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# Effects of rumen-protected, long chain fatty acid, calcium salt supplementation on total lipid, fatty acids, and related gene expression in Korean cattle

**S. A. Belal[1,2], D. R. Kang[1] & K. S. Shim[1,3#]**
[1]Department of Animal Biotechnology, Chonbuk National University, Jeonju-si 54896, Republic of Korea
[2]Department of Poultry Science, Sylhet Agricultural University, Sylhet-3100, Bangladesh
[3]Department of Agricultural Convergence Technology, Jeonbuk National University, Jeonju 54896, Korea

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## Abstract

Rumen-protected linoleic acid-enriched calcium salts (LAE-Ca salts) and oleic acid-enriched calcium salts (OAE-Ca salts) were examined for their effects on total lipids, fatty acids, and lipid metabolism gene expression in Korean steers. A total of 30 animals with a mean body weight of 597 ± 5 kg were divided into three treatment groups: Control, control diet without additional fat (n = 10); LAE-Ca salts fed 50% LAE-Ca salts (n = 10); and OAE-Ca salts, fed 50% OAE-Ca salts (n = 10); 100 g/d/animal on dry matter required basis (DMR). Results revealed that lipid content was substantially lower in the OAE-Ca salts group. Total poly-unsaturated fatty acid (PUFA), $\omega^6$, and $\omega^3$ were substantially higher in the OAE-Ca salts group but the percentage of saturated fatty acids (SAF), mono-unsaturated fatty acids (MUFA), and unsaturated fatty acids (USFA) was not affected. Up-regulation of leptin gene expression was observed in the LAE-Ca salts group. The mRNA levels of genes related to lipogenesis, including lipoprotein lipase (*LPL*), fatty acid synthase (*FAS*), and stearoyl-CoA desaturase (*SCD*), were not statistically different between groups. However, acetyl CoA carboxylase (*ACC*), lipolytic adipose triglyceride lipase (*ATGL*), and fatty acid binding protein (*FABP4*) were substantially higher in the LAE-Ca salt group; hormone-sensitive lipase (*HSL*) was higher in the OAE-Ca salt group. Therefore, our findings indicate that fatty acid-enriched calcium salt diets affect total lipids, fatty acids, and the expression of certain lipid metabolism genes in Korean cattle.

**Keywords:** calcium salts, total lipid, fatty acids, gene expression, Korean cattle
#Corresponding author: ksshim@jbnu.ac.kr

## Introduction

Fat is the most important energy source in animal diets and is supplemented to increase the energy density of rations. Long-chain fatty acids (LCFAs) are physiologically important because they combine to form triacylglycerols and provide energy storage in the adipose tissues of animals. Calcium salts of LCFAs comprise FAs complexed with calcium ions, which makes them insoluble (Frank *et al.,* 2022), prevents problems associated with rumen microbial fermentation and digestion, and have been widely used as a rumen-protected fat in dairy and beef production (Jenkins & Bridges, 2007). Supplemental fats of different origins are used to improve the energy density of ruminant diets. Dietary supplementation with FA calcium salts generally has no or a slight antagonistic effect on dietary digestibility in ruminants (Bain *et al.,* 2016).

Feeding diets with FA calcium salts is a practical way to bypass rumen biohydrogenation via rumen microbes. The rumen biohydrogenation rate depends mainly on two factors: the nature of FAs (e.g., n-3 or n-6) and pH (Loor *et al.,* 2004). Wu *et al.* (1991) stated that in dairy cows, the net biohydrogenation of total unsaturated C18 in diets with added Ca salts of FA and fat (animal-

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vegetable blends) was 57.3% and 87.2%, respectively. Long-chain fatty acid-Ca salts were little protected from ruminal biohydrogenation because the net biohydrogenation of unsaturated FA Ca salts was approximately 50%, whereas that of polyunsaturated FAs ranged from 60 to 90%. Feeding different dietary animal fats or vegetable oils to domestic animals influences the FA composition of muscle tissue. Soybean oil, tallow, and yellow grease were reported to alter the FA composition of the longissimus muscle in steers (Brandt & Anderson, 1990); an inclusion of 5% palm oil increased total intramuscular PUFA in Korean native cattle (Park *et al.,* 2017); the PUFA composition of beef muscle was modified by feeding dietary lipids (soya vs. linseed) in Charolais steers (Choi *et al.,* 2006); whole raw soybeans altered the composition of the beef carcass by altering the unsaturated fatty acid profile (Felton and Kerley, 2004); and lamb meat was improved by increasing muscle n-3 fatty acids by supplementing the diet with either fish oil or fish meal (Ponnampalam *et al.,* 2001). Furthermore, Gillis *et al.* (2004) reported that the addition of rumen-protected conjugated linoleic acid or linoleate-rich corn oil to feedlot cattle diets improved marbling scores and increased leptin content in adipose tissue.

Previous studies have recognized that LCFA-Ca salts have a positive impact on small ruminants and dairy cows. Feeding calcium salts of FAs (vegetable oils) increased milk yield and quality in cows (Purushothaman *et al*., 2008; Andrés *et al*., 2013) and meat quality, such as linoleic acid concentration, in Bali cattle (Bain *et al*., 2016). However, several studies have shown inconsistent results regarding the use of fats in cattle and sheep (Obeidat *et al*., 2012) and a limited number of studies have examined the effect of feeding LCFA-Ca salts on Korean steers. Therefore, the aim of this study was to determine the effects of LCFA-Ca salts on lipid content, FA profiles, and related gene expression, all of which affect meat quality.

## Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee of Jeonbuk National University (approval number: CBNU2018-097), in accordance with the Korean National Law on Animal Care and Use.

Thirty Korean steers were used in this feeding trial with a mean body weight of 597 ± 5 kg. This trial included three dietary treatments: control, a control diet (concentrate mix and rice straw) without additional fat (n = 10); LAE-Ca salts, 50% linoleic acid and 24% oleic acid containing LCFA-Ca salts (n = 10); and OAE-Ca salts, 26% linoleic acid and 50% oleic acid containing LCFA-Ca salts (n = 10); at 100 g/day/animal on a dry matter required basis (DMR). Drinking water and feed were provided *ad libitum*. Before the experiment, all animals were fed an experimental control concentrate for a 2-week adaptation. The nutrient content of the commercial control diet was crude protein, 12.50%; crude fat, 2.50%; crude fibre, 15%; crude ash, 10%; calcium (Ca), 0.70%; phosphorous (P), 1.20%; and total digestible nutrients (TDN), 75%. The control diet was supplied by Nonghyup Feed Co., Ltd (Seoul, Korea). The diets were provided for 180 days (6 months) until the animals were slaughtered. The LCFA-Ca salt was top-dressed at each feeding time. Feed was supplied twice daily in the morning and evening. The concentrate mix diet was fed *ad libitum*, and rice straw was restricted to approximately 10% of the concentrate.

The FA profiles of the supplied LCFA-Ca salts are listed in Table 1. The vital fatty acid components of LCFA-Ca salts were 1) palmitic acid (11.87%), oleic acid (24.84%), linoleic acid (50.03%), stearic acid (7.41%), and 2) palmitic acid (10.80%), oleic acid (50.54%), linoleic acid (26.97%), and stearic acid (3.13%). The ratios of SFAs to unsaturated fatty acids (USFAs) were 19.55:31.86 and 14.15:57.47, respectively.

The animals were sacrificed for sample collection and evaluation. At 27 months of age, steers were withdrawn from the experimental diets 24 h before slaughter, and strip loin samples (*Longissimus dorsi*) were taken from the right side of the carcass. The samples were separated into two parts; one was powdered using liquid nitrogen and processed for gene expression evaluation, and the other was freeze-dried for fatty acid assessment. Samples from all groups were stored at -80 °C until further analysis.

Total lipids were extracted from muscle tissue using a mixture of chloroform, methanol, and distilled water (Bligh & Dyer, 1959). The extraction was performed in duplicate. Briefly, the experimental meat samples were minced with a blender, and 10 g of the obtained mince was transferred into centrifuge tubes. The centrifuge tubes were kept on ice throughout the procedure. Subsequently, 10 mL of distilled water, 20 mL of chloroform, and 40 mL of cold methanol were added to each tube. The mixture was homogenized using a homogenizer (Ultra-Turrax T25, IKA-Labotechnik,

Staufen, Germany) for 2 min at 9000 rpm. Additional amounts of chloroform (20 mL) and distilled water (20 mL) were added separately, and the mixture was homogenized for 30 s after each addition. After homogenization, the tubes were centrifuged (High Speed Centrifuge 2236 HR, Labocene, Seoul, Korea) for 10 min at 11,000 rpm. An aliquot of the chloroform phase (2 mL) was collected from the bottom of each tube and transferred to a pre-weighed glass tube. The glass tube with the chloroform phase was placed in an evaporation unit and kept at 60 °C with $N_2$-stream for 1 h. After evaporation, the tubes were collected, cooled to room temperature, and weighed. The results were expressed as the percentage of total lipids (average ± standard error).

**Table 1** Fatty acid profile of rumen-protected long-chain fatty acid-enriched calcium salts[1, 2]

| Fatty acid | [1] Linoleic acid enriched Ca salts (LAE-Ca salts) | [2] Oleic acid enriched Ca salts (OAE-Ca salts) |
|---|---|---|
| | ------- % ------ | ------- % ------ |
| C14:0 | 0.27 | 0.22 |
| C14:1 | 0.05 | - |
| C16:0 | 11.87 | 10.8 |
| C16:1 | 0.36 | 0.41 |
| C18:0 | 7.41 | 3.13 |
| C18:1 | 24.84 | 50.54 |
| C18:2 | 50.03 | 26.97 |
| C18:3 | 6.38 | 0.69 |
| C20:0 | 0.33 | 0.81 |
| C20:1 | 0.24 | 0.79 |
| C20:2 | 0.04 | 0.04 |
| C22:0 | 0.41 | 0.32 |
| C24:0 | 0.09 | 0.13 |
| Total SAF[3] | 19.55 | 14.15 |
| Total USFA[4] | 31.86 | 57.47 |
| SAF:USFA | 0.61 | 0.24 |

[1] LAE-Ca salts = Control diet with 50% linoleic acid and 24% oleic acid
[2] OAE-Ca salts = Control diet with 26% linoleic acid and 50% oleic acid
[3] SFA, saturated fatty acids
[4] USFA, unsaturated fatty acids

Fatty acids were measured using gas chromatography (GC). Powdered meat samples (0.5 g) were added to 2 mL of boron trifluoride in methanol and 2 mL of methanol in glass tubes. The tubes were capped with teflon-lined caps to prevent loss of volume and the samples were placed in a heating block at 80 °C. After 10 min, the tubes were vortexed every 5 min for 2 h. After 2 h of repeated vortex mixing, the samples were allowed to cool to room temperature. Then, 3 mL of distilled water and 3 mL of hexane were added, and the tubes were capped and mixed by vortexing for 15 s. After centrifugation (2,000 rpm; 5 min) to separate the phases, the supernatant was transferred to a GC vial for analysis. GC was performed for 1-µL **s**amples on a Shimadzu GC-2014 instrument (Shimadzu Co., Maryland, USA) using a FAMEWAX column (Darmstadt, Germany) (30 m × 0.32 mm i. d., 0.25 µm; column temperature, 250 °C) and nitrogen/air as a carrier gas at 53.8 mL/min (split ratio 30:1). The temperature was started at 150 °C and increased to 250 °C with an equilibration time of 3 min.

Total RNA was extracted from the muscle tissue using TRIzol Reagent (Invitrogen, New York, USA), according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific) at 260 nm/280 nm absorbance. The quality of the total RNA was assessed using the Experion™ Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA) with RNA chip kits (Experion™ RNA StdSens Reagents, #700-7259, Bio-Rad). Good quality RNA samples were selected for further reverse transcription. Total RNA (1 µg) was reverse-transcribed into cDNA using an iScript™ cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. The reverse transcription was processed at 25 ºC for 18 s, followed by 11 cycles at 48 ºC for 4 min; the enzyme reaction was inhibited at 55 ºC for 18 sec.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad,) and a CFX96™ Real-Time PCR detection System (Bio-Rad). The cDNA was amplified from each gene, and the reaction was carried out according to the manufacturer's instructions (Bio-Rad). Briefly, PCR was conducted in a 20 µL total reaction volume containing 100 ng cDNA, 10 µl SsoFast™ EvaGreen® Supermix, and 1 µL of 10 pM primers. First-strand cDNAs, as templates, and gene-specific primers were used to amplify genes encoding for leptin, LPL, FAS, SCD, ACC, HSL, ATGL, FABP4, and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) (Table 2). The thermal cycling parameters were: 95 °C for 2 min, followed by 40 cycles at 95 °C for 5 s, 59 °C for 5 s, and 65 °C for 5 s. The ΔΔCT method was used to determine the relative fold-changes, and all data were normalized with the housekeeping gene, *GAPDH*. Statistical analysis of the real-time PCR results was performed using the Ct values ($Ct_{\text{gene of interest}} - Ct_{\text{reference gene}}$). The conversion between ΔΔCt and relative gene expression levels was: Fold induction = $2^{-\Delta\Delta Ct}$, where $2^{-\Delta\Delta Ct}$ is relative gene expression (Livak and Schmittgen, 2001).

All data were analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test using SAS software (SAS version 9.3, USA). Statistical significance was set at $P < 0.05$.

**Table 2** Summary of primers of target reference genes used for real-time quantitative reverse transcription polymerase chain reaction analysis

| Gene | Sequence (5′– 3′) | Size (bp) | Reference |
|---|---|---|---|
| *ACC* | F- AGGAGGGAAGGGAATCAGAA<br>R- GCTTGAACCTGTCGGAAGAG | 69 | Baik *et al.* (2015) |
| *ATGL* | F- TGACCACACTCTCCAACA<br>R- AGTTTCGGACCCACTGTGAC | 100 | Kang *et al.* (2015) |
| *FAS* | F- ATCGAGTGCATCAGGCAAGT<br>R- TGTGAGCACATCTCGAAAGCCA | 92 | Baik *et al.* (2015) |
| *FABP4* | F- GCTGCACTTCTTTCTCACCT<br>R- TTCCTGGTAGCAAAGCCCAC | 140 | Kang *et al.* (2015) |
| *HSL* | F- GATGAGAGGGTAATTGCCG<br>R- GGATGGCAGGTGTGAACT | 100 | Baik *et al.* (2014) |
| *Leptin* | F- TGTGGCTTTGGCCCTATCTG<br>R- CGGACTGCGTGTGTGAGATG | 123 | Warner *et al.* (2015) |
| *LPL* | F- CTTGCCACCTCATTCCTG<br>R- ACCCAACTCTCATACATTCCTG | 119 | Baik *et al.* (2015) |
| *SCD* | F- CCTGTGGAGTCACCGAACC<br>R- CAAAAACGTCATTCTGGAACGC | 92 | Warner *et al.* (2015) |
| *GAPDH* | F- CACCCTCAAGATTGTCAGC<br>R- TAAGTCCCTCCACGATGC | 98 | Yang *et al.* (2012) |

ACC: acetyl-CoA carboxylase alpha; ATGL: adipose triglyceride lipase; FAS: fatty acid synthase; FABP4: fatty acid binding protein4, HSL: hormone-sensitive lipase; LPL: lipoprotein lipase; SCD: stearoyl-CoA desaturase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

**Results and Discussion**

In the OAE-Ca salt-supplemented group, the total lipid content in meat samples was 13.39 ± 0.31% whereas that of the control group was 14.61 ± 0.25% (Figure 1). The lipid content was substantially lower in the OAE-Ca salt group than in the control. Marked differences were also observed between the treatment groups ($P < 0.05$). However, lipid contents were almost similar between the LAE-Ca salt (14.55 ± 0.20%) and control groups.

Lipid content is an important factor and topic of discussion regarding consumer preferences because of its associated health implications. The total lipid content of the muscle is usually termed intramuscular fat or marbling. It plays a vital role in determining the eating quality of beef. Beef lipid content varies from 4–15% on a fresh basis, according to factors including genotype, feeding, and

meat cutting. Hunt *et al*. (2016) stated that marbling is one of the most important factors affecting consumer perception of beef quality because it influences flavour and sensory attributes.

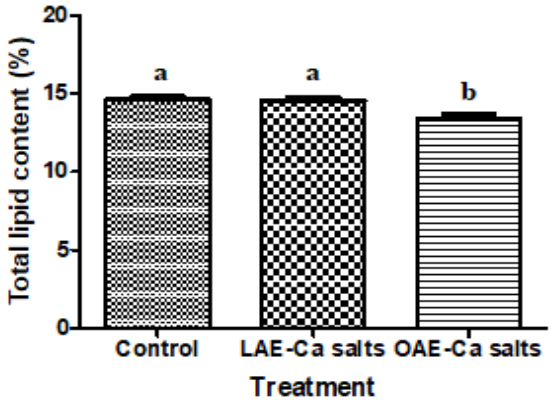


**Figure 1.** Effect of rumen-protected long chain fatty acid (LCFA)-calcium salts on total lipid content in Korean steers. Results are presented as mean ± standard error. The means with a different superscript letter are significantly different ($P$ <0.05).

The fatty acid composition of the meat is presented in Table 3. Fatty acid compositions including PUFA, $\omega^6$, and $\omega^3$ were higher in the OAE-Ca salt group than the other two groups ($P$ <0.05) but did not affect the percentage of SFA, MUFA, and USFA. Oleic acid-enriched Ca salts in the diet increased $\omega^6$ and $\omega^3$ in the current study. In the OAE-Ca salt-fed group, palmitic acid (C16:0) and linoleic acid (C18:3) were increased. These results are consistent with those of a previous study (Suksombat *et al.,* 2016), who demonstrated that SFA, USFA, and MUFA in the *Longissimus dorsi* (LD) muscle of beef steers were unaffected by dietary supplementation with linseed oil. Oleic acid-enriched Ca salts in the diet increased $\omega^6$ and $\omega^3$ in the current study, which concurs with the results of Quinn *et al*. (2008), who included 4% linseed oil in the diets of Holstein–Jersey steers.

Fatty acid profiles in the LD muscle did not differ ($P$ >0.05) between different forms of soybean oil (Santana *et al.,* 2014) and palm fatty acid calcium soap (Warner *et al.,* 2015). The fatty acids affect the taste and quality of meat (Wood *et al*., 2008) and PUFA lowers LDL cholesterol content. Kim *et al.* (2012) reported that palm oil calcium soaps did not affect the percentage of MUFA. Our findings support this hypothesis. Linoleic and oleic acid-enriched calcium salt diets did not affect MUFA or USFA. Feeding 3–5% palm oil calcium soaps to lambs also decreased MUFA content (Castro *et al.,* 2005).

In the OAE-Ca salt group, palmitic acid (C16:0) and linoleic acid (C18:3) increased. A similar response was observed by Chilliard *et al.* (1986), who showed that feeding LCFA calcium salts increased the percentages of palmitoleic acid (C16:1) and linoleic acid (C18:3) in milk. Bhatt *et al.* (2015) showed that FA calcium salt supplementation substantially increased serum non-esterified fatty acids (NEFA) over 90 d, but their content dropped to intermediate levels over 180 d. De Smet *et al.* (2004) also reported that the fatty acid composition of meat was influenced to a greater extent by dietary rather than genetic factors. Thus, our results indicate that OAE-Ca salts can be added to the diet of Korean steers to enrich meat with FAs beneficial to health.

**Table 3** Effect of rumen-protected long chain fatty acid-calcium salts on fatty acid composition in Korean steers

| Fatty acid | [1] Control | [2] LAE-Ca salts | [3] OAE-Ca salts |
|---|---|---|---|
| | % of total fatty acid | | |
| Total SFA | 42.38 ± 0.59 | 40.44 ± 0.81 | 41.49 ± 0.79 |
| Total MUFA | 52.33 ± 0.66 | 51.00 ± 0.43 | 51.83 ± 0.61 |
| Total PUFA | 0.93 ± 0.03$^{a}$ | 0.90 ± 0.05$^{b}$ | 1.13 ± 0.09$^{a}$ |
| Total USFA | 52.44 ± 0.50 | 51.43 ± 0.51 | 52.76 ± 0.55 |
| Total ω6 | 0.54 ± 0.05$^{b}$ | 0.55 ± 0.04$^{b}$ | 0.67 ± 0.04$^{a}$ |
| Total ω3 | 0.37 ± 0.02$^{bc}$ | 0.40 ± 0.03$^{b}$ | 0.51 ± 0.03$^{a}$ |
| ω6/ω3 | 1.47 ± 0.14 | 1.39 ± 0.10 | 1.34 ± 0.07 |

SFA: saturated fatty acid; MUFA: mono-unsaturated fatty acid; PUFA: poly-unsaturated fatty acid; USFA: unsaturated fatty acid; ω6: omega-6 fatty acid; ω3: omega-3 fatty acid
Values are presented as mean ± standard error. Values in rows with different superscript letters are significantly different ($P$ <0.05). The lack of superscript letters indicates no significant difference.
[1] Control = Control diet without additional fat
[2] LAE-Ca salts = Control diet with linoleic acid-enriched calcium salts
[3] OAE-Ca salts = Control diet with oleic acid-enriched calcium salt

Figure 2 shows the expression of the lipid metabolism genes. To clarify the carcass characteristics, the mRNA expression of genes involved in lipogenesis was examined in the LD muscle. In this study, the upregulation of leptin gene expression was observed in the LAE-Ca salt group. No marked changes were observed in the expression of other genes related to lipogenesis, including *LPL*, *FASN*, and *SCD*. However, the mRNA levels of *ACC*, *ATGL* and *FABP4* genes were substantially higher in the LAE-Ca salt group than in the OAE-Ca salt group; however, *HSL* was higher in the OAE-Ca salts group. FAS is the rate-limiting enzyme involved in *de novo* fatty acid synthesis during lipogenesis. However, the current study showed no substantial inhibition of *SCD* gene expression in response to the LCFA-Ca salt diet. Leptin, a protein hormone synthesized and secreted by adipocytes in the white adipose tissue (Della-Fera *et al.,* 2001; Liefers *et al.,* 2002), plays an important role in the regulation of feed intake as an appetite inhibitor and inducer of satiety.

The bovine leptin gene is associated with marbling (Nkrumah *et al.,* 2005); mRNA expression of leptin in the *lumbar multifidus* (LM) muscle was reduced by 50% in the oleic acid-containing diet. This is reasonable because steers fed the 50% linoleic-containing diet had increased marbling scores and, therefore, greater amounts of adipose tissue, which increased leptin production. Geary *et al.* (2003) reported that this hypothesis is reinforced by the positive correlation between marbling score and serum leptin levels in cattle.

LPL and FAS are important enzymes in lipogenesis and their mRNA levels have been found to be higher in the subcutaneous fat than in intramuscular fat (Pickworth *et al.,* 2011). In lipogenesis, FAS is the rate-limiting enzyme involved in *de novo* fatty acid synthesis during lipogenesis. This may explain the similar responses of LPL and FAS expression observed between the treatment groups, which, however, did not reach statistical significance. Stearoyl-CoA desaturase is an enzyme involved in catalysing the desaturation of SFA to MUFA (Li *et al.,* 2013). It has previously been found that increasing the n-3 PUFA composition in the diet causes a substantial reduction in SCD expression in the subcutaneous adipose tissue of Holstein bulls (Hiller *et al.,* 2011). However, the current study showed no marked inhibition of *SCD* gene expression in response to the LCFA-Ca salt diet. Furthermore, MUFA composition of the LD muscle was not affected by USFA supplementation. In another study, Wynn *et al.* (2006) observed similar results and stated that Megalac (high energy rumen-protected fat) supplementation had no effect on SCD mRNA expression in sheep., Other investigators have reported that no correlation between *SCD* gene expression in subcutaneous fat and △9desaturase index (Corazzin *et al.,* 2012). In summary, the current study demonstrates that fatty acid-enriched calcium salt supplements affect the expression of some lipid metabolism genes.

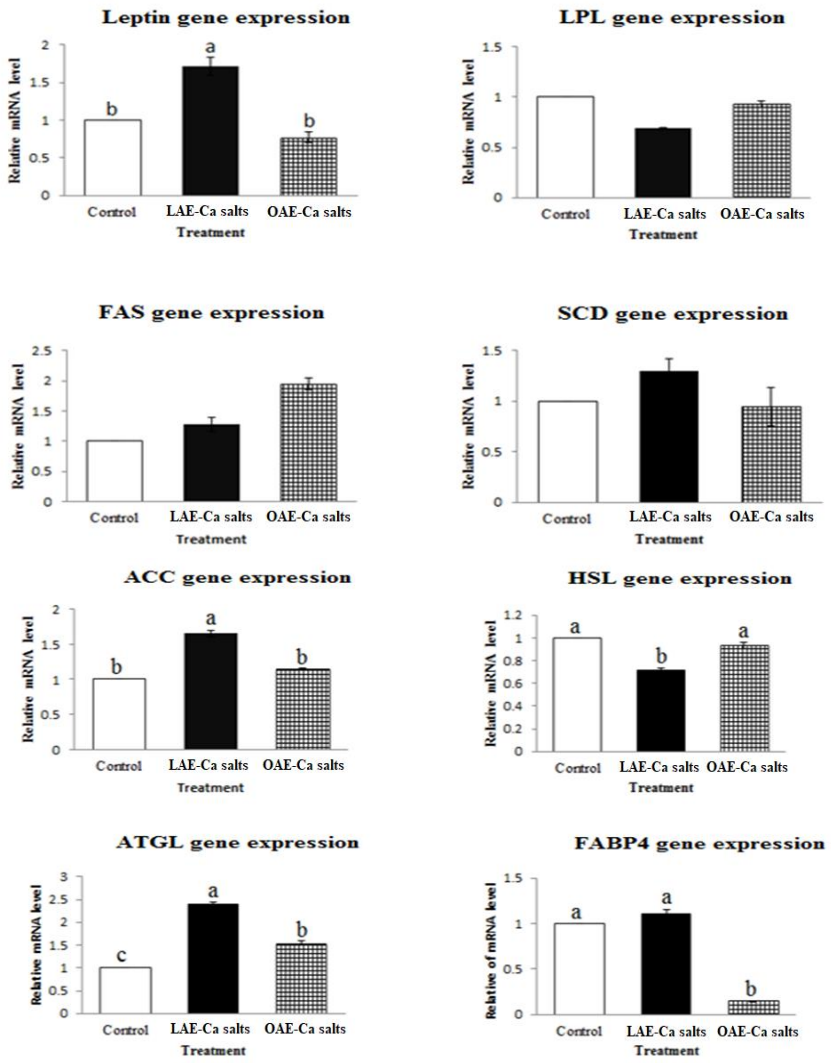


**Figure 2.** Expression levels of lipid metabolism genes in Korean steer muscle tissue. The mRNA levels were compared among the groups (n = 6).
Fatty acid transport lipoprotein lipase (*LPL*), fatty acid synthesis (*FAS*), stearoyl-CoA desaturase (*SCD*), acetyl CoA carboxylase (*ACC*), hormone-sensitive lipase (*HSL*), lipolytic adipose triglyceride lipase (*ATGL*), and fatty acid binding protein (*FABP4*) genes. The mRNA levels were determined using real-time PCR, and the results were normalized with a housekeeping gene. Results are presented as mean ± standard error of the mean. The means with a different superscript letter are significantly different ($P$ <0.05); the lack of letters indicates no significant difference.

## Conclusion

In conclusion, our findings suggest that the addition of linoleic and oleic acid-enriched calcium salts to the diets of Korean steers alters the lipid content and fatty acids; the expression of *ACC*, *ATGL*, and *FAS* genes is increased. Moreover, diets (linoleic and oleic acid-enriched Ca salts) can affect fatty acid composition and related gene expression. Further research is warranted to investigate this association.

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**Authors' Contributions**

SAB participated in laboratory analysis, statistics, interpretation of the data, and manuscript writing. DRK was involved in the data analysis and manuscript writing. KSS participated in designing the study, interpretation of the data, and constructive revision of the manuscript.

**Conflict of Interest Declaration**

The authors declare that they have no conflict of interests

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