*Ann. Anim. Sci., Vol. 21, No. 1 (2021) 109–124* *DOI: 10.2478/aoas-2020-0059*

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# A NON-SYNONYMOUS SINGLE NUCLEOTIDE POLYMORPHISM IN *FASN* GENE ALTERS FASN ENZYME ACTIVITY IN SUBCUTANEOUS AND INTRAMUSCULAR ADIPOSE TISSUE IN HOLSTEIN FRIESIAN STEERS*

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**Abstract**

**The FASN enzyme plays a key role in fatty acids synthesis as the main long-chain fatty acid synthesizer. A non-synonymous SNP (single nucleotide polymorphism) (g.17925A>G) located in the thioesterase domain of this enzyme and an effect in fat deposition has been observed, but has not been evaluated in this breed and, moreover, the reason whereby this occurs remains unclear. The objective of this study was to evaluate the effect of this SNP on the activity of FASN enzyme in subcutaneous and intramuscular adipose tissue from Holstein Friesian steers. To achieve this, 196 animals were sampled in a local abattoir, genotyped for the *FASN* g.17924A>G SNP and characterized for fatty acid profile. Then a sub-sample of 20 animals per genotype were selected to extract the total protein from subcutaneous and intramuscular adipose tissue to estimate the FASN enzyme activity. The FASN activity for each genotyped animal was assessed indirectly by measuring the decrease in the absorbance of NADPH at 340 nm by spectrophotometry in a 24 well plate in the presence of Acetyl-CoA, Malonyl-CoA, and NADPH. To assess the impact of SNP induced amino acid changes in FASN protein structure, *in-silico* simulations were performed. Our results indicated that *FASN* g.17924A>G SNP induces a change in the enzyme activity in subcutaneous adipose tissue, which is higher when the AA genotype is present and lower in the presence of the**

*Work financed from: National Doctorate Grant CONICYT: 21151048.

**AG genotype. The *in-silico* analysis of the amino acid substitution shows that there was a structural change in the dimeric form of the protein between genotypes. Moreover, the global energy between subunits is lower and more favorable when the AA genotype is present and higher and less favorable for the AG genotype. It was also found that the fatty acid profile of subcutaneous adipose tissue was affected when the AG genotype was present, decreasing the C16:0 fatty acid levels and increasing the C18:0 fatty acid levels. The *FASN* g.17924A>G SNP alters the FASN enzyme structure and activity, leading to a variation in the fatty acid composition of subcutaneous adipose tissue in Holstein Friesian steers. Implications: This SNP could be considered as a tool to improve the fat deposition or marbling and the fatty acid profile in cattle.**

**Key words: *Bos taurus*, adipose tissue, enzyme activity, fatty acids, FASN**

Holstein Friesian is the principal dairy breed in Chile (Ostrowski and Deblitz, 2001). The genetics of this breed has an influence in almost 50% of the country cattle population while surplus males from dairy farms supply up to 70% of local meat production (Navarro and Goic, 2003). However, the carcass and meat quality are lower in comparison to other meat breeds, mainly as a result of poor backfat deposition and lower marbling, resulting in reduced overall carcass value (Choi et al., 2000; Paris et al., 2015). Among the factors which regulate fat deposition in cattle, genetics plays a major role (Jeong et al., 2012). Previous studies using Holstein breed cattle have focused on milk production traits without placing emphasis on meat quality traits such as marbling (Ng-Kwai-Hang et al., 1986; Rodríguez-Bermúdez et al., 2017).

The main lipogenic gene that has been evaluated in Holstein breed is the fatty acid synthase (*FASN*) which plays a key role in *de novo* fatty acid synthesis (Ciecierska et al., 2013). In non-lactating ruminants, adipose tissue is the main lipogenic site and has the highest expression level of *FASN* gene (Roy et al., 2005). *FASN* encodes for a multifunctional protein complex with seven different catalytic sites, with the thioesterase domain determining the length of the synthetized fatty acids (Zhang et al., 2008). Functional FASN is a dimeric protein that is responsible for *de nov*o fatty acid synthesis, in particular of saturated fatty acids (SFA) including myristic acid (C14:0) and palmitic acid (C16:0) (Zhang et al., 2008). The *FASN* gene has been associated with fat deposition in several beef cattle breeds (Jeong et al., 2012; Papaleo Mazzucco et al., 2016), and with milk fat composition in dairy cattle breeds (Alim et al., 2014), but there is no information about its role in the quality of meat from Holstein Friesian steers. Many single nucleotide polymorphisms (SNPs) have been identified within the *FASN* gene (Raza et al., 2018; Zhu et al., 2017), but the *FASN* g.17924A>G is the only non-synonymous SNP present in the thioesterase domain (Bhuiyan et al., 2009). This SNP has been reported to influence the fat content and fatty acid composition of milk, but its potential effects on meat quality and fat deposition remain unclear in Holstein cows (Matsumoto et al., 2012). Moreover, there is no information about the effect of this SNP on FASN enzyme activity in adipose tissue from this breed. Therefore, we have hypothesized that SNP g.17924 A>G alters the FASN enzyme activity due to a structural modification induced by the amino acid substitution in the thioesterase domain. We evaluated the effect of the *FASN* g.17924A>G SNP in relation to enzyme activity from samples of

subcutaneous (SAT or backfat) and intramuscular adipose tissue (IAT) in Holstein Friesian steers and assessed the effect of the amino acid substitution in the enzyme structure.

## Material and methods

This study was carried out under the protocol for animal care approved by the Scientific Ethical Committee of Universidad de La Frontera, Temuco, Chile (Protocol number: 033-17).

Samples of the longissimus muscle and subcutaneous adipose tissue were collected from 196 Holstein Friesian steers that were slaughtered at the "Planta Faenadora de Carnes Victoria S.A", located in Victoria, Araucanía region, Chile, 4720897. Samples were quickly frozen in liquid nitrogen and then stored at –80°C until DNA and total protein extraction was carried out. Steers were the same age (18-month-old) and were raised under the same conditions by a local dairy farm "Agricola Ancali", Los Angeles, Bio-Bio region, Chile.

### DNA extraction and SNP genotyping

Each animal was genotyped for the *FASN* g.17924A>G SNP through PCR-RFLP technique using genomic DNA obtained from muscle using a commercial kit (Cat. #D3024, Quick-DNA Miniprep kit, Zymo Research, California, USA). PCR was performed in a total volume of 50 µL containing: PCR Buffer 1X, dNTPs (0.8 mM), primers (10 ng of each) (forward $^{5'}$AGAGCTGACGGACTCCACAC$^{3'}$and reverse $^{5'}$CTGCATGAAGAAGCACATGG$^{3'}$), Paq5000 polymerase (2.5 U), 37 µL of nuclease-free water and 50 ng of genomic DNA. Amplifications were performed using the Paq5000 DNA Polymerase (Cat. #600680, Agilent Technologies, California, USA) protocol as follows: initial denaturation at 95°C for 2 min, 30 amplification cycles (denaturation at 95°C for 20 s, annealing at 56°C for 20 s and extension at 72°C for 30 s) and a final extension period at 72°C for 5 min. The 759 bp amplicon obtained was then digested by the restrictase *MscI* (Cat. #R0534S, New England Biolabs, Massachusetts, USA) which included CutSmart buffer 1X, *MscI* restriction enzyme (3 U), 6.4 µl of nuclease-free water and 10 µl of PCR product (10 ng/µl) in a total volume of 20 µl at 37°C in an overnight reaction and then submitted to agarose gel electrophoresis (1.5% W/V). The 759 bp amplicon containing the AA genotype presented 3 fragments including 342 bp, 251 bp and 166 bp, the AG genotype 4 fragments including 417 bp, 342 bp, 251, 166, and the GG genotype 2 fragments including 417 bp and 342 bp (Figure 1 A).

### Protein extraction by ultracentrifugation

Total protein extraction was performed according to Kumar and Dodds (1981). Briefly, 5 g of the longissimus muscle and subcutaneous adipose tissue samples from each steer (n = 20 per genotype) were homogenized in 38 mL of phosphate-bicarbonate buffer (0.1M and pH: 8.0), then centrifuged at 20,000 g for 10 min, and the supernatant was transferred to a polypropylene tube which underwent ultracentrifu-

gation at 105,000 g for 1 hour. The protein content of resultant supernatants was quantified using the Bradford assay, measuring absorbance at 595 nm in a Synergy HTX spectrophotometer (BioTek, Vermont, USA). Samples were stored at –80ºC.

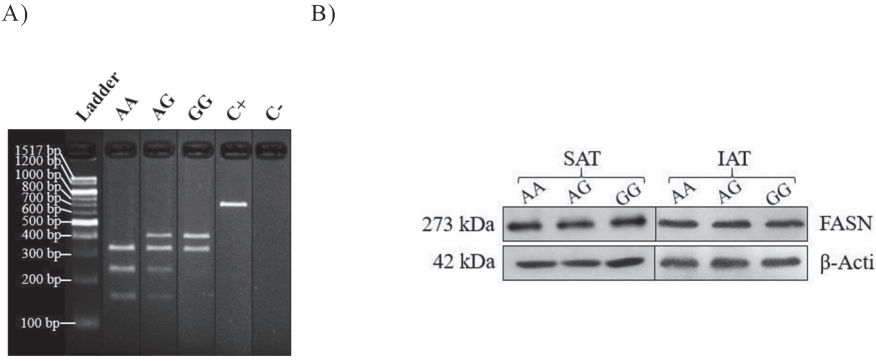


Figure 1. (A) PCR-RFLP assay (agarose gel 1.5%,) showing AA, AG and GG genotypes, positive and negative controls (C+, C-). (B) Western Blot assessing FASN presence in samples of subcutaneous adipose tissue (SAT) and intramuscular adipose tissue (IAT)

**Western Blot assay for assessing the efficiency of FASN enzyme extraction**

After the ultracentrifugation step (105,000 RCF) for protein extraction, 10 samples of each genotype were selected and analyzed using Western blotting, in order to verify that enzyme concentrations were not different between samples. For this, 20 and 100 µg of total protein from supernatant were taken from both tissues (subcutaneous adipose tissue and longissimus muscle, respectively). Samples were denatured in presence of sodium dodecyl sulfate (80 mM), β-mercaptoethanol (360 mM) and glycerol (1.1 M), incubated at 100ºC for 5 minutes. After that, proteins were separated in a 10% polyacrylamide gel, using electrophoresis at 100 volts for 2 and a half hours. Protein wet transference was carried out using a polyvinylidene fluoride membrane at 100 volts for 2 hours and 20 minutes. Then, using the protein ladder (Cat. #26625, Thermo Fisher Scientific, Massachusetts, USA) as references, membranes were cut out in order to perform the immunodetection of FASN and β-actin (used as control). Membranes were then incubated with Anti-FASN (Cat. #OAGA00799, Aviva Systems Biology, California, USA) and Anti-β-Actin (Cat. #OAAB19522, Aviva Systems Biology, California, USA) antibodies respectively, and incubated in blot buffer (Tween 20 (0.1%), bovine serum albumin (10 g/L) and phosphate buffered saline or PBS (1M)) overnight in constant stirring (125 RPM). After incubation, membranes were washed out 4 times for 5 minutes with PBS (1X) and then incubated in buffer blot for 30 minutes in presence of a secondary antibody conjugated to radish peroxidase and then washed 4 times with PBS (1X). Visualization was performed using the SuperSignal™ West Pico PLUS (Cat. #34578, Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer instructions, obtaining a chemiluminescent reaction. Membrane bands were revealed using generic x-ray plates. Results were normalized and assessed with the software ImageJ v1.50.

Data was processed using the ANOVA test to identify differences ($P<0.05$) in the enzyme content between samples.

**Enzyme activity test**

The FASN activity assay was carried out following the method described by Kumar and Dodds (1981). Briefly, the FASN activity was assessed indirectly by measuring the decrease in the absorbance of NADPH at 340 nm by spectrophotometry in a 24 well plate in presence of Acetyl-CoA, Malonyl-CoA, and NADPH. The assay mixture was compounded by 100 µg of total protein extract, potassium phosphate buffer (100 mM at pH 7.0), EDTA (100 µM), Dithiothreitol (1 mM), NADPH (125 µM), Acetyl-CoA (25 µM) and Malonyl-CoA (50 µM). The mixture containing all ingredients but Acetyl-CoA and Malonyl-CoA, was preincubated at 37°C for 8 minutes. After this stabilization, 25 µM of Acetyl-CoA were added, setting a background rate of NADPH oxidation at 340 nm at 37°C. After two minutes of stabilization, 50 µM of Malonyl-CoA was added to the mix to start the synthesis of fatty acids and the oxidation of NADPH to NADP by FASN. The oxidation of NADPH was evaluated by quantifying the reduction in the absorbance at 340 nm for 2, 6, 10 and 15 minutes after the reaction started, and comparing it with a calibration curve. Measurements were made in a Synergy HTX spectrophotometer (BioTek, Vermont, USA). The quantity of enzyme that oxidizes 1 nmol of NADPH per minute is defined as one unit of enzymatic activity (U) (Kumar and Dodds, 1981) and the specific activity is defined as the units of enzymatic activity per milligram of total protein (U/mg).

**Fatty acid analysis**

Total lipids were extracted from the longissimus muscle (intramuscular adipose tissue) and subcutaneous adipose tissue according to the method described by Folch et al. (1957). Fatty acids were submitted to cold methylation method in presence of KOH, methanol and hexane. The fatty acids methyl esters were analyzed using a Clarus 500 gas chromatograph (Perkin Elmer, USA) equipped with an autosampler, a SP™ Fused Silica Capillary Column 2380 (60 m × 0.25 mm × 0.2 µm film thickness, Supelco, USA) and coupled with a flame ionization detector (FID, Perkin Elmer, USA). Nitrogen was used as carrier gas with a flow rate of 45 mL/min. The temperature program was set as follows: pre-heating at 150ºC for 1 min followed by an increase of temperature at a rate of 1ºC/min up to 168ºC (kept for 11 minutes), and a final increase of temperature at a rate of 6ºC/min up to reach 230ºC (kept for 8 minutes), in final cycle of 48.3 min.

***In-silico* simulation of wildtype and mutated FASN**

In order to understand the effect of *FASN* g.17924A>G SNP in the protein structure, leading to a possible change in enzyme activity, an *in-silico* simulation was performed based on the amino acid sequence corresponding to the enzyme fatty acid synthase A of *Bos taurus* (ID: Q71SP7), downloaded from the UNIPROT database (https://www.uniprot.org/) (The UniProt, 2018). Subsequently, an *in-silico* mutation was performed in one amino acid that consisted in the change of $T^{-2264}$ for $A^{-2264}$.

Both protein sequences, mutated (FASN$^-$) and wild (FASN), were modeled with the RaptorX program (Källberg et al., 2012). The stereochemical quality and accuracy of the generated models was evaluated using the SWISS-Model server to analyze the Ramachandran plot (Waterhouse et al., 2018). A molecular docking algorithm based on shape complementarity principles was used on the PATCHDOCK server to perform protein-protein docking simulations with FASN/FASN, FASN/FASN$^-$ and FASN$^-$/FASN$^-$ dimers (Schneidman-Duhovny et al., 2005). The ten best docking models generated by this program were refined with the FireDock program (Mashiach et al., 2008). After that, the 4 most favorable models and their global energy (GE) per genotype were selected and averaged. Generated models were then visualized with PyMOL software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

**Statistical analysis**

An analysis of variance (ANOVA) was performed using the SPSS v.23 statistical package (IBM, USA). The model was set comparing enzyme activity and fatty acid composition of intramuscular fat from longissimus muscle and subcutaneous adipose tissue, using the genotype of *FASN* g.17924A>G SNP as factor, with a confidence interval of 95% (P=0.05). A Pearson correlation was performed comparing the relationship between backfat thickness, intramuscular fat percentage and FASN activity from subcutaneous and intramuscular adipose tissue.

## Results

The mean whole hot carcass weight of animals was 311.5±13.5 kg, with a backfat thickness mean of 2.64±0.8 cm and intramuscular fat percentage mean of 2.9±1.0%.

**Genotyping**

Genotypes are shown in Table 1. All three genotypes of *FASN* g.17924A>G SNP were represented in this study. The AG genotype was found in a higher frequency than the others in sampled animals, while the wild type genotype (AA) had the lowest frequency. The mutated allele was found to occur at a frequency of about 62% within the sampled population.

Table 1. Genotypic and allelic frequencies for *FASN* g.17924A>G SNP

| | Genotypic frequencies (%) | | | Allelic frequencies (%) | |
|---|---|---|---|---|---|
| | AA (*n* = 24) | AG (*n* = *98*) | GG (*n* = 74) | A | G |
| Holstein Friesian | 12.3 | 50 | 37.7 | 38 | 62 |

**FASN enzyme extraction efficiency**

Results for Western Blotting of FASN enzyme extract are shown in Figure 1 B. These results indicate that FASN was present in the ultracentrifugation step super-

natant (105,000 RCF) obtained from both tissues. Bands were observed at 273 kDa for the FASN enzyme and at 42 kDA for β-Actin. B-Actin was used as control, indicating that the same amount of sample was set in all wells. No differences in FASN concentration were found between samples.

**Enzymatic activity assay**

The FASN enzyme activity in subcutaneous adipose tissue differed among all three genotypes ($P<0.05$), with the wildtype genotype having the greatest activity (AA = 273.4±69.4 U/mg), and the heterozygous genotype having the lowest activity (AG = 98.6±23.1 U/mg), while the mutated homozygote had an intermediate level of activity between the other two (GG = 174.68±13.7 U/mg) (Figure 2 A). The FASN activity in intramuscular adipose tissue (Figure 2 B), did not differ between genotypes (AA = 11.71±3.3 U/mg, AG = 7.80±4.4 U/mg, GG = 8.12±1.8 U/mg).

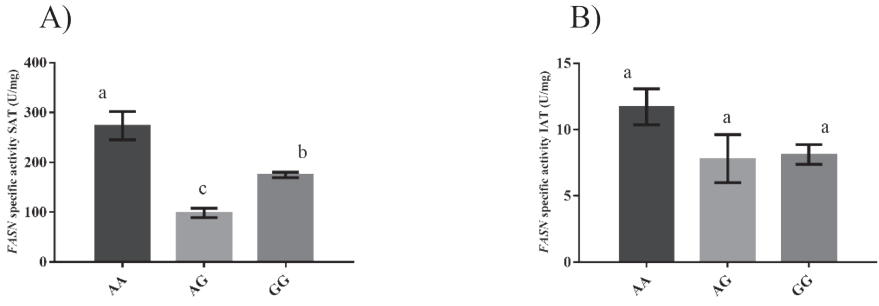


Figure 2. Effect of *FASN* g.17924A>G SNP on FASN enzyme specific activity (U/mg) in Holstein Friesian steers. A: activity of *fatty acid synthase* measured in subcutaneous adipose tissue (SAT). B: activity of *fatty acid synthase* measured in intramuscular adipose tissue (IAT). Columns with different letters differ significantly ($P<0.05$)

**Fatty acid profile**

The myristic acid (C14:0) percentage did not differ between genotypes in subcutaneous adipose tissue ($P>0.05$). However, the percentage of palmitic (C16:0) and stearic (C18:0) acid content was significantly different for this tissue. The percentage of palmitic acid was lower ($P<0.05$) when heterozygous genotype (AG) was present, while the content of stearic acid was higher for the same genotype (Table 2). No additional differences were found among other fatty acids when comparing the genotypes in subcutaneous adipose tissue.

In the intramuscular adipose tissue, the percentage of myristic acid was different ($P<0.05$) between genotypes, where the AA genotype had a higher value (2.39±0.4) and the GG genotype the lowest value (1.73±0.4). Also, a statistical difference ($P<0.05$) was found within both the percentage of polyunsaturated fatty acids (PUFAs) (AA: 7.58±2.03%; AG: 12.63±3.83%; GG: 10.93±2.53%) and the ratio PUFA:SFA (AA: 0.14±0.04; AG: 0.25±0.08; 0.21±0.05) of this tissue.

Table 2. Percentages (%) for C14:0, C16:0 and C18:0 fatty acids according to *FASN* g.17924A>G genotypes of subcutaneous adipose tissue

| | AA (*N* = 20) | AG (*N* = 20) | GG (*N* = 20) |
|---|---|---|---|
| C14:0 (myristic fatty acid) | 3.57±0.4 | 3.52±0.6 | 3.21±0.3 |
| C16:0 (palmitic fatty acid) | 26.93±1.7 a | 23.21±1.8 b | 26.63±0.7 a |
| C18:0 (stearic fatty acid) | 22.87±0.9 a | 26.24±1.9 b | 23.61±3.6 ab |

Values in rows with different letters differ significantly (P<0.05).

Table 3. Percentages (%) for C14:0, C16:0 and C18:0 fatty acids according to *FASN* g.17924A>G genotypes of intramuscular adipose tissue

| | AA (*N* = 20) | AG (*N* = 20) | GG (*N* = 20) |
|---|---|---|---|
| C14:0 (myristic fatty acid) | 2.39± 0.4 a | 1.95±0.3 ab | 1.73±0.4 b |
| C16:0 (palmitic fatty acid) | 26.78±2.6 | 25.92±1.9 | 25.91±0.9 |
| C18:0 (stearic fatty acid) | 22.33±1.2 | 20.39±1.7 | 22.16±2.7 |

Values in rows with different letters differ significantly (P<0.05).

Finally, we found a significant Pearson correlation (P<0.05) between the FASN enzyme activity of both tissues and intramuscular fat percentage (R = 0.53 for SAT and R = 0.55 for IAT) and between FASN activity from SAT and FASN activity from IAT (R = 0.57) (Table 4).

Table 4. Pearson correlation between backfat thickness, intramuscular fat percentage and FASN activity from subcutaneous and intramuscular adipose tissue

| | BFT | IMF% | FASN activity SAT | FASN activity IAT |
|---|---|---|---|---|
| BFT | 1 | | | |
| IMF (%) | 0.23 | 1 | | |
| FASN activity SAT | 0.39 | 0.53* | 1 | |
| FASN activity IAT | –0.05 | 0.55* | 0.57* | 1 |

BFT: backfat thickness, IMF%: intramuscular fat percentage, SAT: subcutaneous adipose tissue, IAT: intramuscular adipose tissue.

*correlation is significant at level 0.05 (bilateral).

### *In-silico* simulation of wildtype and mutated FASN

All three possible genotypes for the FASN enzyme were successfully simulated *in-silico*. Figure 3 shows Ramachandran plots obtained from RaptorX which show the structural differences induced by the amino acid substitution in wildtype (left) and mutated (right) enzyme monomers, and Figure 4 shows predicted interactions, made by FireDock, among 3 possible dimers (Figure 4 A–C) and the structural changes that these interactions made.

The GE values calculated by the FireDock program when monomers interacted, were –9.07, 2.88 and 1.37 kcal $mol^{-1}$ for wildtype dimer, heterodimer and mutated dimer, respectively.

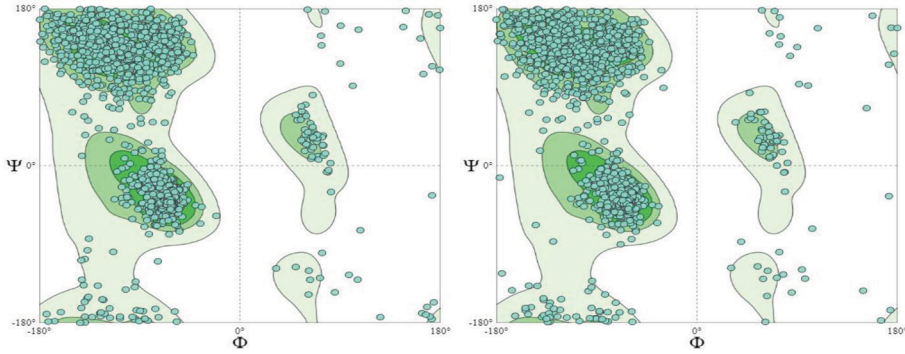


Figure 3. Ramachandran plots of FASN homodimers, wildtype (right) and mutated (left), showing a general view of possible secondary structure of the polypeptide and distribution of residues in favorable areas

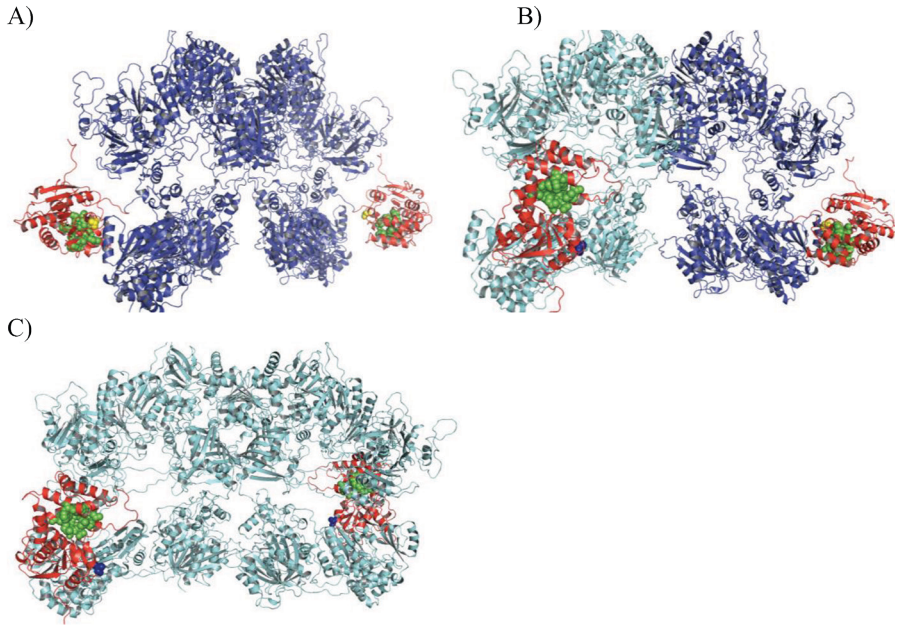


Figure 4. Predicted protein-protein docking performed with FASN models (FASN in royal blue and FASN$^{-}$ in cyan). FASN/FASN (A), FASN/FASN$^{-}$ (B) and FASN$^{-}$/FASN$^{-}$ (C).
Red: thioesterase domain; Green: catalytic residues of thioesterase domain; Yellow and blue: position 2264 where amino acid substitution is located (Threonine/Alanine, respectively)

## Discussion

### Genotyping

Comparing the allelic frequencies for *FASN* g.17924A>G SNP found in Holstein Friesian steers, which is a breed with poor conformation and fatness versus

Aberdeen Angus, the most popular meat cattle breed, with good fat deposition and conformation (Bureš and Bartoň, 2018), it is important to highlight that there is an opposite distribution of alleles for this SNP. In Holstein Friesian, the G allele has the highest frequency (62%), while in Aberdeen Angus it is the A allele (69%) (Papaleo Mazzucco et al., 2016). This could be in part an explanation for the differences in the fat deposition between both breeds and makes more graphic the effect of genetic assisted selection based on polymorphisms in animal breeding, and also for the different distribution of polymorphisms among different breeds.

**FASN enzyme activity**

There are studies assessing FASN enzyme activity in Holstein Friesian cattle related to milk production but there is no information about the impact on meat production in this breed (Matsumoto et al., 2012; Morris et al., 2007).

The SNP g.17924A>G has recently been reported to have a significant effect increasing the marbling in Korean cattle (Oh et al., 2018), but how this SNP induces this effect remains unclear. It has been hypothesized that this SNP could have an effect on FASN enzyme activity as a result of a change in protein sequence and structure caused by the SNP associated amino acid replacement. The monomers of FASN are known to be catalytically inactive while heterodimers with a mutated subunit show dramatically reduced enzyme activity compared to parental homodimers (Joshi et al., 2003). This trend was observed in the results with heterozygous genotype individuals showing less enzyme activity than those with either homozygous genotype. The differences in FASN enzyme activity in this study were found in subcutaneous adipose tissue but not in the intramuscular adipose tissue, probably due to the extreme leanness of the longissimus muscle, and the lack of marbling.

It is known from ruminants this enzyme has the greatest expression level at the adipose tissue and the lowest in muscle (Roy et al., 2005), so the leanness of the muscle and the low presence of this enzyme compared to other proteins in the intramuscular adipose tissue could be the reason why significant differences were not found in activity levels for this tissue. However, the trend of the activity observed for IAT was similar to SAT.

In beef cattle breeds the activity of FASN enzyme has been reported to be greater in Aberdeen Angus, which is a breed with a good marbling and fat deposition (Lunt et al., 1993), intermediate in Japanese Black × Angus, and lower in Limousin, which is a continental breed with good muscling but low marbling and fat deposition (Bonnet et al., 2007)

Other study reports the activity of FASN to be 3 times higher in the subcutaneous adipose tissue than in longissimus muscle and up to 30 times higher when comparing subcutaneous adipose tissue with the semitendinosus muscle (Bonnet et al., 2007).

In this study, FASN activity was, on average, 20 times higher in subcutaneous adipose tissue than in intramuscular adipose tissue, showing the same trend reported by Bonnet et al. (2007).

There is a direct relation between FASN activity and fat deposition as shown by the Pearson correlation between FASN activity and intramuscular fat percentage (Table 4). Jensen-Urstad and Semenkovich (2012) reported that reductions in FASN

activity caused by chemical inhibition have been shown to cause dramatic reductions in fat deposition in mice. This explains why longissimus muscle samples, which have 20 times lower FASN activity than subcutaneous adipose tissue, are extremely lean and highlights that there is a direct relation between FASN activity and fat deposition.

The *FASN* g.17924A>G (T2264A) SNP is located in the thioesterase domain of FASN enzyme and made a change from polar, neutral, and hydrophilic to nonpolar, aliphatic, and hydrophobic in this motif, affecting the structure of the substrate binding site and therefore changing the specific activity of thioesterase domain towards C14-acyl ACP (Acyl carrier protein domain), which transfers the growing fatty acid chain throughout the different domains of this enzyme (Oh et al., 2018; Zhang et al., 2008), affecting its affinity with the fatty acid (Oztabak et al., 2014). Therefore, the thioesterase domain determines the carbon chain length of the final product of this enzyme and any change or variation in this domain could affect the fatty acid composition of meat and fat (Zhang et al., 2008). This was observed in this investigation, where this SNP effectively induces a change in the FASN activity of subcutaneous adipose tissue and in the composition of fatty acids in samples obtained from Holstein Friesian steers.

Based on what was proposed by Joshi et al. (2003), the change in protein conformation of one subunit due to an amino acid replacement will lead to a dysfunction of the enzyme, reducing the total enzyme activity in adipose tissue of animals carrying the heterozygous genotype, but only when different monomers join together, as found.

However, there are no additional references of any work assessing the effect of this specific SNP on FASN activity, therefore, this is the first insight into the effect of the *FASN* g.17924A>G SNP on the FASN enzyme activity.

**Fatty acid profile**

As reported in other studies, this SNP has an effect over the fatty acid composition of adipose tissue (Zhang et al., 2008; Li et al., 2012; Oh et al., 2012; Yeon et al., 2013; Oztabak et al., 2014), and on fat deposition, as found in Korean cattle by Oh et al. (2018), who commented that this SNP could be a casual mutation for increasing fat deposition.

In agreement with this study, Zhang et al. (2008) has reported that the *Longissimus dorsi* from Aberdeen Angus carrying the GG genotype had lower percentage of C14:0 fatty acid (2.92%) compared to AA and AG genotypes (3.46 and 3.26% respectively), and the lowest percentage of C16:0 fatty acid (27.6%) compared to AA and AG genotypes (28.5% and 28.1%, respectively). In another study carried out in Korean cattle (Hanwoo breed), the AA genotype was associated with increased levels of the C14:0, C16:0, and C18:0 fatty acids, and a decrease in the C18:1 fatty acid in longissimus muscle (Oh et al., 2012).

The results showed that myristic acid in both tissues had the same trend, but differences were not significant for SAT. The AA genotype showed the greatest percentage of C14:0 and the GG genotype the lowest in both tissues. This agrees with what was published by Schennink et al. (2009), who reported that this SNP has an

effect on milk fatty acid composition but only for the C14:0 fatty acid, while the AA genotype is the one that shows the highest percentage of this fatty acid and the GG genotype the lowest one (Schennink et al., 2009).

Palmitic acid shows the highest percentage for AA genotype and the lowest percentage for AG genotype in SAT, in agreement with the genotype which has the highest and the lowest FASN enzyme activity, respectively. The AA genotype in IAT is showing the higher percentage of C16:0, but the differences were not significant. This could occur because the variation of IMF (intramuscular fat) content depends on the ability of muscle to utilize circulating lipoprotein-associated triacylglycerols and the intracellular trafficking of fatty acids by the fatty acid-binding proteins known as FABPs (Jurie et al., 2007). Most of those FABPs bind and fix long chain fatty acids ranging from C16:0 to C20:0 (Storch and McDermott, 2009). Therefore, the distribution of these fatty acids in lean muscle could be most influenced by the trafficking and fixation from circulating ones rather than the *novo* synthesis by FASN.

Stearic fatty acid seems to have a different trend when comparing both tissues as well, this is probably due to the reduced production of palmitic acid when the AG genotype is present in subcutaneous adipose tissue. In the case of intramuscular adipose tissue, having the lowest percentage of stearic acids when the AG genotype is present could be probably due to reduced fixation of this acid by FABPs, as described by Storch and McDermott (2009), and to an increase in the proportion of unsaturated fatty acids extracted from phospholipids rather than fat because of the leanness of the longissimus muscle (Karolyi et al., 2009).

In this study, it was found that the reduced activity of the FASN enzyme when the mutation is present, especially in SAT, would reduce the production of palmitic fatty acid. This reduction, in proportion, indirectly increases the percentage of stearic fatty acids, as shown in Table 2 for the AG genotype. On the other hand, the leanness of the longissimus muscle combined with the reduced expression and activity of the enzyme in this tissue (up to 24 times less in IAT than SAT), significantly reduces the quantity of fatty acids methyl esters injected to the gas chromatograph which could decrease the sensitivity of the test for the SFA, which are even present in muscle than fat in lower percentages (Karolyi et al., 2009).

***In-silico* simulation of wildtype and mutated FASN**

The models generated with the RaptorX program showed an excellent quality when they were evaluated with the Ramachandran plot analysis with the FASN model having 92.02% of residues in favored regions and the FASN$^{-}$ model having 91.76% of residues in favored regions. The Ramachandran plot is one of the most reliable methods for determining the quality of protein structures. When models have over 90% of their residues in the favored regions of the Ramachandran plot they are considered as accurate as 2Å resolution crystalline structures (Laskowski et al., 1993). Differences observed in the Ramachandran plots are explained by the change of an amino acid in the thioesterase domain of this enzyme.

Ramachandran plots have been considered as one of the most simple and most sensitive plots for assessing the quality of protein model (Kleywegt and Jones, 1996). As most of the residues are found in an allowed region it is an indicator of a high-

-quality model (Chen et al., 2010). In addition to visualizing all the possible combinations of dihedral angles, phi (f) and psi (Y), of a polypeptide, these plots are a good tool to visualize differences between a wildtype and mutated protein (Gromiha et al., 2002).

Results obtained with FireDock showed a variation in the GE values, where the wildtype dimer of the enzyme showed the most favorable GE between its monomers and heterodimer presented the least favorable GE between its monomers, suggesting a relevant role of this mutation (T2264A) in the interaction between the FASN monomers during dimerization. This is probably due to the change from the polar, neutral, and hydrophilic properties of threonine, to the nonpolar, aliphatic, and hydrophobic properties of alanine affecting the structure of the polypeptide at the dimeric form (Oh et al., 2018).

As the lower the GE the greater the strength of the interaction, we have hypothesized that animals carrying the heterozygous genotype (AG) will have significant less activity than wildtype (AA) and mutated (GG) homozygous genotypes as a result of the monomers having a less stable and more distant join between them caused by the higher GE.

As the acyl carrier protein domain (ACP) of each subunit works with the opposite subunit during the elongation of the fatty acid in synthesis, a more distant and less stable join between subunits could impair the movement of the ACP between catalytic sites of the enzyme and decreasing the affinity with the substrate resulting in lower activity.

Both the Ramachandran plots and GE confirm that the amino acid substitution made a change in FASN structural conformation.

**Conclusion**

This is the first time the effect of the *FASN* g.17924A>G SNP on FASN enzyme activity has been evaluated in Holstein Friesian steers, and it can be confirmed that there is an effect of this mutation on the enzyme activity due to the amino acid substitution in the thioesterase domain, leading to a structural variation in the dimeric form of the enzyme. This leads to a significant difference in the fatty acid composition of subcutaneous adipose tissue. Finally, the FASN enzyme activity shows to be closely related to backfat thickness and intramuscular fat percentage in this cattle breed.

**Acknowledgements**

The first author wishes to thank to Faenadora de Carnes Victoria S.A., and especially to Dr. María Angélica Vásquez for providing the animal samples for this study, and to CONICYT (Comisión Nacional de Investigación Científica y Tecnológica) for funding this doctoral scholarship. This investigation was supported by National Doctorate Grant CONICYT: 21151048.

The authors wish to thank Fondecyt Projects: Post doctorate Fondecyt N°3190287 (John Quiñones) and Fondecyt Initiation in Research Nº11190621 (Rommy Díaz).

We also thank for the corrections and suggestions of Dr. Robert Brinzer that have helped to improve this article.

## Conflicts of Interest

The authors declare no conflicts of interest.

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Received: 10 X 2019
Accepted: 28 V 2020