Buletin Peternakan 49 (1): 13-18, February 2025

ISSTAP
Indonesian Society for Sustainable Tropical Animal Production

Bulletin of Animal Science

ISSN-0126-4400/E-ISSN-2407-876X Accredited: 36a/E/KPT/2016
http://buletinpeternakan.fapet.ugm.ac.id/

Doi: 10.21059/buletinpeternak.v%vi%i.89123

Identification of AHSG Gene Polymorphism and Association With Flavor and Odor Compounds of Indonesian Lamb Meat

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Article history
Submitted: 18 September 2023
Accepted: 1 November 2024

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ABSTRACT

Flavor and odor are important factors for consumers when considering the quality of lamb meat. The Alpha2-Heremans-Schmid Glycoprotein (AHSG) gene is associated with a fatty acid content and is indicated as a gene controlling flavor and odor compounds. The research aims to identify the AHSG gene polymorphism and its association with flavor and odor compounds in Indonesian lamb meat. The sample used in this study was 105 ram samples consisting of 15 Jonggol sheep (JS), 80 Javanese Thin-Tailed Sheep (JTTS), and 10 Javanese Fat-Tailed Sheep (JFTS). The AHSG gene polymorphism was validated using the PCR-RFLP technique utilizing the EagI enzyme restriction. The association of the AHSG gene SNP (g.198655287 G>A) with odor and flavor was analyzed using the analysis of variance (general linear model procedure). In Indonesian sheep, the AHSG|EagI gene's SNP (g.198655287 G>A) showed polymorphic and three genotypes: GG, GA, and AA. The allele distribution was in the Hardy-Weinberg equilibrium. The AHSG significance was associated ($p<0.05$) with skatole compounds (MOA, EOA, MNA, and MI). The GG genotype is recommended for a candidate marker of flavors and odors because it is associated with low compound values. The AHSG gene, specifically the SNP in exons 3, is potentially a candidate genetic marker for lamb meat production by reducing flavor and odor.

Keywords: *AHSG gene, Flavor, Odor, Lamb meat, PCR-RFLP*

Introduction

Lamb meat is a food ingredient that contains many essential nutrients such as protein, minerals, vitamins, and fats that play a role in the human body (Fowler *et al.,* 2019). Lamb meat, classified as red meat, remains a favored choice among consumers in numerous countries owing to its distinctive flavor, texture, and nutritional profile (McAfee *et al*., 2010). In Indonesia, the demand for lamb meat has a favorable rate, reflected by the increase of slaughtered sheep in the last five years, with a percentage increase of 62% from 2017 to 2021 (Ditjen PKH, 2021). However, due to consumer demand, the increase in quantity should align with improving lamb's quality and nutritional content. Nowadays, consumers are more concerned about the quality (aroma, taste, texture, and color), safety, and nutritional values of lamb (Erasmus *et al.,* 2016).

Flavor and odor are essential for consumers to assess lamb quality (Watkins *et al.,* 2014), and flavor is an essential sensory component of meat quality that significantly affects consumer acceptability (Chen, 2012). Consumers tend to complain about lamb's firm, distinctive aroma; therefore, the quality of flavor and odor (taste and aroma) is essential in expanding the lamb business (Listyarini *et al.,* 2018). The components that contribute to flavor and odor are branched-chain fatty acids (BCFA), including 4-methyl octanoic acid (MOA), 4-methyl nonanoic acid (MNA), 4-ethyl octanoic acid (EOA), 3-methylindole (MI), and 3-Methylphenol (MP) (Chen *et al.,* 2012; Watkins *et al.,* 2014; Gunawan *et al.,* 2018).

Improving lamb meat's flavor and odor compounds through genomic investigations represents an effective and efficient approach for identifying genes regulating these qualities. Alpha2-Heremans-Schmid Glycoprotein, or Fetuin-A, is a glycoprotein encoded, mainly secreted by the liver (Fisher *et al.,* 2009). The AHSG glycoprotein, abundant in bone minerals, effectively binds calcium ions (Ca^{2+}), inhibiting the formation of apatite minerals. This protein plays a

vital role in the metabolism of bone cells in humans, as elucidated by Jiang *et al*. (2007). Furthermore, Zhu *et al*. (2021) established a positive association between the AHSG gene and the desirable attributes of tenderness and chewiness in limousin beef. According to Munyaneza *et al*. (2019), the AHSG gene modifies the fatty acid compounds of several Indonesian sheep breeds by decreasing saturated and high unsaturated fatty acids at the SNP g.198655287 G>A. The AHSG gene also regulates insulin sensitivity and body fat, impacting metabolism and body fat composition. Additionally, Fetuin-A, a protein closely related to human plasma cholesterol levels, is circulating due to the AHSG gene (Marechal *et al*., 2011). Previous investigations have not explored the AHSG gene's implications for flavor and odor compounds in Indonesian sheep. Consequently, the primary objective of this research is to determine the polymorphism of the AHSG gene and its potential association with flavor and odor compounds. PCR-RFLP technique can be utilized to acquire this.

Materials and Methods

Animals and Phenotypes

This research used DNA sheep with a total sample of 105 collected from 15 Jonggol Sheep (JS), 80 Javanese Thin Tail Sheep (JTTS), and 10 Javanese Fat Tail Sheep (JFTS). These samples were extracted from the longissimus dorsi muscle of the rams, with the animals being approximately 10-12 mon. old. They were maintained by intensive farming and provided Napier grass and concentrated diet, with water provided ad libitum. The sheep from these 105 animals were positioned at -20°C and later utilized for flavor and odor compounds. All treatments involving animals under permission have been approved by the IPB University's animal ethics commission (approval number: 117-2018 IPB).

Flavor and odor compounds analysis

Samples analyzed included 4-methyloctanoic (MOA), 4-methylnonanoic (MNA), 3-methylindole (MI), 4-methylphenol (MP), and ethyloctanoic (EOA). Five hundred grams of longissimus dorsi muscle samples were used for flavor and odor analysis. The Likens-Nicerson technique, which combines solvent extraction with distillation utilizing Gas Chromatography-Mass Spectophotometry (GC-MS), was used to extract volatile flavor and odor components (Gunawan *et al*., 2013; Watkins *et al*., 2014).

DNA Extraction and Amplification

DNA extraction from Longissimus dorsi samples was performed using the Geneaid gSYNC DNA extraction Kit. The extraction process involved four stages: (1) preparation of the sample, (2) degradation of the protein, (3) degradation of the organic materials, and (4) precipitation of DNA. Sambrook *et al*. (1989) described how the extracted DNA was subsequently preserved at -20 °C. The SNP (g.198655287 G>A), the forward primer (5'-GGA GGA ATC AGG GCA TTT TC-3'), and the reverse primer (5'CCC ATA TCC TTA CGC AAT CC-3') utilized for amplification were observed from the approach outlined by Munyaneza et al. (2019), resulting in a product length of 473 bp. The AHSG gene contains 9 exons and 10 introns and is on chromosome 1. The SNP point (g.198655287 G>A) within the AHSG gene is in exon 3 and classified as a synonymous variant.

The amplification of DNA involves two stages. Initially, 2 µL of the extracted DNA was divided into a 0.2 mL tube, and a solution mixture comprising 6.1 µL of NFW, 0.2 µL of the forward and reverse primer, and 7.5 µL of My Taq HS Red Mix was prepared. This mixture was then thoroughly combined using a centrifuge. Subsequently, combined material was subjected to DNA amplification using an ESCO thermocycler machine during the sample incubation phase. The first denaturation stage of the thermal cycling technique lasted 5 min at 95 °C. The next 35 cycles were as follows: 10 s of denaturation at 95 °C, 20 s of annealing at 58 °C, and 30 s of extension at 72 °C. The process concluded with a final extension step of 5 min at 72 °C to ensure complete primer extension. The results of DNA amplification were completed through electrophoresis on a 1.5% agarose gel.

Polymorphism identification using PCR-RFLP

The PCR-RFLP method utilizes the EagI restriction enzyme to identify polymorphism of the AHSG gene. The mixed samples underwent incubation at 37 °C for 4 h (Thermo Fisher Scientific, EU, Lithuania). Subsequently, 5 µL of DNA was dispensed into a 0.5 mL tube and combined with 2 µL of a mixture containing 0.9 µL of NFW, 0.7 µL of enzyme buffer, and 0.4 µL of EagI restriction enzyme. The samples were visualized by electrophoresis on a 1.5% agarose gel after incubation. The total genotypes of the AHSG with EagIs marker were determined as follows: the AA genotype exhibited a band of 473 bp, the GA genotype showed 473, 273, and 200 bp, and the GG genotype showed 273 and 200 bp.

Statistical Analysis

The Pop Gen 32 software assessed gene polymorphism and Hardy-Weinberg equilibrium, genotype frequency, and allele frequency. The association between the AHSG gene and flavor and odor was determined using the analysis of variance (general linear model procedure) statistical model in SAS 9.2 software.

The approach described by Hartl and Clark (1997) was utilized to calculate Allele frequency and genotype frequency:

$$X_i = \frac{(2n_{ii}+\sum j \neq jn_{jj})}{2N} X_{ii} = \frac{n_{ij}}{N}$$

Note:
Xi = i-th allele frequency
Xii = i-th genotype frequency

N = total number of samples
nii = number of animals with genotype ii
nij = number of animals with genotype ij

The approach described by Hartl and Clark (1997) was utilized to calculate the Hardy-Weinberg equilibrium:

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

Note:
X^2 = chi-square
O = observation value
E = expected value

The association of the AHSG gene genotypes with flavor and odor compounds was described using the analysis of variance (general linear model procedure):

$$Yij = \mu + Gi + eij$$

Yij = observed values variables
μ = the overall sheep mean
Gi = the AHSG genotype fixed effect
eij = the random error

Normality Test

A normality test, either the Kolmogorov-Smirnov or Shapiro-Wilk test, was performed prior to analyzing variance (general linear model procedure). If the data were found to be non-normal, the outlier data were eliminated. After removing all outlier data and ensuring that the data existed non-normal, the data was transformed, and the analysis of variance (general linear model procedure) was carried out (Mishra *et al*., 2019).

Results and Discussion

Polymorphism of the AHSG Gene

AHSG gene with the EagI restriction enzyme resulted in three genotypes: GG, GA, and AA. The selection of the restriction enzyme in this study identified and cleaved the target DNA sequence (Gunawan *et al*., 2017), generating different fragment products (Klug *et al*., 2006) (Figure 1).

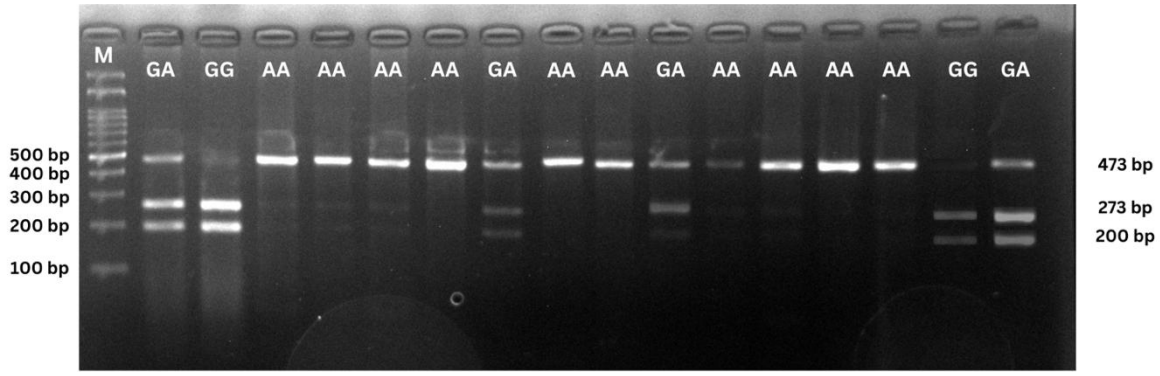

Figure 1. Visualization results of AHSG gene (g.198655287 G>A) in Indonesian sheep with three genotypes: GG, GA, and AA using 2% agarose gel and 100 bp marker.

AHSG gene with the EagI mutation involves a change from G to A base. The GG genotype results in a fragment product that is made up of 200 and 273 bp fragments. By difference, the AA genotype yields a single 473 bp fragment, whereas the GA genotype yields three fragments measuring 473 bp, 273 bp, and 200 bp. The genotype frequency, allele frequency, and Hardy-Weinberg equilibrium of the AHSG gene are shown in Table 1.

Table 1. Genotype and allele frequencies and Hardy-Weinberg equilibrium of AHSG gene in Indonesian sheep population

No.	Breed	N	Genotype Frequency			Allele Frequency		Chi-square
			AA (n)	GA (n)	GG (n)	A	G	(x^2)
1	Javanese Fat-Tailed Sheep (JFTS)	10	1,00 (10)	0,00 (0)	0,00 (0)	1,00	0,00	-
2	Javanese Thin-Tailed Sheep (JTTS)	80	0,53 (41)	0,39 (35)	0,07 (4)	0,73	0,27	1,02
3	Jonggol Sheep (JS)	15	0,40 (4)	0,46 (11)	0,00 (0)	0,63	0,36	5,03
4	Total Population	105	0,55 (55)	0,38 (46)	0,07 (4)	0,74	0,26	1,61

Note: N = Number of samples; JFTS = Javanese fat-tailed sheep; JTTS = Javanese thin-tailed sheep; JS = Jonggol sheep and x^2 (0.05) = 3,84

DNA polymorphism occurs due to mutations in both genes and chromosomes (Gunawan *et al.,* 2017), and mutations are changes in nucleotide bases in genes that cause changes in gene function (Noor, 2010). The AHSG gene with EagI gene shows polymorphism in local sheep, resulting in the GG, GA, and AA genotypes. The GG genotype had a frequency of 0.07, and the GA genotype had a frequency of 0.38, indicating that the AA genotype was dominant, occurring at a frequency of 0.55. This result suggests that the A allele dominates the G allele within the studied Indonesian sheep. The AHSG gene is classified as polymorphic because the overall genotype frequency in the sheep population is less than 0.99, containing the JTTS and JS samples, except for the JFTS sample, which exclusively exhibits the AA genotype. A SNP in a gene is considered polymorphic if it has several alleles with a

frequency value of at least 1% (Rell *et al*., 2013; Gunawan *et al*., 2018).

The AHSG gene in the investigated Indonesian sheep and Javanese thin-tailed sheep (JTTS) in the Hardy-Weinberg equilibrium, except for the Jonggol sheep (JS) population, according to the chi-square analysis, which revealed no significant differences. The allele distribution is not in equilibrium in the populations of Jonggol sheep (JS) and Javanese fat-tailed sheep (JFTS), suggesting that these populations may contain experienced processes of selection, migration, mutation, or selective mating (Hadini *et al*., 2015).

Association of the AHSG Gene with Flavor and Odor Compounds

The SNP (g.198655287 G>A) AHSG gene in exon 3 has a positive association with flavor and odor compounds, including 4-methylnonanoic acid (MNA), 3-methylindole (MI)/skatole, 4-methyloctanoic acid (MOA), and 4-ethyloctanoic acid (EOA). In the case that the AHSG gene is associated with 4-methylnonanoic acid (MNA). The AHSG gene with the A allele indicates a dominant impact on flavor and odor compared to the G allele. This effect is most pronounced in individuals with the AA genotype (homozygous) and the GA genotype (heterozygous) as opposed to those with the GG genotype (homozygous). Consequently, individuals with the AA genotype exhibit a more intense odor than those with the GG and GA genotypes. Chen *et al.,* (2012) explained that 4-methylnonanoic acid (MNA) is the main compound contributing to the 'sweaty' taste of lamb (mutton). The 4-methylnonanoic acid (MNA), one of the components of Branched Chain Fatty Acids (BCFAs), is often associated with 'mutton flavor' in lamb cooking aroma and is influenced by lamb age. Genes that affect fatty acid content influence the quality of flavor and odor (Gunawan *et al.,* 2018). In previous studies, The SNP (g.198655287 G>A) AHSG gene was associated with a fatty acid content, including low saturated, high unsaturated, total fatty acids including the Heptadecanoic acid (C17:0), which was associated with flavor and odor (Munyaneza *et al.,* 2019) The heptadecanoic acid (C17:0) is a good predictor for the 4-Methylnonanoic acid (MNA) (Watkins and Frank 2019). Flavor components in meat are indirectly influenced by fatty acid content in various animal species (Kosowska *et al.,* 2017).

Table 2. Association of AHSG Gene with Flavor and Odor Compounds

Flavor and Odor Compound (µg/µl)	Genotype ($\bar{X}$ ±SE Means)		
	GG	GA	AA
4-Methyloctanoic acid (MOA)	0,87±0,17 (3)^{a,b}	8,52±1,22 (24)^{a}	2,63±0,66 (42)^{b}
4-Ethyloctanoic acid (EOA)	57,37±20,30 (3)^{a}	19,36±4,35 (26)^{a}	13,67±3,66 (45)^{b}
4-Methylnonanoic acid (MNA)	8,68±7,54b (4)^{a,b}	22,12±6,57a (37)^{a}	13,18±3,50ab (52)^{b}
3-Methylindole (MI)	1,63±1,07 (2)^{a,b}	6,32±2,58 (12)^{a}	2,22±0,98 (33)^{b}
3-Methylphenol (MP)	1906,0±1906,0 (2)	5,31±1,45 (19)	9,85±1,30 (38)

Note: $\bar{X}$= means of flavor and odor; SE means= Standard Error; ^{a,b} Mean in the same row with different superscripts differ significantly ($p<0.05$).

Levels of 3-methylindole (MI)/skatole compounds affect the "pasture aroma" of lamb meat after cooking for the sheep reared under pasture management and leguminous feeding, and these compounds resulted in the high smell-like aroma (Castada *et al*., 2017; Watkins *et al.,* 2014; Young *et al.,* 2003). The rumen of ruminants contains indole and skatole chemicals, which are produced by microbial activities that include tryptophan's deamination and decarboxylation. The substances enhance the taste of meat by being absorbed into adipose tissue (Young *et al*., 2003). Essential flavor compounds isolated by adipose tissue and lean meat contribute to the perceived flavor (Grabez *et al.,* 2019). Indole and skatole synthesis is influenced by feed such as grasses and legumes (Schreurs *et al.,* 2008; Devicienzi *et al.,* 2014). Previous studies indicated that several genes, including CYP2A6 (Listyarini *et al.,* 2018) and CYP2E1 (Harahap *et al.,* 2021), contribute to reducing the levels of 3-Methylindole (MI)/skatole compounds in lamb meat. Non-genetic factors such as diet system (grass, legumes, and concentrates) and mature sheep's (mutton) age significantly influence flavor and odor, particularly the unique aroma of lamb meat, which dramatically impacts consumer preference (Watkins *et al*., 2013)

The compounds 4-methyloctanoic acid (MOA) and 4-ethyloctanoic acid (EOA) have been widely significant with good flavor and odor in sheep. The 4-methyloctanoic acid (MOA) and 4-ethyloctanoic acid (EOA) are also compounds of Branch Chain Fatty Acids (BCFAs) that influence 'mutton flavor' in sheep. Age of lamb contributes to increased levels of BCFAs, particularly the compound 4-ethyloctanoic acid (EOA), and increasing age is categorized as a more substantial 'mutton' aroma (>2 years) compared to 'lamb' (<1 year) (Watkins *et al.,* 2010; Watkins *et al.,* 2014). Watkins *et al.,* (2014) explained that the sensory test of meat after cooking is strongly influenced by the 4-methyloctanoic acid (MOA) and 4-ethyloctanoic acid (EOA).

GG genotype is a recommended genetic marker (AHSGIEagI) for flavor and odor compounds, as seen by the low mean values compared to genotypes AA and GA on compound 4-methyl octanoic acid (MOA): 0,87 µg/µL, 4-methylnonanoic acid (MNA): 8.68 µg/µL, and 3-methylindole (MI)/skatole: 1.63 µg/µL. The SNP (g.198655287 G>A) AHSG gene located in exon 3 plays a significant role in the fatty acid composition. Additionally, this study has identified an association between this SNP and flavor and odor. Genetic marker recommendations could be used as

breeding patterns to obtain sheep with reduced flavor and odor compounds (smell-like aroma).

Conclusion

The SNP (g.198655287 G>A) of the AHSG gene showed polymorphism in Indonesian sheep. AHSG|EagI revealed three genotypes: GG, GA, and AA. The GG genotype produces low flavor and odor compounds. The allele distribution was in the Hardy-Weinberg equilibrium. Additionally, a significant association was identified between the AHSG gene and flavor and odor compounds containing 4-methyloctanoic acid (MOA), 4-ethyloctanoic acid (EOA), 4-methylnonanoic acid (MNA), and 3-methylindole (MI)/skatole. It could be speculated that the AHSG gene has the potential to be a candidate marker for breeding programs to obtain sheep with low flavor and odor compounds (smell-like aroma).

Conflict of interest

The authors state that they have no conflicts of interest, whether financial, personal, or affiliative, with individuals or organizations associated with the subject matter discussed in the manuscript.

Funding statement

This research is supported by the Kedaireka Matching Fund program from the Indonesian Ministry of Education and Culture in 2023, with grant number 18977/IT3.L1/HK.07.00/P/T/2023.

Acknowledgment

The authors gratefully acknowledge the support from the Teaching Factory Domba Premium IPB, the Kedaireka Matching Fund program from the Indonesian Ministry of Education and Culture, and Inter-University Program (PUAPT) 2024.

Author's contribution

Asep Gunawan developed the research concept. Ganda Adi Septiyawan conducted the research, processed the data, and drafted the manuscript. Kasita Listyarini, Ronny Rachman Noor, and Katrin Rosita developed the methodology and reviewed and corrected the data and manuscript results.

Ethics approval

This experiment had no adverse impacts on the animal's welfare. All treatments involving animals under permission have been approved by the IPB University's animal ethics commission (approval number: 117-2018 IPB).

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