Identification and association of polymorphism in THOC5 gene with fatty acid composition in Indonesian sheep

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Abstract. THO Complex 5 (THOC5) gene involves in lipid and fatty acid metabolism. The aim of this study was to analyse polymorphism of THOC5 gene and its association with fatty acid composition in sheep. A total of 120 rams at the age 12 month with the average body weight of 25–30 kg was used for identification of gene polymorphism using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). 83 rams, representative from different genotypes of sheep were used for association study using a General Linear Model (GLM). The results identified polymorphism in the THOC5 gene. The THOC5 gene showed two genotypes: CC and CT genotype. Association analysis revealed that THOC5 (g.68234589 C>T) was significantly (P<0.05) associated with fatty acid composition including unsaturated fatty acid: palmitoleic acid (C16:1), and saturated fatty acid: stearic acid (C18:0). The CC genotype was associated with higher level of unsaturated fatty acid and lower level of saturated fatty acid, while the CT genotype was vice versa. This result indicates that THOC5 gene (g.68234589 C>T) may contribute to fatty acid composition in sheep, as well as this polymorphism could be used as a candidate to select sheepmeat with high unsaturated fatty acid and low saturated fatty acid.

1. Introduction
Fat and fatty acids (FA) are essential to meat’s nutritional value and contribute significantly to different aspects of meat quality [1]. The fatty acid can be divided into two general categories: saturated (SFA) and unsaturated (UFA). Meat contains high saturated fatty acid (SFA) levels [2]. A previous study reported that intake of saturated fatty acid (SFA) has strong positive correlations with the incidence of cardiovascular disease (CVD) [3,4]. Conversely, intake of unsaturated fatty acids (UFA has been shown to minimize the risk of CVD [5]. For human health, meat production with high UFA and low SFA content is beneficial [6].

Genetic improvement through selection is recommended since one of the most realistic approaches to producing meat with high UFA and low SFA content. Fatty acid composition in sheep shows moderate to high heritability, indicating that they can modify fatty acid characteristics through selection [7,8]. The THO Complex 5 (THOC5) gene is one the critical gene which contributes to fatty acid metabolism. THOC5 gene has also been found to affect meat tenderness in cattle [9]. The beneficial effects of lipid on meat tenderness are possibly due to the lipid inside perimysium cells that separate muscle fiber bundles [9].
A number of candidate genes for fatty acid composition in sheep have been detected including SCD [10], APOA5 [6], TGFBR2 [11], KIF12 [12], LEPR [13], DGAT1 [14], AHSG [15], and BHMT genes [16]. However, the identification and association study of the THOC5 gene related to fatty acid composition has not been conducted, notably in Indonesian sheep. In sheep, a quantitative trait locus (QTL) affecting the fatty acid composition has been identified. QTL for fatty acid composition was identified for chromosome 2 affecting linoleic acid (18:3 n-3) [17]. Positional and functional research has shown that the THOC5 gene could be significant for the fatty acid composition candidate gene. This study was conducted to analyse the polymorphism and association of the THOC5 gene with fatty acids composition in sheep.

2. Material and Methods

2.1. Animals
A number of 120 samples (Longissimus dorsi and blood) and phenotypes were collected from body-weight rams between 25–30 kg and 12 months of age to identify gene polymorphism. The rams were retained under the same management systems and fattening feed was given ad libitum. A total of 83 slaughtered rams were used for association study in a profit-oriented slaughtered house. The data of carcass and meat quality were gathered in accordance with the Indonesian performance test guidelines. For fatty acid composition analysis, longissimus dorsi was taken for as much as 500 g. 30 μL of blood was taken for DNA extraction. All samples were placed in an ice flask for fatty acid composition analysis and DNA extraction and were stored at a temperature of -20°C.

2.2. Fatty acid composition analysis
Analysis of fatty acid composition was carried out on longissimus dorsi samples. For each sample, the fat and fatty acid composition were measured using AOAC 991.36 (2012) and AOAC 969.333 (2012) extraction methods (2012). The determination of the composition of fatty acids was analyzed by Gas Chromatography (GC), which needs the fat extraction process, followed by methylation, conversion of the FAs into methyl esters afterward (FAMEs), and finally determination process by Gas Chromatography. Measurements of fatty acid composition included saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA).

Table 1. Gen bank accession number and primers.

| Gene name | Accession number | Primer | Size (bp) | Tm (°C) | Enzyme | SNP | Digested fragments length (bp) |
|-----------|-----------------|--------|-----------|---------|--------|-----|-------------------------------|
| THOC5     | NC_019474.2     | F : 5’-CCC AGG AAG GTT TGA TTC TC-3’ | 322     | 60      | Tail  | g.682 | CC = 129, 193 bp |
|           |                 | R : 5’-CAC ACC TAC CAT GTA GTC CT-3’ |         |         |       | 34589 | CT = 129, 193, 322 bp |
|           |                 | C>T    |           |         |       |      | TT = 322 bp                   |

2.3. DNA extraction and PCR-RFLP amplification
Genomic DNA was extracted from blood using the Geneaid Genomic DNA Mini Kit. Using MEGA 6.0 software, gene-specific primers for the THOC5 gene (table 1) were developed. The PCR was conducted with reaction of 15 μL containing 1 μL of genomic DNA, 0.2 μL of forward and reverse primers, 7.5 μL of MyTaq Red Mix and 6.1 μL of ddH2O. THOC5 gene fragment amplification was performed using the ESCO GeneAmp PCR system with the following thermocycling profile: initial denaturation at 94°C for 1 min, then 35 cycles at 94°C for 10 s, 60°C for 15 s, 72°C for 15 s, and final extension at 72°C for
1 min. The DNA amplicon was visualized by 1.5 percent electrophoresis gel. The validation of SNP genotyping was carried out using PCR-RFLP. The DNA amplification product and TaqI enzymes restriction were incubated for 4 hours at 37°C. Using 2 percent agarose gel, the digested products were visualized.

2.4. Data analysis
The value of the allele frequency, genotype frequencies, and the equilibrium value of Hardy-Weinberg were performed according to Nei and Kumar (2000) after genotypes obtained through PCR-RFLP method. Statistical analysis was calculated using SAS ver 9.2. PROC GLM was used to assess the effects of genotype using a fixed effect model (ANOVA) [18].

\[ Y_{ijk} = \mu + \text{genotype}_i + e_{ij} \]

Where:
- \( Y_{ijk} \) = is the sheepmeat compounds (fatty acid composition)
- \( \mu \) = is the population mean
- \( \text{genotype}_i \) = is the fixed effect of i-th genotype (i = CC and CT)
- \( e_{ij} \) = is the residual error

3. Result and discussion

3.1. Phenotype of fatty acid composition in Indonesian sheep
The mean and standard deviation (SD) value of fatty acid composition was presented in table 2. Thirty-one fatty acid compositions were detected, consisting of fourteen SFA and seventeen UFA (PUFA and MUFA). Erucic acid (C22:1n9) showed the lowest mean values (0.0004%) among all the fatty acid composition, whereas oleic acid (C18:1n9c) showed the highest mean values (24.090%). The mean value of fatty acid composition in this study including palmitic acid (C16:0), myristic acid (C14:0), and oleic acid (C18:1n9c), and stearic acid (C18:0) were low in comparison with previous studies [19,20].

The predominant SFAs were palmitic acid (C16:0, 18.425%) and stearic acid (C18:0, 15.703%). These findings are comparable to those reported in prior studies [7,21,22] where these SFA were predominant in sheep. Palmitic acid (C16:0) has been related to increased total plasma cholesterol, particularly LDL (low-density lipoproteins) [23]. The stearic acid (C18:0) improved the plasma cholesterol profile by decreasing total or HDL (high-density lipoproteins) cholesterol ratio compared to other SFA and tended to raise LDL cholesterol, increased the ratio of total HDL cholesterol compared to UFA [24]. The most abundant UFA was oleic acid (C18:1n9c, 24.090%). This result was consistent with the previous study [7,21,25]. The oleic acid (C18:1n9c) has decreased the total plasma cholesterol, however it has not changed plasma triglycerides and non-esterified fatty acids [26].

3.2. Polymorphism of THOC5 gene
A SNP was genotyped in THOC5 gene at position g.68234589 C>T. The SNP was revealed by PCR-RFLP and the THOC5 gene showed two genotypes namely CC and CT genotype. DNA restriction fragments acquired for g.68234589 C>T of THOC5 polymorphism were: 129, 193 bp for CC genotype and 129, 193, 322 bp for CT genotype (figure 1).

The CC genotype was more frequent than CT, and TT genotype was not found in our populations. This result was consistent with a prior study reported with different genes (CYP2A6 and CAST-MspI locus) [27,28] in Indonesian sheep. The TT genotype was not detected, probably due to direct selection or a non-random mating system [29]. The THOC5 gene (g.68234589 C>T) was identified in the Hardy Weinberg Equilibrium (HWE) (P<0.05) as a fundamental concept of population genetics [30]. HWE is used to calculate the number of homozygous and heterozygous carrier variants based on their allele frequency in populations that do not develop [31]. HWE is in an equilibrium state if the frequency of genotypes in the population remains constant among generations in the absence of external factors.
disturbance [30]. The number of animals per genotype and allel frequency of THOC5 was shown in table 3.

**Table 2.** Fatty acid composition (%), mean value and standard deviation (SD) in Indonesian sheep.

| Fatty acid composition (%) | Mean (n=83) | SD |
|----------------------------|-------------|----|
| Fat content                | 4.143       | 3.357 |
| Caprilic acid (C8:0)       | 0.046       | 0.121 |
| Capric acid (C10:0)        | 0.088       | 0.050 |
| Laurie acid (C12:0)        | 0.484       | 0.501 |
| Tridecanoic acid (C13:0)   | 0.011       | 0.013 |
| Myristic acid (C14:0)      | 3.133       | 1.778 |
| Myristoleic acid (C14:1)   | 0.151       | 0.106 |
| Pentadecanoic acid (C15:0) | 0.497       | 0.147 |
| Palmitic acid (C16:0)      | 18.425      | 3.883 |
| Palmitoleic acid (C16:1)   | 1.530       | 0.399 |
| Heptadecanoic acid (C17:0) | 0.936       | 0.340 |
| Ginkgolic acid (C17:1)     | 0.390       | 0.348 |
| Stearic acid (C18:0)       | 15.703      | 5.575 |
| Elaidic acid (C18:1n9c)    | 2.916       | 7.861 |
| Oleic acid (C18:1n9c)      | 24.090      | 9.400 |
| Linoleic acid (C18:2n6c)   | 2.427       | 2.008 |
| Linolenic acid (C18:3n3)   | 0.290       | 0.231 |
| v-Linolenic acid (C18:3n6) | 0.032       | 0.067 |
| Arachidic acid (C20:0)     | 0.117       | 0.101 |
| Eicosenoic acid (C20:1)    | 0.029       | 0.087 |
| Eicosadienoic acid (C20:2) | 0.052       | 0.055 |
| Cis-8,11,14-Eicosatrienoic acid (C20:3n6) | 0.079 | 0.121 |
| Arachidonic acid (C20:4n6) | 1.020       | 1.414 |
| Cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5n3) | 0.166 | 0.195 |
| Heneicosylic acid (C21:0)  | 0.021       | 0.021 |
| Behenic acid (C22:0)       | 0.070       | 0.093 |
| Erucic acid (C22:1n9)      | 0.0004      | 0.002 |
| Cis-13,16-Docosadienoic acid (C22:2) | 0.007 | 0.041 |
| Docosahexaenoic acid (C22:6n3) | 0.051 | 0.076 |
| Tricosanoic (C23:0)        | 0.035       | 0.055 |
| Tetracosanoic acid (C24:0) | 0.054       | 0.105 |
| Nervonic acid (C24:1)      | 0.047       | 0.097 |
| Fatty acid total           | 72.920      | 9.200 |
| Saturated fatty acid (SFA) | 39.625      | 7.637 |
| Monounsaturated fatty acid (MUFA) | 26.210 | 9.600 |
| Polyunsaturated fatty acid (PUFA) | 4.119 | 3.030 |
| Unsaturated fatty acid (UFA) | 30.330     | 9.720 |
3.3. Association of THOC5 gene polymorphisms related to fatty acid composition

Association analysis showed that THOC5 (g.68234589 C>T) was strongly (P<0.05) related to fatty acid composition, which includes unsaturated fatty acid, palmitoleic acid (C16:1), and saturated fatty acid; stearic acid; (C18:0). The CC genotype was associated with a higher level of unsaturated fatty acid and a lower saturated fatty acid level, whereas the CT genotype was vice versa (table 4).

The THOC5 gene (g.68234589 C>T) was significantly (P<0.05) associated with palmitoleic acid (C16:1) and stearic acid (C18:0). This result was consistent with the previous study of various genes in Indonesian sheep [14,16]. Palmitoleic acid (C16:1) has been observed to have potential benefits on increased insulin sensitivity, metabolism of cholesterol, hemostasis, decreased bodyweight, enhanced hyperglycemia, and hypertriglyceridemia [32,33].

Stearic acid (C18:0) has been observed to have potential benefits on decreased cardiovascular and cancer risk different from other saturated fatty acids [34]. The neutral effect of stearic acid (C18:0) on whole blood and low-density lipoprotein (LDL) cholesterol levels has been demonstrated [35]. The THOC5 gene is involved in lipid and fatty acid metabolism [9]. A previous study reported that the THOC5 gene, together with all three study populations, was significantly related to HDL-C in a simultaneous meta-analysis and further studied in an in vitro analysis to elucidate its potentially important role in the metabolism of HDL-C [36]. This study showed the important role of the THOC5 gene in the metabolism of fatty acids. Sheep with a high level of unsaturated fatty acid (palmitoleic acid, C16:1) and a low level of saturated fatty acid (stearic acid, C18:0) may be produced by a selection of sheep with a CC genotype.

### Table 3. The number of animals per genotype and allele frequency

| Sample            | N  | Genotype | Allele | Chi square (χ²) |
|-------------------|----|----------|--------|-----------------|
| Indonesian sheep  | 120| CC       | CT     | TT              |
|                   |    |          |        | 0.94 0.06 0.533 |

Figure 1. PCR-RFLP result for the THOC5 Gene
Table 4. Analysis of THOC5 genotype and association.

| Composition of fatty acid (%) | Genotype |   |   |
|------------------------------|----------|---|---|
|                              | CC       | CT | TT |
| Fat content                  | 4.30±3.39| 1.67±0.82| 0.00±0.00|
| Capric acid (C8:0)           | 0.04±0.12| 0.00±0.00| 0.00±0.00|
| Caprylic acid (C10:0)        | 0.09±0.05| 0.05±0.01| 0.00±0.00|
| Lauric acid (C12:0)         | 0.49±0.51| 0.37±0.13| 0.00±0.00|
| Tridecanoic acid (C13:0)     | 0.01±0.01| 0.01±0.01| 0.00±0.00|
| Myristic acid (C14:0)        | 3.19±1.81| 2.20±0.43| 0.00±0.00|
| Myristoleic acid (C14:1)     | 0.15±0.10| 0.08±0.02| 0.00±0.00|
| Pentadecanoic acid (C15:0)  | 0.49±0.14| 0.47±0.14| 0.00±0.00|
| Palmitic acid (C16:0)        | 18.58±3.92| 15.98±2.09| 0.00±0.00|
| Palmitoleic acid (C16:1)     | 1.55±0.39| 1.17±0.18| 0.00±0.00|
| Heptadecanoic acid (C17:0)  | 0.94±0.34| 0.77±0.09| 0.00±0.00|
| Ginkgolic acid (C17:1)       | 0.39±0.35| 0.29±0.03| 0.00±0.00|
| Stearic acid (C18:0)         | 15.37±5.51| 20.89±3.92| 0.00±0.00|
| Elaidic acid (C18:1n9t)      | 2.80±7.74| 4.62±10.34| 0.00±0.00|
| Oleic acid (C18:1n9c)        | 24.50±9.35| 17.51±8.19| 0.00±0.00|
| Linoleic acid (C18:2n6c)     | 2.42±2.05| 2.40±1.30| 0.00±0.00|
| Linolenic acid (C18:3n3)     | 0.30±0.23| 0.12±0.02| 0.00±0.00|
| v-Linolenic acid (C18:3n6)   | 0.03±0.06| 0.02±0.04| 0.00±0.00|
| Arachidic acid (C20:0)        | 0.03±0.08| 0.12±0.07| 0.00±0.00|
| Eicosenoic acid (C20:1)      | 0.05±0.05| 0.05±0.01| 0.00±0.00|
| Eicosadienoic acid (C20:2)   | 0.05±0.12| 0.08±0.04| 0.00±0.00|
| Cis-8,11,14-Eicosatrienoic acid (C20:3n6) | 0.17±0.19| 0.08±0.05| 0.00±0.00|
| Arachidonic acid (C20:4n6)   | 1.01±1.45| 1.13±0.59| 0.00±0.00|
| Cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5n3) | 0.02±0.02| 0.02±0.01| 0.00±0.00|
| Heneicosylic acid (C21:0)    | 0.06±0.09| 0.07±0.02| 0.00±0.00|
| Behenic acid (C22:0)         | 0.0005±0.002| 0.00±0.00| 0.00±0.00|
| Erucic acid (C22:1n9h)       | 0.17±0.19| 0.08±0.05| 0.00±0.00|
| Cis-13,16-Docosadienoic acid (C22:2) | 0.007±0.04| 0.00±0.00| 0.00±0.00|
| Docosahexaenoic acid (C22:6n3) | 0.05±0.07| 0.04±0.02| 0.00±0.00|
| Tricosanoic acid (C23:0)     | 0.03±0.05| 0.04±0.02| 0.00±0.00|
| Tetraicosanoic acid (C24:0)  | 0.05±0.10| 0.05±0.02| 0.00±0.00|
| Nervonic acid (C24:1)        | 0.04±0.10| 0.03±0.02| 0.00±0.00|
| Fatty acid total             | 73.17±9.33| 68.79±6.03| 0.00±0.00|
| Saturated fatty acid (SFA)   | 39.53±7.72| 41.09±6.54| 0.00±0.00|
| Monounsaturated fatty acid (MUFA) | 26.66±9.54| 19.10±8.30| 0.00±0.00|
| Polyunsaturated fatty acid (PUFA) | 4.13±3.10| 3.94±1.48| 0.00±0.00|
| Unsaturated fatty acid (UFA) | 30.79±9.61| 23.04±9.22| 0.00±0.00|

Mean±SD are the percentage unit of fatty acid composition.
ab Mean value with differ significantly at P<0.05.

4. Conclusion
The THOC5 gene (g.68234589 C>T) is polymorphic in Indonesian sheep. The association analysis shows that the THOC5 gene is significantly (P<0.05) associated with the fatty acid composition, including palmitoleic acid (C16:1), unsaturated fatty acid, and stearic acid (C18:0), saturated fatty acid. The CC genotype was associated with a higher level of unsaturated fatty acid and a lower saturated fatty acid level. These results indicate that the SNP in the THOC5 gene (g.68234589 C>T) may contribute to the fatty acid composition of sheep and could therefore be applied as a candidate gene to select sheep meat with high unsaturated fatty acid and low saturated fatty acid.
Acknowledgment
Authors are grateful to the RISTEKDIKTI for funding this research on the fiscal year 2020, Number: 4185/IT3.L1/PN/2020 of 12 May 2020. The authors are also grateful to those colleagues who have made positive advice for improving this paper.

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