.24-61.89%, and Prayitno et al. (2021) stated that moisture content was around 49.65-50.69%. Moisture content of beef sausage from this study still meets the standard for sausage moisture content, which was a maximum of 67% (SNI 2015).

There was no difference in moisture between nano-calcium fortified sausages and without nano-calcium fortification. This result showed that moisture of beef sausage is not affected by nano-calcium fortification and moisture of beef sausage from this study is good quality. Whereas in other processed meat products, namely meatballs, the presence of calcium fortification of eggshells can increase moisture content of the product (Suryanto et al. 2014).

Protein

Results showed that the fortification with different levels had a highly significant effect ($P<0.01$) on protein content of sausage. Protein content from this study ranged from 15.09-15.41%. Average protein content of sausage from this study was almost the same as the results of Cunningham et al. (2015) that ranges from 13.5-17.4% and El-Nashi et al. (2015) reported that the protein content between 12.72-16.32%. Protein content of beef sausage from this study still meets the standard for sausage protein content, which was at least 13% (SNI 2015).

Prayitno & Rahman (2020) stated that protein content of processed meat products could be influenced by the ingredients used. The protein content from this study showed an increasing value as the level of duck eggshell nano-calcium fortification increased. This can be caused by the higher level of fortification of duck eggshell nano-calcium, the higher the protein that can bind to duck eggshell nano-calcium. Calcium in nanoparticle size has high solubility and reaction ability and calcium ion (Ca^{2+}) as a cation has the ability to interact with meat protein so as to increase the proportion of protein in the product (Prayitno et al. 2016).

Fat

Results showed that the fortification with different levels had a highly significant effect (P<0.01) on fat content of beef sausage. Fat content of beef sausage produced from this study ranged from 11.53-11.86%. This result was almost the same as the results of Cunningham et al. (2015) which ranged from 10 to18.8%, but it was still lower when compared to the results of El-Nashi et al. (2015) which ranged from 16.23 to17.943%. The fat content from this study still meets the standard for sausage fat content, which was a maximum of 20% (SNI 2015).

The fat content in this study showed a decreasing value along with the increasing level of duck eggshell nano-calcium fortification. The fat content showed a value that was inversely proportional to the protein content of beef sausage produced in this study (Table 2). The higher the protein content, the lower the fat content. This was because the higher the level of fortification of duck eggshell nano-calcium, the higher the protein that can bind to duck eggshell nano-calcium (Prayitno et al. 2016). The bond between protein and duck eggshell nano-calcium was strong, it will bind more water than fat during sausage processing so that the fat content of beef sausage in this study was lower with higher protein content of beef sausage, although the moisture content of beef sausage in this study for each treatment did not differ.

Carbohydrate

Results showed that the fortification with different levels did not significantly affect (P>0.01) carbohydrate content of beef sausage. The carbohydrate from this study ranged from 28.31 to 28.48%. The average carbohydrate from this study was higher than the results of El-Nashi et al. (2015) which ranged from 3.29 to14.97%, while according to Leonard et al. (2019) the carbohydrate content was around 9.62-19.31%. Manihuruk et al. (2017) found that the carbohydrate content ranged between 19.82-21.47%. The absence of a significant difference from each treatment to the carbohydrate content of beef sausage can be caused by the ingredients used in beef sausageformulation in this study have almost the same carbohydrate and have a higher carbohydrate content composition when compared to the research conducted by El-Nashi et al. (2015) and Leonard et al. (2019).

The treatments of fortification in this study did not significantly affect carbohydrate content of beef sausage. The absence of differences in the carbohydrate content can be attributed to the absence of starch content of duck eggshell nano-calcium fortification materials used in the processing of beef sausage.

Fiber

Results showed that duck eggshell nano-calcium fortification with different levels did not significantly affect (P>0.01) fiber content of beef sausage. The fiber content in this study ranged from 0.23-0.27%. This result was lower than that of Cunningham et al. (2015) which ranged from 0.4-0.7%, while according to Sánchez-Zapata et al. (2013) sausage fiber content was around 1.46%.

The absence of a significant difference from each treatment to the fiber content of beef sausage can be caused by the ingredients used in this study have almost the same levels and have a lower fiber composition when compared to the research conducted by Cunningham et al. (2015) dan Sánchez-Zapata et al. (2013). The increasing level of duck eggshell nano-calcium fortification in this study could not affect significantly on fiber content of beef sausage.

Table 2. Chemical quality of fortified beef sausage duck eggshell nano-calcium

Variable	Fortification level				
	0%	0.15%	0.3%	0.45%	0.6%
Moisture (%)[ns]	41.70	41.64	41.42	41.34	41.42
Protein (%)	15.09[a]	15.19[b]	15.29[c]	15.32[c]	15.41[d]
Fat (%)	11.86[d]	11.77[cd]	11.66[bc]	11.63[ab]	11.53[a]
Carbohydrate (%)[ns]	28.40	28.37	28.48	28.47	28.31
Fiber (%)[ns]	0.23	0.24	0.27	0.27	0.27
Ash (%)	2.96[a]	3.04[b]	3.14[c]	3.24[d]	3.35[e]
Sugar (%)	3.33[b]	3.29[b]	3.22[a]	3.20[a]	3.19[a]
Calcium (mg/100 g)	25.00[a]	305.50[b]	619.00[c]	921.50[d]	1.165.00[e]
Sodium (mg/100 g)	26.00[c]	23.00[b]	21.50[ab]	21.00[a]	20.00[a]
Energy (kcal/100 g)[ns]	281.00	280.00	280.00	280.00	279.00

[ns]Not significant, [a-d]Different superscripts at the same row indicate highly significant differences (P<0.01)

Ash

Results showed that the fortification with different levels did highly significantly affect (P<0.01) ash content of beef sausage. The ash content in this study ranged from 2.96-3.35%. This result was higher than the results of Cunningham et al. (2015) which ranged from 2.2-2.7% but still lower than the results of the Sánchez-Zapata et al. (2013) which around 4.93%. Gad EL Rab et al. (2019) study which found that the ash content of beef sausage ranges from 6.74-7.20%.The ash content of beef sausage from this study still meets the standard for sausage ash content, which is a maximum of 3% (SNI 2015).

The ash content in this study showed an increasing value along with the increasing level of duck eggshell nano-calcium fortification (Table 2). The increase in ash content from this study could be due to the higher the level of fortification of nano-calcium duck eggshell, the higher the increase in mineral content sourced from duck eggshell nano-calcium. Prasetyo & Prayitno (2020) found that the minerals contained in duck eggshell nano-calcium were phosphorus 0.70%; magnesium 0.41%; sodium 0.35%; and 59.27% calcium.

Sugar

Results showed that the fortification of duck eggshell nano-calcium with different levels had a highly significant effect (P<0.01) on the sugar content of beef sausage. The sugar content in his study ranged from 3.19-3.33%. This result was higher than that of Hadipernata et al. (2016) which ranged between 0.5-1.71% and Coloretti et al. (2019) reported that the sugar content of sausages was around 0.75%.

The sugar content of beef sausage from the results of this study showed a decreasing value along with the increasing level of duck eggshell nano-calcium fortification. This could be seen based on the research results obtained on the sugar content of beef sausage (Table 2). The decrease in sugar content in beef sausage from this study was inversely proportional to the calcium content of beef sausage which increased with increasing levels of duck eggshell nano-calcium fortification.

Calcium

Results showed that the fortification of duck eggshell nano-calcium with different levels did highly significantly affect (P<0.01) calcium level of beef sausage. The calcium content ranged from 25-1.165 mg/100 g. The average calcium content was higher than the results of Irshad et al. (2016) which ranged from 6.48-203 mg/100 g and Gad EL Rab et al. (2019) which ranged from 146.61-246.85 mg/100 g. Processed meat products generally have lower levels of calcium content when compared to processed meat products fortified with calcium materials (Prayitno et al. 2016).

The calcium content showed an increasing value along with the increasing level of duck eggshell nano-calcium fortification (Table 2). The increase in calcium levels from this study could be caused by the higher the level of fortification of duck eggshell nano-calcium, the higher the increase in calcium levels sourced from duck eggshell nano-calcium. The calcium level contained in the duck eggshell nano-calcium was 59.27% calcium (Prayitno, et al. 2020).

Sodium

Results showed that the duck eggshell nano-calcium fortification with different levels did highly significantly affect (P<0.01) sodium content of beef sausage. The sodium content ranged from 20-26 mg/100 g. The average sodium content from this study was lower than the results of Carraro et al. (2012) which ranged from 680.19-998.15 mg/100 g and Stanley et al. (2017) which ranged from 597.3-908.8 mg/100 g. This lower sodium content indicated that the beef sausage from this study has a healthier appeal. Reducing sodium intake in processed meat products can be seen as an effort to reduce risk factors for hypertension and heart disease (Carraro et al. 2012).

The sodium content from this study showed a decreasing value along with the increasing level of the fortification (Table 2). Duck eggshell nano-calcium has a sodium content of 0.35% (Prasetyo & Prayitno 2020). The sodium content in the duck eggshell nano-calcium does not increase the sodium content of the product. The decrease in sodium content in this study was inversely proportional to the calcium content of beef sausage which increased with increasing levels of the fortification fortification.

The absence of a significant difference from each treatment to the energy of beef sausage can be caused by the ingredients used in the formulation of beef sausage in this study have almost the same energy. The increasing level of duck eggshell nano-calcium fortification in this study could not have a significant impact on the energy of beef sausage. Beef sausage carbohydrate has no difference between treatments, so it does not have a significant impact on energy for each beef sausage treatment in this study.

Result showed that the fortification of duck eggshell nano-calcium did highly significantly affect protein, fat, sugar, calcium, sodium, and ash, but did not affect moisture, carbohydrate, fiber, and energy of beef sausage. Fortification of duck eggshell nano-calcium up

to 0.6% could increase the protein, ash, and calcium content but decrease fat, sugar, and sodium content of beef sausage.

CONCLUSION

The results showed that the fortification of duck eggshell nano-calcium had a highly significant effect on protein, fat, sugar, calcium, sodium, and ash, but did not affect on moisture, carbohydrate, fiber, and energy of beef sausage. Fortification of duck eggshell nano-calcium up to 0.6% could increase the protein, ash, and calcium content but decrease fat, sugar, and sodium content of beef sausage.

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Citation in the references:

Literature in the reference is written alphabetically based on the author's name. The same author is written sequentially starting from earlier order.

Example of reference writing

Primary paper:

Bhanja SK, Anjali DC, Panda AK, Sunder GS. 2009. Effect of post-hatch feed deprivation on yolk-sac utilization and young broiler chickens. Asian-Aust J Anim Sci. 22:1174-1179.

Book:

a. Lawrence TLJ, Fowler VR. 2002. Growth of farm animals. 2nd ed. New York (USA): CABI Publishing.
b. Bamualim A, Tiesnamurti B. 2009. Konsepsi sistem integrasi antara tanaman padi, sawit, dan kakao dengan ternak sapi di Indonesia. In: Fagi AM, Subandriyo, Rusastra IW, penyunting. Sistem integrasi ternak tanaman padi, sawit, kakao. Jakarta (Indones): LIPI Press. p. 1-14.
c. Paloheimo M, Piironen J, Vehmaanpera J. 2010. Xylanases and cellulases as feed additives. In: Bedford MR, Partridge GG, editors. Enzymes in farm animal nutrition. 2nd ed. New York (USA): CABI Publishing. p. 12-53.

Proceeding:

Umiyasih U, Antari R. 2011. Penggunaan bungkil inti sawit dan kopra dalam pakan penguat sapi betina berbasis limbah singkong untuk pencapaian bobot badan estrus pertama >225 kg pada umur 15 bulan. Prasetyo LH, Damayanti R, Iskandar S, Herawati T, Priyanto D, Puastuti W, Anggraeni A, Tarigan S, Wardhana AH, Dharmayanti NLPI, editors. Proceeding of National Seminar on Livestock Production and Veterinary Technology.

Bogor (Indones): Indonesian Center for Animal Research and Development. p. 192-199.

Thesis:

Krisnan R. 2008. Kombinasi penggunaan probiotik mikroba rumen dengan suplemen katalitik pada pakan domba (Thesis). [Bogor (Indones)]: Institut Pertanian Bogor.

Electronic magazines:

Wina E, Tangendjaja B, Dumaria. 2008. Effect of *Calliandra calothyrsus* on *in vitro* digestibility of soybean meal and tofu wastes. Livest Res Rural Develop. Vol. 20 Issue 6. http://www.lrrd.org/lrrd20/6/wina 20098.htm.

Institution:

a. [NRC] National Research Council. 1985. Nutrient requirements of sheep. 6th revised. Washington DC (USA): National Academic Press.
b. [CDC] Centers for Disease Control. 2006. Standard operating procedure for the direct Rapid Immunohistochemistry Test (dRIT) for the detection of rabies virus antigen. [accessed December 20th, 2011]. http://www.rabiesblue print.com/IMG/pdf/DRIT_SOP.pdf.

Patent:

Blanco EE, Meade JC, Richards WD. 1990. Ophthalmic Ventures, assignee. Surgical stapling system. United States patent US 4,969,591. 1990 Nov 13.

10. **Citation in text:**

The citation consists of the author's last name and publication year.

Example:

a. One author: ..... grow slower than lamb fed cattle's milk (Supriyati 2012). Supriyati (2012) formulates.....
b. Two authors: ..... expect, end maintenance weight (Khasrad & Rusdimansyah 2012). Khasrad & Rusdimansyah (2012) argued.....
c. Three authors or more: ..... based on DNA mitochondria analysis (Mtileni et al. 2011). Mtileni et al. (2011) report.....
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