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Research Article
Association and Expression Analyses of the Duck FMO3 Gene in Relation to Fatty Acid Composition

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Abstract

Background and Objective: Flavin containing monooxygenase 3 (FMO3) is an excellent candidate gene that affects fishy odor and fatty acid composition. It has been reported that down regulation of FMO3 can inhibit fatty acid oxidation. The aim of this study was to investigate the association and expression of the FMO3 gene as a candidate gene for fatty acid composition in Indonesian Cihateup ducks. **Methodology:** A total of one hundred Indonesian Cihateup ducks were used in this study. Tissues from breast muscles were used for genomic DNA isolation and fatty acid composition analysis. **Results:** Association analysis showed that the SNP g.849A>G was significantly associated with unsaturated fatty acids (palmitoleic, oleic, linoleic, linolenic and arachidonic acid) and saturated fatty acids (lauric, palmitic and arachidic acid). Compared to the GG genotype, the AG genotype ducks exhibited greater levels ($p<0.05$) of lauric acid (C14:0), palmitic acid (C16:0), arachidonic acid (C20:4n6) palmitoleic acid (C16:1), oleic acid (C18:1), linolenic acid (C18:3) and linoleic acid (C18:2, $p<0.05$) but not pentadecanoic acid (C15:0). Furthermore, to analyze the mRNA expression of FMO3 in liver tissues, the ducks were divided into two groups according to the genotypes AG and GG, where AG had relatively favourable unsaturated fatty acid composition. FMO3 mRNA expression was higher ($p<0.01$) in animals with the AG genotype. **Conclusion:** These results will improve the understanding of functions of the FMO3 gene in maintaining muscular fatty acid composition and will shed light on FMO3 as a candidate gene in the selection of ducks with unsaturated fatty acids for meat quality improvement.

Key words: Association, FMO3, duck, meat, PCR, SNP, fatty acid composition

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fatty acid composition plays an important role in meat quality in terms of not only the nutritional value but also the flavor quality of meat[1]. Cameron *et al.*[2] reported that polyunsaturated fatty acids (PUFA), including linoleic acid (C18:2, C20:4 and C22:6), show a positive correlation with the flavor of meat and are associated with human health[2]. The high intake of PUFA and monounsaturated fatty acids (MUFA) can increase hepatic low density lipoprotein (LDL) receptor activity, which decreases the circulating concentration of LDL cholesterol[3]. In contrast, the composition and total amount of saturated fatty acids (SFA) have been identified as dietary risk factors[4], which may be related to various cancers and especially coronary heart disease. A deeper knowledge of the genetic mechanisms of fatty acids is important as it may generate new opportunities for more effective marker assisted breeding, namely, selecting ducks with higher PUFA and lower SFA, leading to economic benefits in the duck production industry. To produce and select animals having higher PUFA and lower SFA in meat, marker assisted selection has been applied to breeding techniques by the direct selection of genes that affect the meat quality. The FMO3 enzyme is the predominant enzyme in the adult human liver involved in the degradation of trimethylamine (TMA)[5]. FMO3 is also reported to play an important role in fatty acid oxidation. Wang *et al.*[6] determined that the down regulation of FMO3 can inhibit fatty acid oxidation. Considering that fat deposition in animals can be attributed to the balance among dietary absorbed fat, endogenous lipogenesis and lipolysis, higher fat deposition may be caused by diminished fatty acid oxidation or increased adipogenesis of adipocytes[7].

Several studies have identified FMO3 as the causative gene of the fish odor traits in cow milk and chicken eggs[8]. A nonsense mutation (R238X) in the cattle FMO3 gene was found to underlie the fishy off-flavor in cow milk[9]. In chickens, a nonsynonymous mutation in the chicken FMO3 gene (T329S) has been verified to be associated with elevated levels of TMA and fishy taint in the chicken egg yolk[8]. Moe *et al.*[10] reported that the nonsense mutation Q319X was significantly associated with the elevated TMA content in the quail egg yolk. In pigs, the FMO3 gene was located in a quantitative trait loci (QTL) region correlated with off-flavor, which indicated an association between the FMO3 gene and off-flavor in pigs[11]. Notably, no study has investigated the association and expression of these genes with regard to fatty acid composition. However, functional and positional studies have suggested that this gene could be important candidate gene for fatty acid composition.

Duck meat generally presents off-flavor due to fatty acids and the FMO3 gene is thought to be the candidate gene for these compounds. Therefore, the present study aimed to study the association of FMO3 with the fatty acid composition in an Indonesian Cihateup duck population. Furthermore, to highlight the functions of FMO3, the mRNA expression differences in the FMO3 gene were investigated in the liver tissues of ducks with divergent unsaturated fatty acid compositions based on genotype.

MATERIALS AND METHODS

Animals: One hundred Indonesian Cihateup ducks were used in this study. The ducks were reared under the same feeding conditions until they were 12 weeks old and had approximately 1.6 kg of slaughter weight per duck. The carcass and meat quality data were collected according to the guidelines of the Indonesian performance test with the number 13-2016 IPB. Tissues from breast muscles were used for genomic DNA isolation and fatty acid composition analysis.

Fatty acid composition analysis: Fatty acid composition was determined for each sample using the extraction method described by Folch *et al.*[12]. Muscle samples (~100 g) were collected and ground for FA composition analysis. The lipids were extracted by homogenizing the sample with a chloroform and methanol (2:1) solution. NaCl at 1.5% was added and the lipids were isolated. The isolated lipids were methylated and the methyl esters were formed according to Kramer *et al.*[13]. The FA composition was quantified using gas chromatography (GC-2010 Plus-Shimadzu AOC 20i autoinjector) with an SP-2560 capillary column (100 m$\times$0.25 mm in diameter with 0.02 mm thickness, Supelco, Bellefonte, PA). The initiating temperature was 70°C with gradual warming (13°C min^{-1}) up to 175°C, holding for 27 min and later, the temperature was further increased by 4°C min^{-1} until 215°C was reached and held for 31 min. The FAs were identified by comparing the retention time of methyl esters of the samples with standards of C4–C24 (F.A.M.E mix Sigma®), vaccenic acid C18:1 trans-11 (V038-1G, Sigma®) C18:2 trans-10 cis-12 (UC 61 M 100 mg), CLA C18:2 cis-9, trans-11 (UC 60 M 100 mg) (Sigma®) and tricosanoic acid (Sigma®). The FAs were quantified by normalizing the area under the curve of methyl esters using the software GS solution 2.42, Shimadzu GC-2010. The FAs were expressed as percentages of the total FA methyl esters. The analysis of the FA composition in meat was performed at the Integrated Laboratory in the Bogor Agricultural University, Bogor, Indonesia.

DNA isolation, SNP identification and genotyping: A nonsynonymous single nucleotide polymorphism detected by Wang *et al.*[14] was used in this study, this polymorphism was an arginine (A) transversion to guanine (G) at g.849A>G in exon 6. For PCR amplification, the primer was designed from the duck FMO3 genomic sequence using the Primer3 tool[15]. For genotyping, genomic DNA was isolated from the breast muscle (BM) tissue of the Indonesian Cihateup ducks according to the standard phenol-chloroform method[16]. *In silico* analysis of the genomic sequence, performed by comparing several sequences from a publicly available database (NCBI), revealed the possible targets for PCR amplification. A working solution with a final concentration of 50 ng μL^{-1} was prepared and stored at 4°C for further analysis. Polymerase chain reactions (PCRs) were performed in a 20 µL volume containing 2 µL of genomic DNA, 1×PCR buffer (with 1.5 mM $MgCl_2$), 0.25 mM of dNTPs, 5 pM of each primer and 0.1 U of Taq DNA polymerase (GeneCraft). The genotyping of the Indonesian Cihateup duck population was performed by the PCR-RFLP method. The PCR product was analyzed using 1.5% agarose gel (Fischer Scientific Ltd.) and digested by using the restriction enzyme AlwNI for FMO3 (New England Biolabs). Digested PCR-RFLP products were resolved in 3% agarose gels. The details of the PCR-RFLP pattern, GenBank, accession numbers and primer sequences used in this study are listed in Table 1.

Statistical analysis: Allele and genotype frequencies were determined for the SNP identified in the FMO3 gene by statistical analyses. The association of the genotypes with the fatty acid composition was calculated by analyzing the variances of the quantitative traits. For these analysis, the generalized linear model (PROC GLM) of SAS (version 9.2 SAS Inst Inc., Cary, USA) was used. The model was as follows:

$$Yijk = \mu + genotypei + sexj + eijk$$

where Yijk is the fatty acid composition, µ is the overall mean, genotypei is the fixed effect of the i-th genotype (i = 1, 2 and 3) sexj is the fixed effect of the j-th sex (j = male/female), which is the combination of location and penning (group, individual) and eijk is the residual error.

Least squares mean values for the loci genotypes were compared by t-tests and p-values were adjusted by the Tukey-Kramer correction[17,18].

mRNA expression study by qRT-PCR: Since fatty acids are metabolized and catabolized in the liver, the liver tissues from

Table 1: Primer sequence used to detect polymorphism and qRT-PCR analysis, enzymes used for PCR-RFLP analysis and length of digested PCR products

Gene number	Accession number	Primer sequence	Application	Size (bp)	Temperature (°C)	Restriction enzyme	SNP location	SNP	Digested fragments length (bp)
FMO3	JX_126807	F: 5′ CAC CGT GGC CTC ACA GGT ATG 3′ R: 5′ CTT GGA ACA CGA CGG ACG TTT C 3′	Genotyping	525	60	AlwyN I	Exon 6	g.849 A>G	AA : 525 AG : 525, 299 and 226 GG : 299 and 226
FMO3	NW_004676298	F:5′ TCT ACC GCA CCG TCT TCA CC3′ R: 5′ TCA GGG CGC TTC CTT ATG TT3′	qPCR	193	60				
GAPDH	XM_005016745.3	F:5′ TCC TCA TCT GCA TCT CTT TT 3′ R: 5′ CAT TCC CGT TAA TCA CAA GT3′	qPCR	217	60				

ducks with divergent phenotypes were selected for the gene expression study. For this purpose, 3 ducks with extremely high FA and 3 ducks with extremely low FA were selected. Total RNA was extracted from the liver tissues of these 6 ducks using Tri-Reagent according to the manufacturer's instructions (Sigma). Total RNA was treated using on column RNase-free DNase (Promega) and was quantified using a spectrophotometer (NanoDrop, ND 8000, Thermo-Scientific). RNA quality was assessed using an Agilent 2100 Bioanalyzer and an RNA Nano 6000 LabChip kit (Agilent Technologies). Furthermore, RNA integrity was examined by 2% agarose gel electrophoresis. In all cases, cDNA was synthesized by reverse transcription PCR using 2 µg of total RNA, SuperScript II reverse transcriptase (Invitrogen) and an oligo(dT) 12 primer (Invitrogen). Gene specific primers for FMO3 for the qRT-PCR were designed by using the Primer3 software[15] (Table 1). In each run, the 96 well microtiter plate contained each cDNA sample and no template control. The qRT-PCR was conducted with the following program: 95°C for 3 min and 40 cycles of 95°C for 15 sec/60°C for 45 sec on the StepOne Plus qPCR system (Applied Biosystem). For each PCR reaction, 10 µL iTaq™ SYBR® Green supermix with ROX PCR core reagents (Bio-Rad), 2 µL of cDNA (50 ng μL^{-1}) and an optimized amount of primers were mixed with ddH_2O to a final reaction volume of 20 µL per well. All samples were analyzed twice (technical replication) and the geometric mean of the Ct values was further used for mRNA expression profiling. The reference gene GAPDH was used for the normalization of the target genes. The delta Ct (Ct) values were calculated as the difference between the target genes and the reference gene (Ct = Ct_{target}- $Ct_{reference\ gene}$) as described previously by Silver *et al.*[19]. The final results were reported as the fold change calculated from delta Ct values. The differences in FMO3 gene expression were analyzed with paired t-tests. The values with $p<0.05$ were considered to be statistically significant.

RESULTS

Fatty acid composition profile: The phenotypic profile in Table 2 shows the descriptive statistics for the fatty acid composition in Indonesian Cihateup ducks. The fatty acid profile analyses detected the composition of 18 fatty acids, including total SFA, PUFA and MUFA in each sample. Total SFA contained seven FAs, namely, lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) stearic acid (C18:0), pentadecanoic acid (C15:0), heptadecanoic acid (C17:0) and arachidic acid (C20:0), with average levels of 0.07, 0.51, 27.80, 5.13, 0.05, 0.10 and 0.17%, respectively. Total MUFA (C16:1, C18:1n9t, C18:1n9c, C20:1) and PUFA (C18:2, C20:4, C18:3n6,C20:2) were calculated by adding each of the 4 FAs. The results also indicated that total SFA was lower than MUFA but higher than PUFA (Table 2).

Table 2: Phenotypes of fatty acid composition

Traits	Mean (46)	SD
Myristic acid (C14:0)	0.52	0.05
Lauric acid (C12:0)	0.09	0.07
Palmitic acid (C16:0)	27.16	2.45
Stearic acid (C18:0)	5.20	0.76
Arachidic acid (C20:0)	0.17	0.11
Pentadecanoic acid (C15:0)	0.05	0.01
Heptadecanoic acid (C17:0)	0.10	0.02
Palmitoleic acid (C16:1)	2.16	0.55
Elaidic acid (C18:1n9t)	0.12	0.03
Oleic acid (C18:1n9c)	44.13	2.78
Cis 11 eicosenoic acid (C20:1)	0.73	0.13
Linoleic acid (C18:2)	18.36	1.60
γ-Linolenic acid (C18:3n6)	0.04	0.01
Arachidonic acid (C20:4)	0.23	0.06
Cis-11,14-eicosedenoic acid (C20:2)	0.12	0.03
SFA	33.28	2.69
PUFA	18.76	1.60
MUFA	47.13	3.03

Gene FMO3 polymorphisms: A nonsynonymous FMO3 SNP at g.849A>G was confirmed in exon 6 of FMO3 in the studied Indonesian Cihateup duck population. Animals of this population were genotyped at g.849A>G in exon 1, which was the SNP segregating within the populations. The SNP was confirmed by PCR-RFLP. The DNA restriction fragments obtained for the g.849A>G SNP of the FMO3-AlwNI polymorphism were 525 bp for the AA genotype, 525, 299 and 226 bp for the AG genotype and 299 and 226 bp for the GG genotype (Fig. 1).

The calculated genotype and allele frequencies of the FMO3 gene in the duck population are shown in Table 3. In this study, two genotypes AG and GG were found with SNPs at g.849A>G in our population. The homozygous GG was more frequent and the heterozygous AG was rare in our population. The chi-square test revealed that the locus of FMO3 was in Hardy-Weinberg equilibrium in this Indonesian Cihateup duck population (Table 3).

Association of FMO3 polymorphisms with fatty acid composition: Association analysis of the g.849A>G SNP with fatty acid composition revealed significant ($p<0.01$) associations with myristic acid (C14:0), stearic acid (C18:0), palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2), palmitoleic acid (C16:1), oleate acid (C18:1) and linoleic acid (C18:2, $p<0.05$). Compared to the GG phenotype ($p<0.05$), the

Table 3: Genotype, allele frequencies and the chi-square test of FMO3 using RFLP in Cihateup duck

		Genotype frequency			Allele frequency			
Sample	Genotype (n)	AA	AG	GG	A	G	He	Ho
Cihateup	100	0.000	0.083 (9)	0.917 (91)	0.042	0.958	0.080	0.083

Table 4: Genotype and association analysis of FMO3 with fatty acid composition

Traits	AG (39)	GG (7)
Myristic acid (C14:0)	0.51±0.07	0.52±0.04
Lauric acid (C12:0)	0.21±0.08[a]	0.07±0.03[b]
Palmitic acid (C16:0)	25.22±4.50[b]	27.50±1.61[a]
Stearic acid (C18:0)	4.57±0.64[a]	4.46±0.75[b]
Arachidic acid (C20:0)	0.28±0.24[a]	0.15±0.06[b]
Pentadecanoic acid (C15:0)	0.04±0.01[b]	0.05±0.01[a]
Heptadecanoic acid (C17:0)	0.10±0.02	0.10±0.02
Palmitoleic acid (C16:1)	3.02±0.36[a]	2.00±0.41[b]
Elaidic acid (C18:1n9t)	0.11±0.03	0.12±0.03
Oleic acid (C18:1n9c)	46.69±3.32[a]	43.67±2.45[b]
Cis 11 eicosenoic acid (C20:1)	0.71±0.14	0.73±0.13
Linoleic acid (C18:2)	19.59±1.09[a]	18.14±1.58[b]
γ-Linolenic acid (C18:3n6)	0.06±0.01[a]	0.04±0.01[b]
Arachidonic acid (C20:4)	0.17±0.06[b]	0.24±0.05[a]
Cis-11,14-eicosedenoic acid (C20:2)	0.12±0.03	0.12±0.03
SFA	30.32±4.21[b]	33.70±2.02[a]
PUFA	19.95±1.08[a]	18.65±1.74[b]
MUFA	49.62±3.10[a]	47.20±2.44[b]

Values with different superscript within rows differ at p<0.05

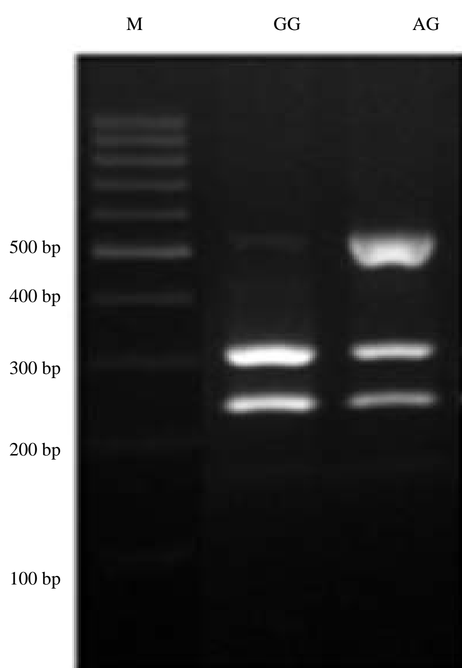

Fig. 1: PCR-RFLP genotyping result for the FMO3 gene

genotype AG exhibited greater compositions of myristic acid (C14:0), palmitoleic acid (C16:1), oleate acid (C18:1) and linoleic acid (C18:2, p<0.05) but not stearic acid (C18:0) (Table 4).

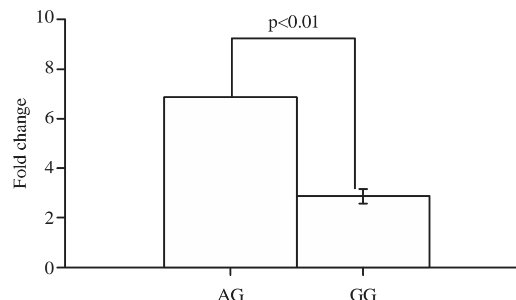

Fig. 2: mRNA expression of FMO3 in liver from high unsaturated fatty acid (AG genotype) and low unsaturated fatty acid (GG genotype)

mRNA expression of FMO3 in divergent unsaturated fatty acid genotypes: Quantitative real-time PCR showed that the FMO3 mRNA was differentially regulated (p<0.01) between animals with the AG genotype (high myristic acid (C14:0), palmitoleic acid (C16), oleic acid (C18:1), linoleic acid (C18:2),low stearic acid (C18:0)), the GG genotype (myristic acid (C14:0), palmitoleic acid (C16), oleic acid (C18:1), linoleic acid (C18:2) and high stearic acid (C18:0)) in the muscles. Higher transcript abundance was detected in the muscles of animals with the AG genotype (p<0.05) compared to that in the muscles of animals with the GG genotype (Fig. 2).

DISCUSSION

The composition of 9 FAs, including SFA, UFA, MUFA and PUFA, was measured in duck muscle sample. The results showed that the total SFA composition was lower than that of MUFA but greater than that of PUFA. The composition of FAs measured in this study is similar to the values measured in broiler chicken populations as reported by Maharani *et al.*[20], in which total SFA was lower than MUFA but higher than PUFA. These findings confirmed that SFA in bird meat is less saturated than ruminant meat. The ratios SFA and UFA levels in membrane fluidity and cell-cell interactions have been affected in a variety of human diseases, such as cancer[21], diabetes[22], obesity[23], hypertension[24] and neurological diseases[25,26]. High levels of MUFAs and PUFAs decrease the circulating concentration of LDL-cholesterol by increasing hepatic LDL receptor activity[27]. In contrast, high intake of SFA

can result in elevated plasma cholesterol, which leads to cardiovascular disease. SFAs such as lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0) most deleteriously influence the cardiovascular health[28].

This study revealed an association of FMO3 with fatty acid composition in Indonesian Cihateup ducks (Table 4). The exonic SNP g.849A>G was found to be significantly associated with fatty acid composition in these ducks, including the UFAs palmitoleic acid (C16:1), oleic acid (C18:1) and linoleic acid (C18:2) and SFAs (myristic acid (C14:0) and stearic acid (C18:0). To our knowledge, this is the 1st association study of an FMO3 polymorphism with fatty acid composition. Previously, several studies reported an association of FMO3 with fishy-taint in several birds and mammals. A nonsynonymous mutation in the chicken FMO3 gene (T329S) had been verified to be associated with elevated levels of TMA and fishy taint in the chicken egg yolk[8]. Furthermore, Moe *et al.*[10] reported that the nonsense mutation Q319X was significantly associated with the elevated TMA content in the quail egg yolk. In cattle, a nonsense mutation (R238X) in the cattle FMO3 gene was found to underlie fishy off-flavor in cow milk[9]. In pigs, the FMO3 gene was located in a QTL region correlated with off-flavor, which indicated an association between the FMO3 gene and off-flavor in pigs[11]. FMO3 is a vital member of the FMO family and is primarily present in the adult human liver, where it plays an important role in the metabolism of xenobiotic-containing TMA[14]. TMA is the main compound causing the human metabolic disorder TMAU[29], the fishy off-flavor in dairy milk and the fishy taint in chicken eggs[8,9]. The highest unsaturated fatty acid values were determined in the AG genotype for palmitoleic acid (C16), oleic acid (C18:1) and linoleic acid (C18:2) but stearic acid (C18:0) values were low in contrast, the GG genotypes exhibited significantly lower UFA values (Table 4). The results indicated that the identified SNP was associated with increases in palmitoleic acid (C16:1), oleic acid (C18:1), linoleic acid (C18:2) and SFA but the SNP was associated with a decrease in stearic acid in heterozygous animals. Palmitoleic acid was considered to have a positive effect on reducing bad cholesterols[30] and on reducing fat deposition in blood vessels and blood clot formation[31]. The ratio of stearic acid to oleic acid is one of the most important factors influencing the balance of membrane fluidity[32,33]. Linoleic acid (C18:2) is considered to have a positive correlation with the flavor of meat and is associated with human health[34,30].

To further explore the association between the FMO3 gene and fatty acid compounds, mRNA expression levels were investigated in the liver tissues collected from AG and GG genotype groups. Higher FMO3 mRNA expression was detected in ducks with high unsaturated fatty acid composition and specifically, there was significant upregulation of FMO3 in the AG genotype compared to that in the GG genotype group (Fig. 1). The FMO family of enzymes converts lipophilic compounds into more polar metabolites and decreases the activity of these compounds[35]. We speculate that the fatty acid composition, especially UFA, was negatively correlated with FMO3 activity. Therefore, it could be postulated that FMO3 plays an important role in producing the fatty acid composition in ducks and that the associated polymorphism could contribute to increase the UFA composition, which is positively correlated with human health. To our knowledge, no mRNA expression of FMO3 has been reported in previous studies related to fatty acid composition. Different isoforms of the FMO gene family member FMO5 are reported to be increased when boar taint is low[36,37]. Falls *et al.*[38] reported FMO1 and FMO3 to be increased and serum testosterone levels to be decreased after castration. The FMO gene family is reported to play a role in sex steroid production[10,39]. Additionally, the FMO gene family is reported to be involved in the metabolism of androstenone in the liver[10,39]. FMO expression was reported to be affected by testosterone and estrogen exposure in mice[38]. In a study conducted by Wang *et al.*[14], using RNA-seq in the adipose tissue of sheep, FMO3 was reported to be down regulated in tissues with higher fat deposition. Furthermore, down regulation of FMO3 can inhibit fatty acid oxidation. Considering that the fat deposition in animals can be attributed to the balance among dietary absorbed fat, endogenous lipogenesis and lipolysis, higher fat deposition may be caused by diminished fatty acid oxidation or increased adipogenesis of adipocytes[7]. The higher expression of FMO3 in ducks with higher UFA composition suggested that FMO3 might be involved in regulating fatty acid metabolism. These results have implications for genomic selection because the FMO3 gene is associated with fatty acid composition. However, the sample size used to determine the association was small, so validation in a larger sample size is necessary.

CONCLUSION

This study demonstrated that polymorphisms in FMO3 at g.849A>G might linked to the muscle UFA compositions of Indonesian Cihateup ducks. The expression levels of FMO3 mRNA and proteins were higher in ducks having higher UFA profiles compare to that of lower UFA compositions in muscle. The association and mRNA expression data have supported the candidacy of FMO3 gene as an important

marker in future molecular breeding techniques to select ducks with higher PUFA and lower SFA in meat. However, these findings should be validated in larger and independent duck populations and additional SNPs should be investigated.

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