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PREFACE

In this edition, Volume 26 No 2, we proudly present articles from animal and veterinary sciences including genetics; reproduction, feed technology, and veterinary. The articles published in this edition are:

"Association of SNP g.232 G>T Calpain Gene with Growth and Live Meat Quality Prediction using Ultrasound Images in Bali Cattle"; "Metabolism Energy and Performance of Several Local Cattle Breeds Fed Rice Straw and Concentrate"; "Application of Plantaricin as an Antimicrobial Substrate in the Milking Process to Maintain Milk Quality in Smallholder Dairy Farm"; "Effect of Chicken Bone Extract Powder on Epididymal Sperm Quality of Male Wistar Rats"; and Avian Beta Defensin 2 (AvBD2) Gene Polymorphism Identification in IPB-D1 Chicken".

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 2021

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LIST OF CONTENT

Association of SNP g.232 G>T Calpain Gene with Growth and Live Meat Quality Prediction using Ultrasound Images in Bali Cattle

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ABSTRAK

Dairoh, Jakaria, Ulum MF, Ishak ABL, Sumantri C. 2020. Asosiasi SNP g.232 G>T gen calpain dengan pertumbuhan dan prediksi kualitas daging hidup menggunakan citra ultrasonografi pada Sapi Bali. JITV 26(2): 49-56. DOI: http://dx.doi.org/10.14334/jitv.v26i2.2701.

Sapi Bali (*Bos javanicus*) adalah ternak asli Indonesia hasil domestikasi dari banteng (*Bibos banteng*). Gen yang memiliki peran penting terhadap kualitas daging adalah proteinase sistein netral yang diaktivasi oleh kalsium, dikenal sebagai calpain (CAPN). Tujuan dari penelitian ini adalah untuk menganalisis keragaman gen calpain dan hubungannya dengan sifat pertumbuhan dan kualitas daging dalam bentuk tebal *longissimus dorsi* (TLD), tebal lemak punggung (TLP), skor marbling, dan presentase lemak intramuskular (PLIM) pada sapi Bali. Keragaman gen CAPN1 dianalisis melalui PCR-RFLP menggunakan enzim restriksi BglII pada sapi Bali (n=52 ekor). Citra ultrasonografi otot longissimus dorsi diambil dengan posisi transversal dan longitudinal diantara tulang vertebrae torakalis ke 12-13 dan dianalisis dengan perangkat lunak Image-J NIH. Hasil penelitian menunjukkan bahwa SNP g.232 G>T gen CAPN1 bersifat polimorfik pada sapi Bali. SNP g.232 G>T gen CAPN1 pada sapi Bali memiliki variasi tinggi yang ditunjukkan dengan nilai heterozigositas 0.48 dan berada dalam keseimbangan Hardy-Weinberg. Keragaman SNP g.232 G>T berasosiasi secara signifikan (P<0.05) dengan bobot badan 730 hari, skor marbling (SM), dan presentase lemak intramuskular (PLIM) pada sapi Bali. Disimpulkan bahwa gen CAPN1 pada sapi Bali adalah kandidat sebagai Marker Assisted Selection (MAS) yang memiliki pengaruh terhadap bobot badan 730 hari, skor marbling, dan presentase lemak intramuskular.

Kata Kunci: Sapi Bali, Gen calpain, Pertumbuhan, Kualitas daging, Ultrasonografi

ABSTRACT

Dairoh, Jakaria, Ulum MF, Ishak ABL, Sumantri C. 2020. Association of SNP g.232 G>T calpain gene with growth and live meat quality prediction using ultrasound images in Bali Cattle. JITV 26(2): 49-56. DOI: http://dx.doi.org/10.14334/jitv.v26i2.2701.

Bali cattle (*Bos javanicus*) are native Indonesian cattle, domesticated from banteng (*Bibos banteng*). Genes that have an important role in meat quality are calcium-activated neutral protease genes, known as calpains (CAPN). The objective of this study was to evaluate the polymophisms of calpain gene SNP g.232 G>T by PCR-RFLP technique and its influence on growth trait and meat quality of Bali cattle detected by ultrasound imaging of *longissimus dorsi* thickness (LDT), back fat thickness (BFT), marbling score (MS), and intramuscular fat percentage (PIMF). The polymorphisms of CAPN1 gene were analyzed by PCR-RFLP using BglII restriction enzyme (n=52 cattle). The ultrasound images of longissimus dorsi muscle were carried out transversally and longitudinal between 12^{th} -13^{th} thoracic vertebrae then analyzed by Image-J NIH software. Result showed that SNP g.232 G>T of CAPN1 gene was polymorphic in Bali cattle. SNP g.232 G>T of CAPN1 gene in Bali cattle has higher diversity which was showed of 0.48 heterozygosity value and was in Hardy-Weinberg equilibrium. The polymorphisms of SNP g.232 G>T was associated significantly (P<0.05) with bodyweight at 730 days, marbling score (MS), and intramuscular fat percentage (PIMF). It suggests that the CAPN1 gene in Bali cattle is a candidate for Marker Assisted Selection (MAS), which influences body weight at 730 days, marbling score, and percentage of intramuscular fat.

Key Words: Bali cattle, Calpain gene, Growth, Meat quality, Ultrasound

INTRODUCTION

In 2020, a total of national meat production was dominated by 59% of chicken, the contribution of beef cattle only 16% into the total national meat production. National beef demand can only be fulfilled by 73.94% of domestic production, and 26.06% of the deficiency is fulfilled by imports (OECD-FAO 2020). Efforts to increase beef production through the breeding program are still focused on growth, while the increasing

demand for meat requires not only quantity but also quality parameters for consumers. The selection of the right beef cattle for slaughter significantly affects meat quality. Therefore rapid method of prediction of optimal fattening period length and measurement to determine the slaughter value in live animal are required (Pogorzelska-Przybylek et al. 2015). Ultrasound imagery is a rapid and non-invasive tool which believed to be an accurate predictor for fat and muscle deposition in livestock (Silva & Cadavez 2012). Ultrasound technology was also used to predict the carcass of beef at 12-13th fat and *longissimus dorsi* thickness (Jakaria et al. 2017). Ultrasonic rump fat and back fat thickness showed a high correlation of 0.64 (Bonin et al. 2015). Ultrasound measurements, including IMF percentage, have shown to be a valuable method for assessing carcass quality characteristics (Nogalski et al. 2018). Non-invasive technology is a well-established method used to assess the quality of live meat prior to slaughter in the cattle breeding program for native cattle.

Bali cattle (*Bos javanicus*), as a genetic resource for native cattle, come from the domestication of banteng (*Bibos banteng*) (Martojo 2012). Bali cattle also have been recognized by the FAO as one of the cattle breeds in the world in 2003 (Directorate General of Livestock Service 2003). Bali cattle have several superiorities, including fertility rates (pregnancy) range from 80% to 90%, birth rates of 75-85% (Wawo 2018) and carcass values of 56%, and good meat quality (Hafid et al. 2019). Bali cattle have the potential to be selected as a premium beef by the influence candidate gene related to meat quality which is Calpains gene (CAPNs). By using the molecular approach, the CAPNs gene can be evaluated for their nucleotide sequence profile. Calpain plays an important role in postmortem proteolysis, which can degrade the myofibril and influence meat tenderness by the proteolytic system (Coria et al. 2018). Calpain activity is regulated by calcium levels and calpastatin as a specific inhibitor. Calpain and calpastatin are two enzymes involved in the process of the proteolytic calpain system. The Calpastatin inhibits the calpain activity in low calcium levels of cytosol (Lian et al. 2013). Muscle growth in meat has a negative effect on the proteolysis activity of calpain, one of which is the muscle growth controlled by the myostatin gene (MSTN). Hypertrophy usually occurs in connective tissue. For example, double muscle is due to deletion of the MSTN gene, which causes a decrease of protein degradation and results in tough meat (Koohmaraie et al. 2002).

The influence of CAPN on meat quality, including in terms of tenderness, muscle fiber characteristic, and fatty acid, has been reported in Japanese quail (Işık 2019), sheep (Zhang et al. 2016), and pork (Lee et al. 2012; Lim et al. 2014). It was also reported in the Korean cattle, namely Hanwoo that calpain has many important roles in marbling score and intramuscular fat content, particularly in untranslated regions (Cheong et al. 2008). If a mutation variant takes place in calpain untranslated region, it can be influenced by the level of the protein product from translation and stabilization of RNA expression (Steri et al. 2018). This process is related to meat quality. The influence of genetic variants in the untranslated region has also been reported: to have an important role in the regulation of the post-transcription process (Araujo et al. 2012), initiates modulator translation (Dacheux et al. 2017), and translation regulation (Mayr 2017). Although the influence of the calpain gene has been widely investigated in the populations of *B. taurus*, *B. indicus*, and *B. taurus* x *B. indicus*, however, it is less studied in the untranslated region of Bali cattle (*Bos javanicus*) up to the present.

Therefore, the presence of calpain genes is necessary to be studied using as a potential candidate of genetic markers in Bali cattle for the selection program. The objective of this study was to evaluate the influence of SNP g.232 G>T calpain gene by PCR-RFLP technique on growth and meat quality traits in Bali cattle.

MATERIALS AND METHODS

Blood sampling

A total of 52 Bali cattle blood samples were used in this study, which was obtained from BPTU-HPT Denpasar of Bali Province. Those blood samples were taken from the *jugular vein* and collected using venoject tubes containing 1.5 ml EDTA. Extraction of DNA was carried out by DNA extraction kits (Geneaid) at the Animal Molecular Genetic Laboratory of the Faculty of Animal Science of IPB University.

Phenotypic data

The phenotypic data observed was including *longissimus dorsi* thickness and back fat thickness (carcass characteristics), marbling score and percentage of intramuscular fat (meat characteristics) measured using a 6.5 MHz transducer of ultrasound images at 12-13th rib position (Ulum et al. 2014). The ultrasound image result was stored in JPEG format and performed by Image-J NIH software (ImageJ, NIH, USA) (Figure 1). The determination of the marbling score (MS) was based on the AUSTRALIAN MEAT and MSA (http://www.wagyu.org.au/marbling/).

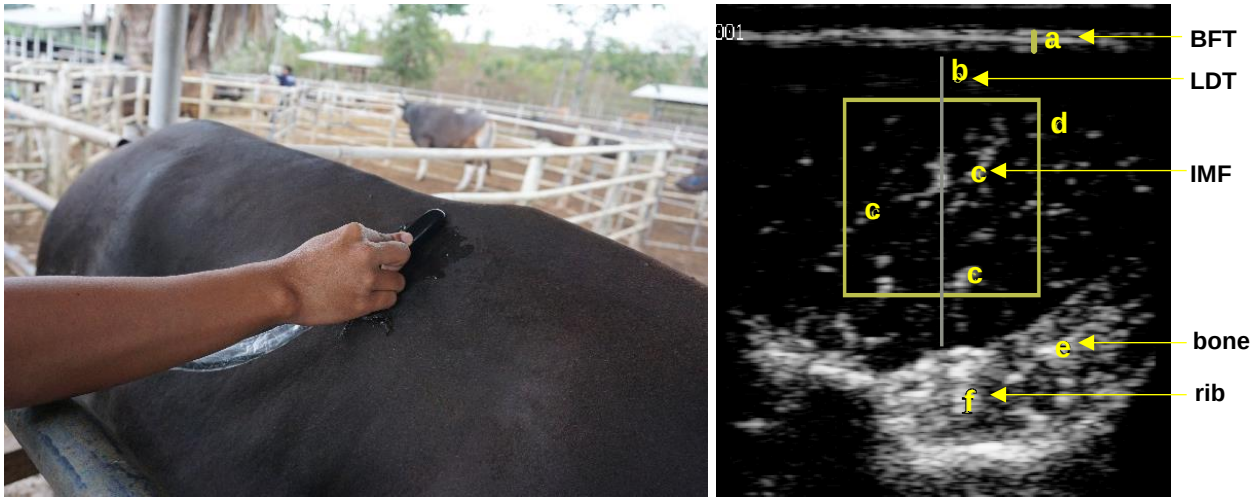

Figure 1. The position of muscle ultrasound measurement at 12-13th rib orientation point (left) and the image analyzed by using Image-J NIH software (right), (line a = back fat thickness (BFT); line b = longissimus dorsi thickness (LDT); c = intramuscular fat content (IMF), rectangle d = measurement area IMF 30 mm x 30 mm; e = bone; f = rib)

Primer design and DNA amplification

The primer sequences of the CAPN1 gene based on Jakaria et al. (2020) were previously designed at the 5'UTR region using GenBank database sequence (NCBI) with access code AH009246.3. The primer sequences included forward and reverse as follows F: 5'-CCCTTCCCACCCAGATAGG-3', R: 5'-CCTGGAGACCGTGAGGAAC-3' and the PCR product was 478 bp. The amplification of the CAPN1 gene at 5'UTR region were conducted using thermocycler AB System machine with PCR condition of pre-denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 57°C for 15 seconds, extension at 72°C for 10 seconds, and a final extension step at 72°C for 3 minutes. PCR product was visualized through 1.5% agarose gel and was observed using UV Transilluminator (Biorad™, California, USA).

PCR-RFLP and genotyping

PCR product was incubated at 37°C for 4 hours with *Bgl*II restriction endonuclease enzyme for digesting SNP g.232 G>T at 5'UTR region. The sample products were then verified using electrophoresis through 2% agarose gel with PeqGreen staining for genotyping. Identification of the genotype was determined by band pattern visualization.

Data analysis

Genetic diversity parameters of allele and genotype frequencies, heterozygosity, and chi-square were calculated by direct counting, namely PopGen 1.32 program. The frequency of alleles and genotypes were calculated using the Nei & Kumar (2000) formula as follows:

$$X_i = \frac{(2n_{ii} + \sum_{i \neq j} n_{ij})}{2N} \qquad X_i = \frac{n_{ii}}{N}$$

Where $\mathbf{x_i}$ is allele frequency, $\mathbf{x_{ii}}$ is genotype frequency, n_{ii} is the number of individuals with genotypes ***ii***, n_{ij} is the number of individuals with genotypes ***ij***; and N is the sample number of individuals.

Furthermore, Genetic diversity is calculated using the Nei & Kumar (2000) formula using observed heterozygosity (H_o) and expected heterozygosity (H_e) value as follows:

$$H_O = \sum_{i \neq j} \frac{N_{1ij}}{N} \qquad H_e = 1 - \sum_{i=1}^{q} x_i^2$$

Where H_o is heterozygosity observation value, N_{1ij} is the number of individuals with heterozygous, N is the observed number of individuals, H_e is heterozygosity expectations value, x_i is frequency of allele, and q is the alleles number.

Hardy-Weinberg equilibrium is analyzed by chi-Square (χ^2) according to Nei & Kumar (2000) as follow:

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Where x^2 is *chi*- Square, O is observed value, and E is expected value.

The association between each of SNPs in CAPN1 gene with carcass and meat characteristic in Bali cattle were performed using SAS 9.4 software by General Linear Model (GLM) procedure (SAS Inst, Inc, Cary, NC). Tukey Multiple Comparison Test ANOVA was compared for the least means square value of

genotypes. The mathematical of GLM model as follows:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where Y_{ij} is observation of phenotypic, μ is overall mean, G_i is effect of genotypes, and e_{ij} is random error.

Meat characteristic and growth traits data were corrected based on age and maintenance system before calculating association using the formula according to Salamena & Papilaja (2010) as follows:

$$\text{Xi corrected} = \left[\frac{\bar{X}_{\text{standard}}}{\bar{X}_{\text{observation}}}\right] \text{ x X observation value i}$$

Where $X_{i\ \text{corrected}}$ is corrected data I, $\bar{X}_{\text{standard}}$ is standard group average, $\bar{X}_{\text{observation}}$ is observation group average, and $X_{\text{observation value i}}$ is observation value data i

Live body weight of Bali cattle was corrected on age and maintenance using formula according to Hardjosubroto (1994) as follows:

$$\text{BWDG} = \left[\frac{\text{Bi-B0}}{\text{Age}}\right] \qquad B_{205} = \left[\frac{\text{Bi-B0}}{\text{Age}} \text{x } 205\right] + \text{B0}$$

$$B_{365} = \left[\frac{\text{Bi-B0}}{\text{Age}} \text{x } 365\right] + \text{B0} \qquad B_{730} = \left[\frac{\text{Bi-B0}}{\text{Age}} \text{x } 730\right] + \text{B0}$$

Where BWDG is body weight daily gain, Bi is body weight at weighing data I, B0 is birth weight, B_{205} is body weight at 205 days, B_{365} is body weight at 365 days, and B_{730} is body weight at 730 days.

RESULTS AND DISCUSSION

Polymorphisms of SNP g.232 G>T 5'UTR Region

The 5'UTR region was successfully amplified at 57 °C, and the PCR product was 478 bp (Figure 2A). The polymorphisms of SNP g.232 G>T were analyzed using the PCR-RFLP. Three distinct-genotype patterns were found in Bali cattle (Figure 2B). *Bgl*II restriction digestion result was in fragment length of 478 bp for genotype GG, and 478 bp, 354 bp, 124 bp for heterozygotes GT, in addition, 354 bp, 124 bp for genotype TT (Figure 2B). The GG genotype is a GenBank reference genotype representing the wild-type allele, whereas the TT genotype represents a mutant genotype with two mutant alleles (National Center for Biotechnology Information 2021). This corresponds to the Ensembl SNP reference with the location code rs44090872 or c.-11 G>T by access code ENSBTAG00000010230 (EnsemblGenomes 2021).

A previous study has been reported that the 5'UTR region of CAPN1 gene was polymorphic in Bali cattle nevertheless quite low nucleotide diversities (Pi) of 0.00632 (Jakaria et al. 2020). Previously, the role of CAPN gene has been reported that the u-calpain is the enzyme proteolysis, contributes to meat tenderness (Avilés et al. 2013; Lozano et al. 2016). The coding region of CAPN1 gene polymorphisms association with meat quality has been widely reported in cattle. Chung et al. (2014) revealed that the CAPN1 gene had a significant effect on meat tenderness in Hanwoo cattle. Furthermore, the polymorphism of CAPN1 gene has been reported to be associated with fatty acid and amino acid in Yanbian Yellow cattle (Xin et al. 2010). However, there was no information about the association of 5'UTR with meat quality in Bali cattle. According to Sihite et al. (2019), Bali cattle with the GG genotype have the highest value of live weight and average daily gain than the GT and TT genotypes.

Genotype and allele frequencies and heterozygosity value of 5'UTR region

Genetic diversity value in a population is stated as a parameter for studying population and evolutionary genetics. The variation of the population can be seen from allele and genotype frequencies. The allele

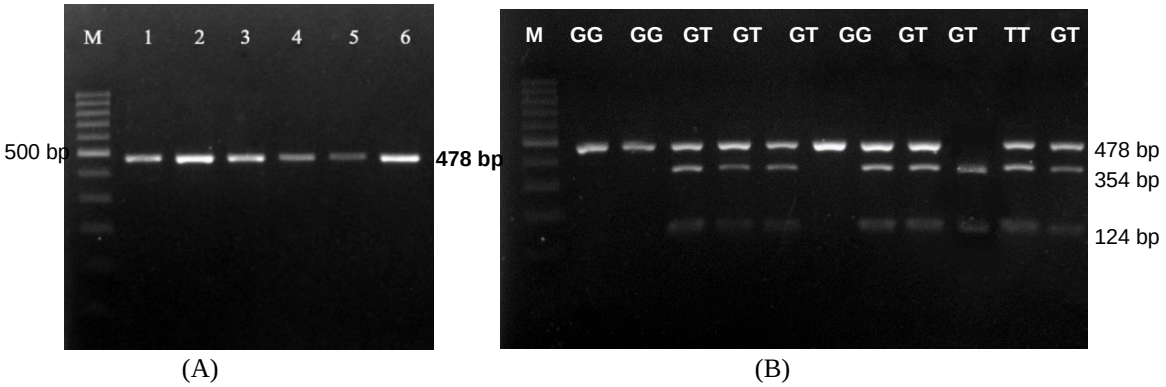

Figure 2. PCR amplification and RFLP genotyping of the polymorphism within CPAN1 gene's 5'UTR region in Bali cattle. (A) electrophoresis 1.5% gel agarose. (B**)** electrophoresis 2% gel agarose showing three PCR-RFLP genotypes pattern. M = 100 bp; line 1-6 = samples

Table 1. Allele frequency, genotype frequency, heterozygosity, and chi-square test SNP g.232 G>T Calpain gene at 5'UTR region in Bali cattle

Breeds	N	Genotype Frequency			Allele Frequency		Ho	He	χ^2
		GG	GT	TT	G	T			
Bali	52	0.46	0.48	0.06	0.70	0.30	0.481	0.423	1.016^{ns}

Note: N = total sample; ns = not significant P>0.05 or χ^2 value < χ^2 (α 0.05 df 1 = 3.84)

Table 2. Association analysis of SNP g.232 G>T polymorphism with meat quality and growth traits in Bali cattle

Traits	Genotype			P-value
	GG (n=23)	GT (n=25)	TT (n=1)	
Weaning weight (kg)	92.67 ± 22.55	86.82 ± 14.79	84.00 ± 0.00	0.544
Body weight at 365 days (kg)	142.30 ± 48.80	120.32 ± 29.68	105.00 ± 0.00	0.145
Body weight at 730 days (kg)	278.90 ± 77.10^{a}	230.20 ± 54.70^{b}	295.00 ± 0.00^{ab}	0.042
Average daily gain (kg)	0.33 ± 0.08	0.29 ± 0.06	0.28 ± 0.00	0.138
	GG (n=24)	GT (n=25)	TT (n=3)	
Longissimus dorsi thickness (mm)	52.95 ± 4.88	51.44 ± 4.47	53.01 ± 7.30	0.527
Back fat thickness (mm)	1.91 ± 0.35	1.68 ± 0.31	1.90 ± 0.31	0.058
Marbling score	1.63 ± 0.69^{a}	1.18 ± 0.26^{b}	1.56 ± 0.71^{ab}	0.024
Intramuscular fat (%)	3.01 ± 1.57^{a}	1.79 ± 0.85^{b}	2.68 ± 1.68^{ab}	0.006

[a-b] Different letters indicate a significant difference between genotypes, P<0.05

frequency, genotype frequency, and heterozygosity value of SNP g.232 G>T polymorphisms in Bali cattle are presented in Table 1. Two alleles and three genotypes of SNP g.232 G>T were found in Bali cattle. Approximately half of the Bali cattle were heterozygous (48%) in SNP g.232 G>T polymorphism of the 5'UTR region. It was assumed that the Calpain/*Bgl*II fragment was polymorphic in Bali cattle. If an allele frequency is 0.99 in a large population or 0.95 in a small population, it is confirmed to be polymorphic (Allendorf et al. 2013).

Based on the chi-square test, Bali cattle were in Hardy-Weinberg equilibrium (P>0.05). Equilibrium of this breed was also described by a comparison of observed and expected heterozygosity values in this study. The observed heterozygosity was higher than expected heterozygosity, and it is suggested that the population was in random mating effect (Sharma et al. 2016). The heterozygosity of Bali cattle was high, about 0.481, which means that Bali cattle have plenty of genetic diversity. Sheriff & Alemayehu (2018) stated that the population with high heterozygosity means lots of genetic variability, of about ≥50%. From these findings, it can be determined that polymorphism of SNP g.232 G>T in Bali cattle was still under the Hardy-Weinberg rule, which there is no mutation, migration, selection, and also random mating (Khan et al. 2018).

Association of genotype with growth and meat quality traits

Table 2 is presenting the mean and standard deviation values for each growth and meat quality of Bali cattle. The result of association analysis between 5'UTR of calpain gene and growth traits showed that the SNP g.232 G>T polymorphisms was associated with body weight at 730 days. Bali cattle with the GG genotype had the highest body weight at 730 days compared to the GT genotype. Sihite et al. (2019) showed that the CAPN1 gene was associated with birth weight and average daily gain, which differs from this present study in terms of the number of samples used. These findings suggested that SNP g.232 G>T polymorphisms potentially be used as the candidate of Marker Assisted Selection (MAS) for selection criteria for growth trait. This finding is supported by Zhang & Li (2011) showed that the Calpain gene has a strong association with bodyweight, weaning weight, and birth weight in Nanyang cattle. Furthermore, Pintos & Corva (2011) stated that one or more genes in bovine

chromosome 29, including the calpain gene, were involved in growth regulation.

The polymorphisms of SNP g.232 G>T at 5'UTR region was associated with marbling score and intramuscular fat percentage in Bali cattle (P<0.05). It showed a strong impact on the different genotypes of these traits. The highest marbling score and intramuscular fat percentage were found in Bali cattle with GG genotypes compared to the TT and GT genotypes. The degree of marbling is generally considered contributing to the sensory quality, in particular the tenderness of cooked meat (Wang et al. 2016). Newlacil et al. (2013) revealed that the marbling score of Charolais and Limousin were 1.54 and 1.27 lower than the marbling score of this study (1.63). This result indicated that the marbling score increase with increasing intramuscular fat content. Lee & Choi (2019) stated that the marbling score and intramuscular fat content have strong correlation ranges from 0.80 up to 0.88 in Hanwoo steer.

Meat quality prediction can be carried out by the actual technique (real-time) or using non-invasive technology as ultrasound imaging. In an actual prediction, male Bali cattle have carcass quality i.e., back fat thickness 3.08 mm, carcass percentage 53.33%, and marbling score of 2.65 (Suryanto et al. 2014). Nellore cattle with a backfat thickness <3 mm had a marbling score of 3.39, while back fat thickness >6 mm had marbling score 3.50 (Malheiros et al. 2015). Hanwoo cattle back fat thickness was 10 mm with marbling score 9.55 (Moon et al. 2003). These results indicate that different breeds can perform different meat quality. Ultrasound imaging is an efficient technology to predict carcass and meat measurement, potentially used in the breeding program. Robinson et al. (1992) stated that the accuracy of ultrasound was obtained from the correlation value with carcass measurement, i.e., rump thickness 92%, rib fat 90%, and longissimus muscle 87%. The backfat thickness and rump fat thickness of Nellore cattle using ultrasound imaging technology were 0.44 and 0.47, respectively (Bonin et al. 2015). Australian Angus cattle heritability value of intramuscular fat was 0.62, and marbling score was 0.46 (Duff et al. 2018)

The association of polymorphism in the UTR region clearly shows a strong association with both meat quality and growth trait (P<0.05) in Bali cattle. The UTR region influences gene expression by affecting mRNA stability and translation efficiency (Gu et al. 2014). There is evidence by Schuster & Hsieh (2019) that two differences of 5'UTR region alternative can increase the expression of the cancer tumor suppressor gene through a transition process. This transition is from the shorter of 5'UTR expression as efficient translation to the longer 5'UTR expression, which contains the secondary structure, and uORF result greatly hinders translation. It is possible that mutation SNPs in the UTR region could affect to the stability of the mRNA and translation process and resulting in a changed function (Juszczuk-Kubiak et al. 2010). Cheong et al. (2008) stated that the polymorphism in the 3'UTR region of the CAPN1 gene had a strong association (P-value: 0.0007) with the marbling score in Hanwoo cattle. Therefore, the polymorphism of the calpain gene in the UTR region is expected in future investigation as a genetic marker in Bali cattle associated with meat quality and growth traits.

CONCLUSION

This study confirmed that the SNP g.232 G>T of calpain gene in Bali cattle was one of the candidate genes for marbling score and percentage of intramuscular fat, which showed a strong association (P<0.0.5). Furthermore, the SNP g.232 G>T polymorphism also has an association with the growth trait of body weight at 730 days. The information of significantly associated SNP g.232 G>T could be used as a Marker Assisted Selection (MAS) candidate in Bali cattle.

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Metabolism Energy and Performance of Several Local Cattle Breeds Fed Rice Straw and Concentrate

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ABSTRAK

Utami ETW, Bata M, Rahayu S. 2021. Metabolisme energi dan performans beberapa bangsa sapi lokal yang diberi pakan jerami padi dan konsentrat. JITV 26(2): 57-64. DOI: http://dx.doi.org/10.14334/jitv.v26i2.2711.

Penelitian bertujuan mengkaji pengaruh bangsa sapi lokal terhadap metabolisme energi dan performans pada kondisi lingkungan dan pakan yang sama. Penelitian menggunakan empat bangsa sapi lokal yang berbeda 40 ekor sapi lokal jantan (2.5 tahun; BB awal 300.30±0.68) yang terdiri dari sapi Madura (M), sapi Sumba Ongole (SO), sapi Bali (B) dan sapi Bali Timor (BT) dan keempat jenis bangsa sapi tersebut sebagai perlakuan (10 ekor/perlakuan). Penelitian menggunaan rancangan acak kelompok (RAK) dengan bobot badan awal ternak sebagai kelompok. Jerami padi diberikan secara adlibitum dan pemberian konsentrat sebanyak 2.5 % dari BB (BK 86.53%). Peubah yang diukur yaitu konsumsi energi (KE), energi tercerna (ET), energi termetabolis (ME), retensi energi (RE), rasio RE terhadap KE, rasio RE terhadap ET, rasio C2/C3, efisiensi konversi heksosa menjadi VFA (EKH) dan rataan pertambahan bobot badan harian (PBBH). Hasil penelitian menunjukan bahwa perbedaan bangsa sapi lokal berpengaruh nyata ($P<0.05$) terhadap KE, ET, ME, RE, rasio RE terhadap KE, rasio RE terhadap ET, rasio C2/C3 dan EKH, tetapi tidak berpengaruh nyata terhadap PBBH ($P>0.05$). KE, ET, ME, dan RE tertinggi pada M masing-masing 139.52 MJ/hari, 99.69 MJ/hari, 65.84 MJ/hari, dan 98.45 MJ/hari, rasio RE terhadap KE tertinggi pada B, sedangkan untuk rasio RE terhadap ET, rasio C2/C3, EKH dan PBBH terbaik pada SO yaitu masing-masing 99.24%, 28.85, 74.97%, dan 1.24 kg. Penelitian dapat disimpulkan bahwa SO memiliki kemampuan terbaik dalam performans dan memanfaatkan energi pakan.

Kata Kunci: Efisiensi energi, Bangsa sapi lokal, Metabolisme energi, Jerami padi

ABSTRACT

Utami ETW, Bata M, Rahayu S. 2021. Energy metabolism and performance of several local cattle breeds fed rice straw and concentrate. JITV 26(2): 57-64. DOI: http://dx.doi.org/10.14334/jitv.v26i2.2711.

This study was conducted to examine the effect of different local cattle breeds on energy metabolism and performance fed on rice straw basal diet. Fourty local male cattle (2.5 years; initial BW 300.30±0.68) of Madura cattle (M), Sumba Ongole cattle (SO), Bali cattle (B), and Bali Timor cattle (BT) were used in this study, where types of local breed were used as treatments (10 animals/treatment). The study used a randomized block design (RBD) with cattle's initial body weight as a group. The cattle were fed on rice straw ad libitum and concentrate 2.5% BW (DM 86.53%). The variables measured were energy intake (EI), digestible energy intake (DEI), metaboloizable energy intake (MEI), energy retention (RE), RE to EI ratio, RE to DEI ratio, C2/C3 ratio, the efficiency of hexose conversion to VFA (ECH) and the average daily body weight gain (ADG). The results showed that the different breeds of local cattle had a significant effect ($P<0.05$) on EI, DEI, MEI, RE, RE to EI ratio, RE to DEI ratio, C2/C3 ratio, and ECH, but had no significant effect on ADG ($P>0.05$). M has the highest EI, DEI, MEI, and RE 139.52 MJ/day, 99.69 MJ/day, 65.84 MJ/day, and 98.45 MJ/day, respectively, but the highest RE to EI ratio at B, while for the best RE to DE ratio, C2/C3 ratio, ECH, and ADG, at SO were 99.24%, 28.85, 74.97%, and 1.24 kg, respectively. It can be concluded that the best local cattle in terms of performance and feed energy efficiency are Sumba Ongole cattle.

Key Words: Energy efficiency, Local cattle breeds, Metabolism energy, Rice straw

INTRODUCTION

Agricultural waste such as rice straw are used as alternative feed for ruminants to overcome limited land for forage cultivation. However, rice straw has a low digestibility only about 40-50% (Suryani et al. 2015). To meet energy requirement of livestock, it is necessary to supply energy source or concentrate feeds. Increased price of conventional feed ingredients has generate efforts to increase feed efficiency due to feed could contribute about 60-70% of the total production costs.

Feed efficiency in ruminants is influenced by the presence of microbes in rumen. About 80% of ruminant energy needs for metabolism are obtained from the fermentation of feed by rumen microbes (Kong et al. 2016). Various studies have been conducted to manipulate rumen microbial environmental conditions, such as changing maintenance patterns, dietary

properties, or adding certain additives into feeds (Vera et al. 2014; Khan et al. 2016; Bata & Rahayua 2017; Soltan & Patra 2020). Although feeds have significant influences on rumen microbial community (Henderson et al. 2015), in adult ruminants, the attempts to manipulate rumen microbes have only able to survive temporarily (Anderson et al. 2016; Malmuthuge & Guan 2017; Weimer et al. 2017; Huws et al. 2018). Recent studies have informed a relationship between cattle breeds and the rumen microbial community (Hernandez-Sanabria et al. 2013; Sasson et al. 2017). Although this difference is only a small part, it affected the host performances, including its energy utilization efficiency (John Wallace et al. 2019).

Indonesia has various local cattle breeds including Pesisir cattle, Aceh cattle, Jabres cattle, Pasundan cattle, PO cattle, Sumbawa cattle, Sumba Ongole cattle, Bali cattle and Madura cattle, and those locals cattle are known to have high adaptability to low-quality feed (Hendri 2013). This low-quality feed adaptability is a distinct advantage for local cattle breeds to develop. Local cattle used in this study were Madura cattle, Sumba Ongole cattle, Bali cattle, and Bali Timor cattle, where Sumba Ongole and Bali Timor cattle are extensively raised and grazed in the pasture (Manu 2013; Palandi & Ngundjuawang 2014), while Bali cattle and Madura cattle are maintained intensively and generally rely on agricultural waste as basal feed (Kutsiyah 2016; Besung et al. 2019). Local cattle breeds are known to have different performances. Sumba Ongole cattle have ADG of 0.8-1.5 kg/day, feed efficiency equal to 10-19%, and percentage of carcass equal to 51-56% (Agung et al. 2015; Bata et al. 2016; Yantika et al. 2016). Bali cattle have ADG of 0.5-1 kg/day (Hau & Nulik 2017; Budiari et al. 2020) with percentage of carcass equal to 50-54% (Suryanto et al. 2017; Neno 2018; Priyono & Priyanto 2018) while Madura cattle have ADG of 0.2-0.6 kg/day (Wisnuwati et al. 2014; Rab et al. 2016) and percentage of carcass equal to 53% (Umar et al. 2011). It's presumably because local cattle breeds have different abilities in energy utilization. However, that performance was shown under different conditions and feeds. Therefore, the research objective was to examine the effect of local breeds of cattle on energy metabolism and performance in the same environment and feed. The information obtained can be applied to improve feed efficiency to support the fulfillment of national meat needs.

MATERIALS AND METHODS

Animal and diets

Fourty local male cattle aged around 2.5 years old were used in this experiment, consisting of: Madura cattle (M), Sumba Ongole cattle (SO), Bali cattle (B), and Bali Timor cattle (BT). Ten of each M, SO, B, and BT were imported directly from Madura, Sumba Island, Bali Island, and Timor Island, respectively. The average initial body weight of cattle used was 300.30±0.68 kg.

The cattle were grouped into ten group base on body weight. There were ten groups of body weight, namely 255-263; 264-272; 273-281; 282-290; 291-299; 300-308; 309-317; 318-326; 327-335; 336-344. Cattle were fed with rice straw and concentrate. Concentrate was composed of 47.60% cassava pulp, 24.00% pollard bran, 10,50% palm kernel meal, 10.00% rice bran, 7.00% soy bean meal, 5.70% coconut meal, 4.00% molasses, 1.6% dolomite, 1.0% salt, 0.60% Urea, and 0.30% mineral mix. The nutrient contents of feed is presented in Table 1.

Table 1. Nutrient of feed during experiment

Nutrient Content	Feed	
	Rice Straw	Concentrate
Dry Matter (%)	73.07	86.53
Ash (%)	23.45	17.16
Crude Protein (%)	4.00	13.82
Crude Fiber (%)	31.16	19.19
Ether Extract (%)	1.3	3.97
NFE (%)	40.09	45.86
TDN (%)	38.21	64.45
NDF (%)	71.43	42.83
ADF (%)	52.95	26.61
Gross Energy (MJ/Kg)	12.79	14.78

Result analysis of laboratory according to AOAC (2019)
NFE: Nitrogen-free extract; TDN: Total digestible nutrient; NDF: Neutral detergent fiber; ADF: Acid detergent fiber

Experimental procedure

The study was conducted for 52 days, consisted of preliminary study for 14 days and measurement period 38 days. Before the preliminary study cattle was dewormed using Dovenix. The rice straw was bought from the rice fields area in Banyumas, Central Java and sun dried. The concentrate was given twice a day at 07.00 WIB and 15.00 WIB with a total daily offered 2.5% of body weight. Drinking water and rice straw were offered ad libitum. During the study, cattle were weighed 3 times, before preliminary, before measurement period, and the end of the measurement period using digital scale (SABB, Type:A1GB-3, Cap: 2 ton x 0,5 kg).

Data collection samples of feed (consemed and refusal), feces, and urine was carried out for 5 days using total collection method (Cole & Ronning 1974). Feed were sampled as much as 250 gr. The feed refusal was taken before morning feeding, weighed, and recorded. The samples of feed offered and the refusal dried in an oven at 60^{o} for 48 h. Feces were collected used a known weight container, sprayed every 4 h using formalin solution to prevent the decomposition process. Feces were collected for 24 h then weighed, and recorded. The feces were sampled ($\pm$3%) and dried in an oven at 60^{o}C for 48 h. Feces and feed that had been collected for 5 d were compiled per individual and subsampled for analysis.

The collection of urine production by seting a diaper/harness made of used tires attached to the part of the cattle's penis that circles its stomach. A plastic hose connected to the bottom of the harness with a jerry can (20 l capacity), 20 ml of H_2SO_4 75% was filled into the can before the urine collection to keep the pH below 3 to prevent evaporation of ammonia. The urine was collected for 24 h, then the volume was measured, and sample was taken for 20ml. Urine samples were stored in a freezer (-20^{o}C) until the sample collection was completed. Total urine collected for 5 d were mixed and stirred until homogen, and then sub samples were taken for analysis.

Rumen fluid samples were taken 3 h after the morning feeding. Sampling was conducted using a rumenocentesis technique (Petrovski. 2017), sterile needle was injected in the rumen position (done by an expert). The samples were taken as much as 3 ml, deposited, and 1.5 ml was separated from the feed sediment. The liquid was then put into the Eppendorf tube and centrifuged at 5000 rpm for 10 minutes. The supernatant was moved into a new tube and stored in a freezer at -20^{o}C until analysis.

At the end of the experiment the cattles were weighed using digital cattle scale and the average daily gain (ADG) was determined by the difference between the final weight and initial weight over the length experiment period. The energy utilization was determined by measuring energy intake (EI), digestible energy intake (DEI), metaboloizable energy intake (MEI), energy retention (RE), RE to EI ratio, and RE to DEI ratio using the total collection method (Cole & Ronning 1974). RE was determined from the difference between digestible energy intake and the total urin energy output. MEI was determined by the difference between energy retention and methane energy output. Methane energy output was calculated using estimation by Ryle & Ørskov (1990), ie. (((2pa+2pb)-pp)/4) x 210.8, pa is the proportion of asetate, pb is the proportion of butyrate, and pp is the proportion of propionate. Concentrations of VFA partial was measured using gas chromatography techniques (Guan et al., 2008). The efficiency of conversion of hexose to VFA (ECH) was calculated using estimates by Ryle & Ørskov (1990), ie. percentage of (0.622 pa+ 1.092 pp+ 1.560 pb)/(pa+pp+2pb), where pa is proportion of asetate, pb is proportion of butyrate, and pp is proportion of propionate.

Chemical analysis

Proximate analysis of feed and analysis of moisture content in samples of feces and refusal feed during collection using the procedure AOAC., (2019). Feed, feces, and urine samples were analyzed using a bomb calorimeter (Dittmann et al. 2014) for gross energy and VFA partial from rumen fluids was analyzed using gas chromatography techniques was following procedures described by Guan et al., (2008).

Statistical analysis

Randomized block design (RBD) (Steel & Torrie 1993) was applied in this study. The treatments were local cattle breeds, namely M, SO, B, and BT with the initial body weight of cattle as a group. Data were analyzed using analysis of variance and further testing using Duncan's Multiple range tests (DMRT) at level 5% performed by IBM SPSS statistic 25.0.

RESULTS AND DISCUSSION

Average Feed Intake, EI, DEI, MEI, RE, RE to EI ratio, RE to DEI ratio, daily fecal energy output, daily urine energy output, methane energy output and the ratio of consumption rice straw and concentrates are presented in Table 2. The variance analysis showed that cattle breeds significantly affected ($P<0.05$) EI, DEI, MEI, RE, RE to EI ratio, RE to DEI ratio.

The EI of B and SO cattle similar, but was significantly lower than M and higher than BT. M cattle reasonable ability to consume feed was thought to be more adaptable to the environment's conditions and the feed given. The maintenance pattern on the cattle's origin area affects the cattle's ability to consume the feed. Several studies had revealed that Madura Island and Bali Island is an island with a relatively high density of livestock population so that another alternative to meet requirement for livestock feed, agricultural waste was as feed ingredients and maintain intensively (Kutsiyah 2012; Nugraha et al. 2015; Kutsiyah 2016; Besung et al. 2019). Liu et al., (2016) study using cannula dairy cows revealed that type of forage affects the dynamics of the microbial composition of rumen, where the presence of *Fibrobacteria,* unclassified *Bacteroidales,* unclassified *Rikenellaceae and* unclassified *Ruminococcaceae* digested more of low-quality forage such as rice straw than alfalfa hay. The ratio consumption of concentrate and rice straw (Table 2), show that M and B were able to consume more rice straw compared to SO and BT.

The lower rice straw consumption in SO and BT due to SO and BT originated from Sumba island and Timor island in which they were raised extensively with source of forage from pasture land, beside that various types of legumes have been planted to improve quality of the pasture (Palandi & Ngundjuawang 2014; Kleden et al. 2015; Hau & Nulik 2017), because SO and BT initially consumes better quality forage, possibly the fewer microbes able to digested low-quality feed. Zhang et al., (2014) mention that forages with more complex nutrients require greater microbial complexity to utilize all the components of the forage efficiently.

In contrast to EI, DEI and MEI showed differences significantly ($P<0.05$) for each local breed. This is due to the difference in energy wasted through feces, urine, and methane. M had the highest DEI and MEI, namely 99.69 MJ/d and 65.84 MJ/d, respectively, while the lowest was in BT at 79.51 MJ/d and 46.91 MJ/d. The low DEI and MEI in BT were due to BT consuming less energy, besides that, the energy lost through excreta and urine on

Table 2. Metabolism of energy on several breeds of local cattle

Parameter	Cattle Breeds			
	M	SO	B	BT
Feed Intake (DM/kg)	9.92±0.01^{c}	8.79±0.02^{b}	9.00±0.06^{b}	7.84±0.03^{a}
Concentrate (DM/kg)	6.34±0.03^{b}	6.32±0.02^{b}	5.57±0.04^{a}	5.60±0.02^{a}
Rice Straw (DM/kg)	3.58±0.03^{c}	2.46±0.03^{b}	3.43±0.02^{c}	2.24±0.03^{a}
C/RS ratio (%)	64:36	72:28	62:38	71:29
Energy Intake (MJ/d)	139.52±0.17^{c}	124.95±0.30^{b}	126.25±0.87^{b}	111.44±0.47^{a}
Energy Intake (kJ/kg $BW^{0.75}$)	1836.35±2.27^{c}	1703.05±4.11^{b}	1557.53±10.73^{a}	1531.32.31±6.46^{a}
Fecal Energy (MJ/kg)	11.78±0.04	11.65±0.06	11.76±0.04	12.56±0.06
Fecal Energy Output (MJ/d)	39.83±0.15^{c}	38.62±0.25^{b}	32.53±0.15^{a}	31.93±0.19^{a}
Fecal Energy Output (kJ/kg $BW^{0.75}$)	524..23±2.03^{c}	526.45±3.35^{c}	401.27±1.89^{a}	438.81±2.62^{b}
Digested Energy Intake (MJ/d)	99.69±0.20^{d}	86.33±0.34^{b}	93.72±0.77^{c}	79.51±0.29^{a}
Digested Energy Intake (kJ/kg $BW^{0.75}$)	1312.12±2.59^{c}	1176.61±4.62^{b}	1156.26±9.51^{b}	1092.77±3.96^{a}
Energy Digestibility (%)	71.45±0.11^{b}	69.09±0.19^{a}	74.22±0.13^{c}	71.35±0.06^{b}
Urine Energy (kkal/g)	0.04±0.001	0.02±0.001	0.04±0.001	0.04±0.002
Urine Energy Output (MJ/d)	1.24±0.02^{b}	0.66±0.02^{a}	1.18±0.02^{b}	1.18±0.05^{b}
Urine Energy Output (kJ/kg $BW^{0.75}$)	16.36±0.26^{b}	8.97±0.25^{a}	14.50±0.22^{b}	16.18±0.64^{b}
Methane Energy Output (MJ/d)	32.61±0.22^{b}	28.77±0.29^{a}	30.76±0.41^{ab}	31.42±0.32^{b}
Methane Energy Output (kJ/kg $BW^{0.75}$)	429.21±2.92^{b}	392.15±3.91^{a}	379.45±5.09^{a}	431.83±4.34^{b}
Metaboloizable Energy Intake (MJ/d)	65.84±0.29^{d}	59.90±0.44^{b}	61.79±0.36^{c}	46.91±0.12^{a}
Metaboloizable Energy Intake (kJ/kg $BW^{0.75}$)	866.55±3.86^{c}	775.49±5.96^{b}	762.31±4.41^{b}	644.77±159^{a}
Energy Retention (MJ/d)	98.45±0.21^{d}	85.67±0.34^{b}	92.55±0.76^{c}	78.33±0.29^{a}
Energy Retention (kJ/kg $BW^{0.75}$)	1295.76±2.74^{c}	1167.64±4.53^{b}	1141.76±9.44^{b}	1076.60±3.97^{a}
RE to EI Ratio (%)	70.56±0.11^{b}	68.56±0.19^{a}	73.29±0.13^{c}	70.30±0.09^{b}
RE to DEI Ratio (%)	98.75±0.02^{a}	99.24±0.02^{b}	98.74±0.02^{a}	98.52±0.06^{a}

M: Madura cattle; SO: Sumba Ongole cattle; B: Bali cattle; BT: Bali Timor cattle; C/RS ratio: Ratio of concentrate and rice straw intake
^{a,b,c,d} The difference superscripts in the same line show a significant effect ($P<0.05$)

Table 3. VFA production and efficiency of the conversion of hexose to VFA(ECH) of various breeds of local cattle

Parameter	Cattle breeds			
	M	SO	B	BT
Acetic acid (mMol)	68.83±4.64	73.67±3.49	56.24±4.51	55.74±1.59
Propionic acid (mMol)	16.58±1.06[a]	25.86±1.21[b]	16.28±1.63[a]	15.27±0.73[a]
Butyric acid (mMol)	9.61±0.67	10.66±0.48	8.14±0.70	7.77±0.27
C2/C3 ratio	4.15±0.10[b]	2.85±0.07[a]	3.45±0.14[b]	3.65±0.11[b]
ECH (%)	72.50±0.14[a]	74.97±0.18[b]	73.69±0.27[ab]	73.26±0.20[ab]
Initial body weight (kg)	302.60±1.79	327.40±2.58	289.10±1.69	282.10±2.47
Final body weight (kg)	340.56±2.33	374.65±2.98	325.35±1.54	317.71±2.43
ADG (kg)	0.98±0.02	1.24±0.02	0.95±0.02	0.72±0.12

M: Madura cattle; SO: Sumba Ongole cattle; B: Bali cattle; BT: Bali Timor cattle
[a,b,c,d] The difference superscripts in the same line show a significant effect (P<0.05)

B was also not small. This is consistent with Van Zijderveld et al., (2011) that if the DE intake is not used much for methane formation, it will increase the ME intake.

Energy retention was affected by cattle breed (P<0.05). According to Amtiran et al. (2016), increased feed intake with high digestibility will increase energy retention in the body, where at the percentage of rice straw 80%, 70%, and 60%, energy consumption and energy retention in female local goat are 1661.11kkal/day (1379.91 kkal/d), 1689.54 kkal/day (1397.02 kkal/day), and 1720.08 kkal/day (1437.59 kkal/day), respectively. The results of this study show the same thing (Table 2), M consume higher feed, so that M had the highest RE, and the lowest was BT. RE to EI ratio was lowest at SO (68.56%), which was significantly different (P<0.05) from the other three breeds of cattle, but at M (70.56%) and BT (70.30%), there was a similar value (P>0.05). Different results were that SO actually had a higher RE to DEI ratio, which was 99.24% (P<0.05), while the other three breeds of cattle did not show any differences (P>0.05). This is because the energy lost through excreta on SO is less so that more energy can be used by cattle for maintenance and producing meat.

Consumption of higher concentrates in SO and BT should lead to lower methane gas formation than M and B. The low production of methane gas (CH_4) is due to the availability of hydrogen (H_2) in the rumen, which is more used for the synthesis of propionic acid (C_3) (Beauchemin et al. 2020). However, Table 2 shows different results. Only SO produces lower methane gas, while BT produces more methane gas compared to B and almost equal to M. This reinforces the statement that the amount of methane gas formed is not only from feed given (Zhou et al. 2011), but other factors have important contribution to the formation of methane gas (Basarab et al. 2013). Several studies have revealed that large amount of methane gas emissions apart from feed is also affected by cattle genetics (Pinares-Patiño et al. 2013; Tapio et al. 2017; Auffret et al. 2018). Roehe et al. (2016) revealed that there is differences in microbial communities with low methane emissions and high methane emissions, where methane gas emissions in sire progeny Aberdeen Angus cattle were smaller than that of limousine cattle. This difference in microbial communities is thought to be due to the presence of core microbes that are genetically inherited and responsible for the formation of methane gas (Wallace et al. 2015; John Wallace et al. 2019; Li, Hitch, et al. 2019; Li, Li, et al. 2019; Abbas et al. 2020).

Methane gas formation is influenced by production of VFA, and it affects the ECH value (Beauchemin et al. 2020). The average concentration of acetic acid, propionic, butyric acid, C2/C3 ratio, ECH, and ADG are presented in Table 3. Differences in local cattle breeds did not affect (P> 0.5) acetate and butyrate concentrations but did affect (P<0.05) propionate concentration, C2/C3 ratio, and ECH. The propionate concentration of M cattle was similar to B and BT (P> 0.05) but lower than SO (P<0.05). It causes the C2/C3 ratio of SO was the lowest than the other three breeds of cattle. The ratio of C2/C3 in SO was also better than Cherdthong et al., (2014), with the same basal feed, namely rice straw, Thai native beef cattle had a C2/C3 ratio of 3.8, because, in that research, the consumption of rice straw was higher than concentrate. The C2/C3 ratio shows the amount of rumen microbes that can form acetic (acetogenic) or propionic acid (glucogenic), and it can be used to measure the efficiency of energy use in ruminants (Syapura et al. 2013). That was consistent with Muktiani et al., (2020) statement that a low C2/C3 ratio also indicates a higher ECH. Table 3. also shows that the ECH in SO is higher than the three other local cattle breeds. ECH on SO higher than M but was not significantly different to B and BT. According to Sari et

al. (2019), the high amount of fermentable organic matter causes a low C2/C3 ratio, this is because of *Propionibacteria sp, Veillonella alkalescens,* dan *Peptostreptococcus elsdenii* will use more lactic acid for the formation of propionic acid, which is a precursor for gluconeogenesis. Increased propionic acid concentration causes an increase in glucose production in the blood (Klau Tahuk et al. 2017). Ladeira et al. (2018) explained that meat production and the quality of marbling formation were influenced by glucose availability in the blood. Although local cattle breeds did not significantly affect ADG (P> 0.05), but ADG on SO was higher than the three other breeds (P = 0.12), namely 1.24 kg. The ADG of SO recorded by Yantika et al., (2016) could reach 1-1.57 kg.

CONCLUSION

Madura, Sumba Ongole, Bali, and Bali Timor cattles' abilities to metabolize feed energy were varied. Sumba Ongole cattle was better than the other three local breed cattle in producing higher ADG even though the differences were not significant, so the best local cattle in terms of performance and feed energy efficiency is Sumba Ongole cattle.

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Application of Plantaricin as an Antimicrobial Substrate in the Milking Process to Maintain Milk Quality in Smallholder Dairy Farm

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ABSTRAK

Wahyuningtyas AN, Arief II, Taufik E. 2021. Aplikasi plantarisin sebagai substrat antimikroba dalam proses pemerahan untuk mempertahankan kualitas susu pada peternakan sapi perah rakyat. JITV 26(2): 65-73. DOI. http://dx.doi.org/10.14334/jitv.v26i2.2718.

Cemaran bakteri patogen yang terdapat pada susu sapi segar dapat disebabkan karena manajemen pemerahan yang kurang baik. Proses pemerahan secara tradisional ini memungkinkan susu terkontaminasi dari bakteri dan kotoran. Pencelupan puting sapi perah menggunakan antiseptik komersial adalah upaya umum yang dapat dilakukan untuk mencegah mastitis. Pencelupan puting dapat dilakukan setelah pemerahan dengan menggunakan bahan antiseptik sintetik seperti povidone iodine dan chlorine. Namun penggunaan antiseptik sintetik sebenarnya dapat menimbulkan efek iritasi ringan dan alergi serta meninggalkan residu. Oleh karena itu, penggunaan antiseptik berbahan dasar alami diharapkan dapat menggantikan antiseptik sintetik. Salah satu antiseptik berbahan dasar alami yang dapat digunakan adalah bakteriosin. Penelitian ini bertujuan untuk menganalisis aplikasi plantarisin IIA-1A5 sebagai pengganti antibakteri sintetik selama proses pemerahan sapi perah terhadap kualitas susu sapi yaitu kualitas mikrobiologi, fisikokimia, dan pengukuran pH. Penelitian dilakukan dengan menggunakan Rancangan Acak Kelompok (RAK) dengan tiga ulangan. Rancangan perlakuan terdiri dari kontrol (tanpa pencelupan), plantarisin 0,0074%, dan povidone iodine 0,2%. Hasil penelitian menunjukkan aplikasi plantarisin IIA-1A5 sebagai pencelupan puting sebelum pemerahan dapat menurunkan populasi Total Plate Count, *Escherichia coli*, dan *Staphylococcus aureus*. Penggunaan plantarisin IIA-1A5 sebagai pencelupan puting tidak mengubah nilai pH dan kualitas fisikokimia (lemak, SNF, laktosa, dan protein) yang berada di bawah Standar Nasional Indonesia (SNI) tentang susu segar. Kemampuan ini sebanding dengan iodine, antibakteri sintetik yang banyak digunakan peternak kecil di Indonesia. Disimpulkan bahwa plantarisin IIA-1A5 dapat digunakan sebagai pengganti antibakteri sintetik iodine untuk pencelupan puting susu sebelum pemerahan.

Kata Kunci: *L. Plantarum* IIA-1A5, Plantarisin IIA-1A5, Susu segar

ABSTRACT

Wahyuningtyas AN, Arief II, Taufik E. 2021. Application of plantaricin as an antimicrobial substrate in the milking process to maintain milk quality in smallholder dairy farm. JITV 26(2): 65-73. DOI. http://dx.doi.org/10.14334/jitv.v26i2.2718

Pathogenic bacterial contamination found in fresh cow's milk can be caused by poor milking management. This traditional milking process allows the milk to be contaminated from bacteria and dirt. Dyeing dairy cows using a commercial antiseptic is a common measure that can be done to prevent mastitis. Nipple immersion can be done after milking using synthetic antiseptic agents such as povidone iodine and chlorine. However, the use of synthetic antiseptics can actually cause a slight irritation and allergic effect and leave a residue. Therefore, it is hoped that the use of natural-based antiseptics can replace synthetic antiseptics. One of the natural based antiseptics that can be used is bacteriocin. This research aimed to analyze the application of the plantaricin IIA-1A5 as a substitute for synthetic antibacterial for teat dipping before milking namely microbiological tests, physicochemical tests, and pH measurements. The study was conducted using a randomized block design (RBD) with three replications. The treatment design consisted of control (without immersion), plantaricin 0.0074%, and povidone iodine 0.2%. Results showed application of plantaricin IIA-1A5 as teat dipping before milking can reduce the Total Plate Count, *Escherichia coli*, and *Staphylococcus aureus* population. The use of plantaricin IIA-1A5 as teat dipping did not change pH value and physicochemical quality (fat, SNF, lactose, and protein), which is below the Indonesian National Standard (SNI) about fresh milk. This ability is comparable to the iodine group, a synthetic antibacterial widely used by smallholder breeders in Indonesia. It is concluded that plantaricin IIA-1A5 can be used as a substitute for synthetic antibacterial (iodine group) for teat dipping before milking.

Key Words: Fresh milk, *L. Plantarum* IIA-1A5, Plantaricin IIA-1A5

INTRODUCTION

Pathogenic bacterial contamination found in fresh cow's milk can be caused by poor milking management (Prihutomo et al. 2015). Dairy farming in Indonesia still uses traditional milking methods or does not use machines. This traditional milking process exposes the milk for contamination of bacteria and dirt.

Dyeing dairy cows using a commercial antiseptic is a common measure that can be done to prevent mastitis. Nipple immersion can be done after milking using synthetic antiseptic agents such as *povidone iodine* and *chlorine* (Tomita et al. 2008). However, the use of synthetic antiseptics can actually cause a slight irritation and allergic effect and leave a residue (Flachowsky et al. 2014). Therefore, it is proposed that the use of natural-based antiseptics can be an alternative to replace the synthetic antiseptics. One of the natural based antiseptics that can be used is bacteriocin.

Bacteriocin is a peptide compound produced by lactic acid bacteria and has antimicrobial activity. These bacteriocins are non-toxic to humans, stable to changes in pH and temperature, and safe for food preservatives because they are easily digested by digestive enzymes (Hata et al. 2010). Therefore, bacteriocins can be used as a biopreservative in fresh and processed food products (Soenarno et al. 2020). *Lactobacillus plantarum* is a bacteriocin-producing lactic acid bacterium known as plantaricin. *L. plantarum* IIA-1A5 is a strain of indigenous lactic acid bacteria from local Indonesian beef that was identified using polymerase chain reaction (PCR) and 16s rRNA sequence analysis (Arief et al. 2012). The utilization of bacteriocins such as plantaricin IIA-1A5 as natural preservatives that contains antimicrobial compounds is expected to destroy and kill pathogenic bacteria, such as *Staphylococcus aureus* in fresh dairy milk.

Several studies have been conducted to determine the function and characteristics of plantaricin. Plantaricin is degraded by the trypsin protease enzyme, survive at temperatures of 80 ºC and 121 ºC for 30 and 15 minutes respectively, remains active in the pH range of 4 to 9 (Arief et al. 2013), and is proven to be able to inhibit the growth of pathogenic bacteria such as *Escherichia coli, Salmonella Typhimurium, Bacillus cereus and Staphylococcus aureus* (Arief et al. 2013). It is suggested that Plantaricin IIA-1A5 inhibits the growth of pathogenic bacteria by damaging the cell membranes of the bacteria. Furthermore, plantaricin IIA-1A5 can be used as a biopreservative for fresh and processed food products (Soenarno et al. 2019).

This study's objectives were to analyze the application of the plantaricin IIA-1A5 as a subtitute for synthetic antibacterial for teat dipping before milking on the milk quality, by testing its value on microbiological tests, physicochemical tests, and pH measurements.

MATERIALS AND METHODS

This research was carried out at dairy farms in Kawasan Usaha Peternakan (KUNAK) Cibungbulang District, Bogor Regency, and at the Integrated Laboratory, Animal Product Technology Division, Department of Animal Production and Technology, Faculty of Animal Science, IPB University. This research was conducted for four months, starting from June to October 2020.

Whey making

Following the procedures of (Soenarno et al. 2019) and (Fatmarani et al. 2018), fresh cow's milk was pasteurized at a temperature of 75 °C for 15 minutes, and then cooled down to a temperature of 37 °C. The rennet was then inoculated into pasteurized milk at a concentration of 0.02 g L^{-1} of milk. The milk coagulated after 60 minutes during the inoculation process and formed a curd. Curd was used to make cheese, and the liquid by-product (whey) was used as a medium for growing *L. Plantarum* IIA-1A5.

Production and purification of plantaricin IIA-1A5

The production of plantaricin IIA-1A5 from *L. plantarum* IIA-1A5 culture was performed according to Arief et al. (2015). The medium used for the growth of *L. plantarum* IIA-1A5 was 8 L of whey sterilized at 115 ºC for 3 minutes. It was then inoculated with 10% (v/v) of *L. plantarum* IIA-1A5 culture (10^8-10^9 CFU mL^{-1}). Incubated for 20 hours at 37 ºC and, centrifuged (Himac CR21G) at 10.000 x g for 20 minutes at 4ºC. The supernatant obtained from centrifugation was filtered with a filter membrane (0.20 µm Millipore Sartorius) and pH was neutralized to 5.8-6.2 with 1 N NaOH. The supernatant was evaporated using a Heidolph VV micro evaporator at temperature of 40-45 ºC until the volume is half of the previous volume.

Partial purification using ammonium sulfate $(NH_4)_2SO_4$ was performed to produce protein deposits, with gradual saturation (20%, 40%, 60%, and 80%), homogenized slowly at 4 ºC, and put in refrigerator for 24-48 hours. The precipitate formed from the saturation process was separated by centrifugation (Himac CR21G) at 20.000 x g for 20 minutes at 4ºC. The crude plantaricin deposits obtained were then subjected to dialysis.

Dialysis

Dialysis was carried out using a dialysis membrane (cellulose) with a diameter of 3.5 µm and immersed in a phosphate buffer (KH_2PO_4 and K_2HPO_4) 20 mM and pH of 6.8 for 24 hours at 4 °C. The phosphate buffer was then replaced four times every 6 hours (Hata et al. 2010).

Antimicrobial test of plantaricin IIA-1A5 against pathogenic bacteria

Staphylococcus aureus ATCC 25923 and *Escherichia coli* ATCC 25922 were selected as representatives of Gram positive and Gram negative pathogenic bacteria according to Arief et al. (2013). Pathogenic bacteria were rejuvenated 2 times. The culture was inoculated in 0.85% NaCl medium so that the concentration was 10^8 CFU mL^{-1} (compared to the Mc.Farland standard solution). The same dilution was carried out again to obtain a bacterial concentration of 10^6 CFU mL^{-1}. 20 mL of MHA (Muller Hinton agar, oxoid) medium was poured into a sterile petri dish. A total of 100 µl of pathogenic bacteria were spread on the MHA media surface, which had hardened in the petri dish. A sterile paper disc was placed on the surface of the MHA media that had been inoculated with pathogenic bacteria, and 50 µl of plantarisin IIA-1A5 solution was dropped onto a sterile paper disc. The plates were incubated for 24 hours at 37 ºC. The antimicrobial activity of plantaricin was characterized by the formation of a clear zone around the paper disc and the diameter was measured.

Application of plantaricin IIA-1A5 as a natural preservative for cow teats

Teat dipping was done before and after milking in the morning for 5 seconds. Three dairy cows Frisian Holstein were randomized into three teat dipping treatments. Teat dipping treatments were control, plantaricin 0.0074% (74 ppm), and *povidone iodine* (0.2%). The milk was stored at room temperature for 6 hours after 1 hour of milking and observed every 2 hours, at 1, 3, 5, and 7 hours of room temperature storage for microbiological, chemical, and pH measures.

Microbiological characteristics of fresh milk

The microbiological characteristics of fresh cow's milk include analysis of Total Plate Count (TPC) and the presence of *E. coli* and *S. aureus* bacteria was measured according to the procedures of Arief et al. (2012). 25 mL of fresh cow's milk was put in 225 mL of sterile Buffered Peptone Water (BPW) solution. Dilutions were carried out to 10^{-4}, 10^{-5}, and 10^{-6} for TPC and to 10^{-1}, 10^{-2}, and 10^{-3} for *E. coli* and *S. aureus*. Plate Count Agar media (PCA), Eosin Methylene Blue Agar medium (EMBA), and media Baird-Parker agar (BPA) added with potassium tellurite was poured into 20 mL petri dishes and homogenized. The frozen petri dishes were incubated upside down for approximately 24 hours at 37 ºC. Aerobic bacterial colonization was indicated by the appearance of white color while . *E. coli* colonization was indicated by the appearance of purple color when exposed to light. Colony counts were calculated based on the number that is feasible to count (25-250 colonies) (Maturin & Peeler 2001).

Chemical characteristics of fresh milk

Chemical characteristics (fat content, Solid Non-Fat (SNF), lactose, protein content) was measured using the Lactoscan tool. 25 mL of fresh milk were taken, and poured into a cuvette (25 mL). The cuvette was inserted into the space provided in the Lactoscan. Lactoscan results appeared in 10 minutes, and the results will be automatically printed.

pH analysis

Ten mL of milk samples were taken for pH measurement using pH meter that had previously been calibrated at pH 4 and 7. The pH value of milk was read and recorded.

Experimental design and statistical analysis

All data were statistically analyzed by analysis of variance (ANOVA) with Duncan as a post hoc test (Steel & Torrie 1996). For this purpose, completely randomized block design (RBD) using 1 control, 2 treatments with 3 replications was applied. Groups were based on different sampling weeks.

RESULTS AND DISCUSSION

Characteristics of crude plantaricin IIA-1A5

The data in Figure 1 shows that the molecular weight of crude plantaricin IIA-1A5 determined by SDS-PAGE is 9 kDa and classified as IIA.

Arief et al. (2015) reported that the molecular weight of crude plantaricin IIA-1A5 was less than 10 kDa and was classified in the IIA classification. The plantaricin IIA-1A5 was successfully purified using cation exchange chromatography, and had a molecular weight of 9.65 kDa (Soenarno et al. 2020). This is

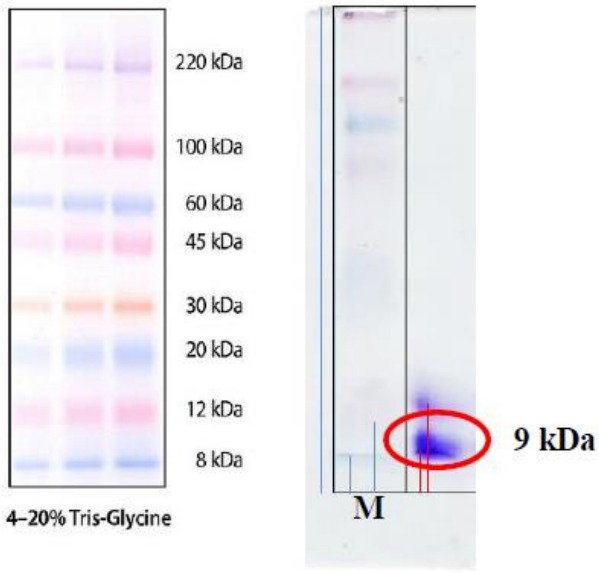

Figure 1. SDS-PAGE of crude plantaricin IIA-1A5

similar to the study of Arifin et al. (2020), that plantaricin IIA-1A5 was successfully purified from ammonium sulfate, and cation exchange chromatography and had a molecular weight of 9.4 kDa. Fatmarani et al. (2018) also researched the production of plantaricin IIA-1A5 from whey cheese, and found a molecular weight of crude plantaricin extract of 9.5 kDa. Lower molecular weights of bacteriocin produced by plantarum IIA-1A5 (6.41 kDa) and by *L. plantarum* FGC12 (4.1 kDa) were reported by Arief et al. (2015b) and Lv et al. (2017). Despite having different molecular weights, these plantaricins were classified as group IIA and relatively heat stable (Zacharof & Lovitt 2012). Another bacteriocin produced by *L. plantarum* in the study of Hu et al. (2013) include plantaricin 163 with a molecular weight of 3.5 kDa, and plantaricin K25 with a molecular weight of 1.7 kDa (Wen et al. 2016). This difference in molecular weight was caused by *L. plantarum* Strain. Kia et al. (2015) suggest that different *L. plantarum* strains greatly affect the characteristics of plantarisin and protein concentrations produced in the SDS-PAGE electrophoresis calculations.

Antimicrobial activity

The diameter of inhibiton zone of crude plantaricin to pathogenic bacteria was presented in Table 1. The antimicrobial activity shown by the inhibition zone's diameter in crude plantaricin IIA-1A5 against *E. coli* and *S. aureus* was not significantly different. The values were less than 3 mm and so the antimicrobial activity was categorized as weak.

These low inhibition zone could be influenced by the storage of bacteriocin plantarisin IIA-1A5 at room temperature. It could also caused by the largest bacteriocin component (Karpinski & Szkaradkiewicz 2013). Todorov et al. (2016) reported that the media's low inhibitory activity could be caused by reduced bacteriocin antimicrobial activity due to the role of organic acids.

The antimicrobial character of plantaricin IIA-1A5 against Gram positive and Gram negative bacteria were closely related to bacterial strains (Arief et al. 2013). According to Arief et al. (2015), that *L. plantarum* IIA-1A5 grown on commercial MRSB media, plantaricin had good antimicrobial activity against *E. coli* ATCC 25922, *S. thypimurium* ATCC 14028, and *S. aureus* ATCC 25923 ranged from 6.86-12.38 mm. Gram positive bacteria were more sensitive, while gram negative bacteria were more resistant (Fatmarani et al. 2018). Based on the research results of Soenarno et al. (2020), that plantaricin IIA-1A5 has a broad spectrum antimicrobial ability against Gram positive and Gram negative pathogenic bacteria.

Microbiological characteristics

Good milk is produced from healthy milking and clean cows udders, normally containing 10^6 CFU mL^{-1} milk (SNI 3141.1: 2011). Temperature control is very important to prevent changes in milk quality associated with bacterial growth. The average total of fresh milk microbes during storage at room temperature is presented in Table 2.

Table 1. Diameter of inhibiton zone of crude plantaricin to pathogenic bacteria

	Protein Yield	*E. coli* (mm)	*S. aureus* (mm)
Crude Plantaricin	96707.77	0.1±0.00[a]	0.1±0.00[a]

Means in the same row with different superscript differ significantly (P<0.05).

Table 2. Population of *E.coli* and *S.aureus* and TPC in fresh cow milk during storage at room temperature

Parameter	Treatment	Population in n-hours (log CFU mL^{-1})			
		1	3	5	7
TPC	Control	5.46±0.03[b]	6.81±0.06	8.01±0.06[b]	8.27±0.02[b]
	Povidone Iodine	5.43±0.02[a]	6.25±0.65	6.86±0.02[a]	8.25±0.03[a]
	Plantaricin	5.42±0.03[a]	5.87±0.66	6.83±0.05[a]	8.25±0.02[a]
E. coli	Control	2.44±0.01[b]	2.59±0.02[b]	3.68±0.00[b]	3.79±0.07[b]
	Povidone Iodine	2.41±0.01[a]	2.45±0.00[a]	2.45±0.01[a]	2.48±0.01[a]
	Plantaricin	2.40±0.01[a]	2.44±0.02[a]	2.44±0.03[a]	2.45±0.01[a]
S. aureus	Control	0.00±0.00	0.00±0.00	1.43±0.04[b]	1.93±0.05[b]
	Povidone Iodine	0.00±0.00	0.00±0.00	0.00±0.00[a]	1.41±0.09[ab]
	Plantaricin	0.00±0.00	0.00±0.00	0.00±0.00[a]	1.02±0.08[a]

Means in the same column with different superscript differ significantly (P<0.05).

Total plate count

Plantaricin IIA-1A5 and *povidone iodine* application to dip the teats suppress bacterial growth significantly (P <0.05) at 1 and 5 hours of storage, compared to controls. At 7 hours of storage, the TPC population trends in plantaricin were comparable to *povidone iodine*.

Plantaricin, which was produced by local Indonesian lactic acid bacteria, had been researched and could be effectively used as a preservative in fresh and processed meat products, such as meatballs and sausages (Arief et al. 2012; Arief et al. 2017). Plantaricin IIA-1A5 produced using commercial media deMann Rogosa Sharp Broth (MRSB) maintained the shelf life of fresh meat for 15 hours at room temperature storage (Sihombing et al. 2015). Application of 0.3% plantaricin could maintain the quality of beef meatballs for 20 hours at room temperature storage (Kia et al. 2015) and effective as a preservative in beef sausage products for six days inside the cold storage (Arief et al. 2017).

Soenarno et al. (2020) stated that plantarisin IIA-1A5 and nisin to fresh milk reduced the increase in the amount of TPC in the first two hours compared to controls. In terms of safety, these results indicated that iodine and plantaricin IIA-1A5 can act as antimicrobials to slow down the total microbial population in fresh milk during the milking process. Although TPC results show a safe microbial population from fresh milk up to 5 hours of storage, TPC was a non-selective medium that may include some pathogenic bacteria that were very harmful to humans, even with low populations. In this experiment, selective media was used to investigate the presence of pathogenic bacteria in fresh milk, including *E. coli* and *S. aureus*. Lactic acid bacteria were known to have the potential for antimicrobial compounds such as bacteriocins that inhibit pathogenic bacteria (Le et al. 2019).

Escherichia coli

Escherichia coli found in control milk was 2.44 log CFU mL^{-1} at 1 hour of storage at room temperature. This population of *E. coli* increased to 3.79 log CFU mL^{-1} after 7 hours of storage. However, with plantaricin IIA-1A5 as a natural antibacterial agent for the milking process, the *E. coli* colonies that grew up to 7 hours of storage at room temperature were successfully reduced by 2.44 log CFU mL^{-1}. Whereas, for 7 hours of storage in fresh milk with *povidone iodine*, *E. coli* was observed to have a population of 2.48 log CFU mL^{-1}, which was still lower than the maximum population allowed for consumption 3.0 log CFU mL^{-1} (SNI 3141.1 2011).

The maximum *E. coli* population allowed in fresh milk products was 3.0 log CFU mL^{-1} (Badan Standardisasi Nasional 2011). Although *povidone iodine* and plantaricin IIA-1A5 resulted in a lower *E. coli*

Table 3. Chemical characteristics and pH analysis in fresh milk during storage at room temperature

Storage Time (Hour)	Parameter	Treatment		
		Control	*Povidone Iodine*	Plantaricin
1	Fat (%)	3.78±0.03	3.81±0.06	3.79±0.05
	SNF (%)	7.57±0.14	6.73±0.35	7.03±0.55
	Lactose (%)	4.17±0.07^{b}	3.72±0.22^{a}	3.76±0.15^{a}
	Protein (%)	2.77±0.05^{b}	2.49±0.14^{a}	2.48±0.03^{a}
	pH	6.72±0.02^{b}	6.61±0.05^{a}	6.67±0.02^{ab}
3	Fat (%)	3.79±0.03	3.82±0.06	3.80±0.05
	SNF (%)	7.68±0.14^{b}	6.95±0.35^{a}	7.18±0.06^{ab}
	Lactose (%)	4.22±0.08^{b}	3.81±0.20^{a}	3.92±0.02^{ab}
	Protein (%)	2.80±0.05^{b}	2.55±0.15^{a}	2.64±0.02^{ab}
	pH	6.67±0.09^{b}	6.57±0.05^{ab}	6.55±0.02^{a}
5	Fat (%)	3.80±0.03	3.83±0.06	3.81±0.05
	SNF (%)	7.48±0.22^{b}	6.89±0.45^{a}	7.28±0.17^{ab}
	Lactose (%)	4.11±0.12^{b}	3.78±0.25^{a}	4.05±0.04^{ab}
	Protein (%)	2.73±0.08^{b}	2.53±0.17^{a}	2.67±0.06^{ab}
	pH	6.63±0.1	6.49±0.03	6.59±0.03
7	Fat (%)	3.81±0.03	3.84±0.06	3.83±0.04
	SNF (%)	7.54±0.15^{b}	7.21±0.05^{a}	7.04±0.08^{a}
	Lactose (%)	4.17±0.11^{b}	3.95±0.02^{a}	3.86±0.05^{a}
	Protein (%)	2.74±0.04^{b}	2.69±0.08^{ab}	2.58±0.02^{a}
	pH	6.61±0.07	6.56±0.07	6.58±0.06

Means in the same row with different superscript differ significantly (P<0.05).

population than the maximum population allowed by the standard, these results suggested that plantaricin IIA-1A5 inhibits *E. coli* much more strongly than *povidone iodine*. The ability of plantaricin IIA-1A5 to inhibit the growth of *E. coli* is in line with (Arief et al. 2012, Arief et al. 2013)). *Escherichia coli* are known to cause the putrefaction of bacteria in food. Plantaricin IIA-1A5 is effective in inhibiting the growth of *E. coli* bacteria from forming a bacterial inhibition zone. Bacteriocins can damage bacterial cell walls, causing the death of *E. coli*. The higher the plantaricin percentage, the larger the inhibition zone produced. This shows that *E. coli* bacteria are inhibited by plantaricin activity. The highest inhibition zone was found in the highest plataricin percentage, namely 50% (Siswara et al. 2019).

Staphylococcus aureus

Staphylococcus aureus populations in control milk ranged from 0.00 to 1.93 log CFU mL^{-1} for 1-7 hours of storage at room temperature. The presence of *povidone iodine* was significantly inhibited the growth of *S. aureus* compared to the control milk. The population of *S. aureus* for 7 hours of room temperature storage after *povidone iodine* was added 0.00-1.41 log CFU mL^{-1}. Interestingly, plantaricin IIA-1A5 demonstrated the ability to inhibit *S. aureus* populations similar to use iodine. Colonies were observed for 1-7 hours of storage at room temperature with a population of 0.00-1.02 log CFU mL^{-1}. The population of *S. aureus* in fresh milk using plantaricin or *povidone iodine* was lower than the maximum level allowed by the standard (2 log CFU mL^{-1}).

Fresh milk that is safe for consumption has a maximum *S. aureus* population of 2 log CFU mL^{-1} with the risk requirements of *S. aureus* for consumption after storage for up to 6 hours at room temperature after 1 hour of milking (Badan Standardisasi Nasional 2011). These results show that up to 7 hours of storage at room temperature, fresh milk with iodine or plantaricin IIA-1A5 is quite safe for consumption. It should be noted that the ability of plantaricin IIA-1A5 to inhibit *S. aureus* is comparable to that of *povidone iodine*,

suggesting the potential use of plantaricin as an *povidone iodine* substitute. Following Arief et al. (2012), plantaricin has antimicrobial activity so that it can be used as a biopreservative in meatball products achieved by inhibiting the total growth of microbes and *E.coli*. The plantaricin used in this study is crude plantaricin, where crude plantaricin has been shown to suppress microbial growth, such as TPC, *E. coli*, and *S. aureus*.

Plantarisin IIA-1A5 with *povidone iodine* has differences in suppressing the number of *S. aureus* bacteria, because the composition of the active or antibacterial substances in both of them is different in reducing the number of these bacteria. Plantarisin IIA-1A5 has antimicrobial properties from organic materials, while *povidone iodine* from inorganic materials.

Chemical characteristics and pH analysis

Results of measurements on the physicochemical quality of fresh milk using the teat dipping method at storage 1, 3, 5, and 7 hours. Results of pH meter measurements of fresh milk using the teat dipping method at 1 and 3 hours storage was significantly different ($P<0.05$). Plantaricin as a natural preservative applied in teat dipping before the milking process does not make any differences in the fat and SNF content. The mean chemical quality and pH analysis resulting from plantaricin and *povidone iodine* treatment was presented in Table 3.Chemical characteristics

Chemical characteristics in fresh milk during storage of 1 to 7 hours at room temperature. Plantaricin as a natural preservative in teat dipping does not make a difference in the fat, and SNF content. In the measurement of fat, SNF, and protein content, all treatments and controls showed values below the SNI 3141.1:2011 level, namely >3% (fat content), >7.8% (SNF), and >2.8% (protein content). Application of plantaricin IIA-1A5 and *povidone iodine* on teat dipping had a higher trend than control. Lactose levels in the plantaricin treatment at 5 hours increased. This shows cause increase of lactose in milk is due to changes in the composition of fat-protein-lactose so that the lactose content in milk has increased.

pH analysis

The three treatments' pH values during storage of 1 to 7 hours at room temperature. The three treatments' pH value was significantly influenced by the treatment and storage time up to 2 hours ($P <0.05$). In general, there was no change in pH for control or teat dipping treatment with *povidone iodine* and plantaricin for 5 to 7 hours. This is because *povidone iodine* contains 14 Polyvinylpyrrolidone active zinc, a strong acid, where the active substance is very useful in coating the nipple hole and can kill bacteria that enter the nipple hole by destroying the metabolism of cells in the cytoplasm to the cell nucleus, so that acidification of milk caused by bacterial activity can be avoided and maintained the pH of the milk at normal. The process of acidifying milk was caused by the fermentation of *Streptococcus lactis* against lactose, which significantly reduce the pH value (Mahardhika et al. 2012). The conversion of lactose caused an increase or decrease in pH into lactic acid by microorganisms and enzymatic activity (Mirdhayati et al. 2008). Marsh et al. (2014) stated that a fermented product's pH was influenced by the buffering capacity with different amounts and different types of protein. The growth of good lactic acid bacteria occurred at pH 6, and the growth rate decreased if the extracellular media became acidic. The decrease in pH resulted from acid accumulation from lactic acid bacteria.

CONCLUSION

Application of plantaricin IIA-1A5 as teat dipping before milking can reduce Total Plate Count, *Escherichia coli*, *Staphylococcus aureus* population and did not change pH value and physicochemical quality (fat, SNF, lactose, and protein). This ability is comparable to the iodine group, a synthetic antibacterial widely used by smallholder breeders in Indonesia. It is concluded that Plantaricin IIA-1A5 can be used as a substitute for synthetic antibacterial (iodine group) for teat dipping before milking.

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Effect of Chicken Bone Extract Powder on Epididymal Sperm Quality of Male Wistar Rats

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ABSTRAK

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Kalsium merupakan salah satu mineral esensial untuk fungsi reproduksi jantan. Kekurangan kalsium mempengaruhi spermatogenesis, fungsi normal sperma dan infertilitas. Pengukuran kualitas sperma tikus yang diberi pakan standar yang mengandung tepung tulang ayam (BEP) dilakukan dalam penelitian ini. Sebanyak dua puluh ekor tikus Wistar jantan berumur 8 minggu ditimbang secara acak dan dibagikan ke dalam dua kelompok yang masing-masing terdiri dari 10 ekor tikus. Pakan yang diberikan secara *ad libitum* merupakan pakan standar yang mengandung kalsium karbonat (CaCO3, kontrol) atau BEP sebanyak 0,5% kalsium dalam keduanya. Pada akhir konsumsi pakan selama 8 minggu, pertambahan bobot badan pada kelompok kontrol (101,33±21,81 g) tidak berbeda secara nyata (P>0,05) dibandingkan kelompok BEP (100,74±26,80 g). Kualitas sperma in vitro dalam hal konsentrasi, motilitas, viabilitas, resistensi terhadap stress hipotonik, kemampuan reaksi akrosomal dan morfologi dapat dibandingkan antara kelompok control dan BEP (P>0,05). Hasil penelitian menunjukkan bahwa BEP yang ditambahkan dalam pakan merupakan sebuah alternative sumber kalsium yang efektif dan murah sebagai CaCO3 - kalsium komersial (fortifikan). Setidaknya, tidak memiliki efek merugikan pada fungsi reproduksi jantan.

Key Words: Kalsium, Tulang ayam, pakan, Tikus, Sperma

ABSTRACT

Buranaamnuay K, Kettawan A, Changsangfa C, Aiemongkot S. 2021. Effect of chicken bone extract powder on epididymal sperm quality of male Wistar rats. JITV 26(2): 74-81. DOI: http://dx.doi.org/10/14334/jitv.v26i2.2729.

Calcium is one of the minerals that are essential for male reproductive function. Calcium deficiency adversely affects spermatogenesis, normal sperm function and results in infertility. The sperm quality of rats fed a standard diet containing chicken bone extract powder (BEP) was assessed in the present study. Twenty male 8-week-old rats, Wistar strain, were randomized by weight into two groups of ten rats each and fed ad libitum a standard diet containing calcium carbonate ($CaCO_3$, control) or chicken BEP; both were equivalent to 0.5% calcium. At the end of the 7-week consumption, the net body weight gains measured in control (101.33±21.81 g) and chicken BEP groups (100.74±26.80 g) were not significantly different (P>0.05). The in vitro sperm quality in terms of concentration, motility, viability, resistance to hypotonic stress, acrosomal reaction ability and morphology was comparable between control and chicken BEP (all were P>0.05). The results suggest that chicken BEP addition into feeds is an alternative calcium source that is as effective but less expensive as $CaCO_3$, a commercial calcium (fortificant). At least, it has no detrimental effect on male reproductive function.

Key Words: Calcium, Chicken bone, Diet, Rat, Sperm

INTRODUCTION

Calcium is one of the minerals essential for the biochemical and physiological processes of life (Veldurthy et al. 2016). Apart from a structural component of bone and teeth when combined with phosphorus, calcium plays a vital role in blood coagulation, muscle contraction, hormone and enzyme secretions, the transmission of nerve impulses, and reproductive function in both males and females (Sittikulwitit et al. 2004; Stewart & Davis 2019). For male reproduction, calcium is necessary for stimulation of sex steroid hormone (i.e., testosterone) production, sperm formation and development, sperm motility, capacitation, acrosome reaction, and fertilization processes (Chung et al. 2011; Valsa et al. 2015). Calcium

deficiency can be associated with depletion of testosterone, inhibition of spermatogenesis, and subsequently, male infertility (Harchegani et al. 2019).

As the body cannot synthesize calcium, it is important to get enough calcium from dietary intake. Calcium-rich diet sources that are well-known and commonly consumed include dairy products (such as milk, yogurt, and cheese), grains, and green leafy vegetables. However, the only problem with these calcium sources is that these foods contain some substances, for example, fiber, phytic acid, and oxalic acid that have a strong binding affinity to calcium, inhibiting its absorption (Atmokotomo et al. 2019). Chicken bone, a by-product from the poultry industry, contains high calcium but does not contain substances that inhibit calcium absorption. In the year 2002, Kettawan et al. prepared high-quality chicken bone extract powder (BEP) using an inexpensive, effective procedure so-called alkaline treatment. Calcium bioavailability of chicken BEP determined by in vitro dialyzability methods was higher than a traditional calcium-rich diet source and several calcium fortificants. Furthermore, when fortified into shrimp chips, chili-paste, bread, and cookies, chicken BEP was well accepted by consumers; the appearance, taste, and texture of the BEP-fortified products were not significantly different from non-fortified counterparts (Kettawan et al. 2002; Sittikulwitit et al. 2004). As a result, chicken BEP could be deemed an excellent alternative calcium source cheaper than imported commercial calcium fortificants such as calcium carbonate ($CaCO_3$). Nonetheless, gastrointestinal (GI) digestion, absorption, metabolism, tissue distribution, safety, and toxicity of chicken BEP assessed in vivo have been reported on a very limited scale and there is no study in male animals. Therefore, the present study was undertaken to determine the effect of chicken BEP on male fertility by appraising the epididymal sperm characteristics of Wistar rats given a standard rodent diet with and without chicken BEP. The outcome of this pre-clinical testing will potentially be valuable data for conducting clinical trials on humans in the future.

MATERIALS AND METHODS

Chemicals

All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. The food-grade $CaCO_3$ powder was supplied by Thai Poly Chemicals, Co., Ltd., Thailand.

Preparation of chicken BEP

Betagro Group, Thailand, kindly provided chicken bone. Preparation of chicken BEP was accomplished by an alkaline treatment method adapted from Kettawan et al. (2002).

Animals

Acclimation phase

Twenty male Wistar rats, 7-week old of age and 250–300 g body weight, were obtained from Nomura Siam International Co., Ltd. At the Institute of Molecular Biosciences (MB), the rats were housed individually in strict hygienic conventional facilities under standard husbandry conditions, i.e., temperature of 22±2°C, relative humidity of 55±15%, and under controlled (12:12 h) light-dark cycle. During the one-week acclimation period, the rats had free access to water and food (standard rodent diet No. 082, C.P. Company, Bangkok, Thailand). All animal experiments were approved by the Animal Care and Use Committee at MB (COA.NO.IMB-ACUC 2020/017).

Experimental phase

After one-week habituation, all rats were weighted by a 2-digit digital scale, divided into two groups of ten animals each, and were given ad libitum for eight weeks on powdered foods containing calcium from two different sources. The rats assigned to group 1 (control) were given a $CaCO_3$ based diet containing 0.5% calcium (Cashman & Flynn 1996). The animals in group 2 were offered a standard powder diet containing 0.5% calcium of chicken BEP. The composition of experimental diets is demonstrated in Table 1. Rats' body weights were measured periodically, on the 1st, 4th, and 8th week of consumption.

Epididymal sperm collection

The methods of epididymal sperm collection were similar to those described by Asadi et al. (2014) and Omirinde et al. (2019), with some modifications. At the end of week 8th, the rats were euthanized by carbon dioxide inhalation. The open castration technique subsequently removed the reproductive organs, i.e., testicles and epididymides. During orchiectomy, the testicles were push up through the incision line at a pre-scrotal area. The tunica vaginalis and spermatic cord were exposed and then scratched. The testicles and epididymides of each rat was placed in a clean container pre-filled with warmed (37°C) 0.9% (w/v)

Table 1. The composition (g/100 g) of experimental diets provided to rats

Ingredients	Group	
	1	2
Cornstarch	46.57	46.57
Casein	14	14
Soybean oil	4	4
Solka Floc-40	5	5
Dextrin	15.50	15.50
Granular sugar	10	10
AIN-93 mineral mix		
$CaCO_3$ formulation	3.50 (0.5% calcium)	
Chicken BEP formulation		3.50 (0.5% calcium)
AIN-93 vitamin mix	1	1
L-Cystine	0.18	0.18
Choline bitartrate	0.25	0.25

sodium chloride (GHP, Pathum Thani, Thailand). At the nearby andrology laboratory, the organs were further washed with the same medium. The epididymides were separated from the testicles by using operating scissors and sharp, serrated tip forceps. Only the caudal part of epididymides was transferred to a small glass dish containing 1.5 mL of phosphate buffer saline (1X, pH 7.4) (Apsalagen, Bangkok, Thailand) and cut into tiny pieces. The dish covered with aluminum foil was placed at 37°C for 10–15 min to wait for sperm release. After that, the sperm-rich fluid (approximately 1 mL of the upper part) was carefully aspirated, deposited into a microtube, and incubated in a 37°C water bath while assessing sperm quality.

Sperm analysis

Sperm count

For sperm counting, 5 µL of the sperm suspension was diluted with formol saline solution [0.9% sodium chloride and 0.1% (v/v) 40% formaldehyde in distilled water] (1:40, v/v). Approximately 10 µL of the diluted solution was transferred into a hemocytometer (Hausser Scientific, Horsham, PA, USA) and let to settle for 5 min. Epididymal sperm number was determined by counting the cells in 5 large squares under a light microscope (Helmut Hund GmbH, Wetzlar-Nauborn, Germany) at 400 × magnification. Sperm concentration was then calculated from average of the two values and reported as ($\times 10^6$) per mL (Badkoobeh et al. 2013).

Sperm motility

To assess the percentage of motile sperm, a small aliquot (10 µL) of suspension was re-pipetted and placed on a glass slide at 37°C; then overlaid with a coverslip. Randomly ten fields from each slide (2 slides/sample) were observed by an experienced evaluator using a blind technique under a phase-contrast microscope (200 ×) (Nikon, Melville, NY, USA). The percentage of sperm motility was recorded based on the appearance of both progressive motile and non-progressive motile sperm (Jaffar et al. 2021).

Sperm viability

Viability of sperm was appraised by one step staining technique comprising 0.6% (w/v) eosin, 5% (w/v) nigrosin and 3% (w/v) sodium citrate dihydrate prepared in distilled water (pH 7.0) (Buranaamnuay 2020). Fifty microliters each of sperm suspension and the dye were mixed on a glass slide. An aliquot of the mixture was spread on another slide and dried on a hot plate with 37°C. An evaluator undertook observation of live (unstained head) and dead (any pink or red coloration) sperm with the aid of a brightfield microscope (× 400 magnification). The percentage of viable sperm was calculated from 200 sperm examined (Iswadi et al. 2012).

Sperm tolerance to hypotonic stress

The present study evaluated the ability of sperm to withstand osmotic stress by determining percentages of

membrane-intact sperm after incubation in a 75 mOsm/kg hypo-osmotic solution (sodium citrate dihydrate and D-fructose dissolved in distilled water) (Buranaamnuay 2019). Fifty microliters of sperm suspension were diluted in 0.5 mL of the solution. Samples were incubated for 20 min at 37°C and then analyzed using phase-contrast microscopy (400 ×). With a total of 200 sperm counted, the number of sperm with curled tails (intact membranes) and non-curled tails (damaged membranes) was recorded as percentages.

Acrosomal reaction ability of sperm

As calcium is necessary for sperm capacitation and acrosome reaction, these physiological processes have been enhanced by adding caffeine in vitro (Harchegani et al. 2019). The present study evaluated the ability of sperm to undergo acrosomal reaction by incubating sperm suspension with 1 mM caffeine prepared in PBS (1:2, v/v) at 37°C (adapted from Funahashi & Nagai 2001).

The protocol developed for assessing the acrosomal status included using an acrosome-specific fluorescent dye – fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; 100 µg/mL), in conjunction with the supravital staining propidium iodide (PI; 340 µM). For this assay, 10 µL of diluted sperm suspension was smeared on a glass slide and dried. The smeared slide was permeabilized in 95% ethyl alcohol, dried, and spread with a combination of fluorescence dyes (10 µL). After incubating in a dark humid chamber at 4°C for 2 h and rinsing with cold distilled water, at least 200 sperm on the slide were observed for the acrosomal status under fluorescence microscopy (600 ×) (DeltaVision Ultra; Cytiva, USA). Sperm with bright green fluorescence over the entire region of the acrosome was judged as acrosome intact. On the other hand, sperm without this staining pattern, i.e., with bright green fluorescence over some part of acrosome or without green color were deemed as acrosome reacted sperm. The percentages of acrosome reacted sperm at 0 and 2 h of caffeine incubation were recorded, as sperm acrosomal reaction ability.

Sperm morphology

Determination of sperm morphology was performed by Diff-Quik rapid staining technique (Natali et al. 2013). A drop (10 µL) of the sperm suspension was spread over the surface of the glass slide. The air-dried slide was fixed in 95% methyl alcohol for an hour and then stained by serially immersed in solution 1 (eosinophilic) for 10 s and solution 2 (basophilic) for 5 s. Eventually, the slides were rinsed in running water to get rid of the excess stain. The stained slides were read under a brightfield microscope at 1,000 magnification with oil immersion (Helmet Hund GmbH, Wetzlar-Nauborn, Germany). A total of 200 sperm for each slide were examined for the appearance of normal and abnormal morphology, e.g., sperm with head, midpiece, and tail defects. However, only the percentage of morphologically normal sperm was reported in the results.

Statistical analysis

A Shapiro-Wilk test in PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) was used to test for normal distribution of data. Normally distributed data were analyzed using Independent-Samples T-Test to determine the level of significance between the control ($CaCO_3$) and experimental (chicken BEP) groups. Values of $P < 0.05$ were considered statistically significant. Results are expressed as means ± standard deviation (SD).

RESULTS AND DISCUSSION

Results

The mean body weights of rats in the control and experimental groups increased progressively throughout eight weeks of the study (Figure 1). Additionally, at the end of week 8th, the net body weight gains measured in both groups were comparable. There was no significant difference ($P > 0.05$) between them, as shown in Figure 2.

Results of epididymal sperm analysis of the rats from both groups are summarized in Figure 3. The sperm quantity (concentration) and quality (the motility, viability, resistance to hypotonic stress, acrosomal reaction ability, and morphology) of rats in the experimental group were not significantly different ($P > 0.05$) from those in the control. Irrespective of group, the representative microphotographs of sperm morphology are illustrated in Figure 4.

Discussion

This present study examined the effect of chicken BEP as a calcium source in rodent diet on the sperm quality of male Wistar rats. Results obtained, with our expectation, will be used as fundamental data extrapolated to humans. Calcium is a significant element that acts as an intracellular second messenger. It is crucial for sperm to maintain the normal functions including spermatogenesis, sperm motility, capacitation, acrosome reaction, chemotaxis, and fertilization (Valsa et al. 2015). Reduced seminal

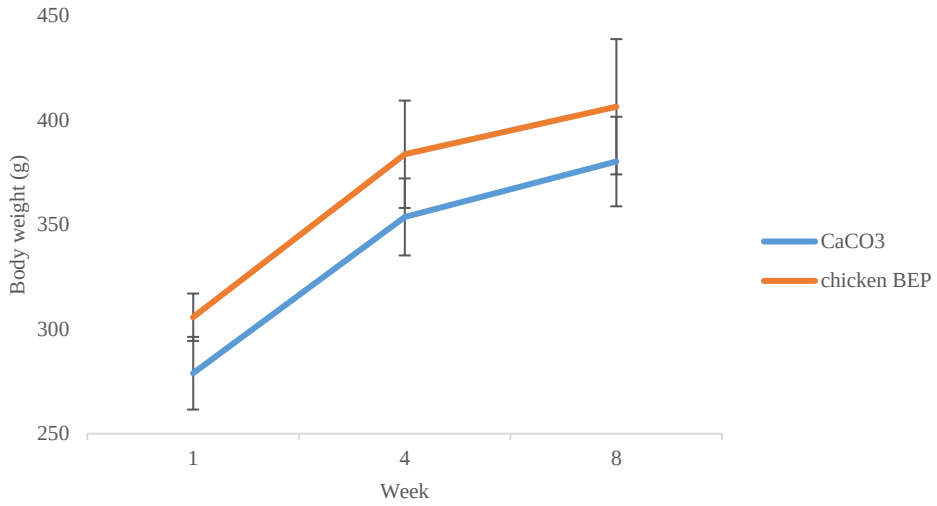

Figure 1. Mean body weight for the period of treatment in male Wistar rats. Values represented are means ± SD.

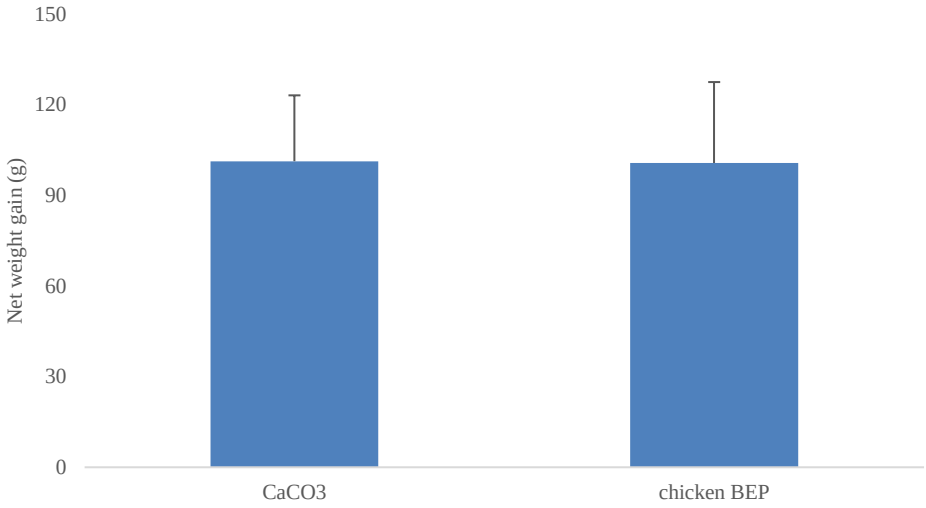

Figure 2. Rats' net weight gain after eight weeks of treatment. Values are represented as means ± SD. P > 0.05 compared to the control ($CaCO_3$).

calcium concentrations were positively correlated with decreased sperm motility and membrane integrity and reduced sperm counts (Talluri et al. 2017). In the present study, male rats were given chicken BEP for eight weeks. This period lasts longer than the duration of spermatogenesis in Wistar rats, i.e., approximately 52-53 days (Clouthier et al. 1996). Therefore, in case of having a positive or negative influence on the reproductive system, there should be some alterations in the sperm quality of rats in the experimental group. However, it was found that epididymal sperm characteristics of rats given chicken BEP were not significantly different from, and even tended to be better than, those in the control Furthermore, throughout the entire study period, rats in the experimental group exhibited normal behavior; they still ate and normally moved (data not shown). Their body weights increased continuously and at the end of the study, net body weight gains of rats in the experimental group were similar to the control. Bodyweight changes suggest that chicken BEP when added into foods, does not decrease the acceptability and palatability of such diets. This suggestion was supported by previous studies undertaken in animals (Atmokotomo et al. 2013) and human subjects (Kettawan et al. 2002; Sittikulwitit et al. 2004).

Moreover, our findings on the unaltered sperm quality imply that the in vivo bioavailability of calcium from chicken BEP was not inferior to that from $CaCO_3$, a commercial calcium salt. The high calcium bioavailability of chicken BEP has also been reported in

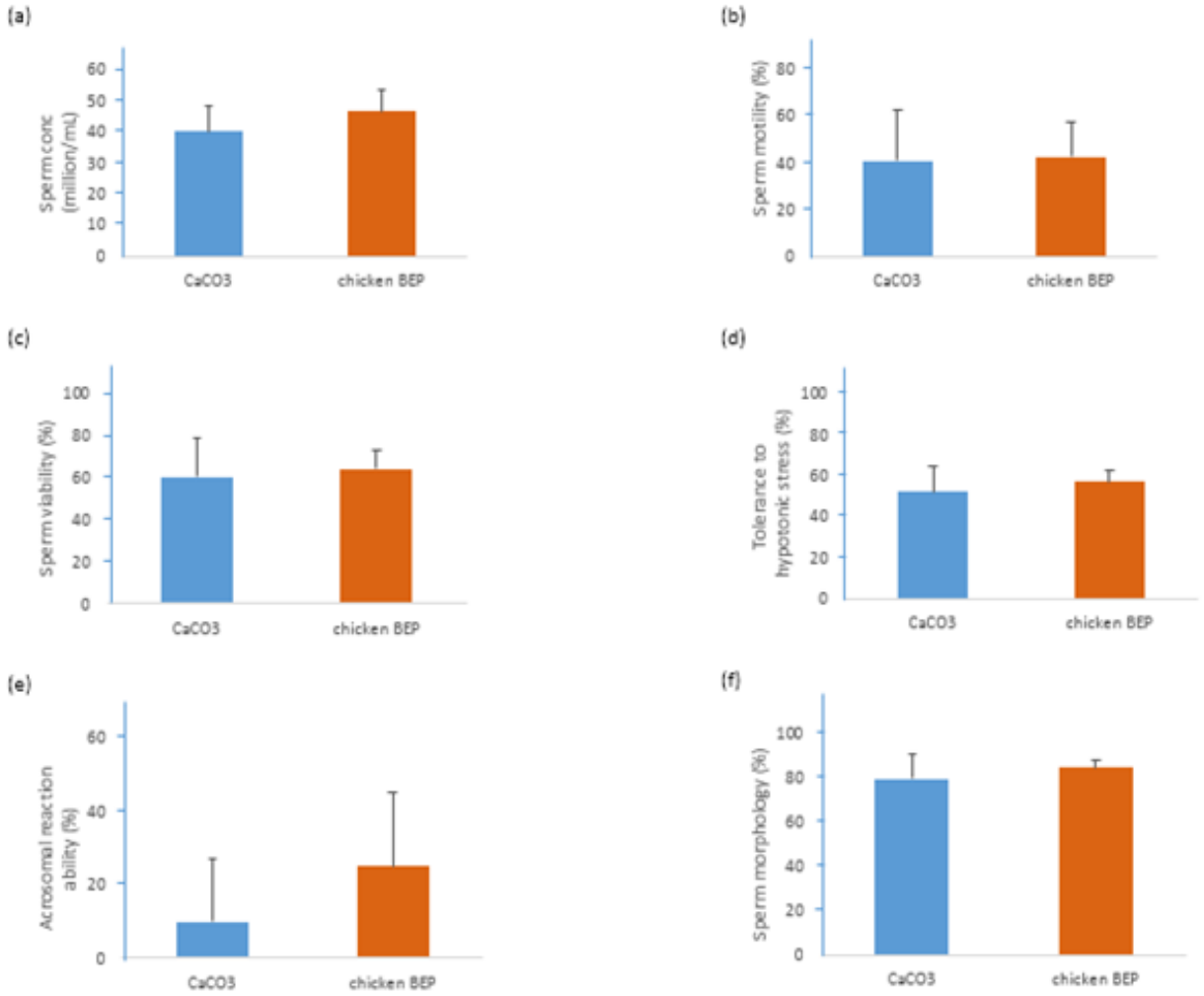

Figure 3. Effect of chicken BEP on epididymal sperm characteristics. (a) Sperm concentration, (b) sperm motility, (c) sperm viability, (d) sperm tolerance to hypotonic stress, (e) acrosomal reaction ability of sperm, and (f) sperm morphology. Values are represented as means ± SD following eight weeks of treatment. All parameters are $P > 0.05$ compared to the control ($CaCO_3$).

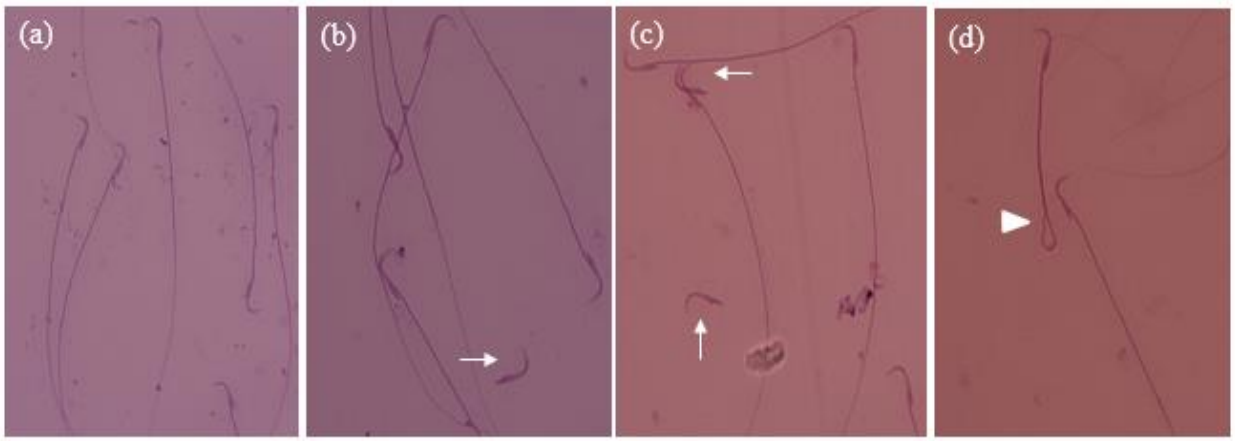

Figure 4. Sperm morphology of Wistar rats using Diff-Quik staining (1000 ×). Sperm with normal (a) and abnormal morphologies (b)–(d). (b) and (c) detached heads (arrows); (d) bent tail (an arrowhead).

female rats. In that study, chicken BEP suspension was given to pregnant Sprague Dawley rats from the first day of pregnancy to the second week of parturition.

The calcium level of rat pups' teeth in the chicken BEP supplementation group was significantly higher than the control (Atmokotomo et al. 2019). The higher calcium bioavailability of chicken BEP, when compared with the same calcium content in traditional calcium-rich diet sources and some forms of commercial calcium salts was explained by the fact that chicken BEP does not contain substances such as fiber, phytate, and oxalate which can bind to calcium becoming insoluble complexing agents and consequently can interfere with the absorption of calcium in the gut (Whisner et al. 2014). Moreover, another explanation is that chicken BEP contains calcium and phosphorus in the appropriate ratio of 2:1, which enhances calcium absorption and retention in the body (Kettawan et al. 2002; Loughrill et al. 2017).

Chicken BEP utilized in the present study is deemed safe for consumption because, using this preparation technique, heavy metal levels, and microorganism and moisture contents in the product were very low. Lead and cadmium were non-detectable; total plate count was less than 10 CFU/g, and water activity (Aw) was just 0.152. A very low Aw can delay microbial growth and food spoilage (Kettawan et al. 2002). Besides the above reasons, chicken BEP is suitable for consumption by humans in every culture unlike pork or cow by-products forbidden for Muslims and some Indians, for example, due to religious restrictions, their belief, and their laws (Brondz 2018; Kennedy et al. 2018).

CONCLUSION

Chicken BEP addition into rodent diet had no adverse effect on the quantity and quality of epididymal sperm in male Wistar rats. This initially suggests chicken BEP being an alternative natural calcium source that is effective and not expensive. However, before stepping up to clinical trials in human participants, the chronic effect of chicken BEP intake on the reproductive and other body systems should be determined. Also, the in vitro and in vivo fertility of sperm from chicken BEP consumers require further study.

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Avian Beta Defensin 2 (AvBD2) Gene Polymorphism Identification in IPB-D1 Chicken

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ABSTRAK

Masrurah, Khaerunnisa I, Murtini S, Sumantri C. 2020. Identifikasi keragaman gen Avian Beta Defensin 2 (AvBD2) pada ayam IPB-D1. JITV 26(2): 82-89. DOI: http://dx.doi.org/10.14334.v26i2.2715.

Gen Avian Beta Defensin 2 (AvBD2) yang berada di kromosom 3 berperan penting dalam sistem imunitas tubuh ayam dengan menghambat perkembangan mikroorganisme seperti bakteri yang mengifeksi jaringan tubuh. Defensin dihasilkan melalui sel epitel segera setelah adanya cidera jaringan atau infeksi, yang kemudian memproses pematangan sel dendritik untuk memulai respon imun di kelenjar getah bening. Tujuan penelitian ini adalah mengidentifikasi polimorfisme gen AvBD2 pada ayam IPB-D1. Metode PCR dan direct-DNA sequencing digunakan untuk mengidentifikasi keragaman intron 1, ekson 2, dan intron 2 gen AVDB2 pada 47 ekor ayam IPB-D1. Perhitungan frekuensi genotipe, frekuensi alel, dan heterozigositas dilakukan untuk mendapatkan informasi polimorfisme gen AvBD2. Sebanyak 10 mutasi titik ditemukan pada gen AvBD2 yang tersebar di intron 1 (g.4843T>A, g.4853G>A, dan g.4859T>C), ekson 2 (g.4881A>G, g.4889G>A, dan g.5002C>T), dan intron 2 (g.5075C>T, g.5111T>G, g.5116G>T, dan g.5177G>T). Seluruh SNP bersifat polimorfik. Mutasi g.5002C>T menyebabkan perubahan asam amino Ala menjadi Val yang berpotensi menjadi kandidat penciri ketahanan penyakit pada ayam IPB-D1.

Kata Kunci: Gen AvBD2, IPB-D1 chicken, Keragaman

ABSTRACT

Masrurah, Khaerunnisa I, Murtini S, Sumantri C. 2020. Avian Beta Defensin 2 (AvBD2) gene polymorphism identification in IPB-D1 chicken. JITV 26(2): 82-89. DOI: http://dx.doi.org/10.14334.v26i2.2715.

Avian Beta Defensin 2 (AvBD2) gene, located in chromosome 3, plays an important role in the immune system of the chicken by inhibiting the development of microorganisms such as bacteria that infect body tissues. Defensins are produced through epithelial cells immediately after tissue injury or infection, which then processes the maturation of dendritic cells to initiate an immune response in the lymph nodes. The purpose of this study was to discover the polymorphism of the AvBD2 gene in IPB-D1 chickens. PCR and direct-DNA sequencing methods were used to identify the polymorphisms of intron 1, exon 2, and intron 2 AvDB2 genes in 47 chickens. Genotype and allele frequency, and heterozygosity calculations were carried out to obtain information of the AvBD2 gene polymorphism. A total of 10 single nucleotide polymorphisms were found in the AvBD2 gene located in intron 1 (g.4843T>A, g.4853G>A, and g.4859T>C), exon 2 (g.4881A>G, g.4889G>A, and g.5002C>T), and intron 2 (g.5075C>T, g.5111T>G, g.5116G>T, and g.5177G>T). All SNPs are polymorphic. The g.5002C>T mutation causes changes in the amino acid Ala to Val which has the potential to be a candidate for characterizing disease resistance in IPB-D1 chickens.

Key Words: AvBD2 gene, IPB-D1 chicken, Polymorphism

INTRODUCTION

Indonesia's native chicken population in 2020 is estimated to reach 308 million chickens, or around 8.8% of the total national chicken population (DGAHP 2020). Application of extensive traditional rearing system shows the low application of biosecurity and sanitation. The lack of attention of local chicken breeders to good rearing management and the application of biosecurity affect the health of local chickens. This condition causes local chicken production to be not optimal. Availability of local chicken breeds that are resistant to disease is very important so that breeders could get disease-resistant chickens maintained in suboptimal biosecurity and sanitation conditions. One of the efforts to provide these breeds is through genetic selection of local chickens that have a good immune system.

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Defensin is part of the immune system that has broad antimicrobial activity (Wilson et al. 2013). Beta defensin is a member of the defensins family found in avian including chickens and has the potential as an alternative to antibiotics due to its role to neutralize various pathogens such as colibacillosis and zoonosis (Derache et al. 2012, Kim & Lillehoj 2019, Zhao et al. 2014). Hong et al. (2012) stated that the pathogens that phagocytes by antimicrobial activity in the AvBD2 gene include *Salmonella enterica* serovar Typhimurium, *C. perfringens*, and *Escherichia coli* found in the digestive tract. Moreover, the AvBD2 gene plays an important role in the level of immunity in chickens (Hong et al. 2012). AvDB2 has been reported to play a role in ileum and cecum mucosal defense in broiler embryos and neonatal chicks (Terada et al. 2018). The AvBD2 gene was isolated from leukocytes, epithelial cells of the skin, digestive tract, and respiratory tract. The role of AvBD2 in resistance to Newcastle Disease virus infection was reported by Liu et al. (2018). Several molecules have also been reported to suppress the action of AvBD2 in defense against NDV, one of which is p38 MAPK (Liu et al. 2018). In addition, localization of AvBD2 and AvBD4 was found in chickens that were vaccinated against ND and Marek's disease (Shimizu et al. 2020).

Defensins are produced immediately after tissue injury or infection. Defensins are released from epithelial cells, including keratinocytes and infiltrating leukocytes. Defensins facilitate the uptake of antigens and processing of immature dendritic cells (iDCs) into mature dendritic cells (mDCs). These mDCs then migrate to the lymph nodes to initiate an immune response (Hazlett & Wu 2011). The chicken AvBD2 gene is located on chromosome 3 and consists of four exons and three introns (Xiao et al. 2004). AvBD2 protein in chickens consists of 22 signal peptides, 6 propieces, and 36 mature peptides, which are equal to other avian such as ostrich, duck, goose, and turkey (Lu et al. 2014).

The polymorphisms of the AvBD2 gene is indicated by the discovery of single nucleotide polymorphysms (SNP) which can be used as genetic markers. Bagnicka et al. (2010) stated that SNPs on Avian beta defensin have a strong correlation to be associated with antibacterial activity against Gram-positive and Gram-negative bacteria. Mommarazi and Habibi (2017) reported that the mutation rate of chicken AvBD2 was 15.4 SNPs/kb, which showed that in every 1000 bp of AvBD2 gene sequence, 15.4 SNPs were found. This shows a high genetic diversity and has a high response to disease resistance (Morammazi & Habibi 2017).

IPB-D1 chicken is Indonesian local chickens that have been released by the Minister of Agriculture of the Republic of Indonesia in 2019 as superior broiler and layer (Sumantri et al. 2020). IPB-D1 chickens are the result of crossing of Pelung x Sentul (male) and Kampung x Cobb strain broilers (female), so they have a blood composition of 75% local chickens (Sumantri et al. 2020). Apart from having superior productivity, IPB-D1 chickens are also developed to have resistance to ND virus (Al-Habib et al. 2020) and Salmonella enteritidis infection (Susanti et al. 2020). Genetic information regarding the diversity of the AVBD2 gene in IPB-D1 chickens has never been reported. The objective of this study is to discover the Single Nucleotide Polymorphism (SNP) of the partial AvBD2 gene in IPB-D1 chicken using direct-DNA sequencing method. Identification of the polymorphism of the AvBD2 gene may be a potential candidate gene for improving genetic quality in IPB-D1 chickens, especially in their immune system.

MATERIALS AND METHODS

Chickens rearing management and blood sample collection

All procedures performed in this study were based on animal research ethics (IPB Animal Care and Use Committee IACUC approval ID: 163-2019). A total of 47 IPB-D1 chickens were used for this study. All chickens were provided by the Animal Genetics and Breeding Division, Faculty of Animal Science, IPB University, Indonesia. Feeding was given twice a day. Chickens were fed 100% commercial feed, 70% commercial feed and 30% rice bran, and 60% commercial feed and 40% rice bran for starter (0-4 weeks), grower (4-12 weeks), and finisher (12-21 weeks), respectively. Clean drinking water is always available. The ND vaccine was administrated to chickens at 3 and 7 days old. At 21 weeks old, blood samples were collected from axillary vein on the wing.

Primer designing

Sequence data for primer designing was obtained from the National Center for Biotechnology Information (NCBI) with the Gen Bank access number AY621317.1 (Xiao et al. 2004). The primer sequences in this study is: forward (F): 5'-CCCACAGAGCATCCATGAGG-3' and reverse (R): 5'-TTGCTGTTGTTGCAGGGTTG-3'. These primers produced a 411 bp DNA sequence covering partial intron 1 to intron 2. The primers were designed using the Primer Designing Tool application (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

DNA isolation and amplification

To obtain genomic DNA template, the blood DNA extraction procedure was performed using a Blood/Cell

DNA Mini Kit (Geneaid) according to the manufacturer's instructions. The amplification of AVBD2 gene fragments was carried out using a PCR machine (Gene Amp PCR System 9700, Applied Bio Systems) in a total of 23 µL premix consisting of 2 µL DNA template, 0.3 µL primers, 12.5 µL GoTaq® Green Master Mix (Promega), and 8.2 µL Nuclease-free Water. The PCR conditions used consisted of three stages. The first stage was the pre-denaturation process (95 ºC for 5 minutes). The second stage carried out 35 cycles consisting of denaturation (95 ºC for 10 sec), annealing (62 ºC for 20 sec), and extension (72 ºC for 30 sec). The third stage is the final extension (72 ºC for 5 minutes). PCR products were visualized in 1.5% agarose gel using PeqGreen DNA/RNA Dye (VWR) staining under UV light (Alpha Imager, Alpha Innotech). Direct sequencing was then carried out for each sample using the 1st Base sequencing services in Selangor, Malaysia.

Data analysis

The sequencing results in the form of a chromatogram were then analyzed using the MegaX (Kumar et al. 2018) and BioEdit (Hall et al. 2011) for DNA sequence alignment and for DNA sequence visualization, respectively. Genetic diversity was analyzed by calculating genotype frequency, allele frequency, and heterozygosity values based on Nei & Kumar (2000).

RESULTS AND DISCUSSION

Identification of mutations in partial AVBD2 gene

This study has successfully amplified the 411 bp partial AvBD2 gene in IPB-D1 chickens (Figure 1). A total of 10 SNPs were found in intron 1 (3 SNPs), exon 2 (3 SNPs), and intron 2 (4 SNPs) of the AvBD2 gene (Table 1). One of the mutations in exon 2 was also reported by Morammazi & Habibi (2017) in local Iranian chicken populations, i.e. g.5002C>T. Meanwhile, the other nine mutations have never been reported previously. All mutations found, the types of mutations and amino acid changes can be found in Table 1.

Six out of ten SNPs found were classified as transition mutations, i.e g.4853G>A, g.4859T>C,

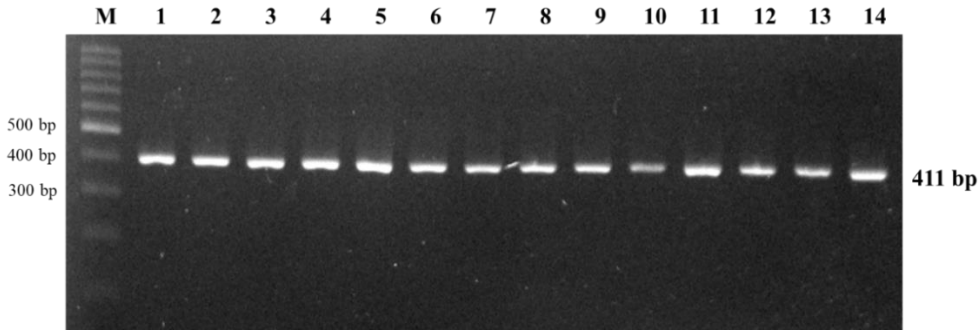

Figure 1. PCR result of partial AvBD2 gene in IPB-D1 chicken. M = DNA ladder 100 bp; 1,2,..14 = sampel

Table 1. Point mutations found in intron 1 to intron 2 of the AvBD2 gene in IPB-D1 chickens along with the amino acid changes occurred

No.	Mutation Position	Location in AvBD2	Mutation Type	Amino Acid (Codon) Change
1.	g.4843T>A	Intron 1	Transversion	-
2.	g.4853G>A	Intron 1	Transition	-
3.	g.4859T>C	Intron 1	Transition	-
4.	g.4881A>G	Exon 2	Transition	-
5.	g.4889G>A	Exon 2	Transition	-
6.	g.5002C>T*	Exon 2	Transition	Ala (GCT) to Val (GTT)
7.	g.5075C>T	Intron 2	Transition	-
8.	g.5111T>G	Intron 2	Transversion	-
9.	g.5116G>T	Intron 2	Transversion	-
10.	g.5177G>T	Intron 2	Transversion	-

*also reported by Morammazi and Habibi (2017)

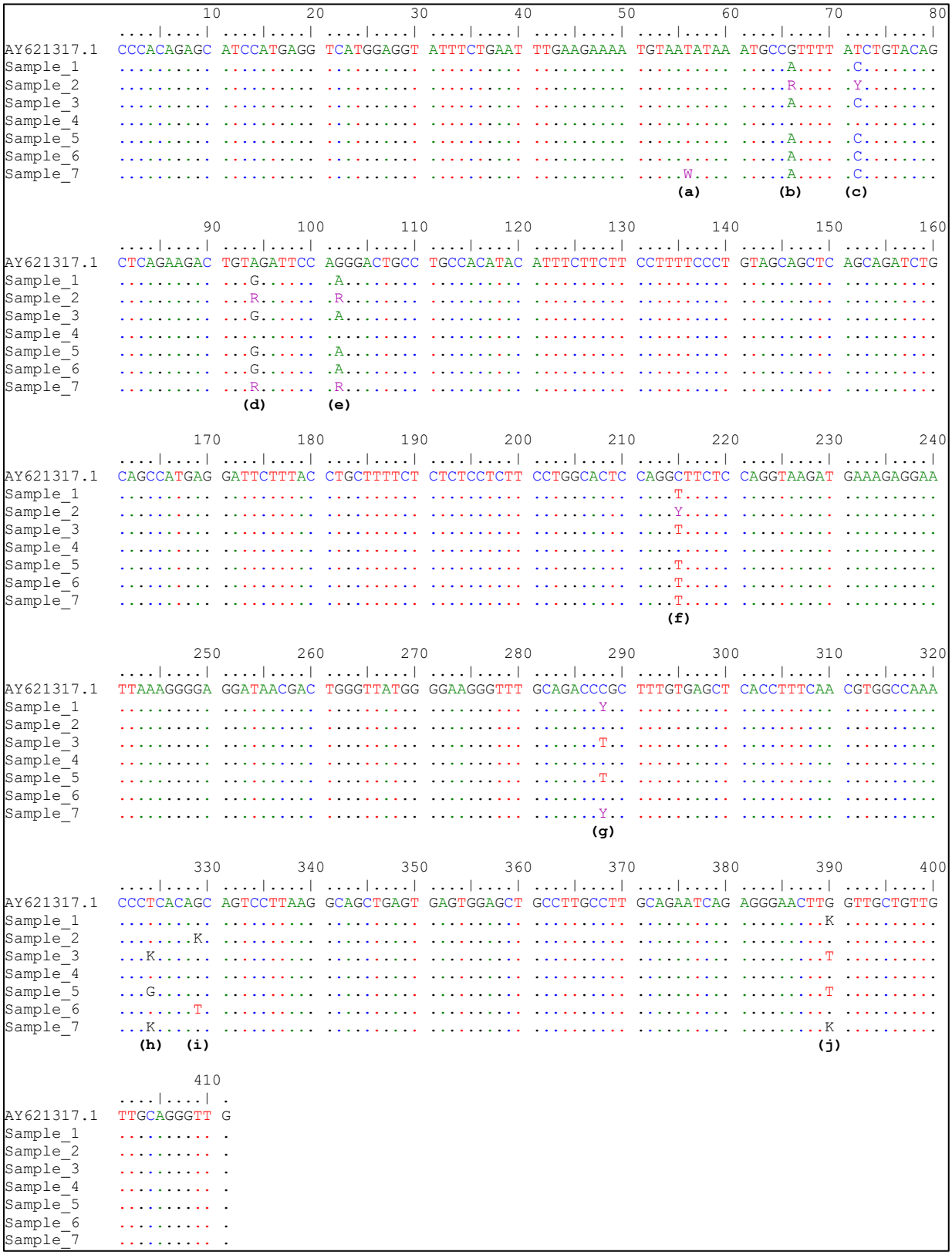

Figure 2. Partial sequence of AvBD2 gene in IPB-D1 chicken including 10 SNPs position, i.e. g.4843T>A (a), g.4853G>A (b), g.4859T>C (c), g.4881A>G (d), g.4889G>A (e), g.5002C>T (f), g.5075C>T (g), g.5111T>G (h), g.5116G>T (i), and g.5177G>T (j)

Table 2. Genotype frequency, allele frequency, and heterozygosityof AvBD2 gene in IPB-D1 chicken

SNP	N	Genotype Frequency			Allele Frequency		Ho	He
g.4843T>A	47	TT	AT	AA	T	A	0.064	0.062
		0.936	0.064	0	0.968	0.032		
g.4853G>A	47	GG	AG	AA	G	A	0.191	0.207
		0.021	0.191	0.787	0.117	0.883		
g.4859T>C	47	TT	TC	CC	T	C	0.170	0.223
		0.043	0.170	0.787	0.128	0.872		
g.4881A>G	47	AA	AG	GG	A	G	0.234	0.268
		0.043	0.234	0.723	0.160	0.840		
g.4889G>A	47	GG	GA	AA	G	A	0.255	0.254
		0.021	0.255	0.723	0.149	0.851		
g.5002C>T	47	CC	CT	TT	C	T	0.191	0.207
		0.021	0.191	0.787	0.117	0.883		
g.5075C>T	47	CC	CT	TT	C	T	0.362	0.500
		0.319	0.362	0.319	0.500	0.500		
g.5111T>G	47	TT	TG	GG	T	G	0.277	0.323
		0.660	0.277	0.064	0.798	0.202		
g.5116G>T	47	GG	GT	TT	G	T	0.255	0.254
		0.723	0.255	0.021	0.851	0.149		
g.5177G>T	47	GG	GT	TT	G	T	0.362	0.499
		0.298	0.362	0.340	0.479	0.521		

N = total sample; Ho=observed heterozygosity; He=expected heterozygosity

g.4881A>G, g.4889G>A, g.5002C>T, and g.5075C>T. While the g.4843T>A, g.5111T>G, g.5116G>T, and g.5177G>T mutations indicated a transversion mutation. Transition mutations are changes in nucleotide substitution that have the same structure, such as substitution between purine with purine (A>G) or pyrimidine with pyrimidine (C>T). This transition mutation does not change hydrogen bonds, while a transversion mutation is a change in nucleotide substitution that has a different structure, ie. in purine to pyrimidine, or pyrimidine to purine (Luo et al. 2016). Transitions are more conservative in their effects on proteins than transversions that may cause a weaker effect of transitions on structure and function of gene regulation (Stoltzfus & Norris 2016). Figure 2 describes the AvBD2 gene sequence alignment in IPB-D1 chickens.

According to Table 1, there are 3 mutations located in exon 2, i.e g.4881A>G, g.4889G>A, and g.5002C>T. Both g.4881A>G and g.4889G>A located in the non-coding region, which are not translated into amino acids. Meanwhile, the g.5002C>T is located in the coding region. This g.5002C>T mutation also alters the amino acid Alanine to Valine. This missense mutation was also reported by Morammazi & Habibi (2017).

Nie et al. (2018) mentioned that Valine is one of 3 branched chain amino acids (BCAAs), including leucine (Leu) and isoleucine (Ile). These BCAAs play an important role in the regulation of energy homeostasis, nutrient metabolism, immunity and disease resistance in humans and animals (Nie et al. 2018). The non-synonymous mutation of the AvBD2 gene plays an important role in the antimicrobials found in different muscle tissues (Hellgren et al. 2010). This Alanine to Valine mutation needs to be further studied to understand its effect on disease resistance in chickens.

Avian beta defensin gene family has been reported to have strong association with chicken bacterial resistance. A total of 5 SNPs at the AvBD5 gene were reported to be associated with Salmonella susceptibility in China local chicken (Zhang et al. 2020). Using same chicken population, four SNPs at the AvBD14 gene were also associated with Salmonella susceptibility (Zhang et al. 2020). To our knowledge, no studies has been reported the association between the AvBD2 gene with bacterial resistance in chicken.

Polymorphism Information of the AVBD2 Gene

Table 2 shows a total of 10 SNPs found in this study, and each SNP has 2 alleles and 3 genotypes except g.4843T>A which has 2 genotypes (TT and AT) of 2 alleles (A and T). Individuals with genotype AA were not found at g.4843T>A. All alleles on each SNP had a frequency higher than 0.01 (1%), which indicated that all SNPs in the AVBD2 gene were polymorphic in the IPB-D1 chicken population. In accordance with the explanation of Nei & Kumar (2000) that a population with 2 or more alleles with a frequency value of more than 0.01 or 1% indicates that the gene is polymorphic. The highest allele frequency at g.4843T>A, g.4853G>A, g.4859T>C, g.4881A>G, g.102G> A, g.5002C>T, g.5111T>G, g.5116G>T, and g.5177G>T are the T, A, C, G, A, T, T, G and T alleles, respectively. Whereas at the g.5075C>T, both C and T alleles have the equal frequency (0.5).

Heterozygosity value calculations were carried out to determine how high the level of genetic diversity at a locus based on allele frequencies (Wang et al. 2015). All loci were found to have Ho values lower than 0.5, this indicated that all loci had a low level of diversity (Allendorf et al. 2013). Apart from being an indicator of the level of diversity, the comparison of Ho and He is used to indicate the occurrence of that mentioned by Allendorf et al. (2013). Of the ten loci found, 7 loci had lower Ho values than He, i.e g.4853G>A, g.4859T>C, g.4881A>G, g.5002C>T, g.5075C>T, g.5111T>G, and g.5177G>T. Ho value which is lower than He may indicate the occurrence of inbreeding in the IPB-D1 chicken population.

Previously, it was explained that the mutation of the nucleotide C to T in the missense mutation g.5002C>T in exon 2 is known to change the amino acid Alanine to Valine. Results of the amino acid sequence prediction showed that the T allele will produce the amino acid Valine which plays an important role in immunity and disease resistance. Based on Table 2, the T allele at the g.5002C>T locus has a much higher frequency (0.883) than the C locus (0.117). This indicates that most of the IPB-D1 chicken population tends to have good resistance. However, this proposal needs to be strengthened by further studies.

CONCLUSION

In this study, a total of 10 SNPs were found in the intron 1 to intron 2 of the AvBD2 gene in the IPB-D1 chicken population. All SNPs are polymorphic. A total of 7 SNPs were found in intron 1 (g.4843T>A, g.4853G>A, g.4859T>C) and intron 2 (g.5075C>T, g.5111T>G, g.5116G>T and g.5177G>T). While the other 3 SNPs were found in exon 2 (g.4881A>G, g.4889G>A, g.5002C>T). A missense mutation was found in g.5002C>T which changed the amino acid Ala to Val. This g.5002C>T has the potential as a candidate marker for disease resistance in IPB-D1 chickens.

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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.
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Proceeding:

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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.

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

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