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Original Research Article

Effect of changing the proportion of C16:0 and *cis*-9 C18:1 in fat supplements on rumen fermentation, glucose and lipid metabolism, antioxidation capacity, and visceral fatty acid profile in finishing Angus bulls

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ABSTRACT

This study evaluated the effects of different proportions of palmitic (C16:0) and oleic (*cis*-9 C18:1) acids in fat supplements on rumen fermentation, glucose (GLU) and lipid metabolism, antioxidant function, and visceral fat fatty acid (FA) composition in Angus bulls. The design of the experiment was a randomized block design with 3 treatments of 10 animals each. A total of 30 finishing Angus bulls (21 ± 0.5 months) with an initial body weight of 626 ± 69 kg were blocked by weight into 10 blocks, with 3 bulls per block. The bulls in each block were randomly assigned to one of three experimental diets: (1) control diet without additional fat (CON), (2) CON + 2.5% palmitic calcium salt (PA; 90% C16:0), (3) CON + 2.5% mixed FA calcium salts (MA; 60% C16:0 + 30% *cis*-9 C18:1). Both fat supplements increased C18:0 and *cis*-9 C18:1 in visceral fat ($P < 0.05$) and up-regulated the expression of liver FA transport protein 5 (*FATP5*; $P < 0.001$). PA increased the insulin concentration ($P < 0.001$) and aspartate aminotransferase activity (AST; $P = 0.030$) in bull's blood while reducing the GLU concentration ($P = 0.009$). PA increased the content of triglycerides (TG; $P = 0.014$) in the liver, the content of the C16:0 in visceral fat ($P = 0.004$), and weight gain ($P = 0.032$), and up-regulated the expression of liver diacylglycerol acyltransferase 2 (*DGAT2*; $P < 0.001$) and stearoyl-CoA desaturase 1 (*SCD1*; $P < 0.05$). MA increased plasma superoxide dismutase activity (SOD; $P = 0.011$), reduced the concentration of acetate and total volatile FA (VFA) in rumen fluid ($P < 0.05$), and tended to increase plasma non-esterified FA (NEFA; $P = 0.069$) concentrations. Generally, high C16:0 fat supplementation increased weight gain in Angus bulls and triggered the risk of fatty liver, insulin resistance, and reduced antioxidant function. These adverse effects were alleviated by partially replacing C16:0 with *cis*-9 C18:1.

1. Introduction

The efficiency of intramuscular fat deposition in beef cattle during the finishing period directly affects the marbling of beef (Ogata et al., 2019). Marbling is closely related to beef tenderness, juiciness and flavor, and is considered one of the most critical factors affecting beef quality (Lee et al., 2019). Increasing dietary energy concentration, such as supplementing fat, effectively increases intramuscular fat deposition (Park et al., 2018). However, individual fatty acids (FA) may have different fattening effects due to different preferential metabolic pathways of oxidative deposition. In lactating dairy cows, palmitic (C16:0) and oleic (*cis*-9 C18:1) acids are widely used to improve milk fat and body condition respectively because of their different effects (De Souza et al., 2018; Western et al., 2020). However, there are few reports on the difference between C16:0 and *cis*-9 C18:1 on the fattening performance of beef cattle. Previous experiments have shown that

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compared with *cis*-9 C18:1, more C16:0 improves beef cattle's growth performance and fat deposition (Bai et al., 2023). Dietary C16:0 and *cis*-9 C18:1 likely follow different oxidative deposition metabolic pathways in the body. Specifically, studies in rodents have shown that dietary saturated FA (SFA) reduce diet-induced thermogenesis by reducing sympathetic nerve activity in brown adipose tissue, thereby promoting fat accumulation in the body (Takeuchi et al., 1995). While ingested, unsaturated FA (UFA) oxidize faster and to a higher degree in the body, resulting in less weight gain (DeLany et al., 2000). However, whether C16:0 and *cis*-9 C18:1 affect beef cattle production through this differential metabolic regulation is not known.

In addition, when supplementing C16:0 as SFA, while effectively improving the weight gain of beef cattle, continuous intake is likely to lead to excessive obesity (Li et al., 2021). Ectopic fat deposition and adipose tissue expansion will release more non-esterified FA (NEFA; Palomer et al., 2018). Excessive blood NEFA and liver fat content have been shown to cause metabolic imbalances in dairy cows, leading to metabolic diseases such as ketosis, fatty liver, and insulin resistance (Hammon et al., 2009). However, medical studies have shown that *cis*-9 C18:1 can counteract or weaken the adverse effects of C16:0 by increasing insulin sensitivity and anti-inflammatory effects (Palomer et al., 2018). Therefore, we hypothesized that when partial *cis*-9 C18:1 is substituted for high C16:0 in fat supplements, the adverse effects of SFA C16:0 can be alleviated while maintaining high weight gain in beef cattle and ultimately maintaining body health.

Therefore, we adjusted the ratio of C16:0 to *cis*-9 C18:1 in fat supplements to evaluate the different effects of C16:0 and *cis*-9 C18:1 on weight gain and fat deposition in beef cattle due to their preferred metabolic pathways. At the same time, we assessed the effects of high C16:0 on glucose (GLU) and lipid metabolism homeostasis, plasma immune indices, and antioxidant function, as well as the alleviating effect of *cis*-9 C18:1 on the negative impact of C16:0.

Table 1
Ingredients and nutrient composition of treatment diets.

Item	Diet[1]		
	CON	PA	MA
Ingredient, % of DM			
Corn grain	45.00	45.00	45.00
Soybean meal	8.50	8.50	8.50
Distillers dried grains with solubles	5.00	5.00	5.00
Corn germ meal	5.50	5.50	5.50
Limestone	1.00	1.00	1.00
Salt (sodium chloride)	0.80	0.80	0.80
Sodium bicarbonate	1.10	1.10	1.10
Mineral-vitamin premix[2]	0.40	0.40	0.40
Magnesium oxide	0.20	0.20	0.20
Peanut hulls	15.00	15.00	15.00
Corn stalk	15.00	15.00	15.00
Zeolite powder	2.50	–	–
Palmitic calcium salt	–	2.50	–
Mixed FA calcium salt	–	–	2.50
Nutrient composition[3], % of DM			
CP	11.56	11.59	11.53
EE	3.74	6.25	6.23
NDF	34.28	34.30	34.28
ADF	20.32	20.36	20.36
Ash	7.97	7.27	7.29
NEm, Mcal/kg	1.76	1.83	1.82
NEg, Mcal/kg	1.15	1.20	1.19
Fatty acids, g/100 g DM			
C16:0	1.47	3.76	2.95
C18:0	0.38	0.65	0.47
cis-9 C18:1	1.15	1.18	1.91
cis-9, *cis*-12C18:2	0.46	0.52	0.68

CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber; NEm = net energy for maintenance; NEg = net energy for gain.
[1] CON, control diet without additional fat; PA, CON + 2.5% DM palmitic calcium salt (90% C16:0); MA, CON + 2.5% DM mixed fatty acid calcium salt (60% C16:0 + 30% *cis*-9 C18:1).
[2] The mineral-vitamin premix provided the following per kilogram of the diet: vitamin A 6,000 IU, vitamin D 600 IU, vitamin E 50 IU, Fe 50 mg, Cu 15 mg, Mn 27 mg, Zn 65 mg, Se 0.1 mg, I 0.5 mg, Co 0.2 mg.
[3] NEm and NEg levels were estimated according to NASEM (2016), and the rest of the nutrient levels were measured.

2. Materials and methods

2.1. Animal ethics statement

All the procedures of this study were carried out following the guidelines approved by the Animal Ethics Committee of Northeast Agricultural University (Protocol number: NEAUEC2023 02 59). Animals were treated humanely in the process of feeding, slaughtering and sampling and this animal experiment complied with the ARRIVE guidelines.

2.2. Animals and experimental design

The design of the experiment was a randomized block design with 3 treatments of 10 animals each. A total of 30 finishing Angus bulls (21 ± 0.5 months) with an initial body weight of 626 ± 69 kg were blocked by weight into 10 blocks, with 3 bulls per block. The bulls in each block were randomly assigned to one of three experimental diets: (1) control diet without additional fat (CON), (2) CON + 2.5% palmitic calcium salt (PA; 90% C16:0), (3) CON + 2.5% mixed FA calcium salts (MA; 60% C16:0 + 30% *cis*-9 C18:1). Both fat supplements are rumen bypass fat. Diets were formulated based on the nutritional needs of this weight period according to NASEM (2016). The zeolite powder in CON was replaced by 2.5% dietary dry matter (DM) in the two fat treatments, and the other ingredients were the same, so the three diets were isoproteic and the PA and MA diets were isocaloric. The composition of diets and fat supplements is shown in Tables 1 and 2. Each bull was kept alone in a 4 m × 3 m pen, fed twice a day (08:00 and 17:00), and the feed amount was adjusted according to the feed intake of the previous day to get about 3% orts. All bulls could drink freely. The experiment lasted for 104 d following a 14-d preliminary feeding period.

2.3. Data and sample collection

2.3.1. Feed, body weight and blood

During the experiment, feed samples were collected once a week and stored at −20 °C until nutrient analysis. Daily orts were collected to determine dry matter intake (DMI). The bulls were weighed before morning feeding on d 0, 52, and 104 to determine weight changes. At the end of the experiment, blood samples were collected from the tail vein of all bulls before morning feeding and then centrifuged at 2,000 × *g* for 15 min at 4 °C to obtain plasma and stored at −20 °C until analysis.

2.3.2. Slaughter samples

After weighing and collecting blood on the 104 d, all bulls immediately were transported to an abattoir (Haosheng abattoir, Harbin, China) 15 km from the finishing farm for slaughter after fasting for 24 h. After slaughter, all the rumen contents of each bull were emptied into a plastic basin and mixed thoroughly. Approximately 500 g of the content was then collected and strained thorough 4 layers of cheesecloth to obtain a representative sample of rumen fluid. The rumen fluid pH was determined immediately with

Table 2
Composition of fat supplements (% of total fatty acids).

Item	Fat supplement[1]	
	Palmitic calcium salt	Mixed FA calcium salt
SFA		
C16:0	90.36	60.45
C18:0	6.78	2.93
Total SFA	97.14	63.38
MUFA		
cis-9 C18:1	1.62	30.87
PUFA		
cis-9, *cis*-12 C18:2	1.07	3.86
Total UFA	2.69	34.73
Others[2]	0.17	1.89

SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids, UFA = unsaturated fatty acids.
[1] After the two kinds of fat supplements were designed, Sanhe Feed Co., Ltd. (Hei Longjiang, China) was entrusted to produce them.
[2] Others included C14:0, C20:0, C16:1, and C18:3 *n*-3.

a pH meter (Mettler, METTLER TOLEDO Instrument Co., Ltd., Shanghai, China). One milliliter of metaphosphoric acid (25 %) was mixed with 5 mL rumen fluid and centrifuged at 3,000 × *g* for 15 min. The supernatant was stored at −20 °C until volatile FA (VFA) was analyzed. Another 10 mL rumen fluid was mixed with 0.2 mL 50% sulfuric acid and stored at −20 °C to analyze ammonia nitrogen (NH_3−N). The liver was weighed, sampled, and stored at −80 °C for subsequent analysis. Ten grams of visceral fat around the kidneys of bulls was stored at −20 °C for FA profile analysis. Fifty grams of longissimus dorsi muscle between the 12th and 13th ribs of the left half carcass of each bull was stored at −20 °C for intramuscular protein and fat content analysis.

2.4. Laboratory analysis

2.4.1. Feed nutrients, intramuscular fat and protein, FA profile

Feed samples were dried at 55 °C for 72 h, then crushed and passed through a 1-mm sieve. The nutrients in the feed were determined according to AOAC (1990). In short, absolute DM was determined by oven-drying at 105 °C for 8 h. The nitrogen (N) content was measured by the Kjeldahl method, and the crude protein (CP) content was calculated by multiplying the N content by 6.25. The ash content was measured after combustion in a muffle furnace (SX2-12-10, Lichen Instrument Technology Co., Ltd, Shanghai, China) at 550 °C for 6 h. The ether extract (EE) content was determined by a Soxhlet apparatus (Ankom TX15, ANKOM Technology, Macedon, NY, USA). The acid detergent fiber (ADF) was determined gravimetrically as the residue remaining after extraction with an acid detergent solution. According to Van Soest et al. (1991), the content of neutral detergent fiber (NDF) in feed was determined by heat-stable α-amylase treatment. The total digestible nutrients (TDN), net energy for maintenance (NEm) and gain (NEg) of feed were calculated according to the NASEM (2016): DE (Mcal/kg DM) = TDN% × 4.409/100; ME (Mcal/kg DM) = DE × 0.82; NEm (Mcal/kg DM) = $1.37ME - 0.138ME^2 + 0.0105ME^3 - 1.12$; NEg (Mcal/kg DM) = $1.42ME - 0.174ME^2 + 0.0122ME^3 - 1.65$. The meat samples were freeze-dried in a freeze-dryer (Pilot2-4LD, Boyikang Laboratory Instrument Co., Ltd, Beijing, China) to determine DM, and the contents of CP and intramuscular fat were also determined by referring to AOAC (1990). The FA profile of feed, fat supplements and visceral adipose tissue were also analyzed according to the descriptions of Choi et al. (2013). In short, total lipids in 5 g of feed, 100 mg of FA supplements and 100 mg of adipose tissue were extracted with chloroform–methanol (2:1, vol/vol). Then, FA methyl ester (FAME) was prepared according to ISO 5009 (2001). C19:0 was the internal standard. FAME was analyzed by a gas chromatography-flame ionization detector (GC-2010 Plus autoinjector-AOC 20i; Shimadzu Scientific Instruments, Kyoto, Japan) equipped with a fused silica column (SP-2560, 100 m × 0.25 mm i.d. with 0.2-μm film thickness; Supelco Inc., Bellefonte, PA, USA). The initial temperature was 100 °C for 13 min, then it was heated to 180 °C at the rate of 10 °C/min for 6 min, then to 200 °C at the rate of 1 °C/min for 20 min, and finally to 230 °C at the rate of 4 °C/min for 10.5 min. Nitrogen was the carrier gas, the shunt ratio was 100:1, and the injection volume was 1 μL. The FA composition of each sample was confirmed by comparing the retention time with the standards (FAME mix of 37 components from Supelco Inc., Bellefonte, PA, USA).

2.4.2. Ruminal fermentation

The VFA in rumen fluid was analyzed by gas chromatography (GC-8A; Shimadzu Corp., Kyoto, Japan), and the NH_3−N concentration was determined by the phenol-hypochlorite method as described by Li et al. (2023).

2.4.3. Plasma indices, liver triglycerides and cholesterol

The aspartate aminotransferase (AST), alanine aminotransferase (ALT), and GLU were detected by an automatic biochemical analyzer (model BS-800; Mindray Medical International Ltd., Shenzhen, China). The NEFA, β-hydroxybutyrate (BHB), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were determined according to the instructions of the colorimetric kit (Jiancheng Bioengineering Research Institute, Nanjing, China). The plasma leptin and insulin were determined according to the standard operation of the ELISA kit (Beijing Sino-UK Institute of Biological Technology, Beijing, China). The liver's triglyceride (TG) content was determined according to the description of Kalaitzakis et al. (2010). First, the total lipids in the liver were extracted with chloroform–methanol solution (2:1, vol/vol), then saponified with 0.5 mol/L KOH and anhydrous ethanol at 70 °C for 60 min. Finally, an enzymatic assay kit (Jiancheng Bioengineering Research Institute, Nanjing, China) was used to determine the TG. The cholesterol in the liver was extracted and determined according to the method of Cong et al. (2012).

2.4.4. Real-time quantitative PCR analysis

The detailed operational steps of the mRNA transcriptional level analysis were reported in Shao et al. (2021). Briefly, Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from 100 mg liver tissue. The total RNA obtained was measured for concentration and purity using a Nanodrop 2000 ultramicro spectrophotometer (Thermo, America), and subsequent reverse transcription was performed based on this concentration. Reverse transcription of RNA into cDNA was done according to the instructions of the biosharp reverse transcription kit (BL699A, biosharp, Anhui, China). Based on the target gene sequence in GenBank, primers were designed using Primer Premier 6.0 software and synthesized by entrusting Sangon Biotech Co., Ltd. (Shanghai, China). The specific primer sequence information is shown in Table 3. Beta-actin was used as an internal reference gene. The instructions of 2× Fast qPCR Master Mixture (green) kit from Dining Company (Beijing, China) were followed strictly to prepare the real-time quantitative PCR reaction system and the reaction was performed in a fluorescence quantitative PCR instrument (Quantagene q225, Kubo, Beijing, China). At the end of the reaction, the amplification curve and fusion curve of the PCR were confirmed, and the gene mRNA expression level was calculated according to the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct_{(sample\text{-}control)}$ = (Ct of target gene − Ct of β-actin)$_{sample}$ − (Ct of target gene − Ct of β-actin)$_{control}$.

Table 3
Real-time quantitative PCR primer sequences.

Genes	Primers	Sequences (5′ to 3′)	Product size, bp	GenBank accession No.
C/EBPα	Forward	TGGACAAGAACAGCAACGAG	133	NM_176784
	Reverse	TCATTGTCACTGGTCAGCTC		
PPARα	Forward	CAATGGAGATGGTGGACACA	95	NM_001034036.1
	Reverse	TTGTAGGAAGTCTGCCGAGAG		
FATP5	Forward	GCATGGTGTGACTGTGATCC	141	NM_001033625
	Reverse	TGGAAGGTCTCCCACACTTC		
FASN	Forward	ATCGAGTGCATCAGGCAAGT	92	NM_001012669
	Reverse	TGTGAGCACATCTCGAAAGCC		
SCD1	Forward	TTATTCCGTTATGCCCTTGG	83	NM_173959
	Reverse	TTGTCATAAGGGCGGTATCC		
DGAT2	Forward	CATTGCCGTGCTCTACTTCA	86	NM_205793.2
	Reverse	AGTTTCGGACCCACTGTGAC		
HMGCR	Forward	CGCAACCTTTACATCCGTTT	117	NM_001105613
	Reverse	GTGCATTTCGGGGAAATACTC		
ATGL	Forward	TGACCACACTCTCCAACA	100	NM_001046005
	Reverse	AAGCGGATGGTGAAGGA		
Beta-actin	Forward	AGCAAGCAGGAGTACGATGAGT	120	NM_173979
	Reverse	ATCCAACCGACTGCTGTCA		

C/EBPα = CCAAT/enhancer binding protein α; *PPARα* = peroxisome proliferator-activated receptor α; *FATP5* = fatty acid transport protein 5; *FASN* = fatty acid synthase; *SCD1* = stearoyl-CoA desaturase 1; *DGAT2* = diacylglycerol acyltransferase 2; *HMGCR* = 3-hydroxy-3-methylglutaryl-CoA reductase; *ATGL* = adipose triglyceride lipase.

2.5. Statistical analysis

All data were analyzed using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). Because the randomized block design was used in this study, a mixed model considered the fat treatment as a fixed effect and the weight block as a random effect. The statistical model was as follows:

$$Y_{ij} = \mu + T_i + B_j + E_{ij},$$

where Y_{ij} is the observation of bulls that belong to the jth weight block and were offered a diet containing the ith fat supplement, μ is the general mean, T_i is the fat supplements effect, B_j is the effect of weight block, and E_{ij} represents random residual error. The least squares means were calculated and separated using the default approach (PDIFF). Differences between fat diets were detected using Duncan's adjustment. Declared significant at $P < 0.05$, and trends were declared at $0.05 \leq P < 0.10$.

3. Results

3.1. Ruminal fermentation

As shown in Table 4, compared with CON, the two fat supplements did not affect rumen fluid pH and NH_3–N concentration ($P > 0.05$) but significantly reduced butyrate concentration ($P = 0.009$). Compared with CON and PA, the concentration of total VFA ($P = 0.012$) and acetate ($P = 0.002$) of the MA were significantly reduced and compared with CON and MA, the PA significantly increased the concentration of propionate ($P = 0.005$). In addition, other VFA (including isobutyrate, isovalerate, and valerate) and the ratio of acetate to propionate were not affected by PA and MA ($P > 0.05$).

Table 4
Effects of fat supplements with different fatty acids (FA) on rumen fermentation in Angus bulls.

Item	Treatment[1]			SEM	*P*-value
	CON	PA	MA		
pH	6.53	6.50	6.48	0.076	0.897
NH_3–N, mg/dL	7.81	8.32	7.98	0.454	0.719
Total VFA, mmol/L	52.24^a	53.28^a	47.94^b	1.242	0.012
Individual VFA, mmol/L					
Acetate	28.37^a	29.34^a	24.64^b	0.897	0.002
Propionate	9.38^b	10.18^a	8.83^b	0.268	0.005
Isobutyrate	0.84	0.89	0.88	0.033	0.476
Butyrate	4.53^a	3.40^b	3.47^b	0.265	0.009
Isovalerate	1.60	1.79	1.61	0.083	0.202
Valerate	0.19	0.24	0.21	0.020	0.283
Acetate/propionate	3.03	2.88	2.84	0.111	0.463

NH_3–N = ammonia nitrogen; Total VFA = total volatile fatty acid.
$^{a, b}$Means within rows with different superscripts differ ($P < 0.05$).
[1] CON, control diet without additional fat; PA, CON + 2.5% DM palmitic calcium salt (90% C16:0); MA, CON + 2.5% DM mixed fatty acid calcium salt (60% C16:0 + 30% *cis*-9 C18:1).

3.2. Homeostasis of glucose and lipid metabolism

As shown in Table 5, there was no difference in plasma NEFA concentration between the two fat supplements and CON. However, compared to MA, the PA tended to increase plasma NEFA concentration ($P = 0.069$). Both fat supplements significantly increased plasma leptin concentration compared to CON ($P < 0.001$). Compared with CON, the PA significantly increased plasma AST activity ($P = 0.030$) and TG content in the liver ($P = 0.014$) of bulls; MA was not significant compared with CON and PA. In addition, fat supplementation had no significant effect on other lipid metabolism indexes in the blood and liver ($P > 0.05$).

PA significantly reduced fasting plasma GLU levels in bulls compared with CON and MA ($P = 0.009$). Compared with CON, the PA increased plasma insulin concentration ($P < 0.001$), while MA did not affect plasma insulin.

3.3. The mRNA relative expression of lipid metabolism genes in liver

As shown in Fig. 1, compared with CON, MA up-regulated the expression of the upstream regulatory factor *PPARα* for lipid synthesis in the liver ($P = 0.014$), and PA had no significant impact on *PPARα*. Compared with CON, both fat supplements up-regulated the expression of direct fat uptake gene *FATP5* ($P < 0.001$). Compared with CON, PA significantly up-regulated *SCD1* ($P = 0.010$), the key regulatory gene of FA desaturation, while MA was not different from CON and PA. Compared with CON and MA, PA significantly up-regulated the expression of the key gene *DGAT2* for triglyceride synthesis in the liver ($P < 0.001$). In addition, fat supplementation did not significantly affect the expression of other lipid metabolism genes in the liver, such as *C/EBPα*, *FASN*, *HMGCR* and *ATGL* ($P > 0.05$).

Table 5
Effects of fat supplements with different fatty acids (FA) on the homeostasis of glucose and lipid metabolism of finishing Angus bulls.

Item	Treatment[1]			SEM	*P*-value
	CON	PA	MA		
Plasma index					
NEFA, mmol/L	0.14	0.16	0.13	0.009	0.069
BHB, mmol/L	0.24	0.23	0.22	0.007	0.377
Leptin, ng/mL	5.37^{b}	6.31^{a}	6.29^{a}	0.166	<0.001
AST, U/L	70.06^{b}	75.86^{a}	72.41^{ab}	1.456	0.030
ALT, U/L	30.52	31.44	27.12	1.665	0.173
AST/ALT	2.35	2.53	2.68	0.135	0.245
GLU, mmol/L	3.92^{a}	3.49^{b}	3.85^{a}	0.097	0.009
Insulin, μIU/mL	13.16^{b}	14.65^{a}	13.26^{b}	0.232	<0.001
Liver index					
Liver weight, kg	7.19	7.42	7.16	0.258	0.737
TG, mmol/g	0.11^{b}	0.13^{a}	0.12^{ab}	0.005	0.014
Cholesterol, mmol/g	0.031	0.028	0.029	0.002	0.403

NEFA = non-esterified fatty acid; BHB = β-hydroxybutyrate; AST = aspartate aminotransferase; ALT = alanine aminotransferase; GLU = glucose; TG = triglycerides.
[a,b]Within a row, values with different superscripts differ significantly at $P < 0.05$, a trend at $P < 0.1$.
[1] CON, control diet without additional fat; PA, CON + 2.5% DM palmitic calcium salt (90% C16:0); MA, CON + 2.5% DM mixed FA calcium salt (60% C16:0 + 30% *cis*-9 C18:1).

3.4. Blood antioxidation

As shown in Table 6, there was no significant difference in activities of SOD between the two fat supplements and CON. However, compared with PA, MA significantly increased the SOD activity ($P = 0.011$). Compared with CON and MA, PA increased MDA content, a lipid peroxidation metabolite ($P = 0.001$). In addition, fat supplementation did not affect T-AOC ($P = 0.101$) and GSH-Px activity ($P = 0.258$).

Table 6
Effects of fat supplements with different fatty acids (FA) on blood antioxidation in finishing Angus bulls.

Item	Treatment[1]			SEM	*P*-value
	CON	PA	MA		
T-AOC, U/mL	2.81	2.65	2.89	0.078	0.101
GSH-Px, U/mL	134.94	133.51	129.03	2.578	0.258
SOD, U/mL	94.18^{ab}	92.41^{b}	96.56^{a}	0.903	0.011
MDA, nmol/mL	3.43^{b}	3.98^{a}	3.68^{b}	0.094	0.001

T-AOC = total antioxidant capacity; GSH-Px = glutathione peroxidase; SOD = superoxide dismutase; MDA = malondialdehyde.
[a, b]Means within rows with different superscripts differ ($P < 0.05$).
[1] CON, control diet without additional fat; PA, CON + 2.5% DM palmitic calcium salt (90% C16:0); MA, CON + 2.5% DM mixed FA calcium salt (60% C16:0 + 30% *cis*-9 C18:1).

3.5. Growth performance and meat quality

There was no significant difference in body weight among the bulls of the three groups at 0, 52, and 104 d (Table 7; $P > 0.05$). However, compared to CON, PA significantly increased the weight gain of bulls throughout the entire experimental period ($P = 0.032$), while MA showed no difference compared to CON and PA. There was no significant difference in DMI during the entire experimental period among the three diets ($P = 0.274$). Compared with CON, the two fat supplements did not affect the muscle protein ($P = 0.577$)

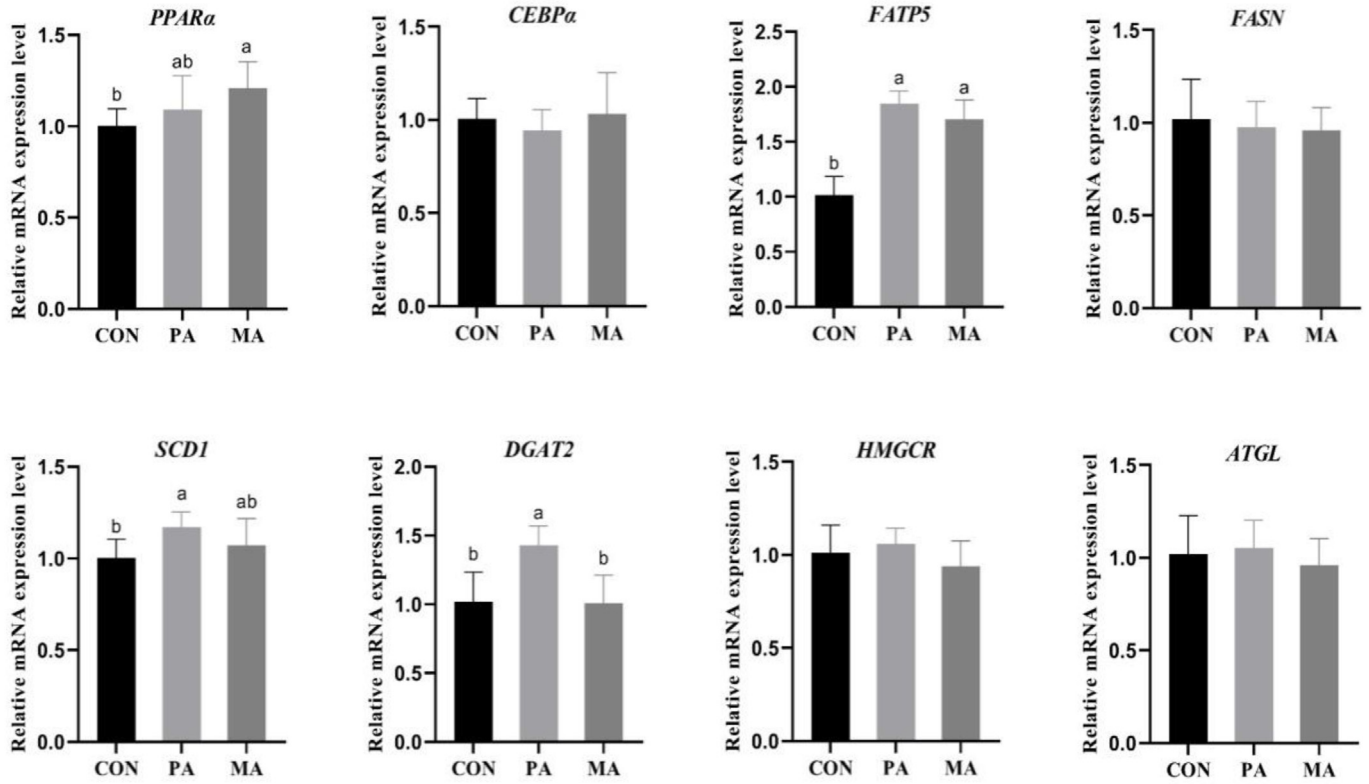

Fig. 1. Effects of fat supplements with different fatty acids (FA) on gene expression of lipid metabolism in the liver of finishing Angus bulls. [a, b]Bars with different letters differ significantly at $P < 0.05$. Data are expressed as means ± SEM. CON, control diet without additional fat; PA, CON + 2.5% DM palmitic calcium salt (90% C16:0); MA, CON + 2.5% DM mixed FA calcium salt (60% C16:0 + 30% *cis*-9 C18:1). *PPARα* = peroxisome proliferator-activated receptor α; *C/EBPα* = CCAAT/enhancer binding protein α; *FATP5* = fatty acid transport protein 5; *FASN* = fatty acid synthase; *SCD1* = stearoyl-CoA desaturase 1; *DGAT2* = diacylglycerol acyltransferase 2; *HMGCR* = 3-hydroxy-3-methylglutaryl-CoA reductase; *ATGL* = adipose triglyceride lipase.

Table 7
Effects of fat supplements with different fatty acids (FA) on growth performance and meat quality of finishing Angus bulls.

Item	Treatment[1]			SEM	*P*-value
	CON	PA	MA		
Growth performance					
DMI, kg/d	12.08	12.46	12.07	0.190	0.274
Initial weight, kg	627.12	623.68	628.69	22.194	0.987
Weight at 52 d, kg	693.53	710.36	700.19	20.447	0.843
Weight at 104 d, kg	753.7	787.4	773.75	17.284	0.395
Weight gain (0 to 104 d), kg	126.58^{b}	163.72^{a}	145.06^{ab}	9.359	0.032
Meat quality					
Muscle protein, %	19.34	19.65	19.89	0.367	0.577
Intramuscular fat, %	6.81	7.40	7.39	0.218	0.100

DMI = dry matter intake.
$^{a, b}$Means within rows with different superscripts differ ($P < 0.05$).
[1] CON, control diet without additional fat; PA, CON + 2.5% DM palmitic calcium salt (90% C16:0); MA, CON + 2.5% DM mixed FA calcium salt (60% C16:0 + 30% *cis*-9 C18:1).

and intramuscular fat ($P = 0.100$) content of longissimus dorsi muscle.

3.6. FA composition of visceral adipose tissue

Table 8 shows the composition of FA in visceral adipose with content greater than 0.1% of total FA. There was no significant difference in visceral fat C16:0 content between the two fat supplements compared with CON. But compared to MA, PA significantly increased the C16:0 content ($P = 0.004$). There were significant differences in C16:1, C18:3 *n*-3, and total *n*-3 FA (Σ*n*-3 FA) content among the three diets ($P < 0.001$), as well as significant differences in the proportion of *n*-6 and *n*-3 FA (*n*-6/*n*-3 FA; $P < 0.001$). Compared with CON, the two fat supplements significantly increased C18:0 ($P = 0.009$), *cis*-9 C18:1 ($P < 0.001$), total UFA (ΣUFA; $P < 0.001$), and total monounsaturated FA (ΣMUFA; $P < 0.001$), while also increasing the ratio of ΣMUFA to ΣSFA (ΣMUFA/ΣSFA; $P = 0.003$). The content of total SFA (ΣSFA) in visceral fat was increased by PA, compared with CON and MA ($P < 0.001$). Compared to CON, MA increased the ratio of ΣUFA to ΣSFA (ΣUFA/ΣSFA; $P = 0.003$), while PA showed no difference compared to CON and MA.

Table 8
Effects of fat supplements with different fatty acids (FA) on the FA profile of visceral adipose tissue of finishing Angus bulls (% of total FA).

Item	Treatment[1]			SEM	*P*-value
	CON	PA	MA		
C14:0	2.97	3.13	2.96	0.073	0.207
C14:1	0.17	0.18	0.19	0.014	0.632
C15:0	0.40	0.41	0.39	0.010	0.567
C16:0	31.68^{ab}	32.56^{a}	30.81^{b}	0.334	0.004
C16:1	1.79^{c}	2.04^{a}	1.91^{b}	0.041	<0.001
C17:0	0.87	0.87	0.86	0.008	0.530
C17:1	0.35	0.33	0.35	0.009	0.717
C18:0	28.04^{b}	30.42^{a}	29.72^{a}	0.512	0.009
cis-9 C18:1	25.55^{b}	28.84^{a}	29.04^{a}	0.445	<0.001
C18:2 *n*-6c[2]	1.67	1.64	1.69	0.056	0.810
C18:2 *n*-6t[3]	0.19	0.18	0.19	0.004	0.891
C18:3 *n*-3[4]	0.19^{b}	0.16^{c}	0.22^{a}	0.006	<0.001
C20:0	0.25	0.26	0.24	0.006	0.319
C21:0	0.36	0.35	0.36	0.010	0.810
ΣSFA[5]	64.57^{b}	67.99^{a}	65.34^{b}	0.593	<0.001
ΣUFA[6]	29.90^{b}	33.39^{a}	33.57^{a}	0.477	<0.001
ΣMUFA[7]	27.86^{b}	31.40^{a}	31.48^{a}	0.485	<0.001
ΣPUFA[8]	2.05	1.98	2.09	0.059	0.460
Σ*n*-3 FA	0.19^{b}	0.16^{c}	0.22^{a}	0.006	<0.001
Σ*n*-6 FA[9]	1.86	1.82	1.87	0.056	0.793
n-6/*n*-3 FA	10.00^{b}	11.13^{a}	8.71^{c}	0.336	<0.001
ΣMUFA/ΣSFA	0.43^{b}	0.46^{a}	0.48^{a}	0.010	0.003
ΣPUFA/ΣSFA	0.032	0.029	0.032	0.001	0.067
ΣUFA/ΣSFA	0.46^{b}	0.49^{ab}	0.52^{a}	0.010	0.003

SFA = saturated fatty acids; UFA = unsaturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; Σ*n*-3 FA = total *n*-3 fatty acids.
Fatty acids detected at < 0.1 % of total fatty acid are not reported.
^{a,b,c}Means within rows with different superscripts differ ($P < 0.05$).
[1] CON, control diet without additional fat; PA, CON + 2.5% DM palmitic calcium salt (90% C16:0); MA, CON + 2.5% DM mixed FA calcium salt (60% C16:0 + 30% *cis*-9 C18:1).
[2] C18:2 *n*-6c = *cis*-9, *cis*-12 C18:2.
[3] C18:2 *n*-6t = *cis*-9, *trans*-11 C18:2.
[4] C18:3 *n*-3 = *cis*-9, *cis*-12, *cis*-15 C18:3.
[5] ΣSFA = C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0.
[6] ΣUFA = C14:1 + C16:1 + C17:1 + *cis*-9 C18:1 + C18:2*n*-6c + C18:2*n*-6t + C18:3*n*-3.
[7] ΣMUFA = C14:1 + C16:1 + C17:1 + *cis*-9 C18:1.
[8] ΣPUFA = C18:2*n*-6c + C18:2*n*-6t + C18:3*n*-3.
[9] Σ*n*-6 FA = C18:2*n*-6c + C18:2*n*-6t.

4. Discussion

4.1. Ruminal fermentation

Due to the toxic effects of UFA on rumen microorganisms, which can reduce the rumen digestibility of fiber (Maia et al., 2007), *cis*-9 C18:1, *cis*-9, *cis*-12 C18:2, and C16:0 are usually complexed with calcium ions to form FA calcium salts (FA-Ca, a typical rumen bypass fat), aiming to minimize the adverse effects of rumen microbial fermentation (Rico et al., 2014). The results of this study are consistent with previous studies on finishing cattle and sheep in which when FA-Ca was supplemented at low levels (2% to 5% DM), rumen pH and NH_3–N were unaffected (He et al., 2018a; Behan et al., 2019). However, the FA-Ca characteristics of rumen protection make scholars neglect its effect on the rumen microorganism because FA-Ca is not completely inert in the rumen (He et al., 2018b). This may partly explain why rumen butyrate concentration decreased significantly after supplementation with FA-Ca (including PA and MA). In particular, MA containing 30% *cis*-9 C18:1 and 4% *cis*-9, *cis*-12 C18:2 significantly reduced acetate and total VFA. Unsaturated FA was shown to be more capable of lowering the rumen abundance of fibrolytic bacteria *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* and Protozoa (Kholif et al., 2018), thus inhibiting the production of VFA. However, we observed that PA with more SFA increased propionate concentration. This is consistent with Behan et al. (2019), who found that a diet with more SFA (78% C16:0) increased propionate concentration in sheep compared to treatments with control and mixed FA (48% C16:0 + 41% *cis*-9 C18:1).

4.2. Homeostasis of glucose and lipid metabolism

Non-esterified FA in the blood comes from the intake of dietary FA or the decomposition of adipose tissue. In our results, PA had a tendency to increase NEFA compared to MA. This may be related to the difference in FA metabolism with different saturation. The saturation of ingested FA has been proven to affect its distribution between oxidation and storage in the body (Piers et al., 2003). Specifically, UFA such as *cis*-9 C18:1 and *cis*-9, *cis*-12 18:2 is oxidized faster than SFA such as C16:0 and C18:0 (Bergouignan et al., 2009) and C16:0 tends to be preferentially stored in tissues. This characteristic of C16:0 may keep the blood NEFA of PA treatment at continuously a high level. In addition, the cumulative positive balance of fat intake will lead to a large amount of body fat accumulation (Piers et al., 2002), including ectopic fat deposition in the

liver; this is a critical cause of fatty liver. The increase in blood AST and liver TG under PA indicates that high C16:0 increases the burden on the liver and the risk of fatty liver disease in bulls. However, AST and TG returned to normal under MA with 30% *cis*-9 C18:1 and 4% *cis*-9, *cis*-12 C18:2 partially replacing C16:0. Consistent with our result, a study on rats showed that C16:0 incubated rat livers tend to use C16:0 for liver TG synthesis, while *cis*-9, *cis*-12 C18:2 incubated rat livers tend to be β-oxidized (Jones and Schoeller, 1988), suggesting that C16:0 is more likely than UFA to cause the liver to over-synthesize TG, resulting in fatty liver. Fatty liver in dairy cows has been reported extensively under the condition of negative energy balance in early lactation (Bobe et al., 2004). Cows with fatty liver also showed a commonality of elevated serum NEFA (Ohtsuka et al., 2001). This is consistent with our results stated above; that is, the plasma NEFA of bulls treated with PA increased with the increased risk of fatty liver. Leptin is an inhibitor secreted by fat cells that prevents excessive fat deposition. Both fat supplements increased leptin in the blood, which may mean that continuous dietary fat supplementation strongly activated leptin feedback regulation, maintaining lipid homeostasis by inhibiting fat production in peripheral tissues and increasing fat oxidation (Chilliard et al., 2005).

Unlike monogastric animals, which mainly absorb GLU through the intestines, ruminants only absorb about 10% of GLU through their intestines. Most of the circulating GLU comes from the liver gluconeogenesis of propionic acid, lactic acid and amino acids (De Koster and Opsomer, 2013). Therefore, when the liver function is damaged, it may affect gluconeogenesis in the ruminant liver. Studies on lactating cows have shown that the increase in liver fat content will interfere with the production capacity of GLU and lead to impairment of gluconeogenesis in the liver (Bobe et al., 2004). In our study, the decrease in fasting plasma GLU concentration in PA-treated bulls was most likely caused by impaired gluconeogenesis in the liver. As previously mentioned, the TG content in the bull's liver under this diet was significantly increased.

Studies on perinatal cows have shown a positive correlation between energy intake and insulin concentration (Gärtner et al., 2019). This effect was also observed in the PA group. However, MA with equal energy to PA did not affect insulin concentration. This led us to infer that not only the amount of fat intake but also fat saturation may affect insulin release. On the one hand, due to the metabolic difference between SFA and UFA mentioned above, bulls treated with PA have higher plasma NEFA and liver TG. These traits, representing obesity in bulls, easily result in insulin resistance, followed by hyperinsulinemia (De Koster and Opsomer, 2013; Czech, 2017). In addition, medical studies have shown that higher C16:0 supplementation will increase the production of harmful complex lipids diacylglycerol and ceramide in the body, thus damaging insulin signaling pathways and causing the development of insulin resistance (Holland et al., 2011). However, C16:0 substituted by *cis*-9 C18:1 can alleviate the adverse effects of C16:0 on insulin resistance and significantly improve insulin sensitivity (Palomer et al., 2018). In our study, MA with 30% *cis*-9 C18:1 is likely to mitigate the adverse effects of C16:0 through this mechanism, thereby maintaining normal fasting insulin levels. However, the specific mechanism still needs to be explored in future studies.

4.3. The mRNA relative expression of lipid metabolism genes in liver

Direct fat intake, de novo synthesis, storage and oxidative utilization are essential for maintaining liver fat balance (Flatt, 1995). The saturation of dietary fat affects lipid absorption and oxidation (Wang and Koo, 1993). From our results, high C16:0 significantly increased the likelihood of fatty liver disease in bulls, while *cis*-9 C18:1 alleviated this symptom. It may be that C16:0 and *cis*-9 C18:1 have different effects on liver lipid metabolism, so we measured liver-related genes. *C/EBPα* and *PPARα* are upstream transcriptional regulators of lipid synthesis. Compared with CON, the upregulation of *PPARα* under MA may be related to the *cis*-9 C18:1 in MA because *cis*-9 C18:1 has been proven to up-regulate the expression of *PPARα* (Belal et al., 2018). The expression of *PPARα* in the liver can up-regulate the key enzymes of FA oxidation and promote liver fat decomposition (Rogue et al., 2014). Both fat supplements up-regulated the expression of the FA direct uptake gene *FATP5* but did not affect the expression of the FA de novo synthesis gene *FASN*. This may mean that extra dietary fat supplement strongly activates the mechanism of the liver to quickly clear blood NEFA through direct intake. The activation of this mechanism may not be related to individual FA but only related to total FA intake and blood flow. Diacylglycerol acyltransferase 2 is the key enzyme for the liver to synthesize TG (Xu et al., 2015). Although *cis*-9 C18:1 and C16:1 are the key substrates of liver TG synthesis (Xu et al., 2023), our results show that MA with 30% *cis*-9 C18:1 does not up-regulate the expression of *DGAT2*. In contrast, PA with high C16:0 up-regulated *DGAT2*. This may be due to the activation of desaturase activity by a large amount of C16:0 and C18:0 ingestion, which desaturated C16:0 and C18:0 into C16:1 and *cis*-9 C18:1, respectively. PA's significant upregulation of the *SCD1* gene confirms this inference because SCD1 is the key enzyme that desaturates C16:0 and C18:0 into MUFA (Orrù et al., 2011). In addition, fat supplementation did not affect the liver cholesterol synthesis gene *HMGCR* and TG catabolism gene *ATGL*. In general, both forms of FA promote direct uptake of the liver. High C16:0 may be desaturated by SCD1 to MUFA as the substrate of DGAT2, resulting in more liver TG. On the other hand, *cis*-9 C18:1 promotes fat decomposition and avoids excessive accumulation of TG by up-regulating the expression of *PPARα*.

4.4. Blood antioxidation

Producing mitochondrial reactive oxygen species (ROS) can induce oxidative stress, which increases the possibility of systemic inflammation and insulin resistance in cattle (Palomer et al., 2018; Sordillo, 2013). Exposure of skeletal muscle cells to purified C16:0 significantly increases the production of ROS by mitochondria (Yuzefovych et al., 2010). Although ROS was not directly measured in our study, MDA was significantly increased in the PA group, which also indicated that the bull was in a state of oxidative stress induced by high C16:0 because MDA is considered to be the final stable product of ROS lipid peroxidation (Canakci et al., 2009). However, when *cis*-9 C18:1 was used to replace part of the C16:0 in PA, MA results showed that MDA in the blood was decreased, while the contents of SOD were increased. This means that supplementation of *cis*-9 C18:1 can reduce lipid peroxidation, improve antioxidant capacity, and protect the body from C16:0-induced oxidative stress (Nakamura et al., 2009). A study on obesity proves this point; that is, the antioxidant defense of cells is inhibited in the presence of C16:0, while *cis*-9 C18:1 supplementation restored this antioxidant mechanism (Sargsyan et al., 2016).

4.5. Growth performance and meat quality

When beef cattle (steer) are in the fattening stage of around 500 kg, fat accounts for over 50% of their weight gain (NASEM, 2016). The most abundant FA in beef cattle's adipose tissue and muscle were *cis*-9 C18:1, C16:0, C18:0, and *cis*-9, *cis*-12 C18:2 (Woods and Fearon, 2009). However, supplementing C16:0 and *cis*-9 C18:1 based on body composition may exhibit different priority metabolic pathways. Specifically, SFA C16:0 reduces diet-induced thermogenesis by lowering the sympathetic activity of brown adipose tissue, thereby promoting fat accumulation in the body

(Takeuchi et al., 1995). The intake of *cis*-9 C18:1 can reduce fat deposition by increasing the β-oxygen of UFA (DeLany et al., 2000; Sanz et al., 2000). However, our study showed that both fat supplements did not significantly affect intramuscular fat content compared to CON. This shows that the primary impact target of metabolic difference between C16:0 and *cis*-9 C18:1 may be the other two fat depots, namely subcutaneous fat and visceral fat because these two fat depots are the primary deposition sites for fat (Schumacher et al., 2022). The bulls on the two fat-supplemented diets were fed diets equal in energy and nitrogen. The results showed that there was no difference in DMI during the whole trial period. Therefore, compared with CON, only PA increased the total weight gain, while MA was insignificant, which may be related to the difference in their FA composition. Because the oxidation rate of individual FA in fat intake can make a difference in weight gain, the weight gain of fast oxidizing *cis*-9 C18:1 was lower than that of SFA (DeLany et al., 2000; Mercer and Trayhurn, 1984). So, it could be the 30% *cis*-9 C18:1 in the MA that lowered the weight gain effect of C16:0 on beef cattle. However, this is only an inference based on a large number of medical and mouse research conclusions. How C16:0 and *cis*-9 C18:1 deeply affect ruminant animal fat deposition and weight gain needs further research.

4.6. FA composition of visceral adipose tissue

In the process of beef cattle growth, visceral fat is deposited first, followed by subcutaneous fat, and finally, muscle fat (Peng et al., 2021). The increase in visceral fat is related to the increase in portal vein NEFA (Smith et al., 2001). Therefore, the dietary intake of FA into the blood circulation is likely to be the first to affect the FA composition of visceral adipose tissue. In general, our results did prove this point, especially that PA significantly increased the C16:0 in the visceral adipose tissue of bulls. This is generally considered to be an adverse effect because increased SFA (especially C14:0 and C16:0) in animal products is thought to increase the risk of atherosclerosis in humans (Daley et al., 2010). Surprisingly, however, both fat supplements increased levels of C18:0, MUFA C16:1 and *cis*-9 C18:1. These FA are thought to be beneficial to human health (Kris-Etherton, 1999; Yu et al., 1995). Both fat supplements were mostly C16:0. This may mean that after dietary intake of abundant C16:0, the regulatory mechanism of adipose tissue is strongly activated, and C16:0 is deposited by lengthening and desaturating to C16:1, C18:0, *cis*-9 C18:1 (Moon et al., 2014). A key role in this process may be the SCD1 enzyme because several previous studies, including our results, showed that the activity of the SCD1 enzyme and mRNA expression was strongly activated in the presence of C16:0 (da Silva-Santi et al., 2016; Li et al., 2021).

Visceral tissue was taken from around the bull's kidney, as the fat in this area has been shown to continuously express uncoupling protein 1 (*UCP1*; a marker of brown fat; Komolka et al., 2017). Uncoupling protein 1 can induce adipose tissue to remove excess fat through thermogenesis and affect energy balance by regulating fat metabolism (Maliszewska and Kretowski, 2021). Saturated FA has been shown to reduce diet-induced thermogenesis by reducing sympathetic nerve activity and *UCP1* expression in brown adipose tissue (Matsuo et al., 2002; Takeuchi et al., 1995), thereby promoting lipid accumulation in adipose tissue. Our results showed increased levels of C16:0, C18:0 and total SFA in adipose tissue treated with PA. It is likely that these elevated SFA promote the accumulation of body fat in bulls through this mechanism and ultimately increase their body weight.

5. Conclusion

High C16:0 fat supplementation increased weight gain in fattening bulls and carried the risk of fatty liver, insulin resistance, and reduced antioxidant function. These adverse effects were alleviated by replacing C16:0 with 30% *cis*-9 C18:1. However, some adverse effects of *cis*-9 C18:1 on rumen fermentation were observed. Therefore, there is a need for future work to investigate the optimal ratio of C16:0 and *cis*-9 C18:1 to maintain both increased weight gain and physical health in beef cattle.

Author contributions

Haixin Bai, **Yonggen Zhang** and **Yang Li:** Writing- original draft, Writing & editing, Project administration, Validation, Supervision. **Haixin Bai**, **Lubo Wang:** Methodology, Software, Investigation, Formal analysis. **Modinat Tolani Lambo:** Writing-review & editing, Language improvement & proofreading.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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