

Novel variant in the leptin receptor (LEPR) gene and its association with fat quality, odour and flavour in sheep

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ABSTRAK

Tujuan dari penelitian ini adalah untuk menginvestigasi asosiasi dan mengukur ekspresi gen LEPR sebagai kandidat gen untuk kualitas sifat perlemakan, *odour* dan *flavour* pada daging domba. Total 47 domba jantan digunakan pada penelitian ini. Komposisi asam lemak, BCFA (Branch Chanin Fatty Acids; MNA, MP, MOA, dan EOA) dan skatole (MI) yang merepresentasikan kualitas perlemakan, sifat *odour* dan *flavour* diukur pada domba jantan umur 10-12 bulan. Identifikasi mutasi baru gen LEPR dan analisis ekspresi gen pada perbedaan kandungan kualitas perlemakan, sifat *odour* dan *flavour* dianalisis berturut-turut menggunakan PCR RFLP dan qRT-PCR. SNP pada daerah genomik g.40854778 A>C gen LEPR berasosiasi dengan kualitas perlemakan; asam lemak jenuh asam trikosanoat (C23:0) dan asam tetrakosanoat (C24:0) dan asam lemak tak jenuh asam dokosaheksaenoat (C22:6n3), sementara mutasi gen LEPR tidak ditemukan adanya asosiasi ($P>0.05$) dengan sifat *odour* dan *flavour* (MNA, MI, MP, MOA, dan EOA) pada daging domba. Hasil ekspresi mRNA gen LEPR lebih tinggi ($P<0.01$) pada domba dengan genotipe AA. Hasil penelitian ini dapat disimpulkan bahwa mutasi gen LEPR berkontribusi terhadap kandungan asam lemak tanpa mempengaruhi sifat *odour* dan *flavour* pada domba.

Kata kunci: flavour, LEPR, ekspresi mRNA, odour, mutasi

ABSTRACT

The aim of this study was to investigate the association and expression of LEPR with fat quality, flavour and odour in sheep. A total of 47 rams were used in this study. Fatty acids composition, BCFA (Branch Chanin Fatty Acids; MNA, MP, MOA, and EOA) and skatole (MI) representing fat quality, flavour and odour in sheep were measured at ages of 10-12 months old. Identification of novel variant and expression analysis of LEPR gene with different fat quality, odour and flavour were performed by PCR RFLP and qRT-PCR, respectively. A SNP in genomic region g.40854778 A>C of the LEPR gene was associated ($P<0.05$) with fat quality of saturated fatty acids (SFA) including [tricosanoic acid (C23:0) and tetracosanoic acid (C24:0)] and poly unsaturated fatty acid (PUFA) [docosaheksaenoic acid (C22:6n3)]. However, the novel variant of LEPR was not significantly associated ($P<0.05$) with fat content, odour and flavour (MNA, MP, MOA, EOA, and MI). The mRNA expression analysis showed

that LEPR mRNA expression was higher ($P < 0.01$) in sheep found at the AA genotype. It could be concluded that variant SNP in the LEPR may contribute to fatty acid composition without influencing odour and flavour traits in sheep.

Keywords: flavour, LEPR, mRNA expression, odour, variant

INTRODUCTION

Fat quality, sheep meat odour, and flavour are regarded as important components of the edible meat quality of sheep (Pleasant *et al.*, 2005; Pethick *et al.*, 2005). Fat quality in these regard fat content and fatty acid composition may decrease in lean meat of animals due to declining levels of saturated fatty acids (SFA) and increased levels of phospholipids and polyunsaturated fatty acids (PUFA) (Wood, 1984). For odour and flavour, two chemical compounds mainly branch chain of fatty acid (BCFA; BCFAs; 4-methyloctanoic (MOA), 4-ethyloctanoic (EOA) and 4-methylnonanoic acids) and skatole (MI; 4-methylindole) have often associated with odour and flavour, respectively (Watkins *et al.*, 2014; Ran-Ressler *et al.*, 2014). Improvement of meat quality including fat quality, odour and flavour is a critical factor in the protection and development of markets for sheep. However, these traits can usually be measured post-slaughter and often had low heritabilities (Boukha *et al.*, 2011). Selection on the basis of molecular genetic of candidate gene approached through identification variant analysis (SNPs) was most effectively implemented for breeding programs (van der Steenet *et al.*, 2005). Identification of novel SNPs within genes influencing fat quality, odour and flavour traits and their association and gene expression could be the important and generally applied tools to predict the genetic merit of sheeps.

As part of the search for genetic contributors to fat quality and sheepmeat odour and flavour, whole genome approaches using RNA sequencing had revealed leptin receptor (LEPR) as a gene with differential expression in sheeps divergent for fatness traits (Gunawan *et al.*, 2018). Leptin receptors (LEPR) were expressed in the olfactory mucosa and upregulated by fasting (Baly *et al.*, 2007). Furthermore, LEPR were found in the olfactory bulb and the piriform cortex (Bennet *et al.*, 1998). In animal model of mice, leptin could modulate olfactory-mediated preingestive behavior through leptin receptors (Gechell *et al.*, 2006). In humans, a gender specific relation

between leptin and the ability to identify an odour was observed (Karlsson *et al.*, 2002). In livestock, SNP detection of the LEPR gene in cattle had been associated with somatic cells count (Kulig and Kmiec, 2009), milk performance (Anton *et al.*, 2012), reproduction traits (Almeida *et al.*, 2003) and growth traits (Kulig and Kmiec, 2009). However, there was no study investigating the association and expression of LEPR with fat quality like fatty acid composition, sheepmeat odour and flavour in sheep, especially in Indonesian sheep. Functional and positional studies suggested that these genes could be important candidate genes for fat quaiity, odour and flavour compounds in sheep. The aims of this study were to analyse the novel variant of LEPR genes and to unravel the association and expression of these genes with fat quality, odour and flavour in sheep.

MATERIALS AND METHODS

Animals and Phenotypes

Forty seven Indonesian sheeps including fat-tailed sheep (JFT, $n=20$), thin-tailed sheep (JTT, $n=17$), and Garut composite (GCS, $n=10$) sheep were used in this study. Indonesian sheeps are dominated with JFT and JTT which are distributed in all of region Indonesia. The sheep were caged in group and were given fattening feed *ad libitum*. Samples were taken from loin tissue and were collected from the rams with body weight between 25-30 kg and ages between 10-12 months old. The animals were slaughtered in the slaughter house according to the standard operational procedure provided in the house. Data of carcass and meat quality were determined according to reference methods for assessment of physical characteristic of meat (Honikel *et al.*, 1998). The loin was taken approximately of 500 grams for fatty acid (FA) analysis, 500 grams for odour and flavour analysis, and 30 mg loin for DNA extraction. All samples for the DNA extraction were immidiately placed in ice and kept at the temperature of -20°C until futher usages.

Fatness Traits: Fat Content and Fatty Acid Analysis

Muscle samples of approximately 100 g were collected for the analysis needs of fatness traits including fat content and fatty acid (FA). Fatty acid content was measured for each sample using the extraction method according to Folch *et al.*, (1957) procedure. The FA composition was quantified using gas chromatography (GC-2010 GC-2010 Plus-Shimadzu according AOAC 2001). The fatness traits were expressed as a proportion of the total FAs included fat content, saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA).

Odour and Flavour Analysis

Due to the limitations of loin muscle samples from sheep, analyses of odour and flavour compounds were carried out on loin samples from javanese fat tailed (JFT, n=22). The odour and flavour compounds were extracted using Likens-Nicerson method which was a combination of distillation and extraction with solvent simultaneously using Gas Chromatography Mass Spectrophotometry (GC-MS) tool. The phenotypes of odour and flavour were measured including MNA (4-methylnonanoic), MP (4-methylphenol), MOA (4-methyloctanoic), EOA (4-ethyloctanoic) and MI (3-methylindole) referred to Listyarini *et*

al. (2018).

SNP Discovery of LEPR Gene

The SNP of LEPR used in this study were based on RNA sequencing study (Gunawan *et al.*, 2018). Using RNA deep sequencing analysis, our group describing several single nucleotide polymorphisms (SNPs) have been described recently by our group by comparing loin muscles unsaturated fatty acids contents. Among them, a SNP of LEPR (g.40854778 A>C) gene was selected for this association study. For this purpose, the Indonesian sheep population (n=47) were genotyped. Amplification of LEPR gene fragments were conducted using polymerase chain reactions (PCR). Amplification process of PCR began denaturation step at 94°C for five minutes. The second phase consists of 35 cycles, each cycle consisting of denaturation process at 94°C for 10 seconds, primer annealing at temperatures 57°C for LEPR gene and DNA extension at 72°C for 30 seconds. The final stage was the primer extension at 72 °C for ten minutes. The genotyping was performed by PCR-RFLP method. The PCR product was analyzed using 1.5% agarose gel (Fischer Scientific Ltd) and digested by using the restriction enzymes of *AciI* for LEPR (New England Biolabs). Digested PCR-

Table 1. GenBank Accession Numbers and Primer Sequences

Gene Name	Accession Number	Primer Sequence	Application	Size (bp)	Tm (°C)	Enzyme	SNP	Digested Fragments Length (bp)
LEPR	NC_019458.2	F: 5'- GAT GAC CTG ACA TAT CCA GG-3' R: 5'- CAA TGA AGT GGG GAA AGG AC-3'	Genotyping	432	60	<i>AciI</i>	g. 40761 672 A>C	AA : 432 CC : 292 and 140 AC : 432, 292 and 140
LEPR	NM_0010097 63.1	F: 5'- GAA GCC TGA TCC ACC ATT AG -3' R: 5'- GAG GAG CAA GAG ATT GGA TG -3	qRT-PCR	239	60			
GAPDH	NC_019460.2	F: 5'- GAG AAA CCT GCC AAG TAT GA -3' R: 5'- TAC CAG GAA ATG AGC TTG AC-3	qRT-PCR	203	62			

RFLP products were resolved in 2% agarose gel. The details of PCR-RFLP pattern, GenBank accession number and primer sequences used in this study were listed in Table 1.

mRNA Expression of LEPR Gene

The sample tissues from nine sheep liver with different fat quality, odour and flavour were isolated for mRNA study base on genotype results. The nine sheep were divided into three groups AA, AC, and CC genotype. The differential gene expression between genotypes was performed using PROC GLM test in SAS. For the qRT-PCR analysis, cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Gene specific primers for LEPR for the qRT-PCR were designed by using the Primer3 software (Rozen and Skaletsky, 2000) (Table 1). In each run, the 96-well microtiter plate contained each cDNA sample and no-template control. The qRT-PCR was performed with the following program according to Gunawan *et al.*, (2013a and 2013b). The housekeeping genes (GAPDH) were used for reference of the target genes (Table 1).

Statistical Analysis for Fatness Traits: Fat Content and Fatty Acid Traits

The association of LEPR gene with fatness traits; fat content and fatty acid compositions was performed by General Linier Model (GLM) using SAS. 9.2 software. The model of GLM was follows:

$$Y_{ijk} = \mu + G_i + B_j + E_{ijk}$$

Where, Y_{ijk} was the fatness traits; fat content and fatty acid traits, μ was overall mean, G_i was the fixed effect of i -th genotype ($i = AA, AC, \text{ and } CC$) and B_j was the fixed effect of j -th breed ($j = JFT, JTT \text{ and } GCS$) and E_{ijk} is the residual error. Least square mean values for the loci genotypes were compared by t-test and p-values were adjusted by the Tukey-Kramer correction (Kayana *et al.*, 2011; Cinar *et al.*, 2012).

Statistical Analysis for Sheepmeat Odour and Flavour

Due to the limitation of odour and flavour analysis, the study was conducted in javanese fat tailed (JFT). The association of LEPR gene with sheepmeat odour and flavour was performed by General Linier Model (GLM) using SAS. 9.2 software. The model of GLM was:

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

Where, Y_{ij} was the odour and flavour, μ is

overall mean, G_i was the fixed effect of i -th genotype ($i = AA, AC, \text{ and } CC$) and E_{ij} was the residual error. Least square mean values for the loci genotypes were compared by t-test and p-values were adjusted by the Tukey-Kramer correction (Kayana *et al.*, 2011; Cinar *et al.*, 2012).

Analysis of LEPR Gene Expression

The delta Ct (ΔCt) method was used for calculating the difference between target gene and the reference genes: ($\Delta Ct = Ct_{\text{target}} - Ct_{\text{housekeeping genes}}$) as described previously by Silver *et al.*, (2003). The results of graph were calculated as fold change calculated from delta Ct-values. The phenotypic differences between high and low fat quality, odour and flavour of LEPR gene expressions were compared using t tests. The significant values ($P < 0.05$) were considered statistically to indicate the significant differences.

RESULTS

Novel Variant SNP of LEPR Gene

SNP of LEPR at position g.40854778 A>C was successfully genotyped in Indonesian sheep populations. The SNP g. 40854778 A>C of LEPR was polymorphism: 432 bp for the CC genotype, 432, 292, and 140 bp for the AC, 292 and 140 bp for AA genotype (Figure 1). Three genotypes of AA, AC, and CC were found in this study. The genotype frequency of LEPR (g.40854778 A>C) was detected in Hardy Weinberg Equilibrium (HWE) ($P < 0.05$). The gene frequencies including genotype and allele frequency of LEPR were presented in Table 2.

Association Analysis between LEPR Genotyped and Fatness, Odour and Flavour of Sheeps

Association analysis of the g.40854778A>C SNP with fat quality revealed significant ($P < 0.05$) associations with saturated fatty acids including tricosanoic acid (C23:0), tetracosanoic acid (C24:0), and polyunsaturated fatty acid (C22:6n3) (Table 3). Sheep with homozygous 'CC' genotypes were associated with higher polyunsaturated fatty acids [docosahexaenoic acid (C22:6n3)] and higher saturated fatty acid [tricosanoic acid (C23:0) and tetracosanoic acid (C24:0)] (Table 3). However, the LEPR gene (g.40854778A>C) had no significant effect ($P > 0.05$) with fat content (Table 3) and any of the sheepmeat odour and flavour compound (Table

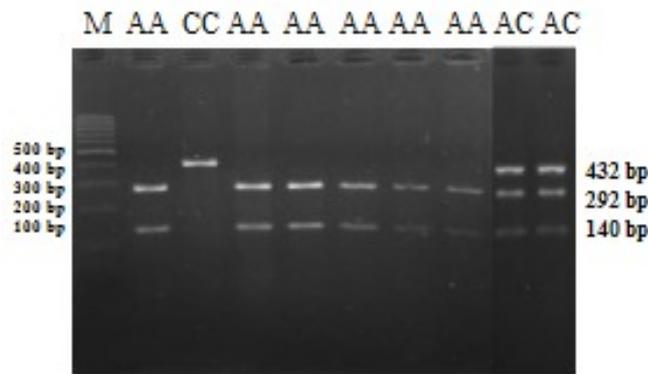


Figure 1. PCR-RFLP Genotyping Result for the LEPR Gene. M = 100bp markers. 1,3, 4,5,6,7 = AA genotype. 3 = CC genotype. 8,9 = AC genotype

Table 2. The Number of Animals per Genotype and Allele Frequency of Each SNP

Sample	N	Genotype Frequency			Allele Frequency		Chi-squared (χ^2)
		AA	AC	CC	A	C	
Indonesian sheep	47	0.66 (31)	0.28 (13)	0.06 (3)	0.8	0.2	0.02*

*=significant at $P < 0.05$

4). This gene was not associated with all the phenotypes including MNA, MP, MOA, EOA, and MI (Table 4).

mRNA Expression of LEPR in Different Fatty Acid Composition

Higher mRNA expression was detected in the loins muscle of sheeps with the AA genotype ($P < 0.05$) compared to the AC and CC genotypes (Figure 2). The genotype of AA (high PUFA docosahexaenoic acid (C22:6n3) and lower SFA tricosanoic acid (C23:0), as well as tetracosanoic acid (C24:0) was different compared to genotypes of AC and CC (low PUFA docosahexaenoic acid (C22:6n3), and higher SFA tricosanoic acid (C23:0), as well as tetracosanoic acid (C24:0) in the muscle.

DISCUSSION

This study showed that the LEPR gene at g.40854778 A>C polymorphism was significantly associated ($P < 0.05$) with saturated fatty acid (SFA), tricosanoic acid (C23:0) and tetracosanoic acid (C24:0), and polyunsaturated fatty acid

(PUFA) docosahexaenoic (C22:6n3). Sheep with AA genotype had lower tricosanoic acid (C23:0) and tetracosanoic acid (C24:0) than AC and CC genotypes (Table 3). However, the highest content of unsaturated fatty acid docosahexaenoic acid (C22:6n3) was sheep with AA genotype. Docosahexaenoic acid (C22:6n3) and oleic acid (C18:1n9c) were able to reduce the toxic effects of 7-ketocholesterol (7KC), thought to contribute to reduce risk of neurodegenerative disease and age-related diseases (Debbabi *et al.*, 2017). Previous study reported an association of LEPR with fatty acid composition in pigs (Oliveira *et al.*, 2017; Rodriguez *et al.*, 2016). Rodriguez *et al.*, (2016) reported that LEPR gene was significantly associated ($P < 0.05$) with saturated fatty acids (SFA); C14:0, C16:0, C18:0, C20:0, monounsaturated fatty acid (MUFA); C20:1n-9, and polyunsaturated fatty acid (PUFA); C20:4n-6 in fattening Duroc pig. LEPR gene in was associated with fatty acid composition in muscle (Oliveira *et al.*, 2017).

The LEPR gene (g.40854778A>C) had no significant effect ($P > 0.05$) with fatness trait fat content (Table 3) and any of the sheepmeat odour

Table 3. Genotypes and Association Analysis of LEPR Gene

Fatness Trait	Genotypes		
	AA (n=31)	AC (n=13)	CC (n=3)
Fat content	6.03 ± 3.93	4.77 ± 2.65	4.75 ± 4.90
Lauric acid (C12:0)	0.53 ± 0.70	0.32 ± 0.41	0.40 ± 0.26
Myristic acid (C14:0)	3.70 ± 2.21	2.36 ± 1.33	3.83 ± 3.26
Myristoleic acid (C14:1)	0.15 ± 0.07	0.08 ± 0.05	0.13 ± 0.07
Pentadecanoic acid (C15:0)	0.50 ± 0.17	0.51 ± 0.12	0.51 ± 0.09
Palmitic acid (C16:0)	19.00 ± 2.80	17.86 ± 1.87	18.21 ± 5.76
Palmitoleic acid (C16:1)	1.73 ± 0.37	1.50 ± 0.28	1.37 ± 0.60
Heptadecanoic acid (C17:0)	1.08 ± 0.35	1.10 ± 0.44	0.94 ± 0.26
Stearic acid (C18:0)	12.51 ± 4.41	15.74 ± 6.24	15.61 ± 5.10
Elaidic acid (C18:1n9t)	0.10 ± 0.11	0.09 ± 0.10	0.05 ± 0.09
Oleic acid (C18:1n9c)	27.99 ± 4.23	27.00 ± 4.19	24.68 ± 8.57
Linoleic acid (C18:2n6c)	2.23 ± 0.96	2.26 ± 0.78	2.42 ± 1.76
Arachidic acid (C20:0)	0.08 ± 0.04	0.10 ± 0.05	0.10 ± 0.11
Eicosadienoic acid (C20:2)	0.04 ± 0.02	0.04 ± 0.02	0.05 ± 0.01
Behenic acid (C22:0)	0.02 ± 0.02	0.02 ± 0.02	0.05 ± 0.07
Arachidonic acid (C20:4n6)	0.44 ± 0.41	0.46 ± 0.33	0.93 ± 1.15
Tricosanoic acid (C23:0)	0.007 ± 0.01 ^b	0.01 ± 0.01 ^{ab}	0.03 ± 0.03 ^a
Tetracosanoic acid (C24:0)	0.006 ± 0.016 ^b	0.01 ± 0.01 ^{ab}	0.03 ± 0.05 ^a
Docosahexaenoic (C22:6n3)	0.04 ± 0.05 ^{ab}	0.02 ± 0.03 ^b	0.09 ± 0.005 ^a
Fatty Acid Total	71.17 ± 5.94	70.55 ± 5.14	70.30 ± 10.10
Saturated Fatty Acid (SFA)	37.57 ± 5.83	38.19 ± 7.50	39.89 ± 5.37
Monounsaturated Fatty Acid (MUFA)	30.37 ± 4.65	29.11 ± 4.65	26.43 ± 9.09
Polyunsaturated Fatty Acid (PUFA)	3.11 ± 1.39	3.12 ± 1.11	3.89 ± 2.99

Mean ± SD are units of percentage fatty acid composition. ^{ab} Mean value with different superscript letters in the same row differ significantly at P<0.05

and flavour compound (Table 4). This gene was not associated with all the phenotypes odour and flavour including MNA (4-methylnonanoic), MP (4-methylphenol), MOA (4-methyloctanoic), EOA (4-ethyloctanoic), and MI (3-methylindole). Odour and flavour differences were not only a matter of LEPR gene function, but also considered in close reliance on memory, learning and sensory function (Stevenson *et al.*, 2010).

However, we could speculate that the LEPR gene may be positively correlated with odour and flavour compounds in sheep.

The mRNA of LEPR was differentially regulated (P<0.05) between sheep with AA genotype (high PUFA docosahexaenoic acid (C22:6n3) and lower SFA tricosanoic acid (C23:0) and tetracosanoic acid (C24:0)) compared to the AC and CC genotypes (low PUFA

Table 4. Genotypes and Association Analysis of LEPR Gene with Flavour and Odour Compounds

Polymorphism	Odour and Flavour Compound (ug/g)	Genotypes ($\mu \pm$ S.E)		
		AA (n=14)	AC (n=5)	CC (n=3)
LEPR	MNA	0.485 \pm 0.183	0.135 \pm 0.112	0.103 \pm 0.081
	MP	20.240 \pm 3.520	27.810 \pm 6.040	20.007 \pm 0.478
	MOA	0.094 \pm 0.029	0.346 \pm 0.320	0.037 \pm 0.021
	EOA	0.394 \pm 0.119	0.471 \pm 0.190	0.068 \pm 0.037
	MI	0.314 \pm 0.092	0.215 \pm 0.081	0.073 \pm 0.043

MNA = 4-methylnonanoic, MP = 4-methylphenol, MOA = 4-methyloctanoic, EOA = 4-ethyloctanoic, MI = 3-methylindol

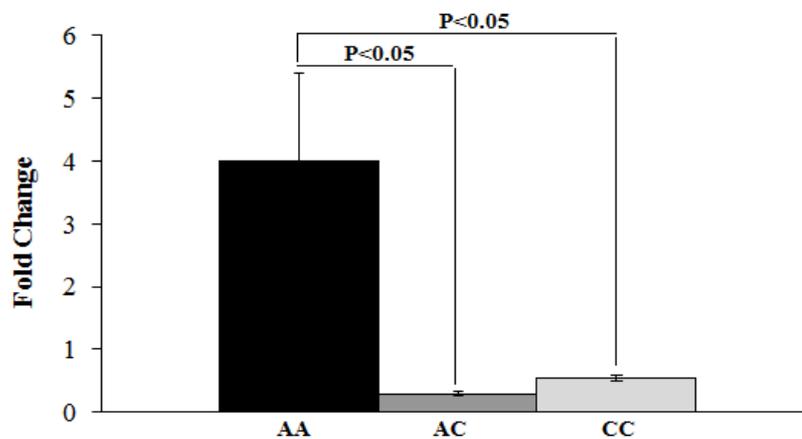


Figure 2. mRNA Expression of LEPR Gene in the Liver with Different Fatty Acid Composition

docosahexaenoic acid (C22:6n3) and higher SFA tricosanoic acid (C23:0), and tetracosanoic acid (C24:0) in the loin muscle (Figure 2). The LEPR was expressed mainly in hypothalamus, the centre of the energy homeostasis and the regulation of secretory organs activity (Matteis *et al.*, 2012). Leptin deficiency leads to extreme obesity in both mice and humans, while leptin treatment of wild-type mice reduced fat mass (Scwartz *et al.*, 2017; Coll *et al.*, 2007). At the levels of LEPR mRNA and plasma leptin were highly linked with adipocyte cell size and cellular fat content (Skurk *et al.*, 2007; Jernas *et al.*, 2006). The LEPR and the stearoyl-CoA desaturase (SCD) were two of genes playing important role in controlling fatness including fatty acid traits. LEPR as a catalyze of the leptin hormone influenced overall fatness

(Houseknecht *et al.*, 1998), while SCD rate-limiting was important enzyme for fatty acid composition for the biosynthesis of MUFA from SFA (Ntambi & Miyazaki, 2004). Higher transcript abundance of LEPR in high PUFA composition might cause shift in fatty acid metabolism via up-regulating the LEPR gene. It could be suggested as the evidence that the Indonesian sheep showed good on fatness traits and were beneficial for human health. These were due to higher PUFA docosahexaenoic acid (C22:6n3) and lower SFA tricosanoic acid (C23:0) and tetracosanoic acid (C24:0). However, this study should need validation in other animal populations in order to evaluate its potential in selective breeding sheeps.

CONCLUSION

Associations and expression of Leptin Receptor (LEPR) gene polymorphisms with fatness traits, odour and flavour were described for the first time in Indonesian sheep, providing evidence that LEPR might be an important candidate gene for fatty acids composition. The LEPR might play a role in fatty acids composition validated through association study and by profiling of mRNA expression in liver tissues. The association study of odour and flavour through LEPR association has not been detected yet. In addition, this study needs to be validated in other animal populations in order to evaluate its potential in selective breeding.

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