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PREFACE

In this edition, Volume 27 No 4, we proudly present articles from animal and veterinary sciences including genetic, reproduction; animal physiology; and veterinary from scientist all over the world. The articles published in this edition are:
"Molecular Characterization and Gene Expression Of TLR4 Gene Associated with Mastitis in Goats"; "Morphological Characteristics Selection of Acid-tolerant *Leucaena leucocephala* Mutant to Addition of IBA Hormone (*Indole butyric acid*) in *Tissue Culture*"; "Impact of Aging on Sperm Quality of Sentul Roosters"; "Polymorphisms of the Thy-1 Gene in IPB D2 Chicken Line: Association with IgY and ND Antibody"; "Analysis of Meat Mineral Content in Cemani Chicken with Homozygous (*Fm/Fm*) and Heterozygous (*Fm/fm*$^{+}$) Genotypes" and "Household Consumer's Perception towards Frozen Beef".

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, December 2022

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LIST OF CONTENT

Molecular Characterization and Gene Expression of TLR4 Gene Associated with Mastitis in Goats

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ABSTRAK

Soquila SS, de Guia ACM, Medina NP, Mingala CN. 2022. Karakterisasi molekuler dan ekspresi gen TLR4 yang dihubungkan dengan penyakit mastitis pada kambing. JITV 27(4):159-169. DOI:http://dx.doi.org/1014334/jitv.v27i4364.

Pada penelitian ini dilakukan karakterisasi gen *Toll-like receptor* 4 (TLR4) pada kambing, pendeteksian polimorpis dalam nukelotida, penentuan hubungan identifikasi genotip dengan kemunculan penyakit mastitis subklinis menggunakan Chi-square dan rasio odds, serta penganalisaan ekspresi gen menggunakan Student's T-test dua sisi. Hasil kajian pertama menunjukan kemiripan yang tinggi (99%) pada sekuen nukleotida TLR4 semua rumpun kambing dengan sekuen *C. hircus* (NM_001285574.1) dan domba (*Ovis aries)*, tetapi memiliki kemiripan yang sedikit lebih rendah dengan sapi *(Bos taurus* dan *Bos indicus*) (96%) serta kerbau air (*Bubalus bubalis*) (95%). Pada kajian kedua, analisis polimorfisme panjang berkas restriksi (RFLP) menunjukan tiga genotip dengan sembilan pola restriksi menggunakan enzim AluI. Gen AA memiliki rasio odds secara berurutan 0,28 dan 0,08 pada semua rumpun dan Anglo-Nubian dengan hubungan yang signifikan (P<0,05). Hal itu mengartikan bahwa sebanyak 0,28 dan 0,08 kemungkinan lebih terjadinya mastitis subkilinis pada semua rumpun dan Anglo-Nubian dibandingkan dengan genotip lainnya. Genotip AB menunjukkan rasio odds secara berurutan 3,83; 13,00 dan 2,40 pada seluruh rumpun, Anglo-Nubian dan yang dimutakhirkan dengan hubungan yang signifikan. Hal ini mengindikasikan peluang paparan mastitis subklinis pada seluruh rumpun, Anglo-Nubian dan yang dimutakhirkan lebih tinggi secara berurutan sebesar 3,83; 13,00 dan 2,40 dibandingkan genotip lainnya. Pada kajian ketiga, ekspresi genetik menunjukkan upregulasi maksimum gen TLR4 adalah 3,63 kali lipat pada kambing tanpa mastitis subkilinis dibandingkan dengan hewan dengan mastitis subkilinis yang hanya 0,65 kali lipat. Hal ini menunjukkan peningkatan fungsi gen TKR4 dalam melindungi hewan dari kemungkinan infeksi.

Kata Kunci: Ekspersi Gen, Genotip, Kambing, Mastitis, PCR-RFLP, Gen TLR4

ABSTRACT

Soquila SS, de Guia ACM, Medina NP, Mingala CN. 2022. Molecular characterization and gene expression of TLR4 gene associated with mastitis in goats. JITV 27(4): 159-169. DOI:http://dx.doi.org/1014334/jitv.v27i4364.

In this study characterization of Toll-like receptor 4 (TLR4) gene of goats; detection of polymorphisms in the nucleotides, and determination of the association of identified genotypes with the occurrence of subclinical mastitis was done using chi-square and odds ratio. Analyzing gene expression using two-sided Student's T-test was also done. Results of Study 1 revealed high similarity (99%) of TLR4 nucleotide sequence of all breeds of goats with that of *C. hircus* (NM_001285574.1) and sheep (*Ovis aries*) sequences and slightly lower similarity with cattle *(Bos taurus,* and *Bos indicus*) (96%), and water buffalo (*Bubalus bubalis*) (95%). In Study 2, restriction fragment length polymorphism (RFLP) analysis revealed three genotypes with nine restriction patterns using AluI enzyme. Genotype AA has odds ratio of 0.28 and 0.08 in all breeds, and in Anglo-Nubian, respectively, with significant association (P<0.05) that inferred 0.28 and 0.08 times greater probability in all breeds, and in Anglo-Nubian, respectively, for subclinical mastitis to occur than those of other genotypes. Genotype AB showed odds ratio of 3.83, 13.00 and 2.40 in all breeds, in Anglo-Nubian, and in Upgraded, respectively, with significant association (P<0.05) that indicated 3.83, 13.00 and 2.40 times more likely in all breeds, in Anglo-Nubian, and in Upgraded, respectively, to suffer subclinical mastitis than those of other genotypes. In Study 3, genetic expression analysis showed a significant upregulation of TLR4 gene up to maximum of 3.63-fold in goats without subclinical mastitis compared to subclinically mastitic animals with only 0.65-fold which suggest a prompt role of TLR4 gene in the protection of animal against possible infection.

Key Words: Gene Expression, Genotype, Goats, Mastitis, PCR-RFLP, TLR4 Gene

INTRODUCTION

Breeding and production of goats (*Capra hircus*) in the Philippines suit most biophysical and socio-economic farming conditions of the agricultural sector. Goats have short gestation, short kidding intervals and lower capital requirements compared to cattle and carabaos. Farmers benefit from the incomes of and/or

consumption of goat's meat and milk (Monteiro et al. 2018). Among infectious diseases in dairy ruminants, mastitis is of major importance because of its high frequency and related costs (Rupp et al. 2014). Subclinical mastitis often goes unnoticed; hence it is left unattended unless it advances to clinical stage. Mastitic goats may often refuse to nourish their offspring because of udder pain. Such animals' milk has changed in chemical composition and physical features, making it unsuitable for processing due to its short shelf life and offflavors (Petlane et al. 2012). Intramammary infection (IMI) can be accurately predicted by somatic cell count (SCC) (Petzer et al. 2017). California Mastitis Test (CMT) is common indirect method of measuring SCC (Duarte et al. 2015) that provides practical means to identify inflammatory infections (Seligsohn et al. 2021) in the field.

Genes associated with immune response have been investigated for presence of single nucleotide polymorphism (SNP) and associations with mastitis related traits. Genes studied in bovine is Toll-like receptor 4 (TLR4). TLR4 gene plays key-role in innate immune system by recognizing components on the surface of various microbes that bind to specific pathogen-associated molecular patterns (PAMPs) that initiates signaling events leading to inflammatory responses and release of antimicrobial agents (Petlane et al. 2012).

Scant literature is available on molecular characterization and gene expression of TLR4 in goats. This study aimed to characterize and detect polymorphisms in TLR4 gene, determine the genotypes associated with subclinical mastitis and assess the expression of TLR4 gene in goats.

MATERIALS AND METHODS

Characterization of TLR4 gene in goats

A total of 99 available goats (17 Native, 33 Upgraded, 29 Anglo-Nubian, and 20 Saanen) were included in the study. All goats were derived from goat farms in Luzon. Goat milk and blood samples were collected. For lactating goats, ribonucleic acid **(**RNA) samples were extracted from milk of animals starting on their 2nd week of lactation and above. This prevented collecting high somatic cell count that normally occurred on the first week of lactation. For non-lactating Native and Upgraded crossbred goats, RNA was extracted from blood. Results showed that genotypes found using deoxyribonucleic acid (DNA) from blood matched those obtained from milk.

RNA extraction from milk and blood samples was done by following the Promega® protocol with some modifications; while RT-PCR kit (TaKaRa ™) was used to synthesize complementary DNA (cDNA) from the extracted RNA samples. Primers 1. Forward: 5'-AGACAGAGGGTCATGCTTT-3' Reverse: 5'-CTGTAAACTTGATAGCCCAGA-3' (440bp), 2. Forward: 5'-ATGCGAAATTAAGATTATTGAAG-3' Reverse: 5'-TACTGAAGGCTTGGTAGCTC-3' (870bp), 3. Forward: 5'-CTCACGGAACGATACAGACT-3' Reverse: 5'-ATGTTCACAAACACAAGCAA-3' (874bp), 4. Forward: 5'-GCAGTTTCAACCGTATCAC-3' Reverse: 5'-GGATTCTCCTCCTCAGGT-3' (887bp) were designed using Primer3 server (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) based on the caprine sequence of TLR4 (NM_001285574.1) mRNA from National Center for Biotechnology Information (NCBI) GenBank. Designed primers were analyzed for self-annealing and loop properties using Oligo analyzer software (https://sg.idtdna.com/calc/analyzer). Primer-BLAST server (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was also used to ensure that each set of primer amplifies specific gene segment that are targeted.

Gene amplification

PCR was performed using a thermocycler (SimpliAmp, Thermofisher). The 20µL reaction volume used for all PCR tests contained 2µL of genomic DNA template, 10 pmol of each primer, and PCR master mix. Amplification cycles were carried out in thermocycler optimized for this study: initial denaturation 94ºC for 10 min, denaturation 94ºC for 1 min, annealing for 1 min: Primer 1, 55ºC (35 cycles); Primer 2, 56ºC (37 cycles); Primers 3 and 4, 57ºC (38 cycles), extension 72ºC for 1 min and final extension 72ºC for 10 min.

After amplification, 1µL of PCR product was electrophoresed in 2 % agarose gel containing 1X TAE buffer at 100 volts for 30 minutes (MYGEL™ mini, Accuris Instruments) and visualized under ultraviolet light using UV transillumination advance imaging system (FlourChem E by ProteinSimple ™). To ensure that amplification products are of the expected size, a 1kb plus DNA ladder was run simultaneously as marker.

Gene sequencing and analysis

TLR4 PCR products were submitted for sequencing. Sequences were assembled using MEGA 7.1 software (Kumar et al. 2016). Each primer's forward and reverse sequences were put together to create contigs. Gene sequences were compared with the TLR4 coding DNA sequence (CDS) of *Capra hircus* (NM_001285574.1), *Ovis aries* (NM_001135930.1), *Bos taurus* (NM_174198.6), *Bos indicus* (KX138607.1), and *Bubalus bubalis* (NM_001290903.1) from the database found in NCBI GenBank.

The DNA nucleotides were conceptually translated using Molecular Evolutionary Genetic Analysis (MEGA) 7.1 software and again compared with database found in NCBI Genbank for caprine TLR4 to detect amino acid changes. Contiguous TLR4 nucleotide sequences were subjected to Basic Local Alignment Search Tool (BLAST) to determine sequence similarity with corresponding regions of other ruminant species. Phylogenetic tree was constructed using the Maximum Likelihood method. Confidence in the groups was estimated by bootstrap of data using 1000 replications. Genetic distances and phylogenetic trees were derived using MEGA 7.1 software. Functional domains of the gene were predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services /SignalP) (Petersen et al. 2011) and Simple Modular ArDZchitecture Research Tool (SMART) server (http://smart.embl-heidelberg.de/) (Letunic & Bork, 2018).

Polymorphism in TLR4 gene in goats

Polymorphism analysis

Amplified TLR4 PCR products (15µL) from Study 1 were subjected to endonuclease digestion. New set of primers were designed to amplify ligand-binding region in exon 3 of TLR4; forward primer: 5'-GTATTCAAGGTCTGGCTGGTT-3' and reverse primer: 5'-GTCATTGAAGCTCAGATCTAAAT-3'.

Restriction enzymes that can cut the fragments were identified using Sequence Manipulation Suite (SMS): Restriction Map, and Restriction Digest (http://www.bioinformatics.org) (Stothard 2000). AluI enzyme (AG/CT) was selected to cut segments of 494 bp ligand-binding region of TLR4 at loci 312, 378, 431and 485. Restriction-digested gene fragments were visualized on UV transilluminator (FlourChem E by ProteinSimple ™) and photographed. Differences in fragment length yielded by the various restriction enzymes that would indicate polymorphism in a particular gene were analyzed and compared.

Association of polymorphism of* TLR4 *gene in goats and the occurrence of subclinical mastitis

A total of seventy-six (76) available lactating does were identified from farms mentioned in Study 1. There were 67 samples used for the association of occurrence of subclinical mastitis. Milk was collected manually for CMT scoring and for SCC evaluation using PortaSCC®. CMT scores for all animal subjects were classified as Non-subclinically mastitic if CMT score result was 1 or lower and subclinically mastitic if CMT score result was 2 or higher. Raw milk samples from the animal with CMT scores of 1 or higher were subjected for somatic cell count determination using the Porta SCC® goat milk test. Results were categorized as non-subclinically mastitic if SCC<1,500,000 cells/mL and subclinically mastitic if SCC ≥1,500,000 cells/mL.

PCR and RFLP

RNA was extracted from milk samples and converted to cDNA and determination of polymorphism of TLR4 gene using RFLP was done. Since TLR4 coding region has 2664 bp, certain regions with high polymorphism were identified for RFLP analysis. Similar to Study 2, sequence covering 494 bp by the new TLR4 Primer was amplified and subjected for RFLP. Genotype frequency of fragments/alleles was identified through direct counting.

Statistical analysis

Univariate analysis on possible association between genotypic frequency and occurrence of subclinical mastitis was examined using Chi-square (X2) test for goodness-of-fit (Petrie & Watson, 2006). Odds ratio were also computed in Microsoft Excel 2013 to determine the strength of association.

Determination of Genetic Expression of TLR4 Gene in Goats with and without subclinical mastitis

Quantitative gene expression analysis

Real time PCR for TLR4 gene was performed in an ABI 7500 sequence detection system with SYBR green PCR master mix (Applied Biosystems, CA). The 10µL reaction mixture for RT-PCR consisted of 1µL cDNA, 0.15µL each of the forward (upstream) primer: 5'-AATGCCCCTACTCAACCT-3' and reverse (downstream) primer: 5'-CTTCGCAGAGTCAATCCA-3' (10nmol/L), 5µL SYBR Green Realtime PCR master mix (2x) and 3.7µL of ddH2O using glyceraldehyde phosphate dehydrogenase (GAPDH) as an endogenous control. The cDNA template (10µL) was used for each gene quantification after sequential dilution in 10 folds. Real-time PCR was run using the diluted samples as gradient and template.

The RT-PCR amplification was performed at 95ºC for 5 mins initial denaturation and 40 cycles denaturation at 95ºC for 15 secs, followed by annealing for 45 secs with 57ºC for TLR4 with reaction efficiency of 90-100, quantification cycle (Cq) value of 30.86 and correlation coefficient (R2) value of 0.998 while GAPDH has annealing temperature of 65ºC with reaction efficiency of 90-100, Cq value of 29.36 and R2

value of 0.996. Melt curve stage of 95ºC for 15 secs followed by 65ºC for 30 secs and extension at 95ºC for 15 secs.

Statistical analysis

Significant differences in TLR4 gene expression between animals with and without subclinical mastitis were compared using a two-sided Student's T-test, Comparative C_T values methods (2 ddCt) were computed based on their mean Ct values with reference to those in non-mastitic counterparts.

RESULTS AND DISCUSSION

Characterization of TLR4 gene in goats

Sequence analysis of TLR4

There were 18 samples that produced good sequences for goat TLR4 gene; five contigs completed in Native, nine in Upgraded crossbreds, two in Anglo-Nubian, and two in Saanen. Native goat TLR4 nucleotide sequence resulted to average of 2603 bp, while there were 2597 bp in Upgraded, 2589 bp in Anglo-Nubian and 2645 bp in Saanen.

Goat TLR4 nucleotide sequences were aligned with other ruminant's TLR4 CDS using database from the NCBI GenBank. Statistical nucleotide pair frequency of the 23 aligned nucleotide sequences showed average of 2452 identical pairs, 61 transitional pairs and 61 transversional pairs, with ratio of 1.0. Between different goat breeds' nucleotide pair frequencies, average identical pairs were 2468 with 48 transitional pairs and 61 transversional pairs, with ratio of 0.8. This finding showed that there was high similarity of nucleotide sequences between breeds of goats studied.

Nucleotide BLAST (BLASTn) of TLR4 CDS of the representative goat sample breeds revealed high degree of similarity between the query (sample) sequence and other ruminants' TLR4 nucleotide sequences available in the NCBI GenBank database (Table 1). Native, Upgraded, Anglo-Nubian and Saanen TLR4 nucleotide sequences had 99% similarity with that of *C. hircus* (NM_001285574.1) and *O. aries* (NM_001135930.1). Lower similarity was seen on *B. taurus* (NM_174198.6), *B. indicus* (KX138607.1) and *B. bubalis* (NM_001290903.1) with only 96%, 96%, and 95%, respectively.

Comparatively, protein BLAST (BLASTp) results showed high percentage similarity (99%) of translated amino acid sequence of Native and Upgraded goats to *C. hircus* (NP_001272503.1) sequence from the NCBI GenBank. Lower similarity was observed (98%) on Anglo-Nubian, while Saanen had the lowest similarity with *C. hircus* (NP_001272503.1) (93%). Lower similarity of Native and Upgraded amino acid sequences were also observed on other ruminant species: *O. aries* (NP_001129402.1), *B. taurus* (NP_776623.5), *B. indicus* (ADL28421.1), and *B. bubalis* (NP_001277832.1), with 98%, 94%, 94% and 93% similarity, respectively. Anglo-Nubian had 98%, 96%, 96% and 96% similarity with other ruminant species *O. aries* (NP_001129402.1), *B. taurus* (NP_776623.5), *B. indicus* (ADL28421.1), and *B. bubalis* (NP_001277832.1) respectively. Again, Saanen breed had the lowest similarity of amino acid sequence compared to *O. aries* (NP_001129402.1), *B. taurus* (NP_776623.5), *B. indicus* (ADL28421.1), and *B. bubalis* (NP_001277832.1) with 92%, 89%, 89% and 87% similarity, respectively.

Result showed that even if nucleotide and amino acid sequences were from various species of ruminants and from different locations, there was still high similarity (99%) of the nucleotide and amino acid sequences of TLR4 gene especially in goats. This is in consonance with the study of Goyal et al. (2012), wherein goat TLRs have 61 to 99% similarity with other mammals. TLR sequences are highly conserved and have common evolutionary ancestor. Furthermore, functional domains are conserved due to constraints imposed by necessity to recognize PAMPs that are present in parasitic, bacterial or viral germs. This recognition process is translated rapidly into a meaningful defense reaction (Karaś et al. 2019).

Phylogenetic analysis of TLR4 nucleotide sequence

Maximum Likelihood algorithm with 1000 bootstrap resampling revealed clustering of *C. hircus* (NM_001285574.1) with TLR4 nucleotide sequence of Upgraded (NxA)_98, Native_4 and Native_2 and with 36 bootstrap value (Figure 1). Native_1, Upgraded (NxU)_11, Upgraded (NxA)_97, Native_3, Upgraded (NxU)_15 and Native_5 clade together with 42 bootstrap value. TLR4 nucleotide sequence of Upgraded(NxS)_102 clade with Upgraded (NxS)_101 with 33 bootstrap value and then clade with Upgraded(NxU)_12, Saanen_49, Upgraded(NxA)_99, Saanen_39, Anglo-Nubian_40, Upgraded(NxA)_100 and Anglo-Nubian_41 clade together with 32 bootstrap value. *O. aries* (NM_001135930.1), *B. bubalis* (NM_001290903.1), *B. indicus* (KX138607.1), *B. taurus* (NM_174198.6) TLR4 sequence on the other hand, separated from the clade of goats with 39 bootstrap value.

Predicted functional domains of TLR4 nucleotide sequence

Native_1 goat TLR4 sequence was used as representative animal of this study; the 2603 bp

Table 1. Nucleotide percentage similarity of TLR4 gene in ruminant species with reference to native, upgraded, Anglo-Nubian and Saanen goats

Species	Nucleotide Sequence			
	Native	Upgraded	Anglo-Nubian	Saanen
Capra hircus (NM_001285574.1)	99%	99%	99%	99%
Ovis aries (NM_001135930.1)	99%	99%	99%	99%
Bos Taurus (NM_174198.6)	96%	96%	96%	96%
Bos indicus (KX138607.1)	96%	96%	96%	96%
Bubalus bubalis (NM_001290903.1)	95%	95%	95%	95%

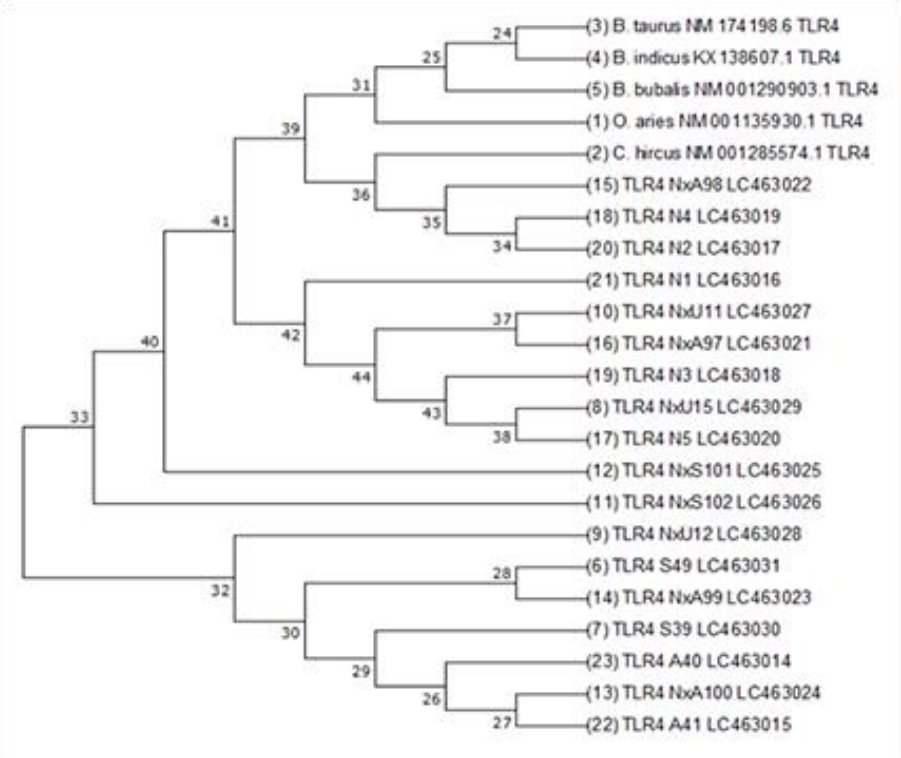

Figure 1. Phylogenetic tree showing relationship between goats and other ruminants' TLR4 nucleotide sequence

nucleotide sequence has corresponding 867 amino acid translation provided by MEGA 7.1 software. Using SignalP 4.1 server, cleavage site was found to be between positions 25 and 26 amino acid location starting from amino acid methionine (http://www.cbs.dtu.dk/services/SignalP). Then, using SMART server, the functional domains were located. Extracellular domains of leucine-rich repeats (LRR) were located starting from amino acid location 95 to 584, followed by single LRRCT (LRR at C-terminal) at positions 597 to 647. Transmembrane protein started at position 653 and ended at 675, followed by globular cytoplasmic domain called the Toll/interleukin 1 receptor (TIR domain) at positions 692 to 837 (http://smart.embl-heidelberg.de). There were typically 13 LRRs present, single globular LRRCT, followed by transmembrane protein and TIR domain. These domains were homologous between breeds of goats, as well as sheep, cattle and water buffalo.

Characterization of TLR4 sequence had elucidated critical regions that were involved in function of the gene. TLR4's function was to bind with specific molecular patterns from pathogens, after which triggered a cascade of events that led to production of

cytokines that initiate further immune response (Mukherjee et al. 2016). This study showed structural domains predicted from translated amino acid. One of these domains was LRR, which contained 20-30 amino acid residues. LRRs are important structural framework for the formation of protein-protein interactions. Proteins containing LRRs included tyrosine-protein kinase receptors, cell adhesion molecules, and extracellular matrix-binding glycoproteins that were involved in variety of biological processes, including transduction, cell adhesion, disease resistance, apoptosis and immune response (Poddar & Banerjee 2020). Result showed 13 LRRs in goat which were similar to that of *O. aries* (NP_001129402.1), while in *B. taurus* (NP_776623.5), *B. indicus* (ADL28421.1), and *B. bubalis* (NP_001277832.1) there is the presence of LRR_TYP (LRR-typical) motif. Dubey et al. (2012) reported that possible variation of LRR motif distribution can occur in various species associated to different diseases with around 21-22 LRRs in buffalo, cattle, sheep and goat.

Polymorphism of TLR4 gene in goats (Native, Upgraded, Anglo-Nubian, and Saanen)

Polymorphism analysis for* TLR4 *gene

From the 2664 bp nucleotide sequence, targeted ligand-binding domain has PCR product of 494 bp that was subjected to restriction enzyme digestion. One heterozygous allele was seen at locus 377 that is part of the ligand-binding region in 494 bp segment. Allele in locus 377 is same as locus 1221, if the start of counting of the nucleotide letters began in exon 1 of the whole CDS of TLR4 mRNA. This polymorphism can either be G or C nucleotide, which can result to either glutamic acid or aspartic acid substitution.

Restriction enzyme AluI that can cut AG/CT nucleotide sequence was selected to examine restriction fragment pattern in the nucleotide sequence of TLR4 gene. Based on SMS: Restriction Digest (http://www.bioinformatics.org) application of the 18 TLR4 complete nucleotide sequence in Study 1, AluI can produce three patterns at nucleotide sequence of 494 bp amplicon. Restriction fragment pattern a, three cuts produced products of 311 bp (1-311), 66 bp (312-377), 107 bp (378-484), and 10 bp (485-494) amplicon sizes; restriction pattern b, four cuts produced products of 311 bp, 66 bp, 53 bp (378-430), 54 bp (431-484) and 10 bp amplicon sizes; and restriction pattern c, three cuts produced products of 311, 119 (312-430), 54 and 10 amplicon sizes. This digestion pattern recognized polymorphism at loci 311, 377, 430, 484 and differentiated animals with allele A or B which can detect amino acid change in locus 377 in the 494 bp amplicon. Thus, restriction digest result fragments with 107 bp will indicate allele B, while absence of cut fragment will indicate allele A.

Figure 2 shows result of digestion of TLR4 gene PCR products using AluI enzyme. From the conceptualized three restriction patterns from nucleotide sequence, nine patterns were observed. Uncut 494 bp, 377 bp (merged of 311 and 66 bp) and 173 bp (merged of 107 and 66 bp) were obtained in addition to conceptualized fragments.

Polymorphism results of this study in ligand-binding region is in consonance with the study of (Singh et al. 2015) using AluI enzyme in PCR-RFLP procedure where polymorphisms in TLR4 gene had been found in Beetal breed but monomorphism in Jamunapari and Black Bengal goats. Furthermore, polymorphism in same domain was also observed by Zhou et al. (2008), who identified five allelic variations in the ligand binding region using PCR-SSCP.

Association of TLR4 Gene in the Occurrence of Subclinical Mastitis in Goats

Detection of subclinical mastitis

A total of 108 milk samples were categorized as non-subclinically mastitic or subclinically mastitic based on CMT scores and PortaSCC® kit results. Non-subclinical mastitis was considered, if CMT score result was 1 or lower, while subclinical mastitis was accounted, if CMT score result was 2 or higher. While in PortaSCC®, results were categorized as non-subclinically mastitic, if SCC<1,500,000 cells/mL and subclinically mastitic, if SCC≥1,500,000 cells/mL.

Association of* TLR4 *gene in occurrence of subclinical mastitis

The 494 bp putative ligand-binding domain described in Study 2 was targeted in this segment of the study. Table 2 shows frequency of TLR4 genotypes in non-subclinically mastitic and subclinically mastitic goats. From the total of 67 milk samples tested using AluI enzyme, 30 was considered subclinically mastitic, while 37 was non-subclinically mastitic. Overall frequency of genotype AA was 17, while genotype AB and BB were 35 and 15, respectively. Frequency of genotype AA in mastitic cases was 4 and genotypes AB and BB were 21 and 5, respectively; while frequency of genotype AA in non-mastitic cases was 13 and genotypes AB and BB were 14, and 10, respectively.

Genotype AA was found to be more frequent in non-subclinically mastitic compared to subclinically mastitic animals. Frequency for occurrence of subclinical mastitis in genotype AA was 4 compared to 13 for non-occurrence of subclinical mastitis. Odds

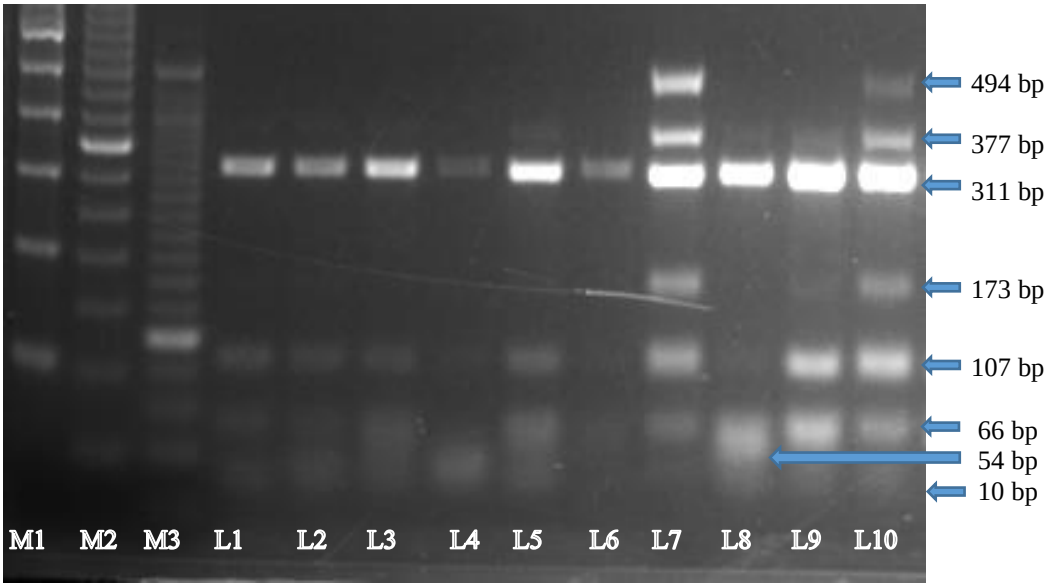

Figure 2. Restriction patterns obtained by digestion of TLR4 gene PCR products using AluI in 2% agarose gel. M1-100bp, M2-50bp, M3-25bp ladders; Lanes 1, 2, 3, 5, 9 –pattern a (fragment size: 311, 107, 66), Lanes 4, 6, 8–pattern b (fragment size: 311, 10 not distinguishable), Lanes 7, 10 – uncut and merged cutting pattern of 494 bp amplicon (fragment size 494, 377 (311+66), 311, 173 (107+66), 107, 66, 10)

Table 2. Frequency of TLR4 AluI-based genotypes in goats with or without SCM

Genotype	Category of Animal		Total	Odds Ratio	95% CI
	Non-SCM	SCM			
AA	13	4	17	0.28*	0.81 to 0.99
AB	14	21	35	3.83*	1.37 to 10.69
BB	10	5	15	0.54	0.16 to 1.80

*Significant association was found, P<0.05

Table 3. Frequency of TLR4 AluI-based genotypes in Anglo-Nubian goats with or without SCM

Genotype	Category of Animal		Total	Odds Ratio	95% CI
	Non-SCM	SCM			
AA	6	2	8	0.08*	0.01 to 0.58
AB	3	13	16	13.0*	1.70 to 99.38

*Significant association has been found P<0.05

Table 4. Frequency of TLR4 AluI-based genotypes in Upgraded goats with or without SCM

Genotype	Category of Animal		Total	Odds Ratio	95% CI
	Non-SCM	SCM			
AA	5	1	6	0.32	0.03 to 3.60
AB	1	4	5	2.40*	1.69 to 341.01
BB	7	1	8	0.17	0.02 to 1.91

*Significant association has been found P<0.05

ratio result in goats with genotype AA was 0.28 with 95% confidence interval (CI) range of 0.81 (lower limit) to 0.99 (upper limit). Significant association was found. This means there was 0.28 times more likely that subclinical mastitis would occur, if the goat had genotype AA than those of other genotypes.

Frequency of subclinically mastitic animals in genotype AB was 21 compared to 14 in non-subclinically mastitic. Odds ratio analysis shows significant association for genotype AB, which was 3.83, thus there were 3.83 times more likely that subclinical mastitis would occur, if the animal had genotype AB than those of other genotypes. Genotype BB had frequency of 5 in subclinically mastitic compared to 10 in non-subclinically mastitic goats. Genotype BB had odds ratio of 0.54 in the occurrence of subclinical mastitis, but no significant association was found.

The frequency of TLR4 AluI-based genotype of Anglo-Nubian breed is presented in Table 3. The frequency of genotype AA with regard to occurrence of subclinical mastitis was 2 and non-occurrence was 6. Odds ratio for occurrence of subclinical mastitis in genotype AA is 0.08, thus, in Anglo-Nubian breed there is 0.08 times more likely that subclinical mastitis would occur, if the goat had genotype AA than with genotype AB. In genotype AB, frequency of occurrence of subclinical mastitis is 13 compared to 3 in non-occurrence. Odds ratio for genotype AB is 13.0 with significant association. Thus, there were 13.0 times more likely that in Anglo-Nubian breed, animal will suffer subclinical mastitis if it has genotype AB than those of genotype AA.

In Table 4, frequency of genotype AB with regard to occurrence of subclinical mastitis in Upgraded goats is 4 compared to 1 in the non-occurrence of subclinical mastitis. Odds ratio result for genotype AB is 2.40 with significant association. Therefore, there were 2.40 times more likely that in Upgraded goats, animal will suffer subclinical mastitis, if it has genotype AB than those of other genotypes. On the other hand, genotypes AA and BB had odds ratio of 0.32 and 0.17, respectively in the probability of occurrence of subclinical mastitis, but no significant association was found.

Frequency of TLR4 AluI-based genotype of Saanen and Native goats are not presented since there was no significant association found. This study in TLR4 using AluI enzyme showed a significant association using odds ratio. Analysis results in the frequency of occurrence or non-occurrence of subclinical mastitis in genotype AA in all breeds of goats and in Anglo-Nubian breed showed odds ratio of 0.28, and 0.08, respectively. Therefore, in all breeds of goats under study, and in Anglo-Nubian breed, likelihood of the animal with genotype AA to suffer from subclinical mastitis is 0.28 times greater in all breeds, and 0.08 times greater in Anglo-Nubian breed, respectively than those of other genotypes. On the other hand, genotype AB showed odds ratio results of 3.83, 13.0 and 2.40 in all breeds of goats, Anglo-Nubian, and Upgraded goats, respectively. This would indicate that in all breeds of goats, there is 3.83 times more likely that they will suffer subclinical mastitis; 13.0 times more probability in Anglo-Nubian breed, and 2.40 times more chances in Upgraded goats to suffer subclinical mastitis if they have genotype AB than those of other genotypes.

These results are in consonance with studies of Gupta et al. (2015) wherein allele A was associated with mastitis resistance, while allele B was associated with mastitis susceptibility in cattle. Although, their study was directed to T4CRBR2, which is part of exon 3, their findings reveal that polymorphism in T4CRBR2 induced C-T SNP at nucleotide 1,397 in exon 3 that led to disease resistance (Novák 2013). In addition, the study of (Gulhane and Sangwan, 2012) in water buffaloes using StyI enzyme showed that allele A was significantly more frequent in group of healthy buffaloes, thus, it was associated with resistance to mastitis. The said allele was found to have nucleotide substitution of cytosine to guanine at 217 nucleotide position in allele B, causing substitution of amino acid arginine by threonine.

In this study, polymorphism in locus 377 of the 494 bp segment of ligand-binding region can be recognized by AluI enzyme. Allele A can be identified by nucleotide substitution from G to C showing no cut in the fragment that indicates amino acid substitution of glutamic acid by aspartic acid.

Determination of genetic expression of **TLR4** *gene in goats with and without subclinical mastitis*

The recurring nature of subclinical mastitis has led to interest on genetic resistance to subclinical mastitis in dairy animals. Though the cause of the condition is multifactorial, resistance still rely on the ability of the immune system to combat the reoccurring infection. Vital immune effectors responsible for early detection and capture of the infectious agent is the TLR4.

Expression analysis of TLR4 gene in lactating goats were performed to give an idea on how these receptors are modulated by the body to confer resistance to infection such as sub-clinical mastitis. The optimized RT-qPCR assay was used to quantify the expression of TLR4 in goats. Figure 3 shows the optimized TLR4 relative expression analysis. Quantification cycles (Qc) of different samples and the gene amplification was validated by its characteristic melting curves showing only one peak.

The relative quantification of mRNA transcript of TLR4 in goat had been evaluated. The fold change is

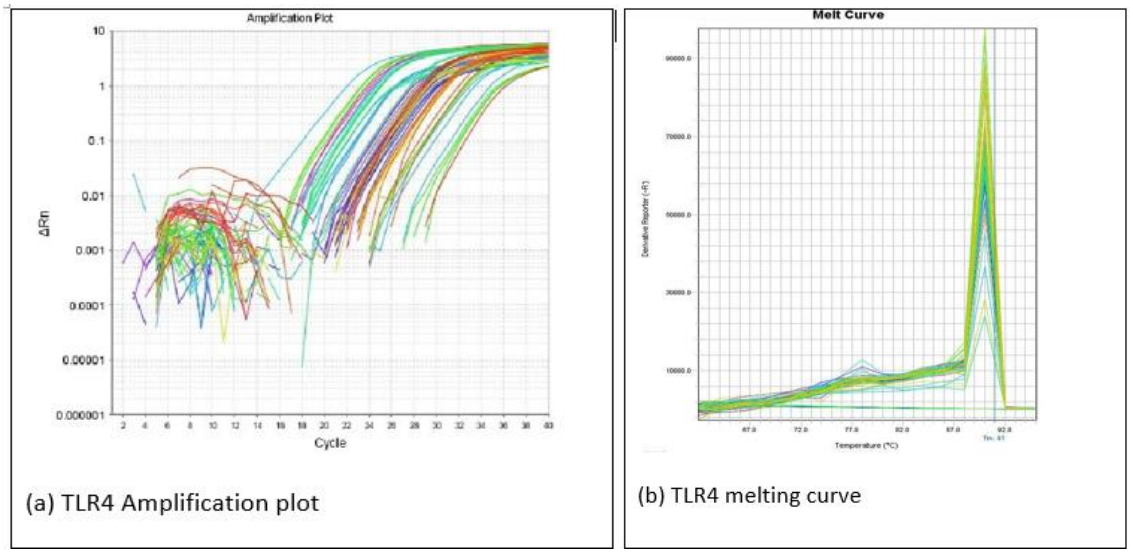

Figure 3. Amplification plot (a) and melt curve (b) of TLR4

the ratio of the normalized mean expression between the positive and negative with subclinical mastitis group. Results showed that TLR4 expression in non-subclinically mastitic goat was 1.40±2.22 fold or maximum expression of 3.63-fold. This was higher to the 0.26±0.39-fold or a maximum of 0.65-fold expression in subclinically mastitic animals. Statistical analysis showed significant difference with P-value of 0.04 ($p<0.05$) between the expression of TLR4 in animals with and without SCM.

This significant result suggests that increase in the expression of the TLR4 gene increased the protective function of the immunologic protein. TLR4 is a part of innate immune response. It is present in all cells and it enables recognition of foreign bodies (Takumi and Taro, 2014). Upon binding of specific lipopolysaccharides of microorganism, it results to a cascade of immune-regulatory process that enables capture and phagocytosis of microorganism. Increase expression of TLR4 protein in non-SCM animal could be attributed to the prompt protection from TLR4 (Vaure & Liu 2014).

The upregulation of the expression of the TLR4 can be explained by the stimulation of recognition of specific pathogen molecules present in bacterial agents. Lipopolysaccharide (LPS) from the cell walls of gram-negative organism are activators of TLR4 activity. TLR4 are receptors present on the surface of tissue macrophages, mast cells and dendritic cells that specifically stimulated by specific pathogen molecular patterns. Upon recognition of specific pathogen through TLR4, macrophages or mast cells would increase the synthesis and secretion of pro-inflammatory cytokines and lipid mediators initiating the inflammatory response through cytokines and chemokine expression (Takumi & Taro 2014; Kany et al. 2019). TLR stimulation of dendritic cells induces the initiation of adaptive immune response (Martin-Gayo & Yu, 2019; Takagi et al., 2016). Studies have shown the significant increase of gene expression of TLR4 in dairy herd with mastitis (Panigrahi et al. 2014).

In this study, the increase in TLR4 expression seen in goats could be attributed by the possible protection induced by the immunoreceptor against causative agent of subclinical mastitis. TLR4 expression was expected to increase in animals with infection ascribing to its role to stimulate host defense by mediating cytokine production and initiate adaptive immunity (Wakchaure et al. 2012). However, according to Zhuang et al. (2020), TLR4 expression is also dependent on the organism present. He observed a significantly lower TLR4 mRNA level in *Staphylococcus aureus* infected primary mammary epithelial cells compared to *E coli* infected cells.

One specific pathogen molecule recognized and that stimulates TLR4 is lipopolysaccharide. Lipopolysaccharide upregulation of TLR4 expression which have been seen in some animals may not be the same to other animals. Vaure and Liu, (2014) explained the phenomenon of LPS tolerance due loss of surface TLR4 expression and rapid induction of the negative feedback regulators of TLR4 pathway. Decrease in TLR4 stimulation could lead to delay in influx of polymorphonuclear neutrophils into the mammary gland after intra-mammary infection. This can result to decrease TLR4 function consequently leading to disease condition (Vangroenweghe et al. 2005)

CONCLUSION

Nucleotide sequences of Native, Upgraded, Anglo-Nubian and Saanen goats had high similarity to *C. hircus* and *O. aries* TLR4 gene sequences from NCBI GenBank. Nucleotide and amino acid sequences of

other ruminants such as cattle and water buffalo had lower similarity to goat nucleotide and amino acid sequences. Ligand-binding domain of TLR4 was highly polymorphic and polymorphisms were identified using restriction enzyme AluI. Significant association had been found in Genotype AA in TLR4 with low odds ratio values in all breeds, and in Anglo-Nubian; while genotype AB had high odds ratio values in all breeds, Anglo-Nubian and Upgraded crossbreds. Expression analysis showed an upregulation of TLR4 in goats without SCM, which suggest a prompt role in the protection of animal against possible infection. TLR4 are receptor proteins that are present in cells specially the macrophages, as part of innate mechanism of immunity, they can be swift in recognizing impending infection.

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Morphological Characteristics Selection of Acid-tolerant *Leucaena leucocephala* Mutant to Addition of IBA Hormone (Indole Butyric Acid) in Tissue Culture

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ABSTRAK

Karti PDMH, Muhklisani, Prihantoro I. Seleksi Karakteristik morfologi mutan *Leucaena leucocephala* toleran asam terhadap penambahan hormon IBA (*Indole butyric acid*) pada kultur jaringan. JITV 27(4):170-176. DOI: http://dx.doi.org/10.14334/jitv v27i4.2960.

Leucaena leucocephala merupakan tanaman pakan jenis leguminosa yang memiliki kandungan protein yang tinggi. Kultur jaringan merupakan salah satu teknik yang dapat digunakan untuk menseleksi mutan tanaman pakan ternak secara in vitro. IBA (*Indole butyric acid*) merupakan salah satu jenis auksin yang dapat menginduksi perakaran dan pertumbuhan pada tanaman. Penelitian ini bertujuan untuk seleksi karakteristik morfologi mutan *Leucaena leucocephala* toleran asam terhadap penambahan hormon pengakaran IBA pada kultur jaringan. Rancangan yang digunakan dalam penelitian ini adalah rancangan acak lengap (RAL) dengan eksplan tanaman lamtoro sebanyak 11 galur mutan toleran asam pH 3,4 hasil irradiasi sinar gamma 400 gy yang telah di berikan perlakuan pada media asam Al^{3+} 300 ppm yaitu galur K1-K11 (mutan+MS+1ppm IBA) dan 2 lamtoro indukan tanpa penyinaran gamma yaitu P0 (lamtoro indukan+MS+0 ppm IBA), P1 (lamtoro indukan+MS+1 ppm IBA), masing masing 15 ulangan, perlakuan yang berpengaruh nyata dilanjutkan dengan uji Tukey. Variabel yang diamati adalah pertambahan panjang akar, tinggi vertikal tanaman, jumlah tunas, persentase tanaman berakar. Peningkatan panjang akar dan peningkatan tinggi vertikal tanaman menunjukkan hasil terbaik pada strain mutan K10, jumlah tunas menunjukkan hasil terbaik pada strain mutan K9 dan K11 dan persentase tanaman berakar tertinggi pada strain mutan K3 dan K11. Penambahan IBA dapat meningkatkan karateritik morfologi mutan *Leucaena leucocephala*.

Kata Kunci: IBA, Leucaena leucocephala, mutan, kultur jaringan

ABSTRACT

Karti PDMH, Muhklisani, Prihantoro I. Morphological characteristics selection of acid-tolerant *Leucaena leucocephala* mutant to addition of IBA hormone (*indole butyric acid*) in *tissue culture*. JITV 27(4):170-176. DOI: http://dx.doi.org/10.14334/jitv v27i4.2960.

Leucaena leucocephala is a legume forage plant that has a high protein content. Tissue culture is a technique that can be used to select mutants for forage plants in vitro. IBA (*Indole butyric acid*) is one type of auxin that can induce rooting and growth in plants. This study aimed to select the morphological characteristics of the acid-tolerant *Leucaena leucocephala* mutant to the addition of the hormone IBA in tissue culture. The design used in this study was a completely randomized design (CRD) with lamtoro plant explants as many as 11 acid-tolerant mutant lines pH 3.4 resulting from 400 gy irradiation which had been treated on 300 ppm Al^{3+} acid media, namely the K1-K11 strain (mutant+MS+1ppm IBA) and 2 parent trees *Leucaena leucocephala* without gamma irradiation, namely P0 as *Leucaena leucocephala* parent+MS+0ppm IBA, P1 as *Leucaena leucocephala* parent + MS + 1 ppm IBA with 15 replicates. The treatment which had a significant effect was continued with the test Tukey. Variables observed were an increase in root length, plant vertical height, number of shoots, and percentage of rooted plants. The increase in root length and increase in plant vertical height showed the best results on the K10 mutant strain, the number of shoots showed the best results on the K9 and K11 mutant strains, and the highest percentage of rooted plants on the K3 and K11 mutant strains. The addition of IBA can increase the morphological characteristics of the *Leucaena leucocephala* mutant.

Key Words: IBA, *Leucaena leucocephala*, Mutant, Tissue Culture

INTRODUCTION

Forage crops are needed by livestock with a fairly high portion. In ruminants, ration consumption covers about 40-80% of the total dry matter of the ration or about 1.5% to 3% of the live weight of livestock (Abdullah et al. 2005). The nutritional content of fodder crops has its proportions and advantages (Geng et al. 2020). Among the animal feed groups, namely Gramineae and legumes, the legume group has a fairly

high nutritional content. Based on its nutritional content, this plant is a source of protein, fiber, and good mineral supplementation for livestock productivity. One of the tree legume varieties is *Leucaena leucocephala*, with a high protein quality of around 15% to up to 38% (Zayed et al. 2014; De Angelis et al. 2021).

The development of plant biotechnology for *Leucaena leucocephala* with tissue culture techniques (in vitro propagation) can maximize the supply of forage seeds that are uniform and have high productivity compared to conventional plant cultivation after the plant already has been selected. Tissue culture techniques can produce plant seeds in large quantities under controlled conditions, and the time required is relatively fast (Loyola-Vargas & Ochoa-Alejo 2018). Lamtoro (*L. leucocephala* cv. Tarramba) embryogenic callus showed an optimal response at a concentration of 2,4-D ZPT 1,5 mg/L, this research is initial research in the assembly of lamtoro to acid soil tolerance (Manpaki et al. 2018).

Adding ZPT (*Growth regulating substances*) in tissue culture media can provide more optimal growth results. IBA (*Indole butyric acid*) is a growth regulator. IBA is a type of auxin hormone with a high ability to initiate rooting (Frick & Strader 2018). This hormone can also synthesize the amino acid tryptophan by positively reacting to callus stimulation, cell growth, and root formation (Wattimena et al. 2011). This research was conducted by Nurbaeti et al. (2020), which stated that the addition of 1 ppm IBA hormone in *Leucaena leucocephala* plants showed the highest percentage of rooted plants.

A mutant candidate from the *Leucaena leucocephala* plant has been produced through the selection process due to 400 gy (gamma-ray) irradiation adapted to acid media pH 3.4 collection of the Agrostology Laboratory of the Faculty of Animal Science, IPB University. Based on this background, it is necessary to conduct further research at the root stage with the addition of IBA to see the characteristics and selection of root growth from the mutants.

MATERIALS AND METHODS

Research material

Materials used as explants in this study were *Leucaena leucocephala* Teramba variety is adapted to Al^{3+} 300 ppm pH 3.4. Materials obtained from the collection of the Plant Tissue Culture Laboratory, Plant and Pasture Science and Technology section, Faculty of Animal Science, Bogor Agricultural University, spirits, 70% alcohol, aqua dest, jelly, sugar, 2% KOH, MS (Murashige Skoog), carbon charcoal active, growth regulator IBA type (Indole butyric acid) is taken with a concentration of 1 ppm.

Tools used are culture bottles, aluminum foil, laminar air flow, spatula, spoon, or pipette, bulb, scalpel, tweezers, beaker, calipers, magnetic stirrer, pH meter, autoclave, heat-resistant plastic, petri dish, timer, bottle, bunsen, analytical scales, weighing containers, thread, scissors, tissue, refrigerated room, and stationery.

Research procedure

Space Preparation

Room temperature and the lighting of the tissue culture room are set automatically with the tool settings. The room is regulated using air conditioning at a temperature of 16ºC, and lighting is regulated using fluorescent lamps for 16 hours a day which functions to carry out the photosynthesis process in plants.

Tool sterilization

Tools in the form of culture bottles, spatula, scalpel, tweezers, Petri dishes, scissors, and bottles were washed with soap and then rinsed clean. After that, the tool is dried and put in a heat-resistant plastic for sterilization. The device was sterilized using an autoclave at a temperature of 121ºC and a pressure of 17.5 psi for 15 minutes, then stored in a tissue culture room. Before the subculture process is carried out, it is necessary to sterilize by heating the planting tool with tweezers and a scalpel on a Bunsen fire until the tool turns reddish.

Mutant preparation

The *Leucaena leucocephala* plant was obtained from the collection of the Agrostology Field Laboratory of the Faculty of Animal Science, IPB University. *Leucaena Leucocephala* mutant plants were acid-tolerant pH 3.4 resulting from 400 gy which had been treated on 300 ppm Al^{3+} acid media as strain numbers K1, K2, K3, K4, K5, K6, K7, K8, K9, K10, K11 (*Leucaena leucocephala* mutant) and control P0, P1 are *Leucaena leucocephala* parent (plants are not irradiated with gamma ray)

Media creation

The media used in this study were basal MS media as control medium (P0) and MS media with 1 ppm IBA (P1, K1, K2, K3, K4, K5, K6, K7, K8, K9, K10, K11). Preparation of control media consisted of MS 4.43 g.L-1 and sugar 30 g.L-1; after that, it was put into a beaker given 1 L of aqua dest and homogenized using a magnetic stirrer. Next, jelly is added to as much as 7

g.L-1 and 1 g.L-1 activated carbon charcoal into the solution; after that, it was heated to boiling using a hot plate magnetic stirrer at a speed of 250 ppm and a temperature of 380^0C. Then the growth regulator IBA type was added according to the treatment. The media was put into culture bottles of 10 ml each in 195 bottles and covered with aluminum foil. Furthermore, the media was sterilized using an autoclave at a temperature of 121ºC and a pressure of 17.5 psi for 15 minutes. The sterile media is stored in the tissue culture room and observed for a week; if contaminated media is not used as a planting medium.

Planting room preparation

The Laminar airflow work area is sterilized using 70% alcohol and then wiped with a tissue; after that, ultraviolet (UV) light is turned on for 15-20 minutes for the sterilization process, then the blower and lights are turned on during the work process.

Multiplication

The main media used MS media with the addition of IBA type growth regulator with a level of 1 ppm in the *Leucaena leucocephala* mutant Tarramba variety was tolerant to acid pH 3.4 (K1, K2, K3, K4, K5, K6, K7, K8, K9, K10, K11) and control *Leucaena leucocephala* is namely with P0 (without added with IBA), and P1 (with added with IBA). Plant explants in shoots and stems were then transferred to the treatment medium through the subculture technique in laminar airflow; each bottle consisted of 1 explant, so the total sample was 195 bottles.

Statistical design and analysis

The design used a completely randomized design (CRD) with *Leucaena leucocephala* explants as many as 11 mutant lines were acid-tolerant pH 3.4 resulting from 400 gy irradiation which had been treated on 300 ppm Al3+ acid media, Namely, the K1-K11 lines were added with1 ppm IBA (mutant+MS+1ppm IBA) and 2 *Leucaena leucocephala* parent trees without gamma irradiation, namely P0 without added with IBA (*Leucaena leucocephala* parent+MS+0 ppm IBA), P1 was added with 1 ppm IBA (*Leucaena leucocephala* parent + MS + 1 ppm IBA), each with15 replicates. The data obtained from the observations were analyzed using ANOVA. If there is a significant difference between treatments, further tests are carried out using the Tukey test; data analysis is carried out using the SPSS application.

RESULTS AND DISCUSSION

Root extension

Roots are important for plant growth because they are needed to absorb nutrients in the media. The sign of root length in *Leucaena leucocephala* is root growth

Table 1. Root length of *Leucaena leucocephala* until 5 weeks after planting

Strain	Week after planting				
	1	2	3	4	5
	……mm……				
P0	0.00±0.00	0.00±0.00	3.70±1.60 ^{bc}	0.42±0.47 ^{e}	1.07±1.19 ^{c}
P1	0.00±0.00	0.00±0.00	4.38±2.23 ^{bc}	3.21±2.91 ^{cd}	3.02±2.82 ^{bc}
K1	0.00±0.00	0.00±0.00	5.24±1.98 ^{ab}	4.48±2.00 ^{bc}	4.20±1.99 ^{ab}
K2	0.00±0.00	0.00±0.00	2.35±1.36 ^{bc}	2.85±1.43 ^{cd}	3.20±1.53 ^{bc}
K3	0.00±0.00	0.00±0.00	3.63±2.46 ^{bc}	6.60±2.36 ^{ab}	5.37±3.42 ^{ab}
K4	0.00±0.00	0.00±0.00	5.22±2.36 ^{ab}	4.22±2.07 ^{bc}	4.63±2.39 ^{ab}
K5	0.00±0.00	0.00±0.00	3.88±2.53 ^{bc}	4.43±1.79 ^{bc}	3.68±2.37 ^{bc}
K6	0.00±0.00	0.00±0.00	1.69±2.64 ^{c}	1.63±1.52 ^{de}	4.72±3.13 ^{ab}
K7	0.00±0.00	0.00±0.00	3.63±3.06 ^{bc}	3.05±1.71 ^{cd}	2.99±2.88 ^{bc}
K8	0.00±0.00	0.00±0.00	3.62±4.07 ^{bc}	3.74±3.25 ^{cd}	3.02±3.11 ^{bc}
K9	0.00±0.00	0.00±0.00	7.54±2.94 ^{a}	7.99±2.94 ^{a}	5.98±1.94 ^{ab}
K10	0.00±0.00	0.00±0.00	3.76±2.54 ^{bc}	4.17±2.50 ^{bc}	7.07±4.29 ^{a}
K11	0.00±0.00	0.00±0.00	1.41±2.53 ^{c}	2.28±2.82 ^{cd}	4.24±2.12 ^{ab}

P0=control+MS+IBA 0 ppm, P1=control+MS+IBA 1 ppm, K1- K11= Mutant+MS+IBA 1 ppm. Different superscripts in the same column showed a significant effect (P<0.05) based on the Tukey test

which can be seen at the bottom of the culture bottle. The increase in the root length that gives the hormone IBA to acid-tolerant *Leucaena leucocephala* is listed in Table 1.

The analysis of variance in the increase of plant root length showed a significant difference (P<0.05). In the first and second weeks, there was no root growth in all plant strains; roots began to form in the third week with the highest increase in the K9 strain with an increase of 7.54 mm and the highest in the fourth week with an increase of 7.99 mm, the fifth week the highest was in the K10 strain with an increase of 7.07 mm, this indicates that the root length increase of the selected mutant plant was higher than P1or P0. The K9 mutant line had the best root length gain with the highest average compared to other plant lines. K9 strain with the addition of IBA can significantly increase root growth. The K10 mutant strain is a response to an increase in root length at 5 weeks after planting. According to Marga et al. (2020) and Manpaki et al. (2017), engineered *Leucaena leucocephala* has a high level of genetic diversity, so the possibility of obtaining superior mutants is also higher. Giving the IBA type of auxin hormone with a concentration of 1 ppm in the growing media can stimulate root growth and increase the number and quality of roots. Following Arlianti et al. (2013) and Zulastri et al. (2020) statements, IBA has activity as a rooting hormone and the fastest root formation time occurs at 1 ppm IBA treatment. The effectiveness of auxin in influencing root length is to expand cell volume by slowing down calcium pectin compounds, causing cell walls to become elastic (Nurbaeti et al. 2020). The expansion of the cell volume results in the exchange of K+ and H+ ions within the cell wall, and this is done to maintain ion balance when the apical meristem elongates. When the elongation has been completed, the auxin hormone will stop its role in inhibiting the calcium pectin (Apriliani et al. 2015). Rafique et al. (2012) said that a significantly high root length (0.96 cm) was found on MS medium supplemented with 1 mM IBA followed by 1.5 mM IBA (0.63 cm).

Plant height

Height gain is one of the variables that describe plant growth to see the response of plant morphology to the treatment given. The results of plant height as treated with IBA on *Leucaena leucocephala* acid-tolerant are listed in Table 2.

In the first week, the highest increase was in the K11 mutant line with an increase of 5.17 mm, the second week the highest increase was in the K5 mutant strain with an increase of 5.55 mm, the third week the highest increase was recorded in K3 mutant strain with an increase of 6.56 mm, while in the fourth and fifth weeks K9 had the highest increase. The K9 mutant strain with the highest increase reaching 8.22 mm indicates that the plant height increase of the selected mutant plant was higher than P1 and P0. The K9 mutant strain had the best vertical height gain and had

Table 2. Plant height of *Leucaena leucocephala* plants until 5 weeks after planting

Strain	Week after planting				
	1	2	3	4	5
	……mm……				
P0	0.61±0.60 ^{b}	1.18±1.53 ^{de}	0.91±1.08 ^{b}	1.16±1.71 ^{cd}	1.95±4.25 ^{c}
P1	0.57±1.06 ^{b}	4.61±4.49 ^{cd}	3.39±3.51 ^{ab}	2.22±2.27 ^{cd}	3.66±3.07 ^{bc}
K1	0.66±1.22 ^{b}	4.99±3.67 ^{ab}	3.19±4.08 ^{ab}	2.96±3.04 ^{cd}	6.52±4.62 ^{ab}
K2	1.06±1.60 ^{b}	5.39±4.39 ^{a}	1.93±2.73 ^{b}	1.74±2.05 ^{cd}	5.05±3.64 ^{bc}
K3	0.78±1.39 ^{b}	4.73±2.40 ^{bc}	6.56±3.22 ^{a}	5.37±2.17 ^{ab}	2.75±2.35 ^{bc}
K4	0.00±0.00 ^{b}	3.97±2.61 ^{de}	1.57±2.97 ^{b}	3.42±3.29 ^{cd}	6.28±4.71 ^{ab}
K5	0.65±1.30 ^{b}	5.55±3.51 ^{a}	4.31±2.46 ^{ab}	4.16±2.53 ^{bc}	2.87±2.45 ^{bc}
K6	1.78±2.85 ^{b}	4.49±2.88 ^{de}	3.29±3.53 ^{ab}	2.25±3.00 ^{cd}	1.69±2.64 ^{c}
K7	0.57±1.06 ^{b}	4.61±4.49 ^{cd}	3.39±3.51 ^{ab}	2.68±2.58 ^{cd}	3.63±3.06 ^{bc}
K8	0.80±1.71 ^{b}	1.43±1.99 ^{de}	3.52±3.20 ^{ab}	3.95±3.80 ^{bc}	3.62±4.07 ^{bc}
K9	0.50±0.82 ^{b}	0.91±1.08 ^{de}	1.16±1.71 ^{b}	8.22±4.74 ^{a}	7.54±2.94 ^{a}
K10	0.28±0.51 ^{b}	0.81±1.05 ^{e}	6.16±5.98 ^{a}	4.00±2.81 ^{bc}	3.76±2.54 ^{bc}
K11	5.17±0.90 ^{a}	4.00±2.81 ^{de}	3.76±2.54 ^{ab}	0.21±0.48 ^{d}	1.41±2.53 ^{c}

P0=control+MS+IBA 0 ppm, P1=control+MS+IBA 1 ppm, K1- K11= Mutant+MS+IBA1 ppm. Different superscripts in the same column showed a significant effect (P<0.05) based on the Tukey test

the highest mean compared to other plant strains. The effectiveness of the hormone IBA in the growth of the plant vertical height had a good effect on the growth of mutant plants of *Leucaena Leucocephala*. Research conducted by Firmansyah et al. (2014) states that the hormone can stimulate the formation of the apical meristem, thereby increasing plant height. According to Supriyanto & Prakasa (2011) and Satbhai et al. (2015), root growth will increase plant height, where nutrients to support plant growth are sufficient, and plants can grow optimally vertically, and horizontally.

Number of shoots

Shoots are one of the new plant organs that grow on each plant. The number of shoots is calculated based on the number of new branches that appear on the plant. The growth of shoots treated with IBA on acid-adapted *Leucaena leucocephala* plants is shown in Table 3.

The analysis of variance in the number of plant shoots showed a significant effect (P<0.05). In the first week and second weeks, the highest shoot number was in the K10 and K11 mutant strains with an average number of 2.93 units and 3.87 shoot units; in the third week, the highest number was in the K11 mutant strain with an average number of 4.27 units, while in the fourth and fifth weeks, the highest was recorded in the K9 and K11 mutant strains with the highest average number of around 5.27 units. The number of shoots of the selected mutant plants was higher than P1 and P0. Mutant strains K9, K10, and K11 had the best number of shoots at 5 weeks after planting, and had the highest average compared to other plant strains. Based on Harahap et al. (2012) research, the time required for explants to grow shoots ranged from 4 to 11 DAP (Days after planting). The effectiveness of the IBA hormone in the formation of plant shoots had a good effect on *Leucaena leucocephala* plant mutants. The hormone auxin's function plays a role in regulating plant growth and development, including growth in shoots (Marga et al. 2020; Zhang et al. 2022). The IBA type of auxin hormone can encourage cell extension and division (Wang & Ruan 2013). The addition of IBA will encourage optimal shoot formation and root initiation (Karti et al. 2019).

Percentage of rooted plants

The percentage of rooted plants from *Leucaena leucocephala* mutant showed different effects in each plant strain were influenced by varying plant responses to adaptation between genetics and hormones. This statement is in line with research conducted by Harahap (2012) which states that a combination of genetic and environmental factors such as hormones will display plant characteristics. The addition of the hormone IBA concentration of 1 ppm provides optimal root growth because it follows the levels of auxin

Table 3. The number of *Leucaena leucocephala* plants shoots until 5 weeks after planting

Strain	Week after planting				
	1	2	3	4	5
	……mm……				
P0	1.00±0.00 ^{c}	1.87±0.35 ^{c}	2.93±0.26 ^{c}	3.00±0.00 ^{e}	3.20±0.41 ^{c}
P1	1.40±0.51 ^{c}	2.93±0.26 ^{b}	2.93±0.26 ^{c}	3.20±0.41 ^{de}	3.87±0.74 ^{bc}
K1	1.00±0.00 ^{c}	2.20±0.41 ^{c}	2.87±0.52 ^{c}	4.27±0.80 ^{c}	4.53±0.64 ^{ab}
K2	1.20±0.41 ^{c}	1.87±0.35 ^{c}	2.93±0.26 ^{c}	3.80±0.41 ^{cd}	3.87±0.35 ^{bc}
K3	1.00±0.00 ^{c}	1.87±0.35 ^{c}	2.93±0.26 ^{c}	3.87±0.35 ^{bc}	3.87±0.35 ^{bc}
K4	1.40±0.51 ^{c}	2.93±0.26 ^{b}	2.93±0.26 ^{c}	3.87±0.35 ^{bc}	3.87±0.35 ^{bc}
K5	1.40±0.51 ^{c}	2.27±0.46 ^{c}	2.93±0.26 ^{c}	4.07±0.59 ^{bc}	4.07±0.59 ^{b}
K6	1.87±0.35 ^{b}	2.93±0.26 ^{b}	2.93±0.26 ^{c}	3.87±0.35 ^{bc}	3.87±0.35 ^{bc}
K7	1.87±0.35 ^{b}	2.93±0.26 ^{b}	3.87±0.35 ^{b}	3.87±0.35 ^{bc}	4.07±0.46 ^{b}
K8	1.87±0.35 ^{b}	2.93±0.26 ^{b}	3.80±0.41 ^{ab}	3.87±0.35 ^{bc}	4.00±0.53 ^{b}
K9	2.27±0.46 ^{b}	2.93±0.26 ^{b}	3.87±0.35 ^{ab}	5.00±0.53 ^{a}	5.27±0.88 ^{a}
K10	2.93±0.26 ^{a}	3.87±0.35 ^{a}	3.87±0.35 ^{ab}	4.47±0.74 ^{ab}	5.13±0.92 ^{a}
K11	2.93±0.15 ^{a}	3.87±0.15 ^{a}	4.27±0.59 ^{a}	5.00±0.93 ^{a}	5.27±0.80 ^{a}

P0=control+MS+IBA 0 ppm, P1=control+MS+IBA 1 ppm, K1- K11= Mutant + MS + IBA1 ppm. Different superscripts in the same column showed a significant effect (P<0.05) based on the Tukey test.

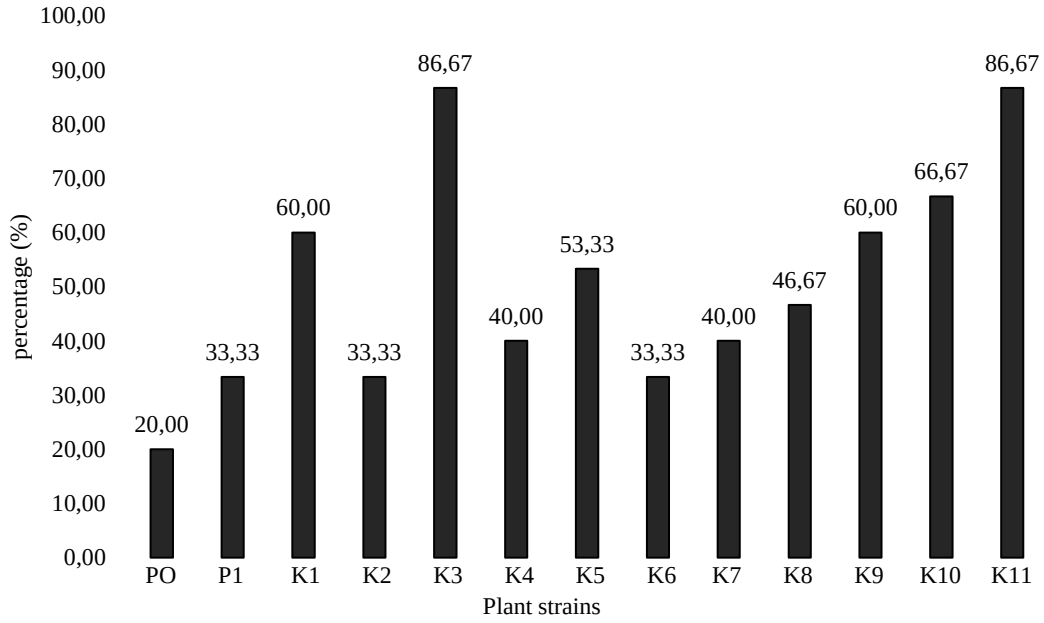

Figure 1. Percentage of *Leucaena leucocephala* rooted plants at 5 Weeks After Plant.

required by the *Leucaena leucocephala* plant (Nurbaeti et al. 2020; Rinaldy et al. 2019). The highest percentage of *Leucaena leucocephala* rooted plants at 5 weeks after the plant is K3 and K11. This research showed that the addition of 1 ppm IBA hormone in the *Leucaena leucocephala* mutant gave a high percentage of rooted plants compared with P0 (without adding IBA). It is different from what (Wijaya & Sudrajad 2019) said that a concentration of 2 ppm IBA showed better root growth compared to others. Concentrations Each plant has a different response to hormones; this is influenced by the concentration given. If the concentration is too low, the hormone will not work effectively. Meanwhile, if the concentration is too high, then the hormone will be inhibited. Adding auxin to plants as a growth regulator can increase plant development by affecting membrane proteins that can accelerate protein and nucleic acid synthesis (Saini et al. 2013), and adding auxin also affects new root formation (Firmansyah et al. 2014). The rooting percentage of the seedling-derived shoots from the Olive plant was 76% for 'Arbequina' and 'Gordal Sevillana' cultivars and 100% for 'Manzanilla de Sevilla' cultivar, whereas, with the electro-pulse method, the rooting percentages were 68, 64 and 88%, respectively (Padilla et al. 2009).

CONCLUSION

Based on the research that has been done, it can be concluded that the increase in root length showed the best results on the K10 strain, the increase in vertical plant height showed the best results on the K9 strain, and the number of shoots showed the best results on K9, and K11 strains. The highest percentage of rooted plants on K3, and K11. Root length, vertical height, shoot number, and the rooted percentage were higher on *Leucaena leucocephala* mutant with added IBA 1 ppm compared with *Leucaena leucocephala* without added IBA.

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Impact of Aging on Sperm Quality of Sentul Roosters

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ABSTRAK

Haryuni N, Hartutik, Widodo E, Tribudi YA, Wahjuningsih S. 2021. Dampak penuaan terhadap kualitas spermatozoa pejantan Sentul. JITV 27(4):177-185. DOI:http://dx.doi.org/10-14334/jitv.v27i4.3015.

Tujuan dari penelitian ini adalah untuk mengetahui dampak penuaan terhadap kualitas spermatozoa pejantan Sentul. Bahan yang digunakan dalam penelitian ini adalah pejantan Sentul umur 48, 58, 68 dan 78 minggu, NaCl, eosin dan aquades. Semen dikumpulkan dengan menggunakan metode pijat abdomen. Penelitian ini menggunakan Rancangan Acak Lengkap (RAL) dengan 4 perlakuan dan diulang sebanyak 5 kali. Analisa statistik menunjukkan bahwa peningkatan umur pejantan memberikan pengaruh yang sangat nyata ($p<0,01$) terhadap penurunan konsistensi semen, gerak masa, konsentrasi spermatozoa dan peningkatan molitilitas spermatozoa. Penuaan pada pejantan Sentul memberikan pengaruh nyata ($P<0,05$) terhadap volume semen tetapi tidak berdampak nyata ($p>0,05$) terhadap pH semen, spermatozoa hidup dan abnormalitas spermatozoa. Rataan volume semen yang dihasilkan dalam penelitian ini berkisar antara 0,54-0,88ml; pH semen 6,80-7,12; skor gerak masa 1,60-3,00; konsentrasi spermatozoa 2,76-4,86 x109/ml; motilitas spermatozoa 66-79%; Spermatozoa hidup 91,75-93,10% dan abnormalitas spermatozoa 1,75-2,51%. Semen dari pejantan Sentul umur 48-68 minggu memiliki konsistensi yang tebal dan berwarna putih keruh. Pada umur 78 minggu didapatkan konsistensi yang bervariasi mulai dari tebal, sedang dan cair. Warna semen pada pejantan Sentul umur 78 minggu juga bervariasi antara putih bening hingga krem. Kesimpulan dari penelitian ini penuaan yang terjadi pada pejantan Sentul menyebabkan gangguan reproduksi yang ditandai dengan rendahnya kualitas spermatozoa. Kualitas spermatozoa yang terbaik dihasilkan oleh pejantan Sentul yang berumur 58-68 minggu.

Kata Kunci: Penuaan, Pejantan Sentul, Kualitas Spermatozoa

ABSTRACT

Haryuni N, Hartutik, Widodo E, Tribudi YA, Wahjuningsih S. 2021. Impact of aging on sperm quality of Sentul roosters. JITV 27(4):177-185. DOI:http://dx.doi.org/10-14334/jitv.v27i4.3015.

This study was done to determine impact of aging on sperm quality of Sentul roosters. Materials used in this study were Sentul males aged 48, 58, 68 and 78 weeks, NaCl, eosin and aquades. Semen was collected by abdominal massage method. Completely randomized design (CRD) was applied in this study with 4 treatments in 5 repetitions. Statistical analysis showed that increasing age of rooster had a very significant effect ($p<0.01$) on decreasing semen consistency, mass motility, spermatozoa concentration and increasing spermatozoa motility. Aging in Sentul roosters affected semen volume significantly ($P<0.05$) but did not significantly affect ($p>0.05$): semen pH, live spermatozoa, and spermatozoa abnormalities. Average volume of semen produced in this study ranged from 0.54-0.88ml; semen pH 6.80-7.12; mass movement score 1.60-3.00; spermatozoa concentration 2.76-4.86 x109/ml; spermatozoa motility 66-79%; Live spermatozoa 91.75-93.10%, and spermatozoa abnormalities 1.75-2.51%. Semen from Sentul males aged 48-68 weeks had a thick consistency and cloudy white in color. At the age of 78 weeks, the consistency varies from thick, medium and liquid. The color of semen in Sentul males aged 78 weeks also varied from clear white to cream. It is concluded that aging in Sentul roosters causes reproductive disorders which are characterized by low sperm quality. The best quality spermatozoa were produced by Sentul roosters aged 58-68 weeks.

Key Words: Aging, Sentul Rooster, Spermatozoa Quality

INTRODUCTION

An unavoidable and inevitable life process in living things is aging. Slowly but surely the aging process that occurs in living things has an impact on the decline in the physiological functions of the organs in the body and the ability to survive and eventually leads to death (Colloca et al. 2020; Das 2021). Aging is defined as a dynamic process related to the existence of damage that comes from within and outside the individual. The theory of aging is generally based on the process of damage that occurs in the individual. This damage is caused by the influence of age or mutations in genes due to stressors or oxidative stress triggers that cause damage to cells (Colloca et al. 2020). Damage to the physiological functions of the organs in the body due to

the aging process makes the tissue unable to maintain its structure so that it loses the ability to functioning. If this kind of damage occurs in the reproductive organs, the hormone cannot be produced optimally and even stops.

Sentul chicken is one of Indonesia's original chickens that has great potential to be developed as a producer of eggs and meat. Sentul chicken originates and grows a lot in Ciamis, West Java, Indonesia (Saleh et al. 2019). The advantages of Sentul chickens compared to other types of local chickens are fast body weight growth, disease resistance, high egg production, high fertility and high hatchability. Sentul chicken is one of superior local rooster because it is able to produce spermatozoa in good quality (Ariyanti et al. 2019).

Development of poultry industry in Indonesia requires the development of technology in the field of reproduction. Artificial insemination (AI) is a technology that is widely used to increase poultry reproduction. This technology is effective to be applied in poultry industry because one male can fertilize dozens of hens (Gao et al. 2021). The success of artificial insemination in poultry depends on the quality of spermatozoa. Artificial insemination in poultry will be successful if the spermatozoa quality is good. Spermatozoa motility plays an important role in the success of the fertilization proces. In breeding industry, it is very necessary to have a selection of roosters used for artificial insemination because the quality of semen reflects its ability to fertilize (Rochmi et al. 2019; Haryuni et al. 2021). The problem that often occurs in the breeding and hatchery industry is that the reproductive performance of roosters is not optimal. The quality of rooster spermatozoa at the age of 50-55 weeks gradually decreased. In aging roosters, there are some important changes in reproductive characteristics such as decreased spermatozoa concentration, motility and antioxidant capacity (Gao et al. 2021). Research is needed to determine impact of aging on the sperm quality of Sentul roosters.

MATERIALS AND METHODS

This study was an experimental study using 20 Sentul roosters with several age variations namely: 48, 58, 68 and 78 weeks. Completely randomized design (CRD) with 4 types of treatment and 5 replications was applied in this study. Evaluation of the semen quality of Sentul's rooster was carried out macroscopically and microscopically.

Bird management and diet

Sentul roosters were placed in cages measuring 50 x 100 x 70 cm where each box contains 1 rooster. Sentul roosters used in this study had body weights ranging from 2.10 to 2.20 kg and were in good health. Feed is given in the morning at 07.00 WIB for 40 g and at 15.00 WIB in the afternoon for 60 g. Drinking water is provided ad libitum. The composition and quality of feed from Sentul roosters is presented in Table 1.

Semen collection

Semen was collected using massage methods with direction from abdomen to the cloaca. The semen collection process was initially carried out by cleaning the cloaca by tissue sprayed with 70% alcohol (Haryuni et al. 2022). Furthermore, the semen secreted was collected in a scale tube. In this study, semen was collected every 5 days. Semen quality was evaluated macroscopically and microscopically in each treatment and the results were statistically analyzed.

Parameters measure

Semen volume

Volume of semen can be determined by measuring the semen produced by Sentul roosters. The steps taken to determine the volume were: the semen that is accommodated was put in a scale tube, the number listed on the scale tube was read and then recorded as the volume of semen.

Semen color

Color of semen can be observed in several steps as follows: semen is put in a scale tube, the color of semen is observed using the five senses and recorded (Mustaqim et al. 2021). The indicators to determine the quality of spermatozoa based on the color as cream for semen with milky white color indicating that the semen has a high concentration of spermatozoa. While clear white for semen with clear color indicating that the semen has a low concentration of spermatozoa.

Semen consistency

Steps taken to determine semen consistency are as follows: semen is put in a test tube, the test tube is tilted, the test tube is then re-enforced, the movement of semen when the test tube is tilted and erected is observed carefully and the results are recorded.

Semen pH

Steps taken to determine pH of the semen are as follows: litmus paper along with a standard to see the pH value is prepared, the tip of the litmus paper is

Table 1. Composition of the diet

Ingredient	Amount	Nutrient	Amount
Corn (%)	50.28	Metabolic energy (kcal/kg)	2,700.9
Soybean meal (%)	19.00	Crude protein (%)	17.90
Rice bran (%)	15.00	Crude fiber (%)	3.65
Meat bone meal (%)	8.00	Crude fat (%)	4.58
Grit (%)	4.90	Methionine (%)	0.40
Milestone (%)	3.20	Lysine (%)	0.92
Premix (%)	0.50	Calcium (%)	3.91
Dicalcium phospate (%)	0.30	Phosphorus total (%)	0.84
Salt (%)	0.10	Phosphor available (%)	0.50
Sodium bicarbonate (%)	0.70	Sodium (%)	0.13

Calculations using Brill Formulation Software

dipped in semen, then waited for 60 seconds, the color changes that occur on the litmus paper are observed, the colors observed are matched with standard litmus paper to determine the pH and recorded.

Spermatozoa mass movement

Mass movement of spermatozoa can be observed using a microscope at 400x or 100x magnification (Usman, Tijjani et al. 2021). The movement of spermatozoa can be assessed as very good (+++) when observed under a microscope it looks like a big thick wave, many, dark and moving actively to form a thick black cloud; good (++), when observed under a microscope it looks like the form of small waves that are thin, rare, moving slowly and seem less clear; not good (+), when observed under a microscope, only the progressive motion of the individual appears and there is no visible mass collection that forms like a wave; bad (0), when observed under a microscope, the only visible movement of individuals in small numbers and looks sluggish

Spermatozoa concentration

Spermatozoa concentrations were calculated using an erythrocyte pipette and haemocytometer using a Neubauer counting chamber. The cement was sucked using an erythrocyte pipette to a scale of 0.5 then added with 3% NaCl solution and sucked up to 1.01. The solution in the pipette is shaken to make it homogeneous with the direction of movement like forming a figure of eight for 2-3 minutes. The next step is to discard the solution as much as 3 drops so that no oxygen is included. After that, the cement was dripped into the Neubauer counting chamber and covered with a cover slip. Observations were made using a microscope at 400x magnification. Spermatozoa concentration was calculated by averaging the number of spermatozoa in 5 Neubauer counting chambers and multiplied by 10^7 per milliliter. The calculation of the number of spermatozoa is done by counting the number of spermatozoa in the counting room (Usman, Tijjani et al. 2021).

$$\textbf{Concentration} = \frac{\sum \mathbf{S(A+B+C+D+E)}}{\mathbf{5}} \mathbf{x\ 10^7}$$

where S is spermatozoa in the counting cahmber, A, B, C, D and E are haemocytometer counting chambers.

Spermatozoa motility

Evaluation of spermatozoa motility was carried out using a microscope. The first stage in this observation is to make preparations by means of which the semen to be evaluated is dropped on a glass object and closed using a cover glass and then observed under a microscope with a magnification of 100x to observe the movement of spermatozoa. Spermatozoa motility is calculated by looking at the movement of spermatozoa that move actively and progressively forward. Spermatozoa motility was observed using a microscope with a magnification of 100x (Mustaqim et al. 2021).

Sperm viability

Measurement of sperm viability was carried out by dripping semen on an object glass, then 2 drops of eosin and observed under a microscope with a magnification of 400X. Indications of live spermatozoa are colorless (transparent), dead sperm will be red. The percentage of live spermatozoa was calculated using the following formula (Najafi et al. 2019).

$$\text{Sperm Viability (\%)} = \frac{\sum \text{ST} - \sum \text{LS}}{\sum \text{ST}} \text{x } 100\%$$

where ST is spermatozoa total and LS is live spermatozoa.

Spermatozoa abnormalities

Spermatozoa abnormalities in general can be observed in terms of shape (head shape, head size, tail

shape, tail size etc.) (Mustaqim et al. 2021). Spermatozoa abnormalities were evaluated by making smear preparations. Review preparations were made using 2 glass objects. The first stage of semen is diluted using 3% NaCl with a ratio of 1:4. The second stage of diluted semen is dripped on a glass object. The next step is to take an empty glass object and the tip is touched to a glass object that has been dripped with semen and lightly rubbed and then allowed to dry. The dried smear preparations were observed under a microscope at 400x magnification. The percentage of spermatozoa abnormalities can be calculated as follows:

$$\textbf{Abnormalities}\ (\%) = \frac{\sum \textbf{ST} - \sum \textbf{AS}}{\sum \textbf{Spermatozoa total}} \textbf{x}\ \textbf{100}\%$$

where ST is spermatozoa total and AS is live abnormal spermatozoa.

Experimental design

The data from the evaluation of cement quality were tabulated and statistically analyzed using a Completely Randomized Design (CRD) with 4 treatments and 5 replications. If there is a significant or very significant different effect, it will be continued with Duncan's test.

$$\mathbf{Y_{ij}} = \mu + \boldsymbol{\delta_i} + \boldsymbol{\varepsilon_{ij}}$$

where Y_{ij} is observation value of treatment i repetition j; μ is mean, δ_i is effect of treatment I; ε_{ij} is effect of error on the i^{th} treatment and j repetition; I is 1, 2, 3, 4, 5, j is 1, 2, 3, 4, 5.

RESULTS AND DISCUSSION

Semen quality that can be observed macroscopically includes semen volume, pH, consistency, color and microscopic quality includes mass movement concentration, motility, live spermatozoa and spermatozoa abnormalities. The impact of aging on Sentul males on the quality of semen is presented in Table 2.

Aging in a biological context is a complex phenomenon that is described as a decrease in the physiological function of organs (Zia et al. 2021). Aging occurs slowly and is difficult to measure qualitatively. Aging involves degenerative and biological processes (Warraich et al. 2020). Aging have an impact on cell damage, changes in cell size and the ability to survive (Rudzińska et al. 2020). Aging has an impact on damage and loss of function in organs, tissues, cells and reproductive abilities in poultry (Tabibzadeh 2021). Aging in Sentul rooster causes the cessation of growth hormone (GH) production and increased glucocorticoid hormone production. This has an impact on low ATP production due to decreased muscle mass (Figure 1). The low production of ATP causes disturbances in energy homeostasis in the brain so that the brain and hypothalamus function decreases. Under these conditions the production of *gonadotropin releasing hormone* (GnRH) decrease. GnRH functions to stimulate the anterior pituitary to synthesize and secrete gonadodropin hormones (luteneizing hormone and follicle stimulating hormone) (Shi et al. 2019). LH (*luteneizing hormone*) will stimulate the interstitial Leidig cells in the testes to synthesize and secrete the hormone testosterone. FSH (*follicle stimulating hormone*) stimulates spermatogenesis in the seminiferous tubules of the testes. Testosterone will increase the development of male sexual organs. Low production of LH and FSH due to the aging process has an impact on spermatogenesis disorders. Based on this explanation, it can be illustrated that the impact of the aging process on the spermatogenesis process is as shown in Figure 1.

Impact of aging on the macroscopic quality of Sentul rooster semen

Volume

Semen is produced by a complex process of synergistic action between the hypothalamus, pituitary gland and testes. Semen can be produced if the hypothalamus, pituitary gland and testes can work normally (Behnamifar et al. 2021). Table 2 above shows that semen production increased with increasing age of the rooster until the rooster was 68 weeks old and semen production decreased at 78 weeks of age. The increase in semen production until the rooster is 68 weeks old is related to the development of reproductive organs. Semen production can be described as a curve where semen production will increase with increasing age of the rooster and after reaching peak production and the rooster getting older the semen production decreases gradually (Khaeruddin et al. 2019; Shi et al. 2019). Aging in Sentul roosters causes disturbances in the reproductive organs and the inability to produce reproductive hormones (Figure 1). The imbalance of reproductive hormones in the male body has an impact on the production of semen in a small volume (Adeoye et al. 2018).

pH semen

The pH obtained in this study ranged from 6.80 to 7.12. Previous research also found that the pH of semen in Sentul roosters was 6.73 (Ariyanti et al. 2019) or 7.24 (Elokil et al. 2019). These results indicate that semen of this research has a good quality. Although statistically the aging process has no effect on semen pH, Table 2 shows a decrease in semen pH. Figure 1 shows that there is an increase in glucocorticoid hormone level in old

Table 2. Average macroscopic and microscopic quality of Sentul rooster semen

Variable	Age of Sentul roosters			
	48 weeks	58 weeks	68 weeks	78 weeks
Macroscopic quality				
Volume (ml)	0.54 ± 0.13[a]	0.74 ± 0.15[b]	0.88 ± 0.19[c]	0.58 ± 0.13[a]
Average pH	7.12 ± 0.16	7.12 ± 0.27	6.80 ± 0.45	6.86 ± 0.49
Consistency	100% Thick	100% Thick	100% Thick	50% liquid, 25% medium and 25% Thick
Color	Cream	Cream	Cream	Cream, clear white
Microscopic quality				
Mass movement	3.00 ± 0.00[b]	3.00 ± 0.00[b]	3.00 ± 0.00[b]	1.60 ± 0.55[a]
Concentration (10^9/ml)	4.86 ± 0.39[b]	4.66 ± 0.39[b]	4.30 ± 0.29[b]	2.76 ± 0.66[a]
Motility (%)	79.00 ± 2.24[b]	79.00 ± 2.24[b]	79.00 ± 2.24[b]	66.00 ± 5.48[a]
Sperm viability (%)	92.20 ± 1.11	92.92 ± 0.63	91.75 ± 3.20	93.10 ± 2.89
Abnormality (%)	1.75 ± 0.42	2.51 ± 0.55	2.12 ± 0.75	1.91 ± 0.41

Different superscripts in the same row indicate significant effect (P<0.05) on semen volume and a very significant effect (p<0.01) on spermatozoa mass movement, spermatozoa conscentration and motility of spermatozoa

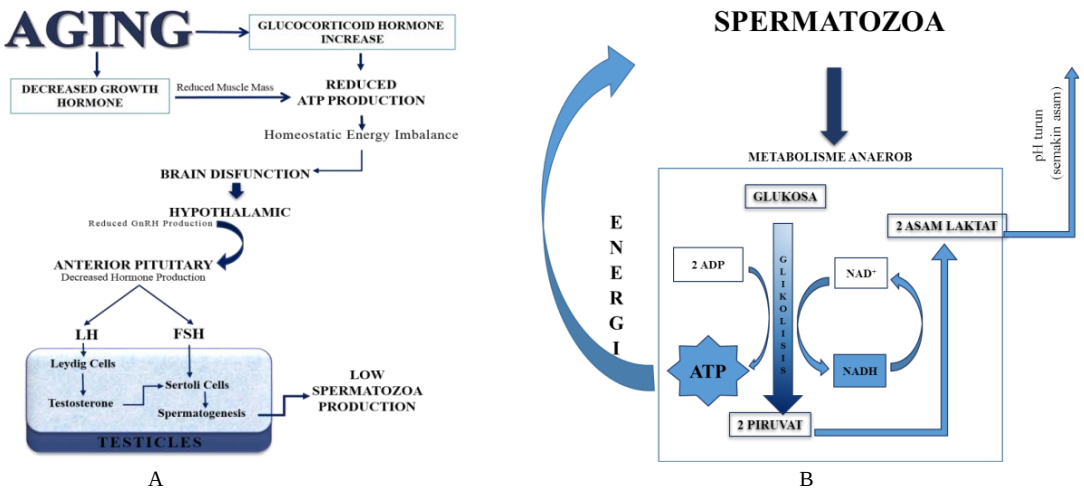

Figure 1. Impact of aging on spermatogenesis (A) and Spermatozoa anaerobic metabolism (B)

age roosters so that it has an impact on increased glucose metabolism. Spermatozoa metabolize anaerobically when they are outside the body to survive. Anaerobic metabolism produces lactic acid which will make semen more acidic. The higher metabolism causes the semen to become more acidic (Figure 2). Spermatozoa that are outside the body in an acidic state can survive only a few hours (Esguerra et al. 2020).

Consistency

Semen consistency is related to the concentration of spermatozoa. Thick semen can be used as an indicator of a high concentration of spermatozoa, however liquid semen indicates that the concentration of spermatozoa is low (Mustaqim et al. 2021). In this study, a thick consistency was found in roosters aged 48-68 weeks with 100% of the replicates showing a thick consistency. In this study, the lowest consistency was found in roosters aged 78 weeks where the consistency of the semen changed to 50% of the repeats the consistency was liquid, 25% medium and 25% thick.

Figure 1 shows that the aging is a complex metabolism that causes changes both morphologically and biochemically and has an impact on body functions that decrease irreversibly and progressively. In old rooster morphological changes occur in the testes. These morphological changes include a decrease in the volume and quantity of germ cells that have an impact on low semen quality (Gao et al. 2021). Aging causes damage to the thermostat in the body and has an impact on disruption of homeostasis in the body. Feed back system in the hypothalamus, pituitary gland and

endocrine glands work very well at a younger age (Tabibzadeh 2021).

Color

Semen color is one of the factors used as an indicator to determine the concentration. Semen that is cream color can be an indicator of a high concentration of spermatozoa. Creamy semen is normal semen and is capable of maximum fertilization (Mustaqim et al. 2021). Semen with a clear white color in a 78-week-old male indicated of low spermatozoa concentrations. Semen with a clear white color in a 78-week-old male indicated of low spermatozoa concentrations. Figure 1 shows that biologically in old roosters (78 weeks) the function of the reproductive organs decreases. Disorders that occur in the reproductive organs cause low semen quality (Inyawilert et al. 2019).

Impact of aging on the macroscopic quality of Sentul rooster semen

Mass movement

The aging process in Sentul roosters had a very significant impact ($p<0.01$) on spermatozoa mass movement. Increasing age in roosters causes an increase in polyunsaturated fatty acids in the plasma membrane and a decrease in antioxidant capacity. High lipid metabolism causes oxidative stress so that ATP production is disrupted (Gao et al. 2021). Decreased ATP production results in the inhibition of the energy source for the movement of spermatozoa (Figure 2). Energy metabolism is a key factor that supports the mass movement of spermatozoa (Qi et al. 2020). Mass movement is a wave of spermatozoa movement together in semen. Spermatozoa in a group will have a tendency to move together in the same direction. Spermatozoa are categorized as good quality if they are active. The movement of individual spermatozoa is reflected in the mass movement, the faster and more active motions of spermatozoa make the mass movement thicker and better. Spermatozoa with good quality will move actively, quickly and progressively towards the ovum. Poor mass movement was found in Sentul roosters aged 78 weeks with an average score of 1.60.

Concentration

The aging process in Sentul roosters had a very significant impact ($p<0.01$) on spermatozoa concentration. The spermatozoa concentration of Sentul roosters found was in the range of previously reported publications from 1.83-3.00 x 10^9/ml (Saleh et al. 2017). Spermatozoa concentration will decrease with increasing age of the male (Khaeruddin. & Amir 2019). The lowest concentration of spermatozoa in this study was found in roosters aged 78 weeks at 2.76x10^9/ml. This result is higher than white leghorn roosters aged 64 weeks of 2.79-2.81 x 10^9/ml.(R Richard Churchil, Lijo John 2019).

Age of the rooster plays an important role in sexual maturity and reproductive performance of the male. Reproduction can be maximized at a certain age. Aging causes degenerative changes in the hypothalamus and is followed by a failure of GnRH secretion (Shi et al. 2019). Disruption in the spermatogenesis process causes poor quality of spermatozoa and low fertility. Factors that influence spermatozoa concentration are age, feed, strain, body weight and frequency of semen collection (Esguerra et al. 2020; Usman, Tijjani et al. 2021).

Motility

Spermatozoa motility is usually used as a parameter in determining the success of the fertilization process. Spermatozoa motility has a great influence on high or low fertility rates (Rochmi et al. 2019). High fertility can be achieved when spermatozoa can move actively and progressively towards the ovum. Quantity of the motile spermatozoa can be observed from the number of active and progressive spermatozoa (Saleh & Mugiyono 2017).

Aging process in Sentul roosters had a very significant impact ($p<0.01$) on spermatozoa motility. Previous reported research found the motility of Sentul rooster spermatozoa were 68-70% (Ariyanti et al. 2019), and 83.33% (Junaedi et al. 2016). Normal spermatozoa motility in chickens was 60-80% (Sigit Mugiyono et al. 2015). The lowest motility in this study was 66% in Sentul roosters aged 68 weeks.

Spermatozoa motility is related to the availability of energy used to move towards the ovum (Rochmi 2019). Homeostatic energy disturbances that occur in old roosters (Figure 1) have an impact on lower energy supply from energy requirements. Adenosine triphosphate (ATP) is a source of energy for advancing spermatozoa resulting from anaerobic metabolic processes (Figure 2). Low ATP causes motility of spermatozoa to decrease. Aging causes a decrease in bone mineral density (BMD) which is associated with loss of trabecular and cortical bone. Another major feature of age-related bone loss is the accumulation of bone marrow fat (Romero-m et al. 2021). Another factor that affects the motility of spermatozoa are microbial contaminants, ambient temperature, cold shock, storage, oxygen and Ca ion content in semen (S. Mugiyono et al. 2015).

Sperm viability

Aging had no significant impact ($p>0.05$) on sperm viability. In previous research with Sentul rooster, it was found that the number of sperm viability were 90.44% (Asmarawati et al. 2019) and 81.72-84.37% (Ariyanti et al. 2019). In old roosters the quality of semen (viability, membrane integrity, motility and volume) produced was low (Inyawilert et al. 2019). Availability of antioxidants plays an important role in the viability of spermatozoa. In old roosters there is an increase in lipid peroxidation in the seminal plasma as a result of increased production of glucocorticoid hormones. The increase in lipid peroxidation in old roosters that is not matched by the intake of antioxidants from feed causes a decrease in the viability of spermatozoa (Hayanti et al. 2022). Viability of spermatozoa can be increased by adding antioxidants as a semen diluent. Antioxidants that can be used include vitamin C. Vitamin C can be used to protect cells from damage caused by free radicals resulting from metabolism when spermatozoa are outside the body (Esguerra et al. 2020).

Based on the report of (Nugrahini et al. 2019), the viability of spermatozoa is related to the fluid balance in the spermatozoa cell membrane. High temperatures or the presence of cold shock can cause high spermatozoa mortality. The ability of spermatozoa to survive outside the male body is influenced by temperature, light, shelf life and nutrients in semen. The acidity of the semen plays an important role in the survival of the spermatozoa (Figure 2). Semen pH that is too high or low has an impact on the death of spermatozoa (Mustaqim et al. 2021). Storage of spermatozoa in the long term causes the viability of spermatozoa to decrease (Karen et al. 2020).

Abnormality

Aging had no significant impact ($P>0.05$) on abnormalities of spermatozoa. The abnormalities of spermatozoa in this study were lower than the abnormalities of Sentul rooster spermatozoa in the previous study which reached 6.87% (Junaedi et al. 2016) and 6.79-6.82% (Ariyanti et al. 2019). Spermatozoa abnormalities have an impact on fertility. Semen has good quality if the spermatozoa abnormality is less than 20% (Saleh & Mugiyono 2017; Haryuni et al. 2021).

Spermatozoa abnormalities indicating morphological disorders of spermatozoa including a double tail, a misshapen shape, head, and crooked (Rochmi et al. 2019). Abnormalities that occur in spermatozoa in principle can be classified into 2 categories which are called primary and secondary abnormalities. Abnormalities caused by low levels of gonadotropins and testosterone are called primary abnormalities (Esguerra et al. 2020). Low levels of gonadotropins and testosterone have an impact on the disruption of the process of spermatogenesis (Figure 1) so that spermatozoa become deformed. Secondary abnormalities are influenced by environmental factors. Secondary defects can occur when spermatozoa pass through the epididymis, mishandling during seollection and damage during preparation of preparations in the laboratory (Feyisa et al. 2018).

Spermatozoa abnormalities found in this study were mostly secondary abnormalities where spermatozoa defects were found on the tail and head. Table 2 shows that in old roosters spermatoza abnormalities were higher than in young roosters (48 weeks). Spermatozoa in old roosters tend to be easily damaged due to the process of spermatogenesis that is not optimal which has an impact on the formation of spermatozoa walls that are susceptible to damage. Aging that occurs in rooster causes a decrease in the production of the LH (*luteneizing hormone*) and FSH (*follicle stimulating hormone*) (Shi et al. 2019).

CONCLUSION

Aging that occurs in Sentul roosters causes reproductive disorders which are characterized by low sperm quality. The best quality spermatozoa are produced by Sentul roosters aged 58-68 weeks.

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Polymorphisms of the Thy-1 Gene in IPB D2 Chicken Line: Association with IgY and ND Antibody

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ABSTRAK

Putri NT, Murtini S, Ulupi N, Khaerunnisa I, Sumantri C. 2022. Keragaman gen Thy-1 pada galur ayam IPB D2: Asosiasi dengan antibody IgY dan ND. JITV 27(4):186-194. DOI: http//dx.doi.org/10.14334/jitv.v27i43029.

Gen Thy-1 berperan penting dalam respon imun, terutama dalam aktivasi sel T. Gen ini mengkodekan protein Thy-1 (CD90) yang berukuran 25–37 kDa. Gen Thy-1 memainkan peran penting dalam respon imun, terutama dalam aktivasi sel T. Tujuan penelitian ini mengidentifikasi keragaman gen Thy-1 dan asosiasinya dengan konsentrasi total IgY dan titer antibodi ND. Sebanyak 123 sampel ayam IPB D2 terdiri dari ayam IPB D2 G0 (generasi awal) 38 sampel dan ayam IPB D2 G2 (generasi kedua) 85 sampel umur 21 minggu digunakan pada penelitian ini. Identifikasi keragaman gen dilakukan dengan teknik PCR dan sekuensing DNA, sedangkan ELISA dan uji HI dilakukan untuk analisis IgY dan titer antibodi ND. Analisis data terdiri dari frekuensi genotipe, frekuensi alel, heterozigositas pengamatan, heterozigositas harapan, dan keseimbangan Hardy-Weinberg. Metode General Linear Model (GLM) dan uji Duncan digunakan untuk mengetahui asosiasi antara keragaman gen dengan konsentrasi IgY dan titer antibodi ND. Hasil penelitian menunjukkan dua titik SNP pada intron posisi basa g.2139 C>T dan g.2542 A>G. SNP g.2139 C>T dan g.2542 A>G bersifat polimorfik, namun tidak berada pada keseimbangan Hardy-Weinberg karena adanya seleksi. SNP g.2139 C>T signifikan berasosiasi ($P<0.05$) dengan konsentrasi total IgY. Semua titik SNP tidak berasosiasi ($P>0.05$) dengan titer antibodi ND. Tidak terdapat asosiasi antara haplotipe dengan konsentrasi total IgY dan titer antibodi ND pada penelitian ini. Kesimpulannya, hasil penelitian ini menemukan bahwa SNP g.2139 C>T bersifat polimorfik dan bisa dijadikan kandidat marker untuk konsentrasi total IgY tinggi. Namun, hal ini perlu divalidasi dengan analisis ekspresi gen (qRT-PCR) dan pada populasi yang lebih besar.

Kata Kunci: IgY, Imunitas, Ayam IPB D2, Titer Antibodi ND, SNP, Gen Thy-1

ABSTRACT

Putri NT, Murtini S, Ulupi N, Khaerunnisa I, Sumantri C. 2022. Polymorphisms of the Thy-1 Gene in IPB D2 Chicken Line: Association with IgY and ND antibody. JITV 27(4):186-194. DOI: http//dx.doi.org/10.14334/JITV.v27I43029.

The Thy-1 gene plays a crucial role in immunological response, particularly in the activation of T cells. This gene encodes a Thy-1 protein (CD90) with 25–37 kDa of size. This study aimed to find a variation in the Thy-1 gene in IPB D2 chickens and its association with the total IgY concentration and ND antibody titers. A total of 123 head of 21 week old IPB D2 chickens consisting of 38 IPB D2 G0 (first generation) chickens and 85 IPB D2 G2 (second generation) chickens were used. PCR methods and direct-DNA sequencing were used to identify the polymorphism of Thy-1 gene. ELISA and HI test were used to analyze total IgY concentration and ND antibody titers. Genotype frequency, allele frequency, observed heterozygosity, expected heterozygosity, and Hardy-Weinberg equilibrium were analized. The General Linear Model and Duncan's Multiple Range Test were used to evaluate association between gene polymorphism and IgY concentration and ND antibody titers. Results discovered two SNPs on the intron (g.2139 C>T and g.2542 A>G). Four haplotypes were created by combining two SNPs. The g.2139 C>T and g.2542 A>G were polymorphic, but not in the Hardy-Weinberg equilibrium because of selection. The SNP g.2139 C>T were significantly associated ($P<0.05$) with total IgY concentration. All SNPs were not associated ($P>0.05$) with ND antibody titer. There was no association between the haplotype polymorphism of the Thy-1 gene with the total IgY concentration and ND antibody titer. It was identified that SNP g.2139 C>T was polymorphic and could be used as a candidate marker for high total IgY concentration. However, further analysis in large population and a validation through gene expression (qRT-PCR) are needed to prove this hypothesis.

Key Words: IgY, Immunity, IPB D2 Chickens, ND Antibody Titer, SNP, Thy-1 gene

INTRODUCTION

IPB D1 chickens are composite local chickens designed by crossing male offspring from F1 Pelung-Sentul chickens with female offspring from F1 kampung chicken-broiler parent stock strain Cobb. The selection of the three types of local chickens (pelung, sentul, and kampung) and broiler chickens for crossbreeding was based on the genetic potential of each breed, including meat production, egg production, disease resistance, and rapid growth (Ulupi et al. 2016; Sumantri et al. 2020). The IPB D1 chickens were officially released as a new line of composite local chickens by the Ministry of Agriculture of the Republic of Indonesia by Decree number 693/KPTS/PK.230/M/9/2019. The purpose of IPB D1 chicken production is to spread it to the local community. One of the initiatives to generate IPB D1 chickens with strong disease-resistance features was the creation of the IPB D2 chicken line. The IPB D2 chicken is a candidate for a new line created by selecting IPB D1 chickens based on immunocompetence and body resistance traits including IgY concentration and ND antibody titer. Candidate IPB D2 chicken line has the advantage of a high IgY concentration above 10 mg/mL, and has an ND antibody titer above 3 log 2 HI units.

Immunoglobulin Yolk (IgY) and Newcasle Disease (ND) antibody are important indicators of disease resistance in chickens. IgY is the main antibody found in poultry. The IgY protein is mostly present in blood and the fluid portion of eggs in chickens. Similar to mammalian IgG, it serves to protect the chicks against pathogens (Munhoz et al. 2014; Gaetani et al. 2017). ND antibody titers are specific antibodies to neutralize ND virus infection. Antibodies can neutralize the virus by tying up with ND virus particles and preventing the virus from adhering to host cells (Kapczynski et al. 2013). Newcastle disease (ND) is a disease in poultry caused by avian paramyxovirus serotype 1 (APMV-1) viruses. The biggest impact of ND is more in rural areas and the production of chickens reared on a free range. In developing countries, local chickes is a very important asset to meet the protein needs of meat and eggs (Alexander 2000; Rahman et al. 2017).

The rearing of local chickens in the community usually uses a free-range pattern that pays less attention to biosecurity. This causes chickens to be more susceptible to viruses and bacteria that may cause disease and even death. The existence of IPB D2 chicken line which has superior disease resistance is very important and may help in improving the genetic quality of Indonesian local chickens. Efforts to improve the genetic quality of Indonesian chickens have been carried out with various breeding programs, such as by selection. Selection of disease resistance traits conventionally is less effective because the heritability value for disease resistance traits in chickens is relatively low (0.20) (Touko et al. 2021). Selection of a molecular approach by utilizing candidate genes as genetic markers is one way to increase the effectiveness of breeding programs (Psifidi et al. 2016). In the selection program and genetical point of view, the concentration of IgY and ND antibody titers can be employed as indicators. Several genes regulate the synthesis of IgY and ND antibodies (Al-Habib et al. 2020).

The Thy-1 (Thymocyte Differentiation Antigen 1) gene or also known as CD90 (Cluster of Differentiation 90) is one of the gene that play a role in antibody production. The Thy-1 gene encodes the protein Thy-1 (CD90), which is a glycoprotein with a molecular size 25-37 kDa. This glycoprotein is expressed on the outside of cell membranes, in a wide variety of cell types, including human fibroblasts, neurons, blood stem cells and endothelial cells, and murine T cells (Lung et al. 2010). The Thy-1 gene in chicken is situated on chromosome 24, measuring 4279 bp and consisting of a promoter region, four exons, three introns, and a flanking region, according to information from the National Center of Biotechnology (NCBI) with access number GenBank NC 006111.4. Thy-1 gene in mice plays a role in T cell activation. Thy-1 gene is similar to CD28 in its capacity to stimulate mouse T cell activation. Activation of mouse T cells via Thy-1 requires an intact and functional TCR/CD3 complex, as Thy-1 triggers do not activate TCR/CD3-deficient T cells (Haeryfar & Hoskin 2004). CD3 molecules are involved in activating T cytotoxic cells (Tc) and T helper cells (Th). Th cells are T cells that play a role in humoral immunity, which can activate naive B cells into plasma cells that are ready to produce specific antibodies against antigens (Wibawan & Soejoedono 2013).

The identification of the Thy-1 gene polymorphism and its association to disease resistance in candidate IPB D2 chicken line has never been reported . This study aimed to analyze the Single Nucleotide Polymorphism (SNP) of the Thy-1 gene and their associations with disease resistance traits, i.e. the total IgY concentration and ND antibody titer in the candidate IPB D2 chicken line. The results of this study are expected to be a candidate for genetic markers (marker assisted selection) to select and accelerate the production of IPB D2 chicken lines that have disease resistance.

MATERIALS AND METHODS

Animals and phenotypic parameters

This study obtained the approval of ethical clearance and animal welfare from the Animal Care and Use Committee (ACUC) of IPB University (Access No: 224-2021 IPB). In this study a total of 38 IPB

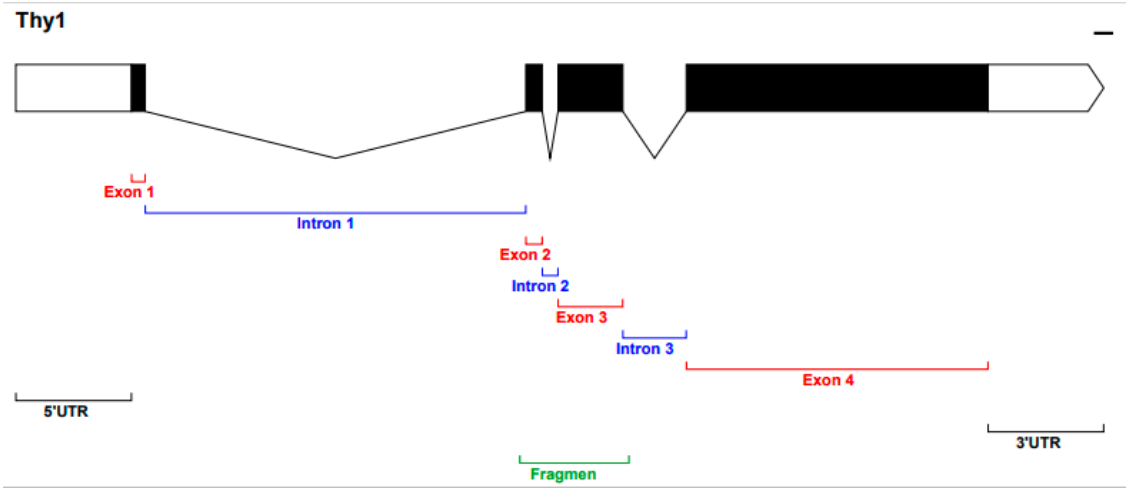

Figure 1. Reconstruction of Thy-1 gene structure in GenBank with NCBI access code: NC_006111.4 and the fragment target of this study (green)

D2 G0 (basic generation) and 85 IPB D2 G2 (second generation) chicken blood samples were collected from the Animal Breeding and Genetic Division of the Faculty of Animal Science, IPB University. Blood samples were collected at 21 weeks old. Those blood samples were drawn from the brachialis vein using a 3 mL syringe, then placed in a tube with 1.5 mL EDTA. The total IgY concentration was analyzed using indirect ELISA method (Hnasko & McGarvey 2015). The ND antibody titer were tested using HI test according to the OIE Manual of Standard Diagnostic Tests (Afonso et al. 2012).

DNA extraction

The phenol-chloroform extraction technique was used to extract DNA. Blood samples were put into a 1.5 mL tube (50 µL), then added with 0.2% NaCl (1,000 µL), and allowed to stand for 5 minutes. The supernatant was discarded after centrifuging samples that had been left to stand for 5 minutes at 8,000 rpm.

The resulting precipitate was added with 20 µL of proteinase K (5 mg/mL), 40 µL of 10% sodium dodecyl phosphate (SDS), and 30 µL 1×STE. The solution mixture was incubated for 2 hours at 55ºC and slowly homogenized. The mixed solution was added with 400 µL of phenol, 400 µL chloroform isoamyl alcohol (CIIA), and 40 µL 5 M NaCl while homogenized for 1 hour at room temperature. The solution combination was then centrifuged at 12,000 rpm for 5 minutes until a distinct DNA phase appeared.

The DNA phase was transferred to a new tube (1.5 mL) of 400 µL, then 800 µL of absolute ethanol and 40 µL of 5 M NaCl were added and stored in the freezer for one night. The DNA samples were centrifuged again for 5 minutes at 12,000 rpm to separate absolute ethanol. The supernatant was removed and the ethanol was allowed to evaporate fully at room temperature. After that, 100 µL of 80% TE were added, and the DNA sample was kept at -20ºC.

Primer design and DNA amplification

The primer used to amplify the THY-1 gene was designed using the Primer Designing Tools Program (http://www.ncbi.nlm.nih.gov/tools/primerblast). The primer sequences in this study were as follows: forward primer (F): 5'-CCTGGCTCACCACATCTCTC-3' and reverse primer (R): 5'-GAACTGGAGGCCATACCCTG-3'. The primer can amplify the exon 2 and 3 of Thy-1 gene along 566 bp, from 2615 bp (intron 1) to 3161 bp (intron 3) (Figure 1).

A total amount of 25 µL was used for DNA amplification. The amplified DNA sample was placed in a 0.2 mL tube with a sample volume of 0.3 µL. The DNA sample was then mixed with 11.9 µL of NFW, 0.15 µL of forward primer, 0.15 µL of reverse primer (all from IDT DNA, Singapore), and 12.5 µL of MayTaq HS RedMix (Bioline Reagents Ltd., London, UK). On an AB System PCR equipment (GeneAmp® PCR System 9700, Applied Bio Systems, Foster City, USA), the PCR procedure was carried out in four phases. The PCR conditions used have been modified, namely initial denaturation at a temperature of 95 ºC for 1 minute and carried out for one cycle. The second through fourth steps are repeated 35 times, with each cycle consisting of a 15-second denaturation process at 95 degrees Celsius, a 10-second annealing process at 60 degrees Celsius, and a 10-second extension process at 72 degrees Celsius.

Electrophoresis

A 45 g agarose powder was mixed with 30 mL 0.5 x TBE solution and microwaved for 3 minutes to make a 1.5 percent agarose gel. After cooling the agarose

solution for 2 minutes at 50 rpm with a magnetic stirrer, 1 µL of fluorosafe was added until the solution was homogenous. The gel solution was then poured into the gel tray for 30 minutes until it solidified into a gel. The gel was put into the electrophoresis machine, which was filled with 0.5 x TBE buffer. 3 µL amplicon was inserted into the gel well and migrated with 100 bp marker as much as 3 µL with a voltage of 100 V for ±35 minutes. The electrophoresis findings were then viewed using a UV Transilluminator equipment (Alpha Imager, Alpha Innotech, Santa Clara, USA). The PCR products of IPB D2 chicken samples were sequenced using the services of Macrogen, South Korea.

Statistical analysis

The results of the sequencing of the THY-1 gene fragment were analyzed using MEGA version 10.0., Bio Edit, and Finch TV programs. The sequencing results were then aligned based on the Gen Bank sequence reference, Gen Bank access code: NC_006111.4. Genetic polymorphism was analyzed using PopGen32 software based on allele and genotype frequencies, Hardy-Weinberg equilibrium, and heterozigosity. The haplotype was analyzed using DNAsp version 6.12.01 software. The association of the SNPs and haplotype of Thy-1 gene with total IgY concentration and ND antibody titer were analyzed by General Linear Model (GLM) procedure using SAS 9.2 software (SAS Institute, Cary, NC, USA) and the least means square values for genotypes and haplotypes were compared by Duncan's Multiple Range test (Zhang et al. 2019). Significant association were declared when P<0.05. The mathematical model follows:

$$\boldsymbol{Yij = \mu + Gi + eij}$$

where Yij is the dependent variable (total IgY concentration and ND antibody titer), µ is the mean square value, Gi is the effect of the I genotype, and eij is the random error.

RESULTS AND DISCUSSION

Detection of Thy-1 gene mutation of candidate IPB D2 chicken line

A single nucleotide polymorphism (SNP) is a pattern in the DNA sequence that displays variations in the base of a single nucleotide, generally one of two potential nucleotides at a given place. SNPs provide several benefits over other forms of polymorphisms when it comes to the genetic dissection of complex characteristics and disorders, as well as investigating population-based genes as they are found across the genome, such as in exons, introns, intergenic regions, promoters, and enhancers. SNPs in the coding area impact protein translation directly, SNPs in the intron region affect splicing, and SNPs in the promotor position affect gene expression (Asaf et al. 2014).

Identification of SNPs from the results of Thy-1 gene sequencing in IPB D2 chicken line candidate found two SNP points with nucleotide changes, i.e, g.2139 C>T and g.2542 A>G (Table 1). Both g.2139 C>T and g.2542 A>G are intron 2 and intron 3, respectively. The part of DNA translated in the protein synthesis process is the exon part, while the intron has an important role in the splicing process (Rogozin et al. 2012). Splicing is the process of removing all noncoding base sequences and combining coding base sequences to obtain mature mRNA molecules, and this process occurs after the transcription process. In the eukaryote genome, there is an alternative splicing phenomenon that aims to increase genome efficiency. Alternative splicing is the main mechanism that causes protein differences and regulates gene expression (Gunawan et al. 2017). According to Maston et al. (2006), Locus control Regions (LCRs) are a group of regulatory elements involved in regulation loci or entire gene cluster. LCRs have the ability to control gene expression from a distance and work regardless of place, and they can also be found inside an intron of the gene. Although the SNPs found in the intron region and introns are not translated into amino acids, mutations that occur in the intron region can also affect gene expression.

Genotype frequency, allelic frequency, and hardy-weinberg equilibrium Thy-1 gene

Table 2 shows the genotype frequency, allele frequency, and Hardy-Weinberg equilibrium of the Thy-1 gene in the candidate IPB D2 chicken line. The Thy-1 gene in the candidate IPB D2 chicken line was polymorphic, according to the findings. According to Allendorf & Luikart (2007), a gene is polymorphic if the allele frequency in a large population is below 0.99 and below 0.95 in a small population.

SNPs g.2139 C>T and g.2542 A>G positions had 3 genotypes. The frequency of the GG genotype at position g.2542 A>G (0.53) in the IPB D2 G0 population showed the highest value in all observed SNPs, it can be said that the probability of the emergence of the GG genotype was higher than other genotypes. The frequency of the CC genotype at position g.2139 C>T (0.54) in the IPB D2 G2 population showed the highest value in all observed SNPs. The difference in genotypes and alleles between the G0 and G2 populations is thought to be due to selection based on disease resistance indicators, thus affecting genetic diversity.

The Hardy-Weinberg equilibrium of the Thy-1 gene in the population was examined using chi-square (χ^2) to

see if the observed data diverged or did not depart from the predicted ratio according to the Hardy-Weinberg equilibrium law. The results showed that SNPs g.2139 C>T and g.2542 A>G were not in Hardy-Weinberg equilibrium. If the estimated value of χ^2 is less than the table value of χ^2, the population is considered to be in Hardy-Weinberg equilibrium. Due to the random merger of gametes in large populations, SNPs in Hardy-Weinberg equilibrium reveal that allele and genotype frequencies remain constant from generation to generation (Allendorf et al. 2013). If there is no selection, migration, mutation, or genetic drift, a big enough population will not change from generation to generation (Noor 2010). The imbalance that occurs indicates the existence of selection in the population. The maintenance of IPB D2 chickens is carried out intensively and selected based on disease resistance indicators so that the possibility of random mating from generation to generation is small.

Heterozygosity value Thy-1 gene

Heterozygosity is one of the essential parameters in determining genetic diversity in a population. High heterozygozygosity suggests a high level of genetic variety, whereas low heterozygozygosity indicates a low level of genetic variation. Inbreeding has occurred in a population when the observed heterozygosity value (Ho) is lower than the predicted heterozygosity value (He) (Zhao et al. 2019). SNPs g.2139 C>T and g.2542 A>G had a lower Ho value than the He value, indicating that inbreeding occurred. This is also consistent with the value of χ^2, which is not in Hardy-Weinberg equilibrium (Table 2). The observed heterozygosity value (Ho) of the three SNPs in this study was found to be low, which is below 50%. Javanmard et al. (2005) explained that a heterozygozygosity score of less than 50% suggests poor gene diversity in the studied population. The difference between the value of Ho and the value of He in the analyzed population can be used as an indicator of an imbalance of genotypes in the population. It is suspected that there is a degree of endogamy (marriage in groups) because it is the result of an intensive selection process (Machado et al. 2003). Inbreeding in a population could be make genetic defect. The number of individuals in a population greatly affects the level of inbreeding, where the smaller the population, the

Table 1. The position of the Thy-1 gene SNPs in the target sequence

No	SNPs position	Location in Thy-1 Gene	Mutation type
1	g.2139 C>T	Intron 2	Transition
2	g.2542 A>G	Intron 3	Transition

Table 2. Genotype frequency, allele frequency, and chi-square value of Thy-1 gene in candidate IPB D2 chicken line

No	SNP/Population	N	Genotype Frequency			Allele Frequency		χ^2
1	g.2139 C>T		CC	CT	TT	C	T	
	IPB D2 G0	38	0.40 (15)	0.13 (5)	0.47 (18)	0.46	0.54	21.30*
	IPB D2 G2	85	0.54 (46)	0.21 (18)	0.25 (21)	0.65	0.35	25.05*
2	g.2542 A>G		AA	AG	GG	A	G	
	IPB D2 G0	38	0.42 (16)	0.05 (2)	0.53 (20)	0.45	0.55	31.28*
	IPB D2 G2	85	0.49 (42)	0.05 (4)	0.46 (39)	0.52	0.48	70.65*

N= Number of samples, *significantly different at $\chi^2_{(0,05;1)} = 3,841$

Table 3. The value of observed heterozygosity (Ho) and expected heterozygosity (He) of the Thy-1 gene in the candidate IPB D2 chicken line

No	SNP/Population	Ho	He
1	g.2139 C>T		
	IPB D2 G0	0.132	0.504
	IPB D2 G2	0.212	0.460
2	g.2542 A>G		
	IPB D2 G0	0.053	0.501
	IPB D2 G2	0.047	0.502

Ho= Observed heterozygosity, He= Expected heterozygosity

greater the inbreeding pressure on a trait. The number of individuals in a population is related to the effective population size, especially those capable of reproduction (Praharani et al. 2018). The maintenance IPB D2 chickens for future breeding it can be done by increasing the population and interbreeding between generations. It aims to reduce inbreeding pressure and increase herterozygosity.

Association of the Thy-1 genotype with total IgY concentration in IPB D2 chickens

Immunoglobulins are glycoproteins secreted by plasma cells in response to exposure to antigens and are considered to be the product that largely influences humoral immunity (Amro et al. 2018). Chicken has three main classes of immunoglobulins (Ig), namely IgM, IgY (IgG), and IgA. Immunoglobulin Yolk (IgY) is the dominant systemic antibody in chicken blood. The association of Thy-1 genotype diversity with total IgY concentration is presented in Table 4.

The result (Table 4) showed that the total concentration of IgY in IPB D2 chickens ranged from 11–13 mg/mL. According to (Oberländer et al. 2020), the average concentration of IgY in blood serum ranges from 5–15 mg/mL, IgM is around 1–2 mg/mL, and IgA antibodies are around 3 mg/mL. Based on the average IgY concentration, IPB D2 chickens had a higher antibody concentration than native chickens (10.07 mg/mL) and purebred chickens (7.89 mg/mL) (Setiani 2016).

Statistical test results showed that SNP g.2139 C>T genotype CT was significantly different from genotype CC and TT ($P<0.05$) in G2 population while in G0 population was not significantly different ($P>0.05$). The CT genotype showed a higher total IgY concentration than the CC and TT genotypes. The change in base C to T did not cause an increase in the total IgY concentration in the TT genotype but in the CT genotype. SNP g.2139 C>T was associated with IPB D2 G2 chickens, while the G0 population had no association. This is presumably due to the selection process that has been carried out from G0 to produce G2 based on disease resistance indicators, so that the action of the Thy-1 SNP gene g.2139 C>T genotype CT appeared in IPB D2 G2 chickens. SNP g.2139 C>T has the potential to be a candidate genetic marker. However, this should be validated by further research through gene expression analysis (qRT-PCR). SNP g.2542 A>G in the two populations based on statistical tests were not significantly different ($P>0.05$) between genotypes, with the AA genotype having a higher total IgY concentration than the AG and GG genotypes.

Table 4. Association of the Thy-1 genotype diversity with total IgY concentration

SNP/Population	N	Total IgY Concentration (mg/mL) (N)		
g.2139 C>T		CC	CT	TT
IPB D2 G0	30	11.77±1.15 (12)	11.45±0.57 (4)	11.75±1.41 (14)
IPB D2 G2	81	$12.56±1.71^{b}$ (45)	$13.63±0.99^{a}$ (16)	$12.56±1.70^{b}$ (20)
g.2542 A>G		AA	AG	GG
IPB D2 G0	31	11.98±1.36 (12)	11.52±1.72 (2)	11.66±1.13 (17)
IPB D2 G2	83	12.96±1.77 (40)	12.48±0.75 (4)	12.60±1.80 (39)

N= number of samples; Numbers in the same line and followed by different superscripts indicate that they are significantly different ($P<0.05$), n: number of samples

Table 5. Association of Thy-1 genotype with ND antibody titers

SNP/Population	N	Titer Antibodi ND (log 2 HI unit) (N)		
g.2139 C>T		CC	CT	TT
IPB D2 G0	35	2.86±2.51 (14)	2.00±0.82 (4)	3.82±2.79 (17)
IPB D2 G2	77	1.58±1.45 (40)	1.22±1.44 (18)	1.37±1.50 (19)
g.2542 A>G		AA	AG	GG
IPB D2 G0	36	4.07±2.40 (14)	4.00±5.66 (2)	2.80±2.55 (20)
IPB D2 G2	77	1.59±1.60 (39)	0.67±1.15 (3)	1.34±1.28 (35)

N= number of samples

Table 6. Haplotype frequency in 566 bp Thy-1 gene amplicons in candidate IPB D2 chicken line

Haplotype	Nucleotide position		Frequency (%)	
	g.2139 C>T	g.2542 A>G	IPB D2 G0 (n)	IPB D2 G2 (n)
Haplotype 1	T	G	23.68 (9)	23.53 (20)
Haplotype 2	T	A	31.58 (12)	16.47 (14)
Haplotype 3	C	G	31.58 (12)	23.53 (20)
Haplotype 4	C	A	13.16 (5)	36.47 (31)

N= number of samples

Table 7. Association of the Thy-1 gene haplotype with IgY concentration and ND antibody titer in IPB D2 chickens

Haplotype	IPB D2 G0		IPB D2 G2	
	IgY mg/mL (N)	ND Antibody Titer (log 2 HI unit) (N)	IgY mg/mL (N)	ND Antibody Titer (log 2 HI unit) (N)
Haplotype 1	11.37±1.02 (7)	3.33±2.12 (9)	13.13±1.73 (30)	1.71±1.61 (28)
Haplotype 2	11.86±1.47 (10)	3.42±2.75 (12)	12.16±1.68 (20)	1.35±1.22 (17)
Haplotype 3	11.84±1.32 (10)	3.27±3.00 (11)	12.51±0.98 (11)	1.23±1.54 (13)
Haplotype 4	12.13±1.73 (4)	3.5±3.42 (4)	12.99±1.84 (20)	1.26±1.37 (19)

N= number of samples

Association of the Thy-1 genotype with ND antibody titer in IPB D2 chickens

An ND antibody titer of above 4 log 2 HI units was categorized as a protective level for vaccinated chickens, while an ND antibody titer below 3 log 2 HI units was categorized as non-protective (Rahman et al. 2017). Based on the results of the study (Table 5), the IPB D2 chicken with vaccination at the age of 3 days and 3 weeks obtained below protective level, except AA genotype and AG genotype of SNP g.2542 A>G in population IPB D2 G2. The second generation (G2) IPB D2 chickens had lower ND antibody titers than the basic generation (G0) or were below the protective level.

In some cases, ND antibody titers were found under the protective category. Lower ND antibody titers may be due to vaccination failure or may be due to the presence of parental antibodies being passed on to the offspring that neutralize the vaccine virus. The higher the ND antibody titer, the lower the susceptibility to clinical infection with the ND virus (Rahman et al. 2017). The relationship between Thy-1 gene polymorphism and ND antibody titer based on statistical tests, showed that the two SNP points found had no significant effect (P>0.05) or were not associated. This study showed that genotype diversity did not affect the ability of IPB D2 chickens to producing specific ND antibodies.

Haplotype

A haplotype is a nucleotide sequence that reflects the genetic variation of each individual within a species (Permana et al. 2015). Two SNP points of the Thy-1 gene were found in IPB D2 chickens, forming four haplotypes. The frequency of the Thy-1 gene haplotype in IPB D2 chickens can be seen in Table 6. Haplotype 4 is a nucleotide sequence that is the wild type according to the Gene Bank (NC_006111.4) with a frequency of 13.16% (IPB D2 G0) and 36.47% (IPB D2 G2). The highest haplotype frequency in IPB D2 G0 was found in haplotype 2 and 3 (31.58%), while the lowest haplotype frequency was found in haplotype 4 (13.16%). The highest haplotype frequency in IPB D2 G2 was found in haplotype 4 (36.47%), while the lowest haplotype frequency was found in haplotype 2 (16.47%). The g.2139 C>T base mutation was found in haplotypes 1 and 2. The g.2542 A>G base mutation was found in haplotypes 1 and 3. Haplotype 1 showed a combination of SNPs at bases g.2139 C>T and g.2542 A>G.

Statistical test results showed that there was no relationship between haplotype and total IgY concentration and ND antibody titer in both G0 and G2 populations (P>0.05) or not associated. Haplotype 1 in population G2 is the haplotype with the highest total concentration of IgY and ND antibody titer and is the haplotype with a combination of SNPs that undergoes mutations, while in population G0 haplotype 4 is the haplotype with the highest total concentration of IgY and ND antibody titer and is the haplotype with the combination of SNPs. without mutation or wild type.

According to (Setyawati et al. 2019), the total IgY concentration is categorized as high if the value is 9.3 mg/mL, while according to Rahman et al. (2017), the ND antibody titer is said to be protective if the antibody titer value is 4 log 2 HI units. Based on Table 6, the results of the average concentration of total IgY on

haplotypes 1, 2, 3, and 4 were in the high category. The average ND antibody titer in the G0 population was close to the protective level, while in the G2 population the ND antibody titer decreased or was below the protective level. Antibodies can be obtained from maternal antibody or direct exposure to the disease. The low value of ND antibody titer in IPB D2 G2 chickens does not mean that the chicken is not resistant to ND disease, but it is suspected that there is no exposure to ND virus in chicken rearing so that it does not trigger the production of ND antibodies. According to Bernardini et al. (2017), the maternal antibody will progressively decline with age and then rise again, depending on the exposure to the illness from the environment. Antibody production is induced by exposure to disease through intricate cell signalling (APC, T cells, and B cells). The development of antibodies and the immunological response of chickens can both be affected by communication problems or mistakes (Al-Habib et al. 2020).

The Thy-1/CD90 gene belongs to the CD (Cluster of Differentiation) group. CD is a protein that functions as a receptor or ligand for agents that expose the body. Not all CD molecules function in cell signaling (communication between cells), some CD molecules only play a role in cell adhesion (Wibawan & Soejoedono 2013). The function of the Thy-1 gene is not fully understood, especially in chickens. Chen et al. (2005) stated that the Thy-1 gene can also function as a cell adhesion molecule. The Thy-1 gene in mice has been implicated in mediating thymocyte adhesion to thymic epithelial cells, regulating thymocyte apoptosis, and in modulating neurite growth in neurons.

CONCLUSION

The results showed two SNPs of the Thy-1 gene in the candidate IPB D2 chicken line, i.e, g.2139 C>T and g.2542 A>G in intron 2 and intron 3. All SNPs are formally defined as polymorphic. The CT genotype of SNP g.2139 C>T can be used as a candidate marker for high total IgY concentration. However, further analysis is needed, namely validation through gene expression (qRT-PCR). No SNPs were associated with ND antibody titer. The combination of 2 SNPs formed four haplotypes. There was no association between the haplotype polymorphism of the Thy-1 gene with the total IgY concentration and ND antibody titer in IPB D2 chickens in this study.

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Analysis of Meat Mineral Content in Cemani Chicken with Homozygous (*Fm/Fm*) and Heterozygous (*Fm/fm*$^{+}$) Genotypes

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ABSTRAK

Safitry RS, Dharmayanthi AB, Kinoshita K, Akiyama T, Darwati S, Kostaman T, Sopiyana S, Khaerunnisa I, Sumantri C. 2022. Analisa kandungan mineral daging pada ayam Cemani dengan genotipe homozigot (*Fm/Fm*) dan heterozigot (*Fm/fm*$^{+}$). JITV 27(4):195-203. DOI : http://dx.doi.org/10.14334/jitv.v27i4.3075.

Ayam Cemani adalah ayam asli Indonesia dengan hiperpigmentasi hitam pada bulu, kulit, paruh, jengger dan daging. Hiperpigmentasi hitam pada ayam Cemani ini dikenal dengan istilah fibromelanosis. Fibromelanosis pada ayam Cemani bersifat semidominant yang menghasilkan dua genotipe: homozigot (*Fm/Fm*) dan heterozigot (*Fm/fm*$^{+}$). Warna hitam pada daging ayam Cemani dapat menunjukkan adanya kandungan mineral daging yang lebih tinggi dibandingkan daging ayam pada umumnya. Penelitian ini bertujuan untuk mendeteksi genotipe homozigot (*Fm/Fm*) dan heterozigot (*Fm/fm*$^{+}$) serta mengetahui kandungan mineral pada ayam Cemani dengan genotipe homozigot (*Fm/Fm*) dan heterozigot (*Fm/fm*$^{+}$). Pada populasi ayam cemani (n= 32), genotipe alel spesifik *Fm* dideteksi dengan Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) dan enzim restriksi *Mlu*I. Kandungan mineral ayam Cemani yang diuji adalah Fe, Zn, Mn, dan Se. Hasil penelitian menunjukkan bahwa ayam Cemani homozigot (*Fm/Fm*) memiliki kandungan mineral Fe dan Zn yang lebih tinggi. Namun ayam Cemani homozigot (*Fm/Fm*) tidak berbeda kandungan mineral Se dan Mn dengan ayam Cemani heterozigot (*Fm/fm*$^{+}$). Penelitian ini menemukan bahwa genotipe ayam Cemani yang berbeda memiliki komposisi mineral yang berbeda. Di masa depan, analisis ini mendukung pemilihan galur ayam dengan kadar antioksidan tinggi.

Kata Kunci: Ayam Cemani, *Fibromelanosis*, Kandungan Mineral, PCR–RFLP

ABSTRACT

Safitry RS, Dharmayanthi AB, Kinoshita K, Akiyama T, Darwati S, Kostaman T, Sopiyana S, Khaerunnisa I, Sumantri C. 2022. Analysis of meat mineral content in Cemani chicken with homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$) genotypes. JITV 27(4):195-203. DOI : http://dx.doi.org/10.14334/jitv.v27i4.3075.

Cemani chicken is an Indonesian native chicken with black hyperpigmentation on feathers, skin, beak, comb, and flesh. Hyperpigmentation in chickens is called Fibromelanosis. Fibromelanosis in Cemani chickens is semi–dominant, producing two genotypes: homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$). Cemani chicken meat's black color may indicate a higher mineral content than regular chicken meat. The study's is aims are to detect genotype homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$) mutations and to determine the mineral content of Cemani chickens with homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$) genotypes. In the Cemani chicken population (n = 32), the *Fm–specific* allele genotype was detected using a Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) and the *Mlu*I restriction enzyme. The mineral contents of Cemani chicken tested were Fe, Zn, Mn, and Se. The results showed that homozygous Cemani chickens (*Fm/Fm*) had higher Fe and Zn mineral content. However, the homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$) Cemani chicken were not different in mineral content of Se and Mn. This study found that different genotypes of Cemani chicken had different mineral compositions. In the future, this analysis supports the selection of chicken strains with high antioxidant levels.

Key Words: Cemani Chicken, *Fibromelanosis*, Mineral Content, PCR–RFLP

INTRODUCTION

Local chickens in Indonesia comprise native chickens and adapted chickens that can be grouped into broiler, laying, dual–purpose and ornamental types (Nataamijaya 2010; Kartika et al. 2016). Ayam Cemani is a native chicken originating from the Kedu region, Temanggung Regency, Central Java (Habsari et al.

2019). The main locations for cemani chicken are in Kedu village, Beji village and Kahuripan village, Kedu region, Temanggung Regency and its distribution in in Kalikuto, Magelang, Central Java (Sartika et al. 2016) Ayam Cemani is distinguished by the blackness of its entire body and internal organs are black. According to Amelia (2019), Cemani chickens was originated from a population of Black Kedu chickens selected for their black color (feathers, skin, cockscombs, beaks, shanks) and jagged single cockscomb forms. The uniqueness of cemani chicken with black color in whole body and its internal organ increases the selling price in the market. In addition, people also believe that ayam cemani can be used for traditional medicine and rituals (Alfauzi & Hidayah 2020).

Black pigmentation in the birds is called *Fibromelanosis*. Abnormal accumulation of eumelanin in tissues causes the *Fibromelanosis* trait in the chickens body and its internal organ (Hutt 1949; Lukanov & Genchev 2013). The presence of a duplication rearrangement in the genomic region containing the Endhotelin3 gene (*EDN3)* on chromosome 20 cause of *Fibromelanosis* (*Fm*) mutation in Cemani and Silkie chickens (Shinomiya et al. 2012; Dorshorst et al. 2010; Dharmayanthi et al. 2017). *EDN3* plays a role in the formation of melanocyte–producing proteins. *EDN3* causes the excessive expression of melanocyte–forming proteins in Cemani chickens, resulting in a black color in the whole body and internal organs of Cemani chickens (Dorshorst et al. 2010).

Shinomiya et al. (2012) crossed White Silkie (WS) chicken (*Fibromelanosis* traits) with Black Minorca Minorca (BM, wild–type phenotype). They found that the F1 between Black Minorca (BM) and WS resulted in different degrees of pigmentation even though they exhibited the *Fm* phenotype. The pigmentation in the internal tissues of the F1 progeny of BM was significantly lighter than that in WS, indicating that hyperpigmentation is more severe in the *Fm/Fm* homozygote than in the *Fm/fm*$^+$ heterozygote**.** A recent study established a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method that for detecting homozygous (*Fm*/*Fm*) and heterozygous (*Fm*/*fm*$^+$) individuals with the *Fm* mutation in the Cemani population (Dharmayanthi et al. 2017; Dharmayanthi et al. 2022).

Pigmentation of the skin and meat is a complex biological process. Ecological factors, diet, and heredity can all have an impact on skin color (Wu et al. 2021). Cemani chicken possesses a meat color that differs from that of regular chickens in general such as broiler or layer chicken. Cemani chickens have a pale black and deep black meat color. Meanwhile, color of the meat in broiler chickens varies greatly between individuals, ranging from slightly yellow to white (Hidayah et al. 2019). Coloration is a phenotypic trait associated with diverse adaptive functions such as thermoregulation, camouflage, and mate selection (Hamilton et al. 2013). Mitić et al. (2012), in a study of heavy metal content of smoked meat, found that meat with a darker color contains almost twice the iron content compared with light–color meat. The iron content in Cemani chicken, an antioxidant cofactor, has not been explored. Other antioxidant minerals are manganese, selenium, and zinc (Rusli 2016). Antioxidants are essential for the body to neutralize free radicals and prevent damage to normal cells, proteins, and fats (Hasanah 2015).

As a result, research into the mineral content of meat in Cemani chickens with homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^+$) genotypes, particularly Fe, is required to understand the differences in mineral composition in Cemani chickens with homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^+$) genotypes. The study's aims are to detect homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^+$) genotypes in the Cemani population and to determine the mineral composition of Fe, Zn, Mn, and Se in each Cemani chicken genotype.

MATERIALS AND METHODS

Blood Sampling

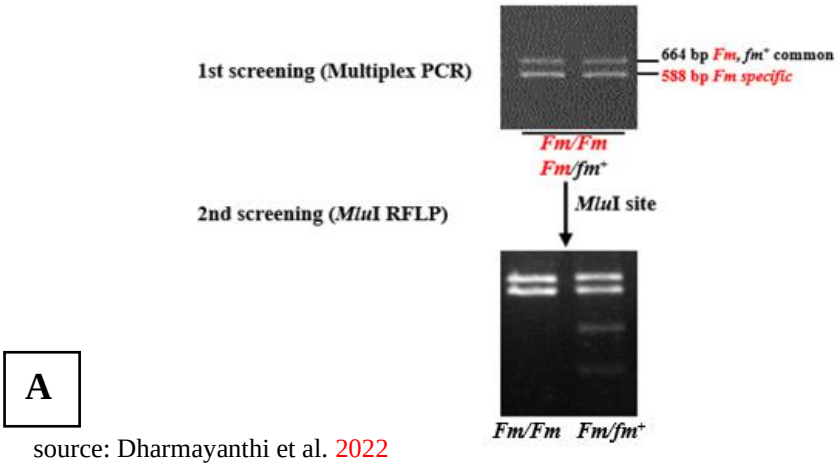
In total, we used 32 blood samples from Cemani chickens; 16 blood samples from PT. Sumber Unggas Indonesia (SUI), Parung–Bogor, Indonesia and 16 blood samples from the Indonesian Research Institute for Animal Production (IRIAP), Ciawi–Bogor, Indonesia. We applied 70% alcohol to the axillary vein on the wing before blood was taken. Blood samples (as much as ±3 mL) were taken using a syringe. The blood samples were placed into a tube containing EDTA and stored in a refrigerator at 4°C before DNA extraction.

DNA Extraction

DNA extraction from Cemani chicken samples was performed using the Genomic DNA Mini Kit (Blood/Cultured Cell) following the kit's protocol.

DNA amplification

The amplification of *Fm*-specific allele was carried out by multiplex PCR technique combining three primers. Three primers used in this study followed previous study (Dharmayanthi et al. 2022) on non-coding genes of Fibromelanosis region: forward primer WT_F : 5'-TTCAGCAGCATTCACTGAAGGC-'3 and reverse primer FMdupl_664_R: 5'-TTCAGCAGCATTCACTGAAGGC-'3 used to amplify 664 bp length of *fm*$^+$ common; forward primer WT_F: 5'-TTCAGCAGCATTCACTGAAGGC-'3 and reverse



source: Dharmayanthi et al. 2022

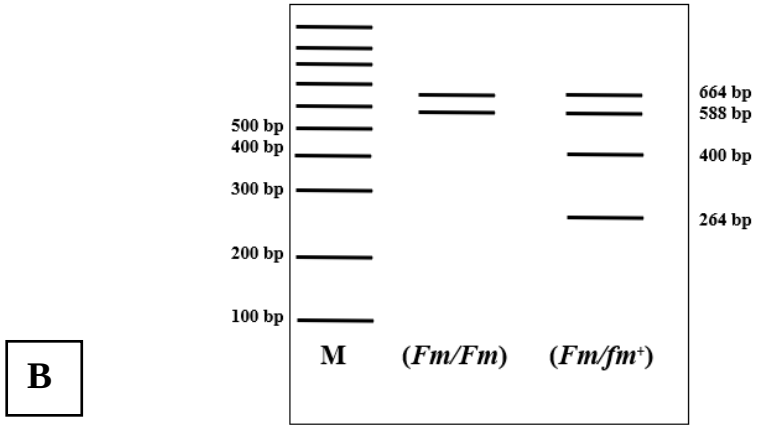


Figure 1. Determination of homozygous (*Fm*/*Fm*) and heterozygous (*Fm*/*Fm*$^+$) genotypes with restriction enzymes *(MluI).* (A) PCR–RFLP amplification workflow to identify genotypes (source: (Dharmayanthi et al. 2017; Dharmayanthi et al. 2022) (B). Visualization results from PCR–RFLP

primer FMspec_588_R: 5'-TGTCCATCTCACATTGC-'3 used to amplify 588 bp length of *Fm* spesific. DNA samples were mixed in a total volume 15ul consisted of primer WT_F 0.4 µL; primer FMdupl_664_R 0.2 µL; primer FMspec_588_R 0.2 µL; nuclease–free water 5.7 µL; and My Taq^{TM} HS Red Mix 7.5 µL. The sample is put into a PCR machine. The amplification process process comprised predenaturation (95°C) for 1 min, followed by 35 cycles of denaturation (95°C) for 15 s, annealing (60°C) for 10 s, and extension (72°C) for 10 s.

PCR–RFLP and genotyping

To identify the *Fm* homozygous (*Fm*/*Fm*) and heterozygous (*Fm*/*fm*$^+$) genotypes of Cemani chickens, a PCR–RFLP genotyping assay was performed using a restriction enzyme (*Mlu*I). The multiplex PCR products were digested at 37°C for 16 h with 10 U of the *Mlu*I restriction enzyme. The digested product was loaded into electrophoresis was performed in 2% agarose gel with a voltage of 50 V for 50 min and then the digested product was visualized using a UV transilluminator. We identified the Cemani chicken genotype based on a previous study by Dharmayanthi et al. 2022 (Figure 1A), and the visualization results are presented in Figure 1B.

Mineral content analysis

Mineral content analysis was performed using the Atomic Absorption Spectrophotometer (AAS) method based on the AOAC (2015) on a Cemani chicken's thigh. The meat's mineral contents tested were Fe, Zn, Mn, and Se. This test was conducted using the analysis services of the Laboratorium Terpadu LSSMKP IPB (Ilfa IPB).

Data analysis

Genotyping data were analyzed by calculating the genotype and allele frequency, Hardy–Weinberg Equilibrium, observed heterozygosity (Ho), and expected heterozygosity (He) using the PopGene 32 program. Allele and genotype frequencies were

calculated using the Nei & Kumar (2000) formula as follows:

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

where x_i is the frequency of the i–th allele, x_{ii} is the frequency of the i–th genotype; 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.

The χ^2 test was used to examine the Hardy–Weinberg Equilibrium, which was calculated using the Nei & Kumar (2000) formula as follows:

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

where χ^2 is the chi–square test, O is the observation value, and E is the expectation value

Genetic diversity was determined from the observed (Ho) and expected (He) heterozygosity values. Observed heterozygosity (Ho) was calculated using the Weir (1996) formula and expected heterozygosity was calculated using the Nei & Kumar (2000) formula as follows:

$$H_0 = \sum_{i \neq j}^{N} \frac{N_{1ij}}{N} \qquad H_e = 1 - \sum_{i=1}^{q} x_i^2$$

where H_0 is the observed heterozygosity value, N_{1ij} is the number of individuals with heterozygous, N is the observed number of individuals, H_e is the expected heterozygosity value, x_i is the frequency of allele, and q is the allele number.

T–test was used to compare the genotypes pure Cemani chicken with homozygous *Fm/Fm* and Cemani chicken with incomplete *Fm* trait (heterozygous *Fm/fm*$^+$) with a 95% confidence interval were calculated using the Mattjik & Sumertajaya (2013) formula as follows:

$$t = \frac{(\bar{x}_A - \bar{x}_B)}{\sqrt{\frac{S_{A\,B}^2}{n_A} + \frac{S_B^2}{n_B}}}$$

where $\bar{x}_A$is the mean of sample A, $\bar{x}_B$ is the mean of sample B, n_A is the number of individual sample A, n_B is the number of individual sample B, S_A is the standard deviation of sample A, S_B is the standard deviation of sample B.

RESULTS AND DISCUSSION

Genotyping the Ayam Cemani population

The PCR amplification results for identifying the *Fm* allele in Cemani chickens showed that two fragment lengths, namely, 664 and 588 bp, were amplified in all Cemani chicken samples (Figure 2A). The results of RFLP using the *Mlu*I restriction enzyme showed two different genotypes found in the Cemani chickens (Figure 2B). PCR–RFLP identified 21 Cemani chickens that were homozygous for the *Fm*/*Fm* genotype and produced two undigested bands (664 bp and 588 bp), and 11 Cemani chickens with heterozygous *Fm/fm*$^+$ genotype produced four bands of 664, 588, 400, and 264 bp (Table 1).

Genotype frequency, allele frequency, and heterozygosity values

The PCR–RFLP results showed that there were two genotypes (homozygous *Fm/Fm* and heterozygous *Fm/fm*$^+$) and two alleles (*Fm* allele and wild type *fm*$^+$) in this study. The values of genotype frequency, allele frequency, heterozygosity, and Hardy–Weinberg test on the *Fm*–specific alleles shown in Table 2. The genotype frequency of 11 individual SUI Cemani chickens out of 16 SUI Cemani chickens showed that half of the Cemani chicken population possessed an incomplete *Fm* trait or heterozygous *Fm/fm*$^+$ (69%) and 5 individual SUI Cemani chickens were pure Cemani chickens or homozygous *Fm/Fm* (31%). Meanwhile, all individual Cemani chickens in the IRIAP were pure Cemani chickens or homozygous *Fm/Fm* (100%). The frequency of alleles in the Cemani chicken population in SUI is was less than 0.99, indicating that the Cemani population is polymorphic. Meanwhile, the Cemani chicken population in IRIAP was 1, and population of Cemani chickens in IRIAP is valued at 1, the population can be assumed to be monomorphic. According to Allendorf et al. (2013), polymorphism is a population with an allele frequency of less than 0.99. Thus, polymorphism in SUI Cemani chickens indicated a population, whereas monomorphism in IRIAP Cemani chickens indicated a nondiverse population.

Hardy–Weinberg equilibrium analysis is used to determine the presence of evolution in a population (Panggabean 2016). This analysis was calculated using the chi–square test (χ^2). The Hardy–Weinberg results showed that the IRIAP Cemani chickens in IRIAP had a value of 0.000, which means that that they cannot be analyzed because only one allele in existx the population. By contrast, SUI Cemani chickens were not significant (P>0.05), indicating gene mutations, migration, and selection in the population. Factors influencing Hardy–Weinberg's condition include random mating, no gene mutations, no migration, and no selection (Nugroho et al. 2016). In a population, He and Ho calculations are used to estimate genetic diversity and to select livestock for the next generation (Putri et al. 2021). In this study, the Ho value was 0.68 and that of He was 0.46 in SUI Cemani chickens, whereas in IRIAP Cemani chickens, the Ho and He values were 0. Populations with heterozygosity of below 0.5 or closer to the margin indicate a low

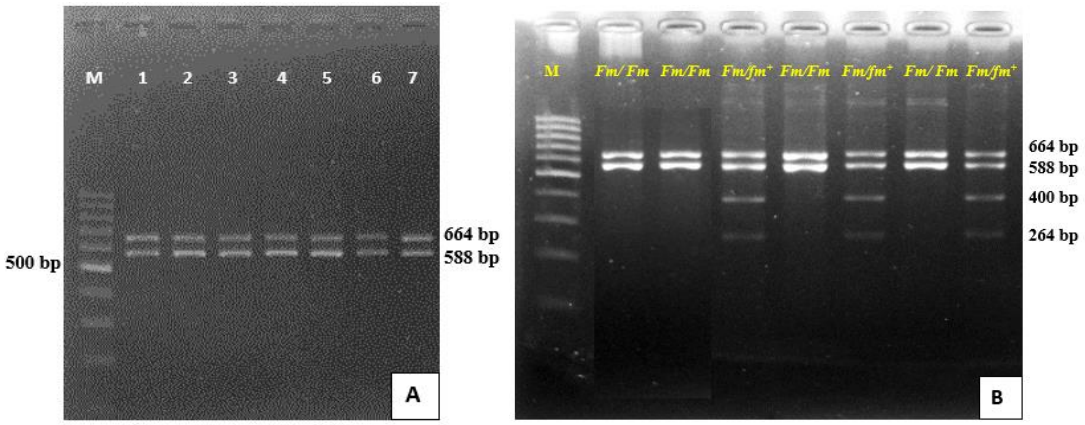


Figure 2. Gel electrophoresis of the PCR–RFLP of the *Fm*–specific allele in Cemani chickens. (A) PCR products amplified by multiplex–PCR primers. (B) *Mlu*I–digested pattern of multiplex–PCR primer–amplified products

Table 1. The result of Cemani chickens genotyping using PCR-RFLP

No	IRIAP Cemani chickens (n = 16)			SUI Cemani chickens (n = 16)		
	Sample Code	Phenotype (skin color)	Genotypes	Sample Code	Phenotype (skin color)	Genotypes
1	47	Deep black	*Fm/Fm*	CM1	–	*Fm/fm*$^+$
2	84	Pale black	*Fm/Fm*	CM2	–	*Fm/Fm*
3	85	Pale black	*Fm/Fm*	CM3	–	*Fm/fm*$^+$
4	88	Deep black	*Fm/Fm*	CM4	–	*Fm/Fm*
5	192	Deep black	*Fm/Fm*	CM5	–	*Fm/fm*$^+$
6	193	Deep black	*Fm/Fm*	CM6	–	*Fm/fm*$^+$
7	194	Deep black	*Fm/Fm*	1A	–	*Fm/Fm*
8	199	Deep black	*Fm/Fm*	5A	Pale black	*Fm/fm*$^+$
9	200	Pale black	*Fm/Fm*	12A	Pale black	*Fm/fm*$^+$
10	1019	Pale black	*Fm/Fm*	14A	Pale black	*Fm/fm*$^+$
11	1037	Pale black	*Fm/Fm*	21A	Pale black	*Fm/fm*$^+$
12	1061	Pale black	*Fm/Fm*	25A	Pale black	*Fm/fm*$^+$
13	1072	Deep black	*Fm/Fm*	26A	Pale black	*Fm/fm*$^+$
14	1096	Deep black	*Fm/Fm*	27A	Deep black	*Fm/Fm*
15	21018	Pale black	*Fm/Fm*	30A	Pale black	*Fm/fm*$^+$
16	21077	Pale black	*Fm/Fm*	33A	Deep black	*Fm/Fm*

Table 2. Genotype, allele frequency, heterozygosity, and Hardy–Weinberg test of the *Fm*–specific allele

Types of Chickens	N	Genotype Frequency		Allele Frequency		H_o	H_e	χ^2
		Fm/Fm	*Fm/fm*$^+$	*Fm*	*fm*$^+$			
SUI Cemani chickens	16	0.3125 (5)	0.6875 (11)	0.66	0.34	0.6875	0.4657	3.9^{ns}
IRIAP Cemani chickens	16	1 (16)	0	1	0	0	0	na

Fm/Fm = homozygous Cemani, *Fm/fm*$^+$ = heterozygous Cemani, Ho= observed heterozygosity; He= expected heterozygosity; χ^2= chi–square test ; ns= non–significant (P > 0.05), na= not analyzed

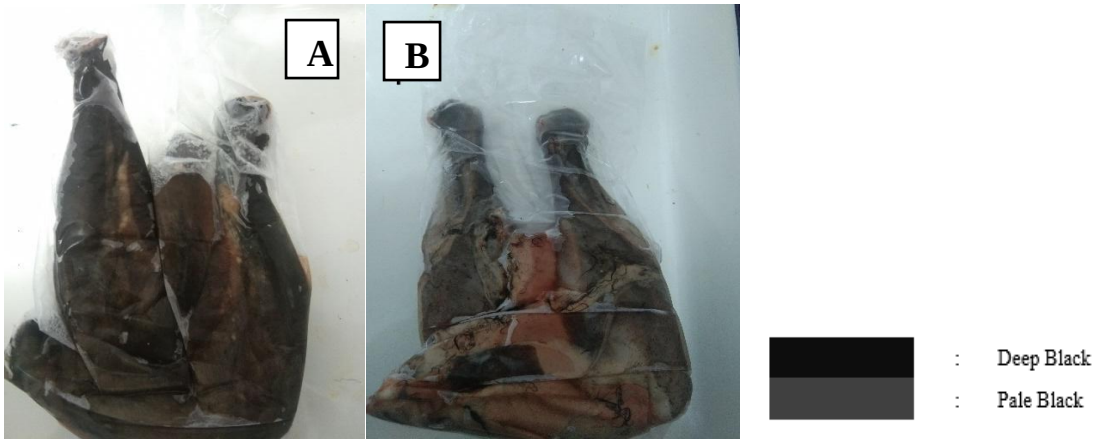


Figure 3. Degree of blackness in the meat of Cemani chickens. (A) Deep black. (B) Pale black

Table 3. Mineral content of Cemani chicken meat

Mineral (mg/kg)	Genotype		P–value
	Pure Cemani (homozygous *Fm/Fm*) (n = 21)	Cemani chickens incomplete *Fm* trait (heterozygous *Fm/fm*$^+$) (n = 11)	
Iron	22.94±9.67	11.55±2.31	0.001*
Zinc	16.43±5.86	14.60±4.66	0.000*
Manganese	0.26±0.19	0.52±0.61	0.378
Selenium	0.40±0.64	2.43±1.88	0.092

*= significant (P<0.05)

variation (Sheriff & Alemayehu 2018). Unlike IRIAP Cemani chickens, SUI Cemani chickens have a higher level of heterozygosity, indicating that the Cemani population in SUI has a high genetic variation. In addition, the significant difference between Ho and He shows that the population has genetic imbalances (Tambasco et al. 2003; Harbison & Nguyen 2017). The population mated randomly because Ho was higher than He (Sharma et al. 2016). Random mating in the Cemani population in SUI suggests that a large number of Cemani chickens have incomplete *Fm* traits.

Degree of the blackness of Cemani chicken meat

In this study, we divided the blackness level of Cemani chicken's into two, namely, deep black and pale black (Figure 3). The results of the observations of 25 Cemani chicken meats indicated that Cemani chickens with deep black and pale black were 40% and 60%, respectively. The black color difference in Cemani chickens is caused by the semi–dominant *Fibromelanosis* gene (Shinomiya et al. 2012). Gene mutations control color expression in chickens (Zhang et al. 2015). In addition, a recent study discovered that the different genotypes in the Cemani population, i.e. homozygous *Fm/Fm* and heterozygous *Fm/fm*$^+$, have differing levels of blackness (Dharmayanthi et al. 2017; Dharmayanthi et al. 2022). Pigmentation in poultry caused by the amount of melanin and muscle size. The higher level of melanin pigments in the skin could darken the skin because the skin color was primarily controlled by the melanin content (Yamaguchi et al. 2007). There are parts of Cemani chicken with a pale black color that have dark black meat, particularly the meat under the skin. The different level of melanin pigment probably responsible for this different deep black color in the meat. In the skin, The higher level of melanin pigments in the skin could darken the skin because the skin color was primarily controlled by the melanin content (Yamaguchi et al. 2007). In the study of Nganvongpanit et al. (2020), the melanin pigmentation in Thailand black bone chicken was present in all of the tissue layers of most organs. However, the distribution of melanin pigmentation in the organs was found to be different except in some tissue samples, such as those obtained from the liver. In the muscle, the uneven distribution of darkness was also due to the accumulation of the different pigment melanin (Kriangwanich et al. 2021). Moreover, different the age of chicken is also caused the different melanin content in breast, drumstick, wing and skin samples (Buasap et al. 2021). This dark–colored chicken meat had higher total pigment, myoglobin, iron and redness (Buasap et al. 2021).

Mineral content of Cemani chicken meat

On the basis of the PCR–RFLP results, the meat mineral content in Cemani chickens was divided into pure Cemani chicken with homozygous *Fm/Fm* and Cemani chicken with incomplete *Fm* trait (heterozygous *Fm/fm*$^+$). Table 3 shows the results of the mineral content analysis of iron (Fe), zinc (Zn), manganese (Mn), and selenium (Se) in the Cemani chicken thigh meat.

The four mineral contents in Cemani meat were chosen for this study because they function as antioxidants in the body. Antioxidants are the substances that inhibit oxidation or "free radical scavengers" as antioxidants form minor reactive species via radicals (Neha et al. 2019). The function of Iron (Fe) in metabolic processes is to activate the enzymes peroxidase and catalase. The enzyme catalase stimulates the hydrolysis of hydrogen peroxide into oxygen and water molecules, whereas peroxidase is used with hydrogen peroxide to stimulate the oxidation of organic and inorganic compounds (Al–Lamei et al. 2020). Peroxide reactions can be characterized by oxidative halogenation and dehydrogenation, oxygen transport and hydrogen peroxide decomposition (Shivakumar et al. 2017). The rest of the Fe metabolism is stored in the body, including the liver, bone marrow, spleen, and skeletal muscles (Prasetiyo et al. 2014). Meanwhile, Zn plays a role in the body's digestion, carbohydrate metabolism, and nucleic acid production of over 70 enzymes (Imanto et al. 2018). Mn serves as a synthesis of carbohydrates, mucopolysaccharides, and enzyme systems for the growth and reproduction of livestock (Prasetiyo et al. 2014). In addition, the Se also plays an essential role in the body's defence system by helping neutrophil activity in the primary defense to protect the body from bacterial infections (Pratiwi et al. 2018). Table 3 shows that samples of pure Cemani chickens with homozygous *Fm/Fm* were significantly different from Cemani chickens incomplete *Fm* trait with heterozygous *Fm/fm*$^+$ in Fe (22.94 mg/kg) and Zn (16.43 mg/kg). However, Cemani chickens with incomplete *Fm* trait (heterozygous *Fm/fm*$^+$) were not significantly different from Cemani homozygous *Fm/Fm* in Mn (0.52 mg/kg) and Se (2.43 mg/kg) minerals content. The results of this study are in accordance with those Mitić et al. (2012), who revealed that black meat has a high mineral content of Fe and Zn. In addition, iron can be found in muscle tissue (myoglobin) (Wijaya et al. 2015) and affects the Fe value in cemani chickens, whereas mineral Zn accumulates highly in bone tissue. The high Mn and Se mineral contents found in Cemani chickens with an incomplete *Fm* trait and a heterozygous *Fm/fm*$^+$ indicate that the meat contains high antioxidant levels. Mn and Se minerals function as antioxidants in the body. Mn mineral in meat acts as a cofactor enzyme (Wijaya et al. 2015), whereas Se mineral acts as an antioxidant in meat and can increase tocopherol activity (vitamin E) (Wijaya et al. 2015). The factors that affect the composition of meat include genetics, gender, physiology, age and body weight, diet, and meat type.

In the future, mineral content analysis can be used to create pure Cemani chicken strains that are high in antioxidants. Further research should explore feed analysis, chicken age uniformity, physicochemical testing, and antioxidant activity.

CONCLUSION

The analysis of genetic diversity in the *Fm*–specific alleles in the Cemani populations in two different chicken farm, SUI and IRIAP, revealed that Cemani chickens in SUI have high genetic diversity, as evidenced by a higher Ho value compared to Cemani chickens in IRIAP. We found two genotypes of Cemani chicken in this study: homozygous *Fm/Fm* genotype showed deep black and some chicken showed pale black meanwhile all heterozygous *Fm/fm*$^+$ with pale black. Mineral content analysis revealed that Cemani chickens with homozygous *Fm/Fm* have higher content of Fe and Zn minerals than Cemani with heterozygous *Fm/fm*$^+$. However, Cemani chickens with heterozygous *Fm/fm*$^+$ have Mn and Se that were not different from Cemani with homozygous *Fm/Fm*. This study found that different genotypes of Cemani chicken had different mineral compositions. In the future, this analysis supports the selection of chicken strains with high antioxidant levels.

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Household Consumer's Perception towards Frozen Beef

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ABSTRAK

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Pemerintah Republik Indonesia melakukan impor daging sapi beku dari negara lain untuk memenuhi kecukupan daging sapi di Indonesia. Namun, sebagian besar masyarakat Indonesia lebih suka membeli daging segar (daging sapi yang baru disembelih) daripada daging sapi beku. Penelitian ini bertujuan untuk mengetahui persepsi konsumen rumah tangga di wilayah Jakarta dan Bogor terhadap daging sapi beku dan menganalisis faktor-faktor yang mempengaruhi konsumen untuk mengambil keputusan dalam pembelian daging sapi. Total 200 responden rumah tangga diwawancarai secara langsung menggunakan kuesioner terstruktur. Kuesioner dibagi menjadi tiga bagian, yaitu karakteristik responden (umur, tingkat pendidikan, pekerjaan, pendapatan, dan jumlah keluarga), perilaku pembelian yang dianalisis secara deskriptif, dan persepsi responden terhadap daging sapi beku yang dianalisis dengan menghitung nilai rata-rata persepsi yang kemudian dikelompokan berdasarkan level kategori persepsi. Hasil penelitian menunjukkan bahwa persepsi konsumen terhadap daging beku dari beberapa aspek memiliki skor yang rendah (skor akhir rata-rata 2.37) yang mengindikasikan bahwa konsumen memiliki persepsi yang buruk terhadap daging beku. Lebih lanjut, sebagian besar responden yang membeli daging sapi adalah ibu rumah tangga yang membeli daging di pasar tradisional yang hanya menyediakan daging sapi yang tidak dibekukan, mereka berasumsi bahwa daging yang tidak dibekukan adalah daging segar yang mempunyai kualitas sangat bagus karena berasal dari sapi yang disembelih pada hari yang sama. Responden tersebut menyatakan pembekuan menyebabkan efek negatif pada daging sapi yaitu dapat menurunkan kualitas dan kandungan gizi daging sapi. Berdasarkan hasil penelitian diperoleh bahwa sosialisasi tentang daging beku dan proses penanganan daging beku diperlukan bagi konsumen rumah tangga, sehingga kualitas daging sapi dapat dipertahankan dan meningkatkan kepercayaan konsumen untuk membeli daging beku.

Kata Kunci: Persepsi Konsumen, Daging Sapi Beku, Konsumen Rumah Tangga, Perilaku Pembelian

ABSTRACT

Apriantini A, Arief II, Cyrilla L. 2022. Household consumer's perception towards frozen beef, Indonesia. JITV 27(4): 204-214. DOI:http//dx.doi.org/10.14334/jitv.v27i4.3003.

Demand of beef in Indonesia is higher compared to the domestic beef supply, hence the Indonesian government has established policy to import frozen beef from other countries in order to support the high demand of Indonesia people. However, mosf of Indonesian people prefer to purchase fresh meat (freshly slaughtered beef) rather than frozen beef. The aims of this study were to identify the characteristics of household consumers who bought beef meat and their perceptions towards frozen beef; including to analyze the relationship between perceptions and consumer characteristics of frozen beef, and to analyze the consumer behavior in making decision to buy frozen beef in Bogor area and DKI Jakarta. Total about 200 households were directly interviewed using structured questionnaire. The questionnaires includes general characteristics of respondents (age, education level, occupation, income, number of family member), purchasing behavior which was analyzed descriptively, and respondents perceptions toward frozen beef which was analyzed by calculating the average value of perception and then categorized based on the level of perception category. Results showed that consumer's perceptions toward frozen beef according to several aspects had a low score (average final score 2.37), indicated that consumers had a poor perceptions towards frozen beef. Furthermore, most of the respondents who buy beef were housewives who buy meat in traditional markets which only provide fresh meat, they assumed that fresh meat had very good quality because the meat came from slaughtered beef. Those respondents think that freezing causes negative effects on beef, freezing treatment reduce the quality such as changes of meat color and flavour; and reduce nutritional content of beef. It was discovered that socialization about frozen beef and the process of handling frozen meat is required for household consumers to control the quality of beef as well as to increase the consumer's trusts in purchasing frozen beef.

Key Words: Consumer Perception, Frozen Beef, Household Consumer, Purchase Behavior

INTRODUCTION

Livestock Sub-sector plays an important role in fulfilling community nutrition, due to livestock produces a lot of food products with high protein content, which one of those products is beef. Beef contain a lot of nutrition subtances, especially protein, which is important for child growth and also important for damaged cells repairment (Prasetyo et al. 2013). However, Beef consumption in Indonesia is still below the world average in 2021 (OECD 2021). The average consumption of Indonesian people is 2.2 kg per capita while the world average is 6.4 kg per capita. Therefore, Indonesian government conduct some efforts to increase beef consumption into minimum 20 kg beef/capita/year by increasing beef supply through encouraging domestic beef production (especially beef from NTT), and importing frozen beef. The demand in Indonesia is higher than the domestic supply, hence the government made policy to import frozen beef from other countries for supporting the high beef demand in Indonesia. Indonesia is an archipelago country, cold beef chain management become the main factor in achieving the govenment's goal. However, Indonesian society in general prefers "warm beef" (freshly slaughtered beef) than frozen beef. Fresh beef or "warm beef", which is sold and marketed in room temperature, will be very vulnerable and has a very short shelf life. Another problem in beef consumption pattern of the most Indonesian society, was low public awareness in health problem, especially the danger of consuming the "warm beef".

Meat is skeletal muscle and its associated tissues derived from mammalian, commonly slaughtered for human consumption (Boler and Woerner 2017). There are various forms of beef available in the market, such as fresh beef, cold beef, and frozen beef. Fresh beef is the meat that still has a time span of 1-2 days after slaughtered (Nafiasari and Handayani 2019). Meanwhile, frozen meat is chilled meat subjected to freezing in appropriate equipment in such a way that the product is maintained at a temperature of -18° C or lower (Aidani et al. 2014). The freezing process can inhibit the growth of microorganisms, thus the meat products have long shelf life (Aidani et al. 2014; Ockerman and Basu 2014). The appearance of frozen meats after thawing are not much different from fresh beef (Aidani et al. 2014; Pham 2014; Coombs et al. 2017).

Beef consumers, in this case household consumers, were concentrated in DKI Jakarta, Banten, and West Java which Bogor is one of the areas in West Java that is close to DKI Jakarta. In 2019, West Java is a province that has the largest total consumption of beef for household consumer compared to other provinces, which reached around 41.91 thousand tons followed by East Java at 48.54 thousand tons and DKI Jakarta 23.4 thousand tons (Badan Pusat Statistik 2019). Meat consumption for household consumers in DKI Jakarta and West Java has increased about 0,73 kg/capita and 0.05 kg/capita, respectively, from 2017 to 2019 (Badan Pusat Statistik 2019). This meat consumption also can be represented by per capita expenditure per month for meat products which increased from Rp 28.144 to Rp. 37. 231 in 2015 (Badan Pusat Statistik 2015). Whereas, meat consumption in DKI Jakarta is represented by percentage of expenditure per capita per month for meat products which increased from 2.34% in 2019 to 2.36% in 2021 (Badan Pusat Statistik 2021).

Household consumers are individual consumers who directly consume or process beef (Sumarwan 2015). These consumers will go through several stages to finally decide whether or not to consume the products. The final decision of purchase is influenced by various factors, including consumer perceptions, consumer characteristics, and product attributes such as price, brand and origin of the product (Kotler and Amstrong 2012). Consumer perception is the result of information processing, which is the initial view of consumers of a product. A good perception will lead consumers to consume a product (Istiqlal 2013; Sumarwan 2015). Perception is a process in selecting, organizing, and interpreting stimuli after receiving sight, feeling, hearing, smelling and touching to produce an image. The decision to buy the goods is influenced by individual's perception of a certain situation and sometimes what is perceived can be different from the objective reality (Agyekum et al. 2015; Venkatachalam and Surumbarkuzhali 2018). Therefore, the perception of each individual can be different or subjective. According to Dermawan (2016), perception is a cognitive process which can make it easier to understand something.

The aims of this study were to identify the characteristics of household consumers who bought beef and their perceptions towards frozen beef; including to analyze the consumer behavior in making decision to buy frozen beef in Bogor area and DKI Jakarta. In order to meet the increasing of beef consumption in Indonesia as described above, the government has implemented a policy to import frozen meat, therefore the research about consumer perceptions and behavior of frozen beef is important, in this case is household consumers in DKI Jakarta and Bogor areas, because both regions have the highest consumption of beef compared to other regions in Indonesia. Results of this study can be used to make strategies in determining market segmentation and establishing the right marketing strategy for frozen beef. Thus, the government policy can be targeted and can increase meat consumption in Indonesia.

MATERIALS AND METHODS

This study was conducted in Jakarta and Bogor, Indonesia. Those areas were selected as study location due to Jakarta and Bogor reflect the urban areas in Indonesia in which the community have high regional minimum wage, thus they have more ability to buy and consume beef compared to community in other area. Moreover, based on BPS 2019, DKI Jakarta and West Java (in this case Bogor) have the highest beef consumption compared to other regions in Indonesia. Data collected consisted of primary data and secondary data. The primary data was collected using questionnaire both open and closed questions as a tool and guidance in conducting interviews. The secondary data was collected from the BPS of Jakarta and Bogor, Trade and Industry Serives of Jakarta and Bogor.

The questionnaires in this study included characteristic of respondents such as age, occupation, income, level of education, and total of family member; purchasing behavior, and respondents perceptions toward frozen beef.

Sample

Total of 200 household respondents were used in this study. Respondents were selected with non probability sampling using purposive sampling technique. The respondents were women over 18 years old, who purchased frozen or fresh beef. In addition, it also depends on the willingness of respondents to be interviewed. The respondens were chosen by the Slovin method with the following formula (Riyanto and Hatmawan 2020):

$$n = \frac{N}{1 + Ne^2}$$

where n is the amount of sample, N is the amount of population and e is error tolerance limit.

Data analysis

The descriptive analysis was used in this study to describe consumers' perceptions, consumer characteristics, and purchasing decision processes by consumers. The data was presented as frequency, mean, percentage, and average score. Relationship between perceptions and characteristics of business consumers was analyzed using Rank Spearman correlation test.

Test of validity and reliability

Validity test was carried out in order to find out whether the questionnaire is able to measure the variables. Validity test would be valid if it has strong support for the total score. The correlation of the questionnaire items must be strong and the chances of errors are not too large (maximum 5%), and the correlation must have a positive direction, the r score higher than r table (Yusup 2018). The validity test was conducted on 30 respondents, as a minimum requirement for validity test. Test of validity was analyzed using statistical SPSS software. The results showed that the assessment of 30 respondents, the data was 100% valid, which indicated that the questions had the r score higher than r table.

In addition, reliability testing was carried out on the research questionnaire to see the level of confidence in the questionnaire. If the results of repeated measurements produce relatively the same results,thus these measurements have high reliability (Yusup 2018). The test was analyzed using SPSS software on a reliable test obtained a high Cronbach's alpha value, this indicated that the questions on the questionnaire were reliable, thus the research can be continued.

Perception score

Calculation of the final perception score was carried out by leveling the aspects of perception, including Nutrition and Health aspects, Product quality, Product Handling, and Price (Table 11). The perception score was calculated using a Likert scale with a range of 1 to 4 (Joshi et al. 2015). The statement strongly does not agree had score 1, the statement does not agree had score 2, the statement agrees had score 3 and strongly agree statement had score 4. Determination of the scale was done using the following formula:

$$\text{Scale range} = \frac{\text{the highest score - the lowest score}}{\text{number of scales}} = \frac{4\text{-}1}{4} = 0.75$$

Table 1. Scale of respondents' perceptions based on the calculation of scale range

Perception level	Score
Very Poor	1.00-1.75
Poor	1.76-2.50
Good	2.51-3.25
Very good	3.26-4.00

RESULTS AND DISCUSSION

Characteristics of household consumer

Characteristics of consumer were used to determine the diversity of consumers based on age, education, occupation, income, and number of family members. The general characteristics will provide an overview of

the consumer condition who make a purchase of beef in DKI Jakarta and Bogor and also to find out the correlation between consumers characteristics and consumers' perceptions about frozen and fresh meat. According to Agyekum et al. (2015), consumers have different ideas or perception on the product quality based on their ages, income levels, and educational background and this goes a long way to influence them on the criteria used in determining the quality of product when making a purchase. The general characteristic of respondents is presented in table 2.

Age as a demographic characteristic that will influence on how consumers behave, act, and think. Based on Table 2, it is cocluded that the majority of consumers who buy beef were above 50 years old, representing 39% from total respondents. Ages above 50 years are classified as advanced adults. This result showed that the elderly were financially able to buy beef as daily meals for their family. They are more established and can reach higher food prices than other respondents. Moreover, consumers who buy beef were family members who usually arrange food menus and make decisions in fulfilling household needs which are usually carried out by mothers or older people in the house.

Most household consumers in DKI Jakarta and Bogor had a high level of education. There were 37% of consumers of beef had experience in bachelors's degree (Table 2). The level of education will relate to informations they received and would determine a person's decision to make a purchase. Higher education respondents will receive more information and knowledge related to the nutrion of food then it will affect their decision in purchasing beef. According to Sumarwan (2015), consumer with ation will be more responsive in processing information. Consumers with the higher education will look for more information regarding what products to buy, thus consumer needs will change as education increases. Education is an individual (personal) factor that can indirectly influence consumers in making decisions, consumers with higher education have different views about the assessment of a product compared to consumers with lower education (Rondonuwu 2013).

About 40.5% of respondents were housewives (Table 2). The high number of respondents who were housewives could be related to the high percentage of respondents who were above 50 years old. Housewife consumers were family members who buy beef because they arrange a daily food menus for family and make decisions in fulfilling household needs.

The study also analyzed income level of the respondents. This analysis will help the income level of respondent who buy beef base on price. Income is an important source for consumers to be able to meet their needs. Almost 80% of respondents had a monthly income above IDR 3,500,000 which according to the Central Statistics Agency/ Badan Pusat Statistik (2013), people with income more than IDR 3,000,000 per month were classified as a very high income people. High income people tend to have ability to purchase beef. Income is one of the factors that determine the consumption of a product by consumers. There is a relationship between income and consumption. High income can increase interest in consuming a product. Thus, the higher the income will increase the purchase of products (Hasanah et al. 2018). Financial capability is one of the important things that influence consumer behavior in consuming products.

Number of family members affect households'decision in purchasing decisions. Most of respondents (54% of respondents) had 3-4 members in family (Table 2). Number of familiy members and the existence of children in family would influence the decisions in consuming beef where the parents have a willingness to provide nutritious and healthy food for their children (Adiana and Karmini 2012; Akbari et al. 2016; Jafrinur et al. 2018).

Consumers behavior in purchasing beef meat

Consumer behavior is an activity related to the process of buying an item or service. Consumer behavior can appear after getting a perception. Consumers, in deciding to buy the food product, will consider some aspects such as product quality, packaging, price, function or use, promotion and place of the product (Rajan et al. 2021). This study will disccus factors that influence consumers behavior in purchasing frozen beef and fresh beef.

Needs recognation

The process of needs recognition arises when consumers face a problem (Sumarwan 2015). In this case, consumers face the problem to fulfill the protein source and one of the option available in the market is beef, either frozen or fresh beef. Respondents had various reasons to consume beef. The majority of respondents, about 72% chose to consume beef due to beef is good nutrition for health and had been recommended by many nutritionists. Education may also influence consumers who buy beef for good nutritional reason, because most of the respondents had a bachelor's education. Furthermore, DKI Jakarta and Bogor are urban areas, thus very easy to access information related to food and health issues.The distribution of reasons for respondents in consuming beef can be seen in Table 3.

Information source of product purchased

The main factor in product marketing is providing product information in various media that can be easily

Table 2. The characteristics of household consumer

No	Characteristics	Variable	Frequency	Percentage (%)
1	Age (years old)	<20	1	0.5
		21-30	24	12.0
		31-40	53	26.5
		41-50	44	22.0
		>50	78	39.0
	Total		200	100.0
2	Educational status	Elementary school	2	1.0
		Junior high school	10	5.0
		Senior high school	63	31.5
		Diploma	32	16.0
		Bachelor	74	37.0
		Post Graduate	9	4.5
	Total		200	100.0
3	Occupation	Housewife	81	40.5
		Employee	49	24.5
		Government employees	50	25.0
		Entrepreneur	10	5.0
	Total		200	100.0
4	Income (Rp)	<3,500,000	39	19.5
		3,500,000-7,000,000	86	43.0
		7,000,000-20,000,000	54	27.0
		>20,000,000	21	10.5
			200	100.0
5	Number of family members	2	200	100.0
		3-4	26	13.0
		5	108	54.0
		7	63	30.5
			3	1.5
	Total		200	100.0

Table 3. The reason for consuming beef meat

Reasons for consuming	Frequency	Percentage (%)
Good in nutrition content	145	72.5
Price	5	2.5
Good in taste	46	23.0
Others (such as feast day, lifestyle, allergy etc.)	4	2.0
Total	200	100.0

accessed by consumers (Nadaraja & Yazdanifard 2013). Information is important in influencing purchasing decisions because more knowledge obtained. Moreover, the information of product can lead consumer in making decisions to buy a product (Dewati and Saputro 2020). Generally, consumers would search the information about the product of frozen beef and fresh beef before doing the purchase of beef. Information about a product can be obtained through two sources, including internal sources by remembering their experience and knowledege and external sources such as internet, news, television or other people experiences. There are many factors that influence the information search process including the characteristics of consumers which consist of consumer knowledge and experience, consumer personality and demographic conditions (Sumarwan 2015). Ibrahim & Adinugraha (2020) found that higher intensity of someone to search the product information can cause high posibility they buy the product.

About 73% of respondents in this study received information about frozen beef and fresh beef through personal experience (Table 4). They believe that frozen meat is not as tasty as fresh meat, the quality of meat decrease because it has been kept for a long time. They prefer to buy fresh beef meat and then store the meat in freezer by themselves or directly cooked. Consumers who already have a lot of experience may not be motivated to find more information.

Alternative evaluation

Alternative evaluation is an activity in which consumer process information about their choices to make the final decision (Istiqlal 2013). Consumers will look for the positive and negative effects before buying frozen beef. Results of this study showed that around 21.48% of respondents considered nutrition content of frozen beef before buying it, followed by flavour, colour, appearance and microbial content, 19.69%, 18.16%, 16.62%, 14.59%, respectively (Table 5). This result related to the result above that based on their experiences the quality of frozen meat such as flavour,

Table 4. Information sources of product purchased

Information source	Frequency	Percentage (%)
Consumers experiences	146	73.0
Family	29	14.5
Friend	10	5.0
Newsprint and television	15	7.5
Total	100	100.0

Table 5. Factors considered by consumers in consuming frozen beef

Attributes	Frequency	Percentage (%)
Nutrition content	84	21.48
Microbial content	57	14.58
Tenderness	37	9.46
Flavour	77	19.69
General Appearance	65	16.62
Colour	71	18.16
Total	391*	100.00

*Respondents could choose more than one answer

colour, and appearance were not as good as fresh meat. This result also in line with the study of Tzimitra-Kalogiani (1996), that most of people especially older people believed that frozen meat had negative effects for health due to nutritional reasons, and also change meat's characteristics. They believed that frozen meat was harmful, it did not looks nice, it has been kept for a long time, and because the conditions of its preservation cause unpleasant odor.

Family member who responsible in making decision to buy beef

The next process after alternative evaluation, the consumer would decide whether to buy beef or other meat. The decision to consume beef in household consumers were made by one of member in the family. 88.5% of the family member who responsible in making purchase decision was mother (Tabel 6). Housewife consumers or mother were family member who buy beef because they arrange a daily food menus for family and make decision in fulfilling household needs. Akbari et al. (2016) reported that the existence of children in family would influence decision in consuming beef where the parents, in this case mother, have a willingness to provide safe and healthy food for their children.

The place to buy beef

The desire to buy beef will encourage consumers to look for the seller. Sumarwan (2015) stated that the purchasing process is divided into four stages, such as dealing with the seller, looking for the products, transactions and consumption. Contact with the sellers will determine the place where consumers buy the beef. The distribution of beef seller is presented in Table 7.

The result showed that 60% of respondents bought beef in traditional markets. The respondents might

Table 6. Family member who responsible in making decision to consume beef meat

Person in charge	Frequency	Percentage (%)
Mother	177	88.5
The oldest children	8	4.0
Servant	15	7.5
Total	200	100.0

Table 7. Place to buy beef meat

Place	Frequency	Percentage (%)
Traditional market	120	60.0
Supermarket	79	39.5
Slaughterhouse	1	0.5
Total	200	100.0

Table 8. Amount of beef purchase

Beef quantity	Frequency	Percentage (%)
<1 kg	57	28.5
1 kg	59	29.5
2 kg	79	39.5
3 kg	5	2.5

Table 9. Frequency for buying beef

Purchasing frequency	Frequency	Percentage (%)
Everyday	41	20.5
Once a week	70	35.0
Once a month	58	29.0
One a quarter	16	8.0
Every Religion Holiday	31	15.5
Total	200	100.0

Table 10. Respondents opinions about the effect of the freezing process

Respondents opinions	Frequency	Percentage (%)
Positive	67	33.5
Negative	83	41.5
No effect	50	25.0
Total	200	100/0

believe traditional markets was the only place that provide unfrozen beef, they assume that unfrozen beef was fresh meat that had very good quality because the meat came from slaughter house. Those respondents assumed that freezing causes negative effects on beef, freezing treatment can reduce the quality and nutritional content of beef (Tzimitra-Kalogiani 1996). Meat sold in traditional markets was a fresh meat, which slaughter on that day, and had very good quality and condition. In addition, respondents can bargain the prices and choose meat directly. This study also in line with Agustina's reseach (2018), people tend to choose traditional markets as a place to shop because the consumers were able to bargain the products with the seller directly, thus they can get the products with the lower prices than the other places or modern market.

In addition, traditional markets also provide convenience in finding the products needed. According to Angriva and Sunyigono (2020), the consideration of buying place is one of consumer behavior in making purchases of a product. Furthermore, the easiness of access and obtaining a product can save consumers time and energy, thus consumers do not have to go far for shopping.

Quantity of beef purchased

The majority of respondents, about 39.5% respodents bought beef 2 kg in one purchase. The distribution of the amount of beef purchases is presented in Table 8. The respondents who generally housewives were not always cook the beef meat directly. They stored the beef meat after they bought from the market. They will store the meat in freezer as food stock.

Frequency for buying beef

About 35% of respondents bought beef meat once a week. Household consumers did not cook the meat immediately after they bought. Respondents would store the beef in the freezer and cooked the meat later. The distribution of frequency for buying beef is presented in Table 9.

Consumer behavior after decision making

Consumer will have experiences and knowledges of the products after doing the purchase and consume the beef meat, both fresh beef meat and frozen beef meat. This process is referred to as an alternative evaluation process. The majority of respondents about 41.5% said freezing cause a negative effect on beef. According to respondents in this study, freezing can reduce the quality and nutrition content of beef. However, based on the research of Ernawati et al. (2018) showed that nutrition content did not differ between frozen and fresh beef meat in both traditional markets and supermarkets. Moreover, frozen meat will lose nutrients during the thawing process and when cooked, not when freezing process (Saraswati and Karang 2016).

About 33.5% of respondents thought that the freezing process had a positive effect on beef, which extends shelf life and easier to process. While 25% of respondents thought that freezing did not change the condition of beef (Table 10).

Household consumer perception of frozen beef

Consumer perceptions of frozen beef were in terms of several aspects, such as nutritional and health values, product quality, product handling, and price (Rahman 2020). The average score of respondent's perceptions is shown in Table 11. The aspects of nutrition and health content had score about 2.28, this value was categorized as poor perception based on the scale of perception levels in Table 1. This result might related to consumer's education and the easiness to access the information in urban areas about frozen food issues. This result in line with the study of Tzimitra-Kalogiani (1996) about the Greece perception toward some different meats, that most of people believe frozen meat was as nourising as fresh meat frozen meat due to nutritional content issue. According to Saraswati and Karang (2016) protein denaturation can occur during the freezing process as a result of increased ionic strength in intracellular tissue followed by water migration to extracellular tissue. Fatty levels drop during frozen processing and storage due to loss of triglyceride fraction caused by fat oxidation. However, according Ernawati's research on the quality of macronutrients in frozen meat and fresh meat, showed that nutrient content did not differ between frozen fresh beef (Ernawati et al. 2018).

According to Permana (2013), product quality becomes one of the factor to consider in making purchasing decisions by consumer. The average score of consumer perception from the whole aspects of product qualities was 2.29 which categorized as poor perception (Table 11). The respondents assumed that freezing treatment on meat causes pale meat color. The pale color of frozen meat indicates a decline in quality of meat. The chemical color of fresh meat is bright red oxymyoglobin (Dewi 2012; Gunawan 2013). The consumer perception in this study was in contrast to research conducted by Gunawan (2013) which showed that frozen meat will retain its quality, which can be seen in the meat color that still have a bright red color during storage.

The decreasing of meat fat can affects the taste of meat. Respondents who have poor perceptions toward

Table 11. Average score of respondent perception toward frozem beef meat

No	Aspects	Sub aspects	Variable	Average Score
1	Nutrition and health aspects	Nutrition content	Frozen beef has good nutritional content	2.30
		Health	Frozen beef is good to be consumed	2.26
2	Product quality	Physical quality	Frozen beef has good quality	2.24
			Frozen beef has soft texture	2.20
			Frozen beef has good taste	2.30
			Frozen beef has typical fragrance of meat	2.23
			Frozen beef has attractive colour	2.13
		Cleanliness	Frozen beef is a clean and hygene meat	2.61
3	Average aspect score			2.29
	Product handling		Frozen beef is not easy to rot	2.60
			Frozen beef could be stored for a long period	2.39
	Average aspect score			2.50
	Price		Frozen beef has economies price	2.40
	Average aspect score			2.40
	Average final score			2.37

frozen beef because they thought frozen beef not only cause change in color but also cause bad taste, bad odour and bad texture. This poor perception might be caused by bad experiences. According Haq et al. (2015), factors that can affect meat quality after slaughtered were not only influenced by storage methods but also other factors such as carcass pH, cooking methods and meat aging. However, the responden still believed that frozen beef meat was a clean and hygene meat because freezing can restrain the rate of microbial growth during storage. Thus the responden still had good perception (score 2.61) for the cleanliness.

Consumer perceptions of frozen beef were also reviewed from the aspect of product handling. This perception was measured by the statement that "frozen beef is not easy to rot and can be stored for a long time". Respondents had an average perception score of 2.51 which was classified as a range of poor perception scores (Table 11). This misperception occurred because of a lack of information on consumers that the freezing process is one of the preservation technologies that can extend the shelf life of beef (Rahman 2020).

Price is one of the important attributes of a product (Sumarwan 2015). The factors that influence beef demand in Indonesian are economic factor and price (Puradireja et al. 2021). Measuring perceptions of prices used the statement that "frozen beef has a more affordable price". The average perception score in the price aspect, which was 2.40 which indicated that respondents had poor perception of frozen beef prices. Based on the average aspects of the four aspects above, the final average score of perception was 2.37 which indicated that the general perception of household consumers on frozen beef meat for whole aspects in Bogor and DKI Jakarta was poor (Table 11). This result was contrary to other research about the perception of business consumers towards frozen beef which had a good perception of frozen meat. Business consumers such as restaurants, hotels and modern markets consider frozen meat to be more hygienic, less perishable, longer shelf-life, easier to store and handle, and had a more affordable price. The shorter distribution chain causes the price of frozen beef to be cheaper than fresh beef (Apriantini et al. 2021).

CONCLUSION

The consumer's perceptions toward frozen beef according to several aspects was low indicated that consumers had a poor perceptions towards frozen beef. Furthermore, most of the respondents who buy beef were housewives who buy meat in traditional markets

which only provide fresh meat. The attitude that frozen meat does not look nice was mainly taken by the older people with age above 50. It was found that those respondents had a negative perception towards frozen beef meat for nutritional content and meat quality. The findings suggest that counseling and campaign about the quality, the safety and handling of frozen beef are needed for household consumers, thus the quality of beef could be maintained and increases the consumers trusts in buying frozen beef.

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UDC: 636.293.2

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Produksi susu kerbau yang disuplemenasi larutan ragi teraktifasi dab ransum dengan bubur singkong fermentasi ragi (Milk Yield of dairy buffaloes supplemented with activated yeast solution and fed ration with yeast-fermented cassava pulp)

(Org: Eng)

JITV 27(1): 1-9

Feed resource availability and quality are two of the major factors limiting dairy production in the Philippines. Utilization of microbial-based feed additives and agricultural by-products such as cassava pulp aided by fermentation technology can help provide the needed resource. This study aimed to determine the effect of activated yeast (*Saccharomyces cerevisiae*) solution (AYS) and yeast-fermented cassava pulp (YFCP) on milk production and feed cost-efficiency in dairy buffaloes. The study followed RCBD design using 63 dairy buffaloes at the Philippine Carabao Center in Ubay Stock Farm, Bohol. The average daily milk yield (ADMY) of buffaloes supplemented with 0.5L AYS and 1L AYS twice daily were greater than that of control buffaloes by 0.67L and 0.69L, respectively (P = 0.0039).On the other hand, the ADMY of buffaloes fed with YFCP and YFCP+AYS were greater than that of control buffaloes by 0.64 and 0.68L, respectively (P = 0.0320). Supplementation of AYS and feeding YFCP yielded the lowest cost per liter of milk produced at PhP 20.25 and PhP 16.24, respectively. It is recommended to supplement milking dairy buffaloes with AYS or feeding YFCP in areas with cassava pulp to increase feed resource, increase milk production and improve feed cost-efficiency thereby increasing significantly the farmer's income.

(Author)

Key Words: Buffaloes, Cassava, Milk, Pulp, *Saccharomyces cerevisiae*

UDC: 636.58.033

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Performa pertumbuhan dan keuntungan ayam broiler yang diberi pakan mengandung tepung daun *Ipomoea asarifolia* (Growth performance and cost benefits of broilers fed diets containing *Ipomoea asarifolia* leaf meal)

(Org: Eng)

JITV 27(1):10-17

This experiment was conducted to determine growth performance and cost benefit of including cooked Ipomoea asarifolia leaf meal (CIALM) in broiler diets. Eight diets were compounded by including Ipomoea asarifolia leaf meal at 0, 2.5, 5 and 7.5% to form T1, T2, T3 and T4 respectively of both starter and finisher diets. One hundred and twenty broiler birds were used in a Complete Randomized Design experiment that lasted for eight weeks. Daily feed intake and weekly weight gain were measured. Average daily feed intake, average daily weight gain, total feed consumed, feed conversion ratio and cost benefit parameters were calculated. In starter phase results showed that there were significant differences ($P<0.05$) between treatment means in all the parameters except initial weight of the birds. There were significant differences ($p<0.05$) between treatment means in all the parameters measured during the finisher phase including the cost analysis parameters except that feed cost decreasing with increasing levels of CIALM and net profit decreased from T1 to T4 because of the decrease in total weight gain from T1 to T4. Final weight gain, total weight gain, average daily feed intake, total feed intake, average daily weight gain, feed conversion ratio, feed cost and feed cost per kg weight gain all decreased with increasing levels of CIALM. Results control diet performed better than other treatment diets which suggest that lower levels of inclusion that is less than 2.5% may give positive results.

(Author)

Key Words: Broiler Chicken, Cost Benefit, Growth Performance, *Ipomoea asarifolia*

UDC: 577.112.385

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Pengaruh *in-ovo* injeksi l-arginine terhadap daya tetas, kualitas anak ayam, performan dan histologi otot ayam lokal (Effect of *in-ovo* injection of l-arginine on hatchability, chick quality, performances and muscle histology of native chicken)

(Org: Eng)

JITV 27(1): 18-27

This study aimed to determine the effect of *in-ovo* injection of L-arginine on hatchability, chick quality, performances, and muscle histology of native chicken. *In-ovo* injection was carried out on the 10th day. A total of 375 fertile eggs with an average weight ranged 39-43 g were grouped into 5 treatments. The first treatment was without injection (negative control), the second treatment was injection of NaCl solution 0.9% (positive control), the third treatment was injection of L-arginine solution 0.5 g per 100

ml of NaCl 0.9% (0.5%, m/v) The fourth treatment was injection of 1.0 g L-arginine solution per 100 ml of NaCl 0.9% (1.0%, m/v), and the fifth treatment was injection of 1.5 g L-arginine solution per 100 ml of NaCl 0.9% (1.5%, m/v). The injection was carried out at the pointed area of the egg in a vertical position (pointed below, blunt above). The Injection was carried out with a depth of 10 mm from the eggshell using an automatic injector. The results showed that all treatments produced the same hatchability. *In-ovo* injection of L-arginine has a beneficial effect on chick quality and post-hatch performance, the concentration of L-arginine solution used did not cause embryo death. *In-ovo* injection of 0.5% L-arginine increased hatching weight, weekly body weight, muscle mass, and myofiber size.

(Author)

Key Words: *In-Ovo*, L-arginine, Performances, Myofiber

UDC: 613.2.099

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Pemanfaatan *Tenebrio molitor* L dalam pakan terhadap kualitas dan kandungan omega-3 telur (The influence of *Tenebrio molitor* L supplementation on egg quality and omega-3 content)

(Org: Eng)

JITV 27(1): 28-34

Tenebrio molitor L is one of the alternative feed ingredients because it is rich in nutrients, namely protein, vitamins, minerals (calcium), energy, and fat. *Tenebrio molitor L* also contains 33.64±0.22% omega-3, so it is hoped that the eggs produced contain omega-3. In this study 300 Lohman Brown laying hens of 20-week-old were used. Completely randomized design (CRD) was applied in this study with 3 treatments and 10 replications, each replication contained 10 laying hens. Treatments were: P0= Feed containing 5% MBM, P1= Feed containing 2.5% MBM + 2.5% *Tenebrio molitor L*, and P2= Feed containing 5% *Tenebrio molitor L*. This research was conducted for 6 months. The variables observed were egg production, egg weight, egg shape index, shell weight, shell thickness, Haugh unit, yolk index, and omega-3. Treatment had no influence on egg physical quality but had a significant influence on egg weight. Treatment P0 prodeced the lowest egg weight that was 59.02±0.53 g. Treatment P2 had higher omega-3 contents than P0 and P1 that was 88±0.12 mg 100 g^{-1}. It was concluded that *Tenebrio molitor* L could replace MBM up to 5% in laying hens feed, improve eggs quality, and omega-3 content in eggs.

(Author)

Key Words: *Tenebrio molitor* L, Egg Quality, Omega-3

UDC: 636.58.033

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Kandungan kimia dan kecernaan *in vitro litter* ayam broiler yang difermentasi dengan waktu pemeraman yang berbeda (Chemical content and *in vitro* digestibility of broiler litter fermented at different ripen time)

(Org: Eng)

JITV 27(1): 35-44

The aim of this study was to examine effect of length of chicken litter fermentation on chemical content and *in vitro* digestibility. Completely randomized design was applied in this study with 4 treatments and 4 replications. The treatments were T0 = no fermentation; T1 = fermentation of chicken litter for 3 weeks; T2 = fermentation of chicken litter for 6 weeks; and T3 = fermentation of chicken litter for 9 weeks. Parameters observed were chemical content and digestibility value of fermented chicken litter. Different fermentation time affected the chemical content of fermented chicken litter, namely water, fat, BETN and TDN content, but did not affect ash content and fiber content. Different fermentation time affected dry matter, protein, fiber fraction digestibility (ADF, NDF, Hemicellulose), but did not affect organic matter digestibility, VFA concentration, NH_3 concentration and total protein production of chicken litter. Based on dry matter, ADF, NDF hemicellulose digestibility and VFA concentration, it is concluded that recommended ripen time for chicken litter fermentation is 6 weeks.

(Author)

Key Words: Broiler Litter, Chemical Content, Digestibility, Fermentation, *In vitro*

UDC: 611.36

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Efek protektif asam galat (GA) dan Kurkumin (Cur) pada transaminase hati, parameter plasma darah dan kadar hormon hipofisis-testis pada tikus yang diberi NiNPs [Protective Effects of Gallic acid and Curcumin on Serum Levels of Hepatic Transaminases, Blood Plasma Parameters and Pituitary-testicular hormones in rats treated with nickel nanoparticles]

(Org: Eng)

JITV 27(1): 45-56

Nickel nanoparticles (NiNPs) have toxic effects on body cells due to the production of free radicals. The purpose of this research was to investigate the protective effects of Gallic acid (GA) and Curcumin (Cur) on hepatic transaminases, blood plasma parameters and pituitary-testicular hormones levels in NiNPs-treated rats. Seventy adult male Wistar rats were divided in 7 groups of 10 including control, Ni50 mg/kg, Ni50+GA150 mg/kg, Ni50+GA300 mg/kg, Ni50+Cur150 mg/kg, Ni50+Cur300 mg/kg and Ni50+GA300+CUR300 mg/kg. NiNPs, GA and Cur were administered orally by oral gavage for 28 days. At the last phase of the study, the samples of blood were taken directly from heart and serum levels of hepatic transaminases (alanine aminotransferase (ALT) and aspartate aminotransferase (AST)), blood plasma parameters (Glucose, total protein (TP), bilirubin (Bil), albumin (Alb), creatinine (Cr), Blood urea nitrogen (BUN), triglyceride, cholesterol, HDL, LDL

and alkaline phosphatase (ALP)) and pituitary-testicular hormones (FSH, LH, testosterone and dihydrotestosterone) were assessed. NiNPs administration increased serum levels of glucose, ALT, ALP, AST, Bil, BUN, Cr, triglyceride, cholesterol and LDL compared to the control group ($p<0.05$) and in contrast, it decreased serum levels of FSH, LH, testosterone, dihydrotestosterone, Alb, TP and HDL ($p<0.05$). However, co-administration of GA and Cur at doses of 300 ml/kg in NiNPs -treated rats improved all blood plasma parameters compared to the control group ($p>0.05$). The findings of this study suggest that co-administration of GA and Cur at a dose of 300 mg/kg can reduce and improve the damaging effects of NiNPs on blood plasma parameters, hepatic transaminases and pituitary-testicular hormones in adult rats.

(Author)

Key Words: Curcumin, Gallic Acid, Hepatic Transaminases, Nickel Nanoparticles, Testosterone

UDC: 577.213

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Korelasi antara kualitas semen, libido dan konsentrasi testosteron pada pejantan sapi Bali (Correlation between semen quality, libido, and testosterone concentration in Bali bulls)

(Org: Eng)

JITV 27(2): 57-64

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

(Author)

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

UDC: 599.735.51

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Penggunaan termografi inframerah sebagai penentu mastitis sub-klinis pada kambing perah Sapera (Application on infrared thermography as a determinant of sub-clinical mastitis in Sapera dairy goats)

(Org: Eng)

JITV 27(2): 65-73

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

(Author)

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

UDC: 636.58.033

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Kesan penambahan serbuk daun misai kucing (*Orthosiphon stamineus*) dalam makanan ke atas prestasi pertumbuhan ayam pedaging (Effect of supplementing ground leaf of misai (*Orthosiphon stamineus*) in diet on growth performance of broiler chickens)

(Org: Eng)

JITV 27(2): 74-83

The use of herbs in animal nutrition is one of the important approaches in overcoming the disadvantages of excessive use of artificial chemicals in animal nutrition. The present study was done to evaluate response of broilers feeding on a diet supplemented with the ground leaf of misai (*Orthosiphon stamineus*). The birds in this, study were 160 one-day-old male broiler chickens, given ad libitum water

and feed for up to 20 days. Treatments were given to 21-day-old male broiler chickens. Data was collected and evaluated after slaughtering 42-day- male broiler chickens. It was shown that supplementing broiler diets with *O. stamineus* powdered leaf at a rate of 8 g/kg resulted in growth performance comparable to tetracycline and Vitamin E supplementation. It was also shown that supplementing the diet with 8 g/kg *O. stamineus* had a blood enzyme-lowering effect. In broilers receiving tetracycline supplementation, however, significant serum enzyme activity was observed. Results also showed that 8 g/kg of ground *O. stamineus* leaf in the diet was equivalent to 200 mg/kg Vitamin E supplementation. Therefore, *O. stamineus* leaf powder can promote organic, safe, and sustainable broiler chicken production, and as diet supplement.

(Author)

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

UDC: 615.35

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Efektifitas enzim BS4 kering dan basah dalam peningkatan proforma ayam broiler yang diberi pakan kepadatan pakan yang berbeda (The effectivity of dry and liquid BS4 enzymes in improving performance of broiler chickens fed different nutrient density diet)

(Org: Eng)

JITV 27(2): 84-92

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

(Author)

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

UDC: 591.51

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Perubahan kondisi psikologi ayam broiler yang disemprot dengan air sebelum transportasi (Changes in physiological condition of broiler chickens sprayed with water before transportation)

(Org: Eng)

JITV 27(3): 93-99

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

(Author)

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

UDC: 637.054

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Pengaruh fortifikasi nano kalsium kerabang telur itik terhadap kualitas kimia sosis sapi (Influence of duck eggshell nano-calcium fortification on the chemical quality of beef sausage)

(Org: Eng)

JITV 27(2): 100-106

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

(Author)

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

UDC: 636.2.003

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Keragaman SNP c.795A>G gen PLAG1 dan asosiasnya terhadap bobot lahir pada sapi Bali (Diversity of SNP c.795A>G PLAG1 gene and its association with birth weight of Bali cattle)

(org: Eng)

JITV 27(3): 107-113

PLAG1 gene is one of those that regulate growth and body size. This study aimed to look at the PLAG1 gene polymorphism and its relationship to birth weight in Bali cattle using PCR-RFLP. The total sample used was 104 samples consisting of 66 Bali cattle from BPTU-HPT Denpasar and 38 Bali cattle from BPT-HMT Serading, each of which had birth weight data. PLAG1 gene polymorphism was analyzed using PCR-RFLP and the Sac1 restriction enzyme. The genotype and allele frequencies, heterozygosity, and Hardy-Weinberg equilibrium were all examined using Popgen32. General Linear Model was used to analyze the association of SNP 795A>G PLAG1 gene with birth weight in Bali cattle. Amplification of the PLAG1 gene resulted in 776 bp fragments and two alleles. The PLAG1 gene had three genotypes: AA (562 bp and 182 bp), AG (562 bp, 182 bp, and 104 bp), and GG (562 bp, 182 bp, and 104 bp). Based on the results, the PLAG1 gene in Bali cattle was polymorphic. The alleles frequency of Bali cattle was in Hardy-Weinberg equilibrium. The SNP c.795A>G PLAG1 gene genotype were associated with birth weight in Bali cattle. The A allele is a determinant of high birth weight in Bali cattle where the AG genotype has the highest birth weight.

(Author)

Key Words: Bali Cattle, PCR-RFLP, PLAG1 Gene, SNP

UDC: 57.017.5

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Pengaruh konsentrasi gliserol sebagai krioprotektan pada semen beku sapi Pasundan (Effectiveness of various glycerol concentrations as a cryoprotectant in frozen semen of Pasundan cattle)

(Org: Eng)

JITV 27(3): 114-121

This study was conducted to determine the effectiveness of Glycerol as a cryoprotectant with various concentrations on the quality of Pasundan cattle semen. Semen was collected from seven bulls of Pasundan Cow using an artificial vagina twice a week for three months. The semen sample was added with a TRIS-Egg Yolk Extender containing 20% (v/v) egg yolk and treated with the addition of Glycerol with five different concentrations (G5= 5%, G6= 6%, G7= 7%, G8= 8%, and G9= 9%) were then performed with cryopreservation. A Completely Randomized Design (CRD) was used to examine the effect of five different concentrations of Glycerol on motility, intact plasma membrane (IPM), the integrity of acrosome cap (IAC), abnormalities, and recovery rate (RR) of spermatozoa after cryopreservation (post-thawing). The results of diluted Pasundan cattle semen evaluation showed that the addition of 7% Glycerol (G7) resulted in the best motility and IAC values (83.68% and 72.84%), the addition of 7% and 8% Glycerol (G7 and G8) resulted in the best IPM values (85.00% and 84.50%). The addition of 6%, 7%, 8%, and 9% Glycerol (G6, G7, G8, and G9) resulted in the lowest abnormality values (1%). On the post-thawing Pasundan cattle semen evaluation, the addition of 7% Glycerol (G7) resulted in the best motility, IAC, IPM, and RR values (54.49%, 38.57%, 54.29%, and 72.28%). Meanwhile, adding various Glycerol concentrations did not significantly affect the abnormality value of post-thawing spermatozoa. Generally, the addition of 7% Glycerol in semen extenders shows optimal results as a cryoprotectant.

(Author)

Key Words: Cryoprotectant, Glycerol, Pasundan Cattle, Semen Quality

UDC: 576.372

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Pengujian status akrosom sperma, konsentrasi malondialdehid dan enzim aspartat aminotransferase dari semen beku sapi Limousin dan Simmental dalam berbagai pengencer komersial (Assesment of sperm acrosome status, malondialdehyde and aspartate aminotransferase enzym concentration of frozen semen from Limousin and Simmental bulls in different commercial diluents)

(Org: Eng)

JITV 27(3): 123-130

Sperm cryopreservation is the process of preserving sperm cells at low temperatures, so that its frozen semen can be used in the future. The quality of the frozen sperm is affected by the diluent. The objective of this study was to compare the effects of commercial diluents on acrosome status, malondialdehyde (MDA) and aspartate aminotransferase (AspAT) enzyme concentration of thawed Limousin and Simmental bull semen. Semen was collected twice weekly using an artificial vagina. The fresh semen processed into frozen semen had sperm motility of >70%. The one-step procedure was used for the dilution methods. Andromed®, Optixcell® and Steridyl® were used as diluents. Data were analyzed by analysis of variance (ANOVA) followed by Tukey HSD 5% confidence interval. The result showed no interaction (P>0.05) between two factors on acrosome status. The sperm acrosome damage of Simmental in Steridyl® was significantly lower than others (P<0.05), although all diluents showed low sperm acrosome damage. Also, no interaction between the type of diluent and breed on MDA and AspAT enzyme concentrations was detected (P>0.05). The results suggest that three commercial diluents have equal efficacy in protecting acrosome status and maintaining MDA and AspAT enzyme concentrations of frozen Limousin and Simmental bull semen. Therefore, all commercial diluents can be an alternative for Limousin and Simmental frozen semen.

(Author)

Key Words: Acrosome Status, AspAT, MDA, Limousin and Simmental Bull

UDC: 591.133.2

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Respon fisiologis, performa, tingkah laku dan kesejahteraan domba Garut yang dipelihara pada sistem semi-intensif di Indonesia (Physiological responses, performance, behaviour, and welfare of Garut sheep raised in semi-intensive system in Indonesia)

(Org: Eng)

JITV 27(3): 130-141

Sheep farming in Indonesia mainly relies on an intensive, labor-intensive system, with limited wiggle room and the number of livestock. On the other hand, developed countries have developed a semi-intensive system that can minimize the number of workers and provide animal welfare to their sheep. This study aimed to compare performance, physiological responses, behavior, and animal welfare of reared Garut sheep in semi-intensive management with an outdoor and full indoor pen, employing a T-test experimental design using ten replicates (n= 10). This experiment used 20 ewes sheep (one year old) with an average body weight of 18.74±2.53 kg. This experiment found no difference in dry matter intake (gram/head/day) or average daily gain (gram/head/day) between both treatments (P>0.05). Ewes with access to an outdoor pen had better physiological status, especially heart rate and respiratory rate, particularly in the morning and afternoon (P<0.05) compared to ewes with the full indoor pen. The ratio of blood N/L for ewes with an outdoor barn showed better results (P<0.05), and the rearing environment did not show any difference nor induce stress on livestock with access to an outdoor pen (P>0.05). Ewes' welfare increases, and they become more active after being provided access to an outdoor pen. A semi-intensive system with an outside enclosure enhances Garut ewes' performance, blood parameters, and welfare index.

(Author)

Key Words: Farming System, Garut Sheep, Livestock Welfare, Pen, Physiology

UDC: 616.98

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Kontribusi mamalia terhadap transmisi infeksi *Schistosoma japonicum* di Lore Barat, Poso, Sulawesi Tengah, Indonesia (Mammalian contribution to transmission of *Schistosoma japonicum* infection in West Lore, Poso, Central Sulawesi, Indonesia)

(Org: Eng)

JITV 27(3): 142-151

Studies on the role of domestic animals in the transmission of schistosomiasis japonica in the West Lore Sub-district, Poso District, are still limited despite its importance as zoonosis. This study used a cross-sectional design to determine schistosomiasis prevalence in domestic mammals and identify the relative contribution of each mammalian species' schistosomiasis transmission in the West Lore Sub-District. Fecal samples were obtained from 209 animals (seven buffaloes, 70 dogs, 44 cattle, 86 pigs, and two horses). The Danish Bilharziasis Laboratory technique was used to detect the occurrence of S. japonicum egg in feces and the intensity of schistosomiasis infection. The examination of 1852 human fecal samples using the Kato-Katz method was carried out by the Laboratory of Schistosomiasis Lengkeka. The measurement of environmental pollution with S. japonicum eggs and the relative contribution of each species in the transmission was performed. The highest prevalence of S. japonicum infection in animals was in horses (100%; 2/2), cattle (54.55%; 24/44),

and pigs (51.16%; 44/86). The prevalence in buffaloes and dogs was 28.57% (2/7) and 32.86% (23/70). Cattle (69.74%) were the main contributors to S. japonicum eggs contamination in the environment, followed by pigs (21.95%) and buffaloes (4.71%). This study reported a high prevalence of schistosomiasis in animals (45.46%) while low human schistosomiasis prevalence (0.59%).

(Author)

Key Words: Animals, Coprology, Humans, *Schistosoma japonicum*, Zoonosis

UDC: 577.27

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Pengaruh *Lactobacillus casei* dan tepung bawang putih terhadap performa, respon imun dan profil darah ayam broiler (Effect of *Lactobacillus casei* and garlic powder on broiler performance, immune response and blood profile)

(Org: Eng)

JITV 27(4): 152-158

Broiler litter waste is increasing as the population of This study aimed to examine effect of giving a mixture of *Lactobacillus casei* and garlic powder (LGP) on broiler performance, immune response and blood profile. One hundred and forty-day old broilers with an average body weight of 43.70±0.88 g were placed randomly in 20 experimental units. Completely randomized design was used in this study, with 4 treatments and 5 replications. The treatment was conducted when the chickens were 22-35 days old (finisher phase). The chickens were given a mixture of *Lactobacillus casei* and garlic powder (LGP) at concentration levels of: 0, 1, 2, and 3%. Parameters measured were blood profile, weight of lymphoid organs (bursa of Fabricius, spleen, and thymus), and carcass production. Data were analyzed for variance and if there was a significant effect, then Duncan's multiple range test was followed at the 5% level. Results showed that the administration of LGP improved the immune response, carcass weight and final body weight of broiler chickens. The administration of 3% LGP improved immune response and achieved the best broiler performance.

(Author)

Key Words: Blood, Broilers, Garlic, Immune, *Lactobacillus*

UDC: 58.088

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Karakterisasi molekuler dan ekspresi gen TLR4 yang dihubungkan dengan penyakit mastitis pada kambing (Molecular characterization and gene expression of TLR4 gene associated with mastitis in goats)

(Org: Eng)

JITV 27(4): 159-169

In this study characterization of Toll-like receptor 4 (TLR4) gene of goats; detection of polymorphisms in the nucleotides, and determination of the association of identified genotypes with the occurrence of subclinical mastitis was done using chi-square and odds ratio. Analyzing gene expression using two-sided Student's T-test was also done. Results of Study 1 revealed high similarity (99%) of TLR4 nucleotide sequence of all breeds of goats with that of *C. hircus* (NM_001285574.1) and sheep (*Ovis aries*) sequences and slightly lower similarity with cattle *(Bos taurus*, and *Bos indicus*) (96%), and water buffalo (*Bubalus bubalis*) (95%). In Study 2, restriction fragment length polymorphism (RFLP) analysis revealed three genotypes with nine restriction patterns using AluI enzyme. Genotype AA has odds ratio of 0.28 and 0.08 in all breeds, and in Anglo-Nubian, respectively, with significant association ($P<0.05$) that inferred 0.28 and 0.08 times greater probability in all breeds, and in Anglo-Nubian, respectively, for subclinical mastitis to occur than those of other genotypes. Genotype AB showed odds ratio of 3.83, 13.00 and 2.40 in all breeds, in Anglo-Nubian, and in Upgraded, respectively, with significant association ($P<0.05$) that indicated 3.83, 13.00 and 2.40 times more likely in all breeds, in Anglo-Nubian, and in Upgraded, respectively, to suffer subclinical mastitis than those of other genotypes. In Study 3, genetic expression analysis showed a significant upregulation of TLR4 gene up to maximum of 3.63-fold in goats without subclinical mastitis compared to subclinically mastitic animals with only 0.65-fold which suggest a prompt role of TLR4 gene in the protection of animal against possible infection.

(Author)

Key Words: Gene Expression, Genotype, Goats, Mastitis, PCR-RFLP, TLR4 Gene

UDC: 636.082.4

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Seleksi Karakteristik morfologi mutan *Leucaena leucocephala* toleran asam terhadap penambahan hormon IBA (*Indole butyric acid*) pada kultur jaringan (Morphological characteristics selection of acid-tolerant *Leucaena leucocephala* mutant to addition of IBA hormone (*indole butyric acid*) in *tissue culture*)

(Org: Eng)

JITV 2(4): 170-176

Leucaena leucocephala is a legume forage plant that has a high protein content. Tissue culture is a technique that can be used to select mutants for forage plants in vitro. IBA (*Indole butyric acid*) is one type of auxin that can induce rooting and growth in plants. This study aimed to select the morphological characteristics of the acid-tolerant *Leucaena leucocephala* mutant to the addition of the hormone IBA in tissue culture. The design used in this study was a completely randomized design (CRD) with lamtoro plant explants as many as 11 acid-tolerant mutant lines pH 3.4 resulting from 400 gy irradiation which had been treated on 300 ppm Al^{3+} acid media, namely the K1-K11 strain (mutant+MS+1ppm IBA) and 2 parent trees *Leucaena leucocephala* without

gamma irradiation, namely P0 as *Leucaena leucocephala* parent+MS+0ppm IBA, P1 as *Leucaena leucocephala* parent + MS + 1 ppm IBA with 15 replicates. The treatment which had a significant effect was continued with the test Tukey. Variables observed were an increase in root length, plant vertical height, number of shoots, and percentage of rooted plants. The increase in root length and increase in plant vertical height showed the best results on the K10 mutant strain, the number of shoots showed the best results on the K9 and K11 mutant strains, and the highest percentage of rooted plants on the K3 and K11 mutant strains. The addition of IBA can increase the morphological characteristics of the *Leucaena leucocephala* mutant.

(Author)

Key Words: IBA, *Leucaena leucocephala*, Mutant, Tissue Culture

UDC: 612.616.2

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Dampak penuaan terhadap kualitas spermatozoa pejantan Sentul (Impact of aging on sperm quality of Sentul roosters)

(Org: Eng)

JITV 27(4): 177-185

This study was done to determine impact of aging on sperm quality of Sentul roosters. Materials used in this study were Sentul males aged 48, 58, 68 and 78 weeks, NaCl, eosin and aquades. Semen was collected by abdominal massage method. Completely randomized design (CRD) was applied in this study with 4 treatments in 5 repetitions. Statistical analysis showed that increasing age of rooster had a very significant effect ($p<0.01$) on decreasing semen consistency, mass motility, spermatozoa concentration and increasing spermatozoa motility. Aging in Sentul roosters affected semen volume significantly ($P<0.05$) but did not significantly affect ($p>0.05$): semen pH, live spermatozoa, and spermatozoa abnormalities. Average volume of semen produced in this study ranged from 0.54-0.88ml; semen pH 6.80-7.12; mass movement score 1.60-3.00; spermatozoa concentration 2.76-4.86 x109/ml; spermatozoa motility 66-79%; Live spermatozoa 91.75-93.10%, and spermatozoa abnormalities 1.75-2.51%. Semen from Sentul males aged 48-68 weeks had a thick consistency and cloudy white in color. At the age of 78 weeks, the consistency varies from thick, medium and liquid. The color of semen in Sentul males aged 78 weeks also varied from clear white to cream. It is concluded that aging in Sentul roosters causes reproductive disorders which are characterized by low sperm quality. The best quality spermatozoa were produced by Sentul roosters aged 58-68 weeks.

(Author)

Key Words: Aging, Sentul Rooster, Spermatozoa Quality

UDC: 636.082

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Keragaman gen Thy-1 pada galur ayam IPB D2: Asosiasi dengan antibody IgY dan ND (Polymorphisms of the Thy-1 Gene in IPB D2 Chicken Line: Association to IgY and ND antibody)

(Org: Eng)

JITV 27(4): 186-194

The Thy-1 gene plays a crucial role in immunological response, particularly in the activation of T cells. This gene encodes a Thy-1 protein (CD90) with 25–37 kDa of size. This study aimed to find a variation in the Thy-1 gene in IPB D2 chickens and its association with the total IgY concentration and ND antibody titers. A total of 123 head of 21 week old IPB D2 chickens consisting of 38 IPB D2 G0 (first generation) chickens and 85 IPB D2 G2 (second generation) chickens were used. PCR methods and direct-DNA sequencing were used to identify the polymorphism of Thy-1 gene. ELISA and HI test were used to analyze total IgY concentration and ND antibody titers. Genotype frequency, allele frequency, observed heterozygosity, expected heterozygosity, and Hardy-Weinberg equilibrium were analized. The General Linear Model and Duncan's Multiple Range Test were used to evaluate association between gene polymorphism and IgY concentration and ND antibody titers. Results discovered two SNPs on the intron (g.2139 C>T and g.2542 A>G). Four haplotypes were created by combining two SNPs. The g.2139 C>T and g.2542 A>G were polymorphic, but not in the Hardy-Weinberg equilibrium because of selection. The SNP g.2139 C>T were significantly associated ($P<0.05$) with total IgY concentration. All SNPs were not associated ($P>0.05$) with ND antibody titer. There was no association between the haplotype polymorphism of the Thy-1 gene with the total IgY concentration and ND antibody titer. It was identified that SNP g.2139 C>T was polymorphic and could be used as a candidate marker for high total IgY concentration. However, further analysis in large population and a validation through gene expression (qRT-PCR) are needed to prove this hypothesis.

(Author)

Key Words: IgY, Immunity, IPB D2 Chickens, ND Antibody Titer, SNP, Thy-1 gen

UDC: 637.5.03

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Analisa kandungan mineral daging pada ayam Cemani dengan genotipe homozigot (*Fm/Fm*) dan heterozigot (*Fm/fm*$^{+}$) [Analysis of meat mineral content in Cemani chicken with homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$) genotypes]

(Org: Eng)

JITV 27(4): 195-203

Cemani chicken is an Indonesian native chicken with black hyperpigmentation on feathers, skin, beak, comb, and flesh. Hyperpigmentation in chickens is called Fibromelanosis. Fibromelanosis in Cemani chickens is semi–dominant, producing two genotypes: homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$). Cemani chicken meat's black color may indicate a higher mineral content than regular chicken meat. The study's is aims are to detect genotype homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$) mutations and to determine the mineral content of Cemani chickens with homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$) genotypes. In the Cemani chicken population (n = 32), the *Fm–specific* allele genotype was detected using a Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) and the *Mlu*I restriction enzyme. The mineral contents of Cemani chicken tested were Fe, Zn, Mn, and Se. The results showed that homozygous Cemani chickens (*Fm/Fm*) had higher Fe and Zn mineral content. However, the homozygous (*Fm/Fm*) and heterozygous (*Fm/fm*$^{+}$) Cemani chicken were not different in mineral content of Se and Mn. This study found that different genotypes of Cemani chicken had different mineral compositions. In the future, this analysis supports the selection of chicken strains with high antioxidant levels.

(Author)

Key Words: Cemani Chicken, *Fibromelanosis*, Mineral Content, PCR–RFLP

UDC: 637.5'62

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Persepsi konsumen rumah tangga terhadap daging sapi beku (Household consumer's perception towards frozen beef, Indonesia)

(Org: Eng)

JITV 27(4): 204-214

Demand of beef in Indonesia is higher compared to the domestic beef supply, hence the Indonesian government has established policy to import frozen beef from other countries in order to support the high demand of Indonesia people. However, mosf of Indonesian people prefer to purchase fresh meat (freshly slaughtered beef) rather than frozen beef. The aims of this study were to identify the characteristics of household consumers who bought beef meat and their perceptions towards frozen beef; including to analyze the relationship between perceptions and consumer characteristics of frozen beef, and to analyze the consumer behavior in making decision to buy frozen beef in Bogor area and DKI Jakarta. Total about 200 households were directly interviewed using structured questionnaire. The questionnaires includes general characteristics of respondents (age, education level, occupation, income, number of family member), purchasing behavior which was analyzed descriptively, and respondents perceptions toward frozen beef which was analyzed by calculating the average value of perception and then categorized based on the level of perception category. Results showed that consumer's perceptions toward frozen beef according to several aspects had a low score (average final score 2.37), indicated that consumers had a poor perceptions towards frozen beef. Furthermore, most of the respondents who buy beef were housewives who buy meat in traditional markets which only provide fresh meat, they assumed that fresh meat had very good quality because the meat came from slaughtered beef. Those respondents think that freezing causes negative effects on beef, freezing treatment reduce the quality such as changes of meat color and flavour; and reduce nutritional content of beef. It was discovered that socialization about frozen beef and the process of handling frozen meat is required for household consumers to control the quality of beef as well as to increase the consumer's trusts in purchasing frozen beef.

(Author)

Key Words: Consumer Perception, Frozen Beef, Household Consumer, Purchase Behavior

AUTHOR GUIDELINES

Indonesian Journal of Animal and Veterinary Sciences or IJAVS contains:

(i) Primary scientific manuscript of unpublished research results.
(ii) Review research methods and innovative techniques useful for research development.

AUTHOR GUIDANCE

The manuscript is written in proper English, accompanied by an abstract in English and Indonesian. The manuscript is typewritten on the A4 paper size using Times New Roman 10 with single spaces distance and 4 cm from the left side, 3 cm from the right side, and 3 cm from the top and bottom sides. We provide you with IJAVS Template that you can find on our website: http://medpub.litbang.pertanian.go.id/index.php/jitv.

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Citation in the references:
The literature in the reference is written alphabetically based on the author's name. The same author is written sequentially, starting from earlier order.

Example of reference writing

Primary paper:
Barbato O, De Felice E, Todini L, Menchetti L, Malfatti A, Scocco P. 2021. Effects of feed supplementation on nesfatin-1, insulin, glucagon, leptin, T3, cortisol, and BCS in milking ewes grazing on semi-natural pastures. Animals. 11:682. DOI:10.3390/ani11030682.

Book:
a. Alshelmani M, Abdalla E, Kaka U, Basit M. 2021. Advances in poultry nutrition research. In: Kumar Patra A, editor. Adv Poult Nutr Res. London (UK): IntechOpen; p. 19–32. DOI: 10.5772/intechopen.91547.
b. Reece W. 2015. Respiration in mammals. New Jersey (USA): Willey-Blackwell.
c. Van Soest P. 2018. Nutritional ecology of the ruminant. 2nd ed. New York (USA): Cornell University Press.

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Damayanti R, Wiyono A, Dharmayanti N. 2021. Pathogenicity study of ducks infected with a local isolate of highly pathogenic avian influenza-H5N1-clade 2.3. . In: Inounu I, Priyanti A, Burrow H, Morris S, Min R, Suhubdy, Sutaryono Y, editors. Proc 4th Int Semin Livest Prod Vet Technol. Bogor (Indones): Indonesian Center for Animal Research and Development; p. 277–288.

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Mwasame DB. 2020. Analysis of the socio-

economic contribution of donkey ownership and use to houshold livelihoods in Kiambu country, Kenya (Thesis). Nairobi (KE). University of Nairobi

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Maranga B, Kagali R, Omolo K, Sagwe P. 2022. Effect of growth substrates on water quality, catfish (*Clarias gariepinus*) culture, and spinach (*Spinacia oleracea*) propagation under the aquaponic system. Livest Res Rural Dev.:82. http://www.lrrd.org/ lrrd34/9/3482mara.html.

Institution:

a. [PSA] Philippine Statistics Authority. 2016. Dairy Industry Performance Report, January – December 2015. Quezon City (Philiphine): Philippine Statistics Authority. P. 1-11
b. [FAO] Food and Agriculture Organization. 2021. Gateway to dairy production and products. Food Agric Organ United Nations. [accessed August 10, 2021]. https://www.fao.org/dairy-production-products/production/feed-resources/en/.

Patent:

Raab RM, Lazar G, Shen B. 2022. AGRIVIDA Inc, assignee. Engineered phytases in animal feed. 2022 Feb 8.

10. **Citation in text:**

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

Example:

a. One author: ..... ranging from 84 to 135 per minute (Scott 2015). Scott 2015 stated.....
b. Two authors: ..... in glucocorticoid production, primarily cortisol (Narayan & Parisella 2017). Narayan & Parisella (2017) stated that stress caused an increase in ……
c. Three authors or more: ..... in milk production without affecting the ewe's weight (Barbato et al. 2021). Barbato et al (2012) reported .....
d. The same author cited from 2 different papers: (Purwadaria et al. 2022a, 2022b).
e. The author with the same family name is written consecutive: (Dawson J 1986; Dawson M 1986).
f. Several different authors are written consecutively: (Damayanti et al. 2021; Leonard et al. 2022; Motlagh RK 2022).
g. Institution: FAO (2021).....

11. **Table:**

a. The standard word used is Times New Roman with 1 space distance and 11 of the font size.
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c. Each column from the table should have a heading. Its unit is separated from the title by a comma, in parentheses, or at its bottom.
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e. The dividing line is made in the form of horizontal.

12. **Figure and graphic:**

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b. Line in the graphic should show clearly the difference between one and others if there is more than one curve.
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