

## Article

# Guanidinoacetic Acid Supplementation Promotes Skeletal Muscle Fiber Type Transformation from Fast-to-Slow-Twitch via Increasing the PPARGC1A Based Mitochondrial Function and CaN/NFAT Pathway in Finishing Pigs

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**Abstract:** Guanidinoacetic acid can improve pork quality. Previous studies have demonstrated that pork quality is closely linked to the muscle fiber type mediated by PPARGC1A. Therefore, this study aimed to evaluate the influence of dietary GAA supplementation on the skeletal muscle fiber type transformation. A total of 180 healthy Duroc × Landrace × Meishan cross castrated male pigs with a similar average weight ( $90 \pm 1.5$  kg) were randomly divided into three treatments with five replicates per treatment and 12 pigs per replicate, including a GAA-free basal diet and basal diet with 0.05% or 0.10% GAA for 15 days. Our results showed that 0.10% GAA supplementation increased the contents of  $\text{Ca}^{2+}$  in sarcoplasm ( $p < 0.05$ ). Compared with the control group, both GAA supplementation groups upregulated the expression of Troponin I-ss ( $p < 0.05$ ), and 0.10% GAA supplementation downregulated the expression of Troponin T3 ( $p < 0.05$ ). GAA supplementation increased the expression of peroxisome proliferator activated receptor- $\gamma$  coactivator-1 $\alpha$  (PPARGC1A) ( $p < 0.05$ ), and further upregulated the mitochondrial transcription factor A (TFAM), increased the level of membrane potential, and the activities of mitochondrial respiratory chain complex I, III ( $p < 0.05$ ). The 0.10% GAA supplementation upregulated the protein expression of calcineurin catalytic subunit  $\alpha$  (CnA $\alpha$ ) and nuclear factor of activated T cells (NFATc1) ( $p < 0.05$ ). Overall, dietary GAA supplementation promotes skeletal muscle fiber types transformation from fast-to-slow-twitch via increasing the PPARGC1A based mitochondrial function and the activation of CaN/NFAT pathway in finishing pigs.

**Keywords:** guanidinoacetic acid; muscle fiber type transformation; PPARGC1A; mitochondria; CaN/NFAT; finishing pig

## 1. Introduction

In mammals, different fiber types, which are the basis of muscle plasticity in response to a variety of functional demands, can be distinguished in skeletal muscle. Moreover, the muscle fiber characteristics are the major factors for the evaluation of meat quality, especially the fiber type composition [1]. According to the diverse structural properties and myosin heavy chain subtypes, muscle fiber can be classified into four fiber types (MyHC-I, MyHC-IIa, MyHC-IIx, MyHC-IIb), and these muscle fiber types can be transformed into each other ( $I \leftrightarrow IIa \leftrightarrow IIx \leftrightarrow IIb$ ) [2]. MyHC-I slow-twitch muscle fiber (oxidative fiber), has an oxidative phosphorylation profile. Fast-twitch type fibers include IIa, IIx, and IIb muscle fibers [3]. It is easy to bring about the production of more lactic acid in animals after

slaughter. Meanwhile, the excessive accumulation of postmortem lactic acid could further lead to a decline in pH and meat quality. As a consequence, it is particularly important to promote the transformation of skeletal muscle fibers from fast-twitch to slow-twitch. Moreover, skeletal muscle fiber transformation can be caused by some factors, such as heredity, exercise, and nutrition [4]. Meanwhile, the Troponin I-ss and Troponin T3 are specifically expressed in the slow and fast muscle fibers, and their content can indicate the proportion of the slow and fast muscle fibers, respectively [5]. Previous studies showed that skeletal muscle can be changed via decreasing the proportion of fast fibers and increasing slow fibers through some nutritional modulation strategies [6,7].

Mitochondria contain a variety of aerobic metabolism enzymes and all components of the electron-transport system. Therefore, the changes of mitochondrial content and function will affect the aerobic metabolism of the body [8]. It is worth noting that type I fibers have more mitochondria and a high capacity for the oxidation of energy substrates. In addition, type IIb fibers are poor in mitochondria, whereas rich in glycolytic enzymes, resulting in a higher potential for glycolysis [9]. A previous study has demonstrated that the mitochondria function played an important role in the muscle fiber-type transformation [10]. On the other hand, peroxisome proliferative activated receptor gamma coactivator 1 alpha (PPARGC1A) is involved in the regulation of energy metabolism and the transformation of skeletal muscle fiber-type [11]. PPARGC1A can promote the biosynthesis of mitochondria and the improvement of mitochondrial function during the metabolic process of skeletal muscle cells [12]. Once the increase of intracytoplasmic  $\text{Ca}^{2+}$  content increases, the expression of PPARGC1A would be upregulated [13]. The high expression of PPARGC1A can upregulate the DNA content of mitochondria and the activity of mitochondrial transcription factors A (TFAM) and DNA junctions, and then promote the biosynthesis of mitochondria [14]. Calcineurin (CaN)/nuclear factor of activated T cells (NFAT) signal pathway is a crucial signal transduction pathway to regulate the differentiation of skeletal muscle and the transformation of muscle fiber [13]. Moreover, PPARGC1A, as the substrate of CaN, may also mediate the conversion of muscle fibers from fast-twitch to slow-twitch through the modulation of CaN/NFAT signaling pathway [15].

Guanidinoacetic acid (GAA), which is synthesized from the glycine and arginine in the kidney, is the main endogenous substance of creatine synthesis in animals [16]. Creatine participates in the creatine phosphate system and generates ATP to provide energy for the body [17]. It was reported that dietary GAA supplementation significantly improved the meat quality of livestock and poultry [18]. It is well known that the skeletal muscle fiber types are highly related to the meat quality. A previous study found that creatine subjects demonstrated significant increase in muscle fiber type I [19]. Meanwhile, Zhu et al. have also found that GAA improved the meat quality by regulating the change of muscle fiber type [20]. However, the mechanism was not clear. Therefore, this study was conducted to evaluate the effects of dietary GAA supplementation on the skeletal muscle fiber type transformation, mitochondrial function, and CaN/NFAT pathway in finishing pigs.

## 2. Materials and Methods

### 2.1. Experimental Design, Animals, and Treatment

All of the experimental procedures involving the use of finishing pigs were in alignment with the Animal Care and Use Committee of Nanjing Agricultural University under the protocol number of SYXK 2017-0027 and its scope of application involves rabbit, dog, and pig. A total of 180 Duroc  $\times$  Landrace  $\times$  Meishan cross castrated male pigs with a similar average weight ( $90 \pm 1.5$  kg) were randomly allocated to three treatments with five replicates (pens) per treatment and 12 pigs per pen. All of the pigs were fed in the form of Jiangsu MingTian Agriculture and Animal Husbandry Technology Co., LTD (Nanjing, China). In addition, all of the pigs were housed in solid concrete floor pens in an environmentally controlled room (temperature:  $22 \pm 2.5$  °C, humidity:  $65 \pm 2.4\%$ ). These experimental treatments included the following: The control group was fed with a GAA-free basal diet, and two GAA groups were fed with basal diets with 0.05% and 0.10% GAA,

respectively. The experiment lasted for 15 days. The GAA additive was obtained from Beijing Gendone Biotechnology Co., Ltd. (Beijing, China). All of the trail finishing pigs were allowed free access to feed and water. The formulation of basal diet (Table 1) was referred to the nutritional requirements of NRC (1998).

**Table 1.** Composition and nutrient level of basal diet.

Composition	%	Nutrient Level	%
Maize	72	Digestible energy (MJ kg <sup>-1</sup> )	13.91
Soybean meal	20	Crude protein	15.81
Wheat bran	4	Calcium	0.88
Dicalcium phosphate	1.4	Total phosphorus	0.57
Limestone	1.05	Lysine	0.98
Lysine-HCl	0.2	Methionine	0.23
Sodium chloride	0.35	Threonine	0.58
Premix <sup>A</sup>	1	Tryptophan	0.18
Total	100		

Note: <sup>A</sup> The premix provided the diet per kg as follows: 100 mg Fe, 100 mg Zn, 30 mg Mn, 10 mg Cu, 0.3 mg Se, 0.5 mg I, 5000 IU VA, 1000 IU VD3, 20 IU VE, 3.0 mg VK, 2.0 mg VB1, 6.0 VB2, 3.0 mg VB6, 30 µg VB12, 20 mg nicotinic acid, 8 mg calcium pantothenate, 0.5 mg folic acid, 300 mg choline.

## 2.2. Sample Collection

After 15 days of the feeding trial, 30 pigs (10 pigs per treatment, 2 per pen with medium bodyweight) were chosen and transported to abattoir (Su Meat Products Co., Ltd., Huaian, Jiangsu, China) for slaughter, according to the slaughtering procedures after a 12 h fast except for water. All of the pigs were slaughtered by exsanguination after electrical stunning (in front of the ears, at the temporal fossae: 50 Hz, 1.40 A, and 350 V). The *longissimus thoracis* muscle and *semitendinosus* muscle samples of the right carcass were collected in frozen tubes, and immediately placed in liquid nitrogen and stored at −80 °C for the determination of all indices included in this study.

## 2.3. Antibodies

The primary antibodies were as follows: Anti-peroxisome proliferator activated receptor-γ coactivator-1 alpha (PPARGC1A) (ABclonal Technology, Cat. No. A12348, Wuhan, China); anti-nuclear factor of activated T cells (NFATc1) (ABclonal Technology, Cat. No. A1539, Wuhan, China); anti-troponin I slow type (Troponin I-ss) (CLOUD-CLONE CORP, Cat. No. PAD227Hu01, Wuhan, China); anti-troponin IIb fast skeletal type (Troponin T3) (Abbkine, Cat. No. ABP60722, Wuhan, China); anti-calcineurin catalytic subunit α (CnAα) (Proteintech Group, Cat. No. 13422-1-AP, Chicago, IL, USA); anti-mitochondrial transcription factor A (TFAM) (Affinity biosciences, Cat. No. AF0531, Changzhou, China); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Servicebio Technology, Cat. No. GB11002, Wuhan, China).

## 2.4. Measurement of Sarcoplasmic Ca<sup>2+</sup> Contents

The concentration of Ca<sup>2+</sup> in sarcoplasm was detected using the modified method of Wang et al. [21]. About 2 g of muscle sample was homogenized twice in 5 mL of ice-cold 0.15 M KCl at a speed of 12,000 rpm for 1 min each time, and then centrifuged at 27,000× g for 10 min at 4 °C to obtain the supernatant. Supernatants (4 mL) were obtained and added to 10% trichloroacetic acid to a final concentration of 0.8%. After 10 min, strontium chloride (5.4 mg) was added. After centrifugation at 10,000× g for 10 min at room temperature, the supernatants were collected to determine the concentration of sarcoplasmic Ca<sup>2+</sup> with the method of Inductively Coupled Plasma (iCAP, 7000, SERIES, Thermo Fisher Scientific, Waltham, MA, USA).

## 2.5. Measurement of Mitochondrial Membrane Potential, Mitochondrial Respiratory Chain Complex I, and Mitochondrial Respiratory Chain Complex III

The mitochondrial membrane potential (MMP) was detected using a commercial kit (JC-1, Cat. No. C2006, Beyotime, Nanjing, China). First, the mitochondria were separated from the muscle using a commercial kit (Cat. No. C3606, Beyotime, Nanjing, China): About 80 mg of muscle sample was placed in a centrifuge tube with 800  $\mu$ L pre-cooled PBS, and ice-chilled for 3 min. The muscle samples were precipitated by centrifugation at  $600\times g$  for 15 s, and the supernatant was discarded. Then, 640  $\mu$ L of precooled mitochondrial separation reagent B was added. An ice bath homogenate was performed 25 times, and then centrifugation was performed at  $600\times g$  for 5 min at 4  $^{\circ}$ C. The obtained supernatant was transferred to another centrifugal tube and centrifuged at  $11,000\times g$  for 10 min at 4  $^{\circ}$ C. The precipitates were mitochondria. The 0.9 mL of a JC-1 staining solution was added to the 0.1 mL mitochondria. In addition, the fluorescence spectrophotometer was used for MMP detection (excitation wavelength: 485 nm, emission wavelength: 590 nm).

Mitochondrial respiratory chain complex I and mitochondrial respiratory chain complex III were measured using kits, according to the manufacturer's instructions (Cat. No. D799471-0500 and D799480-0100, respectively, Sangon Biotech Co., Ltd., Shanghai, China). About 0.1 g of muscle sample was homogenized using the extract at 4  $^{\circ}$ C. The supernatant was centrifuged at  $600\times g$  for 10 min at 4  $^{\circ}$ C, and then transferred to another centrifuge tube, centrifuged at  $11,000\times g$ , 4  $^{\circ}$ C for 15 min. The obtained precipitation was added into a 400  $\mu$ L extract, which was used for activity detection after ultrasonic crushing.

## 2.6. Gene Expression Analysis

Total mRNA from *longissimus thoracis* and *semitendinosus* muscle samples (about 70 mg) was isolated with RNAiso plus reagent (Takara Biotechnology Co., Ltd., Dalian, China). The mRNA was reverse transcribed into cDNA using a commercial kit (Takara Biotechnology Co., Ltd., Dalian, China). The reaction system volume was 40  $\mu$ L and the conditions of the program were operated at 37  $^{\circ}$ C for 15 min, and 85  $^{\circ}$ C for 5 s.

The RT-qPCR was performed on an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). The PCR cycle conditions used were at 95  $^{\circ}$ C for 30 s, 40 cycles at 95  $^{\circ}$ C for 5 s, and 60  $^{\circ}$ C for 30 s, then 95  $^{\circ}$ C for 15 s, 60  $^{\circ}$ C for 1 min, and 95  $^{\circ}$ C for 15 s. The primer sequences designed by the NCBI Primer blast are listed in Table 2. The targeted genes expressions were quantified using the method of  $2^{-\Delta\Delta C_t}$  with GAPDH as an endogenous control.

**Table 2.** Primer sequence of target genes and housekeeping gene.

Genes	Prime Sequences	Product Lengths	GenBank Numbers
PPARGC1A	F:AAGGATGCGCTCTCGTTCAA R:TGAACGTGATCTGGCGCAC	184	XM_021100442.1
TFAM	F:GAAAGTCAGGAGCGGACCTC R:TTGTACACCTGCCAGTCTGC	289	NM_001130211
NRF1	F:TCCTTCATCTCCTGAAGACACC R:ACATGAGGCCGTTTCCGTTT	156	XM_021079000.1
SIRT1	F:AGGTTTGAAGAATGTTGCCTGC R:TGGACTCTGGCAAGTTCCAC	256	NM_001145750
TFB1M	F:ATGGAACGCACTGGGAAACT R:TTTCTGACGTGTGCCTGAG	175	NM_001128475.1
GAPDH	F:CCCGCCAACATCAAATGGG R:ACTTCTCATGGTTCACGCC	175	NM_001206359.1

Note: PPARGC1A: Peroxisome proliferator activated receptor- $\gamma$  coactivator-1alpha; TFAM: Mitochondrial transcription factor A; NRF1: Nuclear respiratory factor 1; SIRT1: Silent information regulator 1; TFB1M: Mitochondrial transcription factor B1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

### 2.7. Western Blot Analysis

Total protein of muscle samples (about 30 mg) was extracted using the RIPA buffer. The content of total protein was measured using a bicinchoninic acid (BCA) kit (Cat. No. C503061-1250, Sangon Biotech Co., Ltd., Shanghai, China), and then adjusted to 4 µg/µL. Each protein sample was mixed with 5 × loading buffer at the ratio of 1:4, incubated at boiling water for 5 min.

The protein sample was separated with 12.5% sodium lauryl sulfate-polyacrylamide gel (Cat. No. PG113, EpiZyme Biotechnology Co., Ltd., Shanghai, China) electrophoresis (100 V, 1.5 h), and then transferred to a polyvinylidene fluoride membrane (Millipore; Billerica, MA, USA). After blocking with 5% skimmed milk solution for 2 h at room temperature, the membranes were incubated with a primary antibody overnight at 4 °C before incubation with the secondary antibody for 1 h at room temperature. The secondary antibody was purchased from Cell Signaling Technology Inc. (Cat. No. 7074S, Danvers, MA, USA) and diluted by 1 × TBST at the ratio of 1:3000. The membranes were visualized using the ChemiDoc™ Imaging System (BIO-RAD, Singapore). The densities of the target bands were quantified using ImageJ software (1.6.0-20, 1.53e, National Institutes of Health, Bethesda, MD, USA) and were normalized with GAPDH as an endogenous control. Using the computer's own photo function, the western blot images were cropped. The antibodies of PPARGC1A, NFATc1, Troponin I-ss, Troponin T3, CnAα, and TFAM were diluted by 5% skimmed milk at the ratio of 1:1000, 1:1000, 1:530, 1:800, 1:4000, 1:1000, respectively. The ECL substrate used was purchased from Vazyme Biotech Co., Ltd., Nanjing, China (Cat. No. E412-02).

### 2.8. Statistical Analysis

All of the data analyses were performed by the one-way ANOVA procedure using the IBM SPSS Statistics 20.0 software (SPSS Inc, Chicago, IL, USA). The experimental data were analyzed with pen as the experimental unit [ $n = 5$  (the means of two pigs per pen were used to represent the pens)]. Data were expressed as the mean ± SEM.  $p < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Skeletal Muscle Fiber Type Transformation

According to Figure 1A,B, the GAA treatment increased the troponin I slow type (Troponin I-ss) protein level in *longissimus thoracis* and *semitendinosus* muscle ( $p < 0.05$ ), as compared with the control. Compared with the control group, the GAA addition down-regulated the expression of troponin IIb fast skeletal type (Troponin T3) in *semitendinosus* muscle ( $p < 0.05$ ). Likewise, as shown in Figure 1A, the expression of Troponin T3 was the lowest in the 0.10% GAA group of *longissimus thoracis* muscle among all of the treatments ( $p < 0.05$ ). The full blots of Troponin T3 were shown in Supplementary Figures S1 and S2.

### 3.2. Sarcoplasmic $\text{Ca}^{2+}$ Contents

Compared with the control group, the 0.10% GAA treatment increased the sarcoplasmic  $\text{Ca}^{2+}$  content in *longissimus thoracis* muscle ( $p < 0.05$ ; Figure 2A). However, no significant difference was observed in the sarcoplasmic  $\text{Ca}^{2+}$  content of *semitendinosus* muscle among the three groups ( $p > 0.05$ ; Figure 2B).

### 3.3. Gene and Protein Expressions of PPARGC1A

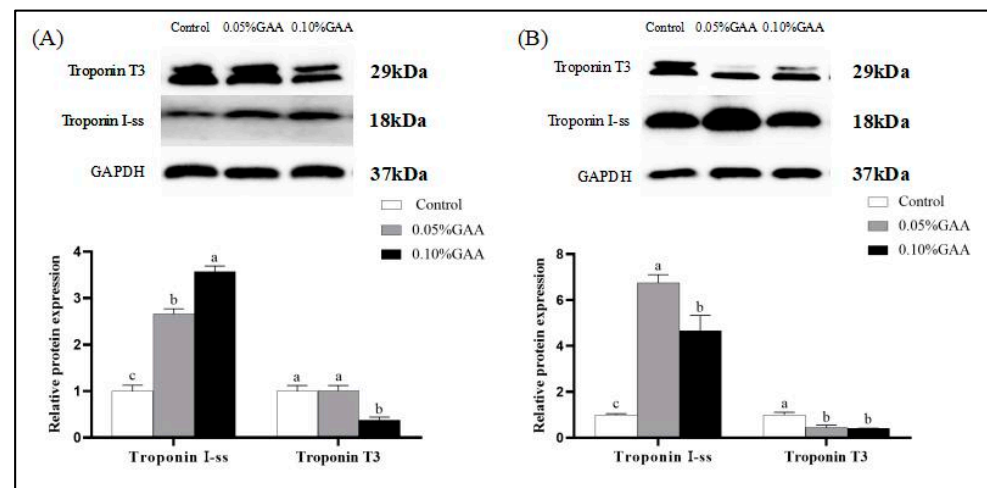
Dietary 0.05% GAA supplementation upregulated the mRNA expression of PPARGC1A in *longissimus thoracis* muscle compared with the control ( $p < 0.05$ ; Figure 3A). Meanwhile, the GAA supplementation also increased the protein level of PPARGC1A in *longissimus thoracis* muscle ( $p < 0.05$ ; Figure 3B). Moreover, the 0.10% GAA addition upregulated the protein expression of PPARGC1A in *semitendinosus* muscle ( $p < 0.05$ ; Figure 3C).



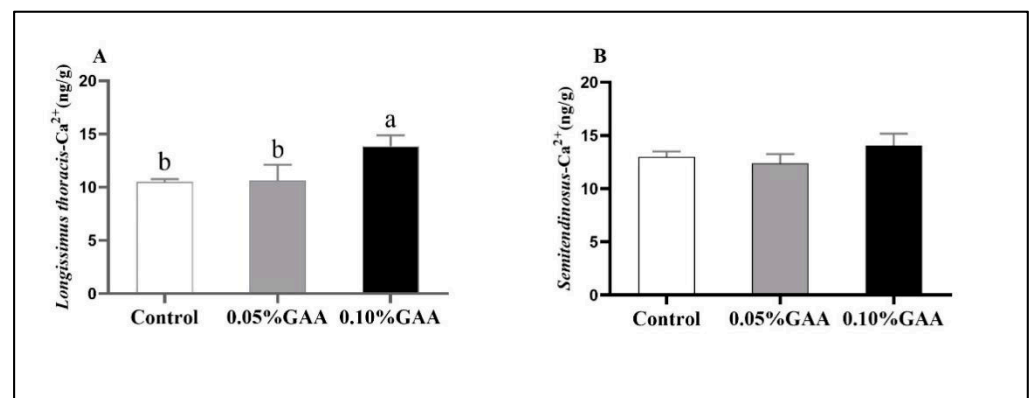
### 3.4. Indexes Related to Mitochondrial Function

As shown in Figure 4B, compared with the control group, GAA supplementation increased the level of mitochondrial membrane potential in *semitendinosus* muscle ( $p < 0.05$ ).

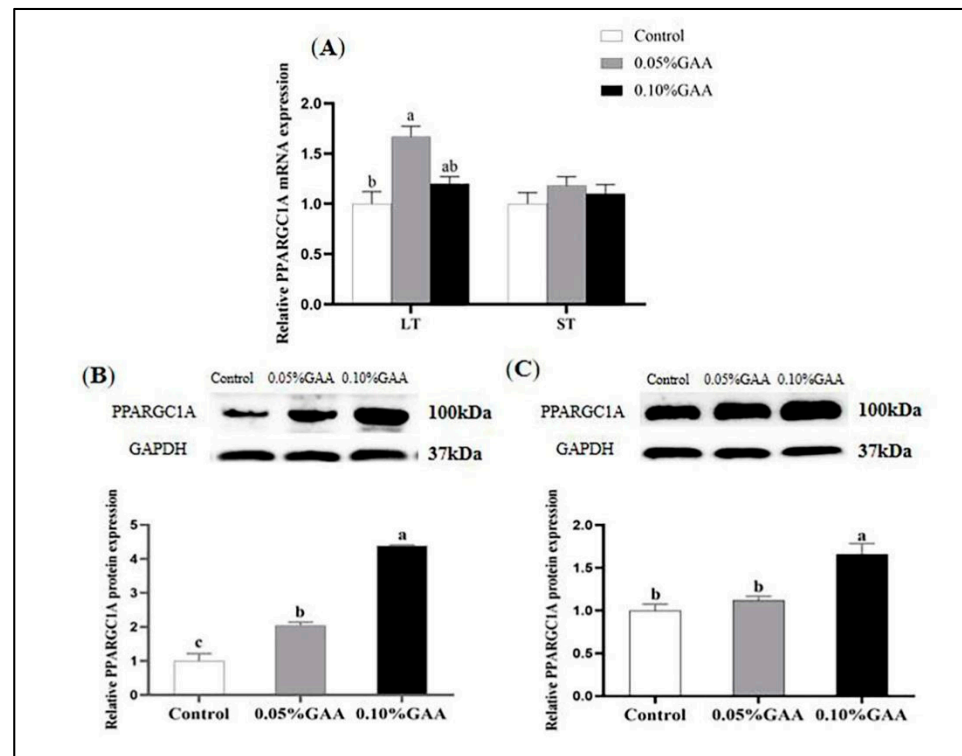
Compared with the control group, the addition of GAA increased the activity of mitochondrial respiratory chain complex III in *longissimus thoracis* muscle ( $p < 0.05$ ; Table 3). Meanwhile, dietary 0.10% GAA increased the activity of mitochondrial respiratory chain complex I in *semitendinosus* muscle ( $p < 0.05$ ).



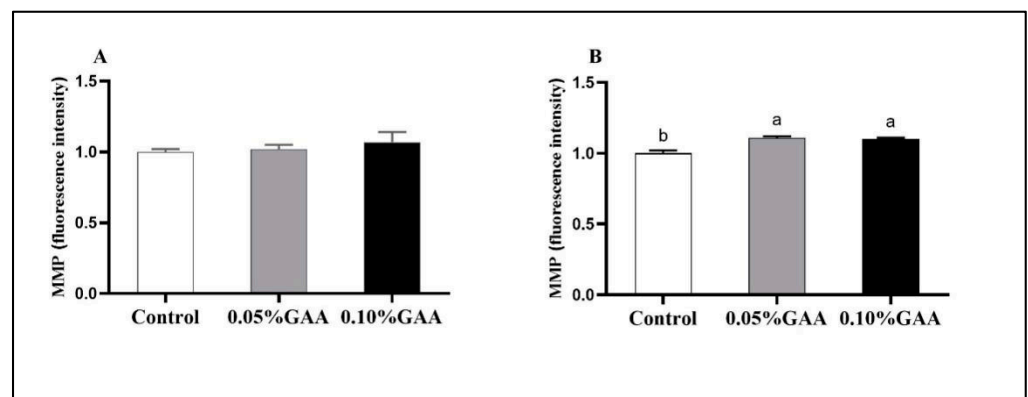
**Figure 1.** Effects of guanidinoacetic acid supplementation on the protein expressions of Troponin I-ss, Troponin T3 of (A) *longissimus thoracis* muscle, and (B) *semitendinosus* muscle in finishing pigs. Means with different letters differ significantly ( $p < 0.05$ ). Values are presented as the mean  $\pm$  SEM ( $n = 5$ ). Control: Finishing pigs were fed a basal diet; 0.05% GAA or 0.10% GAA: Finishing pigs were fed a basal diet supplemented with GAA at 500 or GAA at 1000 mg kg<sup>-1</sup>; Troponin I-ss: Troponin I slow type; Troponin T3: Troponin IIb fast skeletal type; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.



**Figure 2.** Effects of guanidinoacetic acid supplementation on the sarcoplasmic Ca<sup>2+</sup> contents in (A) *longissimus thoracis* muscle and (B) *semitendinosus* muscle of finishing pigs. Means with different letters differ significantly ( $p < 0.05$ ). Values are presented as the mean  $\pm$  SEM ( $n = 5$ ). Control: Finishing pigs were fed a basal diet; 0.05% GAA or 0.10% GAA: Finishing pigs were fed a basal diet supplemented with GAA at 500 or GAA at 1000 mg kg<sup>-1</sup>.



**Figure 3.** Effects of guanidinoacetic acid supplementation on the mRNA (A) and protein expressions of PPARGC1A of *longissimus thoracis* muscle (B) and *semitendinosus* muscle (C) in finishing pigs. Means with different letters differ significantly ( $p < 0.05$ ). Values are presented as the mean  $\pm$  SEM ( $n = 5$ ). Control: Finishing pigs were fed a basal diet; 0.05% GAA or 0.10% GAA: Finishing pigs were fed a basal diet supplemented with GAA at 500 or GAA at 1000 mg kg<sup>-1</sup>. LT: *Longissimus thoracis* muscle; ST: *Semitendinosus* muscle; PPARGC1A: Peroxisome proliferator activated receptor- $\gamma$  coactivator-1 $\alpha$ .



**Figure 4.** Effects of guanidinoacetic acid supplementation on the mitochondrial membrane potential (MMP) of (A) *longissimus thoracis* muscle and (B) *semitendinosus* muscle in finishing pigs. Means with different letters differ significantly ( $p < 0.05$ ). Values are presented as the mean  $\pm$  SEM ( $n = 5$ ). Control: Finishing pigs were fed a basal diet; 0.05% GAA or 0.10% GAA: Finishing pigs were fed a basal diet supplemented with GAA at 500 or GAA at 1000 mg kg<sup>-1</sup>.

**Table 3.** Effects of guanidinoacetic acid supplementation on the activities of mitochondrial respiratory chain complex I and III in the *longissimus thoracis* and *semitendinosus* muscle of finishing pigs.

Item	Control	0.05%GAA	0.10%GAA	SEM	p-Value
<i>longissimus thoracis</i>					
mitochondrial respiratory chain complex I (U mg <sup>-1</sup> protein)	0.63	0.65	0.98	0.08	0.181
mitochondrial respiratory chain complex III (U mg <sup>-1</sup> protein)	0.23 <sup>b</sup>	0.48 <sup>a</sup>	0.51 <sup>a</sup>	0.05	0.009
<i>semitendinosus</i>					
mitochondrial respiratory chain complex I (U mg <sup>-1</sup> protein)	0.62 <sup>b</sup>	0.81 <sup>b</sup>	1.36 <sup>a</sup>	0.12	0.006
mitochondrial respiratory chain complex III (U mg <sup>-1</sup> protein)	0.21	0.28	0.22	0.02	0.622

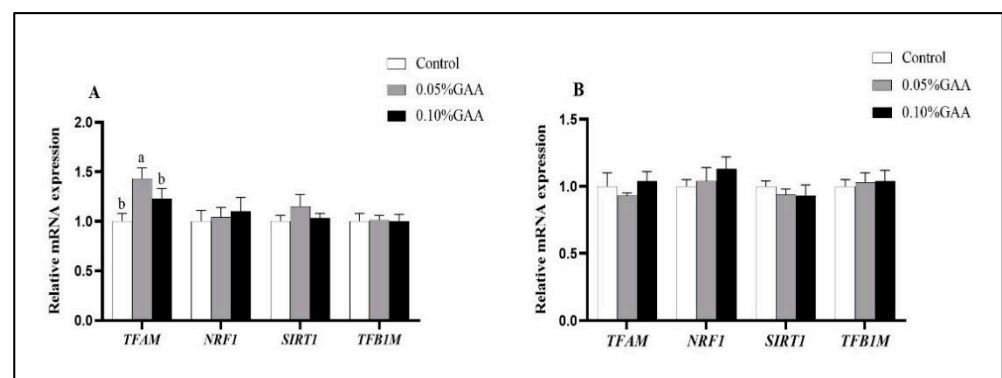
Means with different letters differ significantly ( $p < 0.05$ ). Values are presented as the mean  $\pm$  SEM ( $n = 5$ ). Control: Finishing pigs were fed a basal diet; 0.05% GAA or 0.10% GAA: Finishing pigs were fed a basal diet supplemented with GAA at 500 or GAA at 1000 mg kg<sup>-1</sup>.

### 3.5. Related Genes and Protein Expressions of Mitochondrial Synthesis

According to Figure 5A, compared with the control group, 0.05% GAA supplementation upregulated the mRNA expression of *TFAM* in *longissimus thoracis* muscle ( $p < 0.05$ ). Moreover, as shown in Figure 6B, the 0.10% GAA supplementation upregulated the protein expression of TFAM in *semitendinosus* muscle compared with the control group ( $p < 0.05$ ).

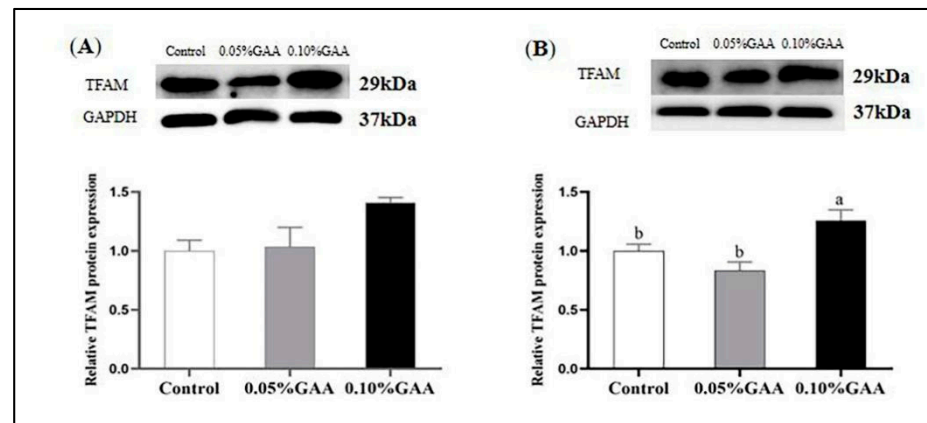
### 3.6. Protein Expressions of CnA $\alpha$ and NFATc1

As exhibited in Figure 7A, dietary 0.10% GAA upregulated the protein expression of CnA $\alpha$  in *longissimus thoracis* muscle compared with the control group ( $p < 0.05$ ). Meanwhile, 0.10% GAA treatment increased the protein expression of NFATc1 in *semitendinosus* muscle, as shown in Figure 7B ( $p < 0.05$ ).

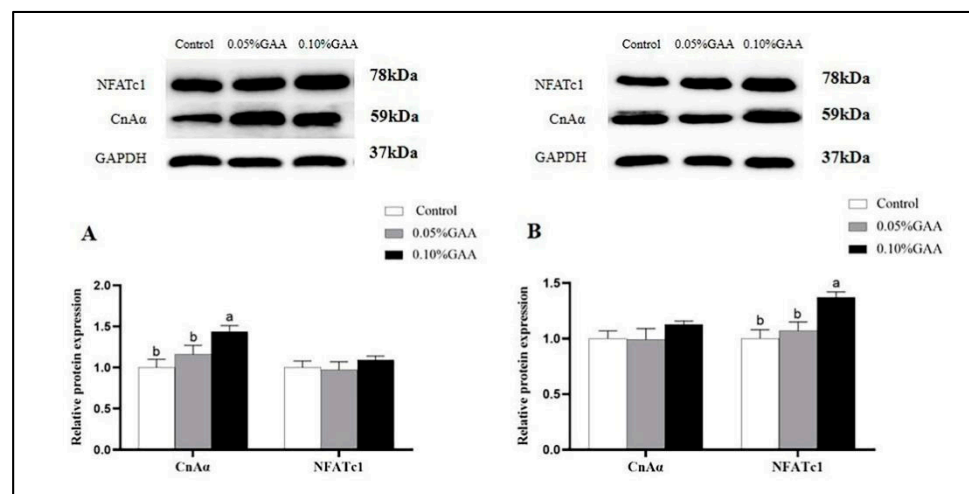


**Figure 5.** Effects of guanidinoacetic acid supplementation on the related genes of mitochondrial synthesis of (A) *longissimus thoracis* muscle and (B) *semitendinosus* muscle in finishing pigs. Means with different letters differ significantly ( $p < 0.05$ ). Values are presented as the mean  $\pm$  SEM ( $n = 5$ ). Control: Finishing pigs were fed a basal diet; 0.05% GAA or 0.10% GAA: Finishing pigs were fed a basal diet supplemented with GAA at 500 or GAA at 1000 mg kg<sup>-1</sup>; TFAM: Mitochondrial transcription factor A; NRF1: Nuclear respiratory factor 1; SIRT1: Silent information regulator 1; TFB1M: Mitochondrial transcription factor B1.





**Figure 6.** Effects of guanidinoacetic acid supplementation on the protein expression of TFAM of *longissimus thoracis* muscle (A) and *semitendinosus* muscle (B) in finishing pigs. Means with different letters differ significantly ( $p < 0.05$ ). Values are presented as the mean  $\pm$  SEM ( $n = 5$ ). Control: Finishing pigs were fed a basal diet; 0.05% GAA or 0.10% GAA: Finishing pigs were fed a basal diet supplemented with GAA at 500 or GAA at 1000 mg kg<sup>-1</sup>; TFAM: Mitochondrial transcription factor A.



**Figure 7.** Effects of guanidinoacetic acid supplementation on the protein expressions of CnA $\alpha$ , NFATc1 of (A) *longissimus thoracis* muscle, and (B) *semitendinosus* muscle in finishing pigs. Means with different letters differ significantly ( $p < 0.05$ ). Values are presented as the mean  $\pm$  SEM ( $n = 5$ ). Control: Finishing pigs were fed a basal diet; 0.05% GAA or 0.10% GAA: Finishing pigs were fed a basal diet supplemented with GAA at 500 or GAA at 1000 mg kg<sup>-1</sup>; CnA $\alpha$ : Calcineurin catalytic subunit  $\alpha$ ; NFATc1: Nuclear factor of activated T cells.

#### 4. Discussion

Muscle fiber is the basic constituent unit of skeletal muscle. Based on the expression of the predominant MyHC isoforms, the mammalian muscle fibers can be generally classified into four types, including MyHC-I, MyHC-IIa, MyHC-IIx, MyHC-IIb. [22]. Meanwhile, the proportion of different muscle fiber types directly affects the meat quality [23]. Therefore, an investigation of the effects and potential mechanisms of feed additives on skeletal muscle development and fiber type transformation in the production of livestock and poultry has extremely vital roles in improving meat quality. Without affecting the growth performance, we found that the dietary supplementation with GAA can increase the troponin I slow type (Troponin I-ss) protein level and downregulate the expression of troponin IIb fast skeletal type (Troponin T3) in finishing pigs. Moreover, we found this function of GAA involved in promoting the PPARGC1A-based mitochondria function and CaN/NFAT pathway.

The composition of muscle fiber types is an important factor to determine the meat quality. Generally, meat quality is positively correlated with the ratio of slow muscle fibers [24]. Troponin I-ss and Troponin T3 are two subtypes existing in slow and fast muscle fibers, respectively, and their expression levels can reflect the content of slow and fast muscle fibers in muscle [25,26]. Our unpublished data demonstrated that GAA supplementation increased  $\text{pH}_{45\text{min}}$  and decreased the drip loss in *semitendinosus* muscle significantly, which was consistent with the muscle fiber types transformation in our present study. In addition, our unpublished data have also found that dietary GAA significantly increased the mRNA expression of MyHC-I and decreased the expression of MyHC-IIb in *longissimus thoracis* and *semitendinosus* muscle. Meanwhile, our present data revealed that GAA addition increased the expression of Troponin I-ss and downregulated the expression of Troponin T3 in *longissimus thoracis* and *semitendinosus* muscle, suggesting that GAA could promote the muscle fiber type transformation from fast-to-slow twitch. Similarly, Zhu et al. [20] found that dietary GAA improved the meat quality and upregulated the MyHC-I mRNA expression in finishing gilts, which was in accordance with our results.

The transformation of muscle fiber types is mediated by several independent signaling pathways, and PPARGC1A is one of the most important regulatory pathways [27]. The expression of PPARGC1A is higher in slow muscle type than in fast muscle type [28]. Russell et al. [29] found that PPARGC1A transgenic mice increased mitochondrial concentrations and the level of oxidative phosphorylation in skeletal muscle and contained more MyHC-I and MyHC-IIa fibers. The increase of PPARGC1A expression can be achieved by AMPK activation [30]. On the other hand, a previous study has also found that the signaling of  $\text{Ca}^{2+}$  can activate the expression of PPARGC1A in skeletal muscle [31]. Theoretically, the change of  $\text{Ca}^{2+}$  levels depends on the activity of  $\text{Ca}^{2+}$ -ATPase, which is closely related to cellular energy metabolism [32]. With the increase of intracellular ATP concentration, the activity of  $\text{Ca}^{2+}$ -ATPase increases. This can in turn activate the calcium pump on the sarcoplasmic reticulum to release more  $\text{Ca}^{2+}$  into the cytoplasm [32]. The increased concentration of  $\text{Ca}^{2+}$  has the potential to activate PPARGC1A. A previous study has demonstrated that dietary GAA can significantly increase the content of ATP in animal cells [33]. Meanwhile, the present study indicated that GAA treatment increased the concentration of  $\text{Ca}^{2+}$  in *longissimus thoracis* muscle. Finally, our data showed that the mRNA and protein levels of PPARGC1A were upregulated in GAA supplementation treatments, suggesting that GAA could promote the expression of PPARGC1A through the  $\text{Ca}^{2+}$  signal.

There are four fiber types found in the skeletal muscle (MyHC-I, MyHC-IIa, MyHC-IIx, MyHC-IIb). MyHC-I is called slow-twitch fiber, which has a predominantly oxidative metabolism. MyHC-IIb is based on anaerobic metabolism due to the lower mitochondria and higher glycogen, which leads to a decrease in meat quality [2]. In other words, the change in muscle fiber type distribution from fast-to-slow twitch is the result of changes in key oxidative phosphorylation and glycolytic enzymes. Mitochondria contain cytochrome and enzymes for oxidative phosphorylation [34]. Therefore, the changes in mitochondrial content and function would affect the body's aerobic metabolic functions [34]. A previous study showed that the improvement of mitochondrial biosynthesis and function could lead to an increased oxidative phosphorylation capacity of fibers, and finally upregulated the proportion of slow muscle fibers [35]. In addition, PPARGC1A is involved in energy metabolism regulation. It has been demonstrated that PPARGC1A plays a vital role in the improvement of mitochondrial biosynthesis and function [36]. Furthermore, the improvement of mitochondrial biosynthesis and function promotes the conversion of skeletal muscle fiber types [37].

To prove the improvement of mitochondrial function in muscle due to GAA addition, the present study measured the mitochondrial membrane potential (MMP) and the activities of mitochondrial respiratory chain complex I and III. In the process of respiratory oxidation, mitochondria store the generated energy as electrochemical potential energy in the inner membrane of mitochondria. This state results in asymmetric distribution of ion concentrations on both sides of the inner membrane, and then forms the MMP [38].

The normal MMP is a necessary condition to maintain the mitochondrial function, oxidative phosphorylation, and ATP production [39,40]. On the other hand, the main effect of mitochondrial respiratory chain, representing the basic function of mitochondria, is to form energy through hydrogen and electron transmitters and a series of redox reactions. Moreover, mitochondrial respiratory chain complex I is the main element to drive electrons into the respiratory chain and complex III is the essential protein of mitochondrial oxidative phosphorylation and the gatekeeper of respiratory chain [41]. In our study, dietary GAA significantly increased the level of MMP in *semitendinosus* muscle and upregulated the activity of mitochondrial respiratory chain complex I, III in *semitendinosus* and *longissimus thoracis* muscle, respectively. Additionally, we detected the levels of mitochondrial synthesis-related genes and proteins to evaluate the state of mitochondrial synthesis. Mitochondrial transcription factor A (TFAM) is a key factor in the replication of mitochondrial DNA and mitochondrial formation [42]. In mitochondria, TFAM exerts its transcriptional activation ability through the recruitment of mitochondrial RNA polymerase and mitochondrial transcription factor B1 (TFB1M) [43]. Previous evidences indicated that the increased expression of PPARGC1A upregulated the DNA content of mitochondria and promoted the activity of TFAM with DNA junctions, and then increased the biosynthesis of mitochondria [44]. Our present results indicated that GAA significantly increased the mRNA expression of TFAM in *longissimus thoracis* muscle. Meanwhile, 0.10% GAA group upregulated the protein expression of TFAM in *semitendinosus* muscle. To summarize, these results indicated that the increased expression of PPARGC1A promoted the improvement of mitochondrial biosynthesis and function, which may further increase the proportion of slow muscle fibers.

CaN/NFAT is one of the most important pathways that regulates the transformation of skeletal muscle fiber types after animal birth [45]. In skeletal muscle, once the concentrations of intracytoplasmic  $\text{Ca}^{2+}$  elevates, the catalytic subtypes  $\text{CnA}\alpha$  of CaN will integrate  $\text{Ca}^{2+}$ , resulting in the excitation of CaN [46]. A previous study demonstrated that PPARGC1A, as the substrate of CaN, also mediated the conversion of muscle fibers from fast-twitch to slow-twitch through CaN/NFAT signaling pathway [47]. PPARGC1A directly interacts with myocyte enhancer factor 2 (MEF2), which physically binds to the NFATc1 site to upregulate the expression of slow-twitch fibers genes. [48]. Myogenic differentiation (MyoD) and myogenic factor 5 (Myf5), as the downstream genes of CaN/NFAT signal pathway, play a crucial role in regulating the proliferation and differentiation of muscle cells [49]. Our unpublished data found that GAA treatment significantly upregulated the mRNA expression of MyoD in *longissimus dorsi* muscle. Lu et al. [50] also found that dietary GAA enhanced the expression of MyoD and Myf5 in *longissimus thoracis* muscle of finishing pigs. In our previous results (unpublished data), GAA addition upregulated the mRNA expressions of *CnA* and *NFATc1* in *semitendinosus* muscle. Moreover, in the current study, the protein expressions of  $\text{CnA}\alpha$  and NFATc1 were upregulated in the muscle of finishing pigs from the GAA supplementation groups, which were also in accordance with the increased expression of PPARGC1A.

## 5. Conclusions

Dietary GAA supplementation promotes the skeletal muscle fiber type transformation from fast-to-slow-twitch in finishing pigs by increasing the expression of PPARGC1A to improve the mitochondrial function and the mitochondrial biosynthesis, and by upregulating the key factors of CaN/NFAT pathway. These findings may expand our knowledge regarding the function of dietary GAA in finishing pigs.

**Supplementary Materials:** The following supporting information can be downloaded at: <http://www.mdpi.com/xxx/s1>. Figure S1: Effects of guanidinoacetic acid supplementation on the expressions of Troponin T3 of longissimus thoracis muscle in finishing pigs ( $n = 5$ ), Figure S2: Effects of guanidinoacetic acid supplementation on the expressions of Troponin T3 in *semitendinosus* muscle in finishing pigs ( $n = 5$ ).

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**Data Availability Statement:** The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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## References

1. Lefaucheur, L. A second look into fibre typing—relation to meat quality. *Meat Sci.* **2010**, *84*, 257–270. [\[CrossRef\]](#)
2. Talmadge, R.J.; Roy, R.R.; Edgerton, R.V. Muscle fiber types and function. *Curr. Opin. Rheumatol.* **1993**, *5*, 695–705. [\[CrossRef\]](#)
3. Schiaffino, S. Muscle fiber type diversity revealed by anti-myosin heavy chain antibodies. *FEBS J.* **2018**, *285*, 3688–3694. [\[CrossRef\]](#)
4. Men, X.M.; Deng, B.; Xu, Z.W.; Tao, X.; Qi, K.K. Age-related changes and nutritional regulation of myosin heavy-chain composition in longissimus thoracis of commercial pigs. *Animal* **2013**, *7*, 1486–1492. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Wei, B.; Jin, J.P. TNNT1, TNNT2, and TNNT3: Isoform genes, regulation, and structure-function relationships. *Gene* **2016**, *582*, 1–13. [\[CrossRef\]](#)
6. Xu, M.; Chen, X.; Huang, Z.; Chen, D.; Yu, B.; Chen, H.; Luo, Y.; Zheng, P.; Yu, J.; He, J. Grape seed proanthocyanidin extract promotes skeletal muscle fiber type transformation via AMPK signaling pathway. *J. Nutr. Biochem.* **2020**, *84*, 108462. [\[CrossRef\]](#)
7. Luo, P.; Luo, L.; Zhao, W.; Wang, L.; Sun, L.; Wu, H.; Li, Y.; Zhang, R.; Shu, G.; Wang, S.; et al. Dietary thymol supplementation promotes skeletal muscle fibre type switch in longissimus thoracis of finishing pigs. *J. Anim. Physiol. Anim. Nutr.* **2020**, *104*, 570–578. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Zeng, M.; He, Y.; Du, H.; Yang, J.; Wan, H. Output Regulation and Function Optimization of Mitochondria in Eukaryotes. *Front. Cell Dev. Biol.* **2020**, *8*, 598112. [\[CrossRef\]](#)
9. Shen, L.Y.; Luo, J.; Lei, H.G.; Jiang, Y.Z.; Bai, L.; Li, M.Z.; Tang, G.Q.; Li, X.W.; Zhang, S.H.; Zhu, L. Effects of muscle fiber type on glycolytic potential and meat quality traits in different Tibetan pig muscles and their association with glycolysis-related gene expression. *Genet. Mol. Res.* **2015**, *14*, 14366–14378. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Yan, Z.; Okutsu, M.; Akhtar, Y.N.; Lira, V.A. Regulation of exercise-induced fiber type transformation, mitochondrial biosynthesis, and angiogenesis in skeletal muscle. *J. Appl. Physiol.* **2011**, *110*, 264–274. [\[CrossRef\]](#)
11. Popov, D.V. Adaptation of Skeletal Muscles to Contractile Activity of Varying Duration and Intensity: The Role of PGC-1 $\alpha$ . *Biochemistry* **2018**, *83*, 613–628. [\[CrossRef\]](#)
12. Ying, F.; Zhang, L.; Bu, G.; Xiong, Y.; Zuo, B. Muscle fiber-type conversion in the transgenic pigs with overexpression of PGC-1 $\alpha$  gene in muscle. *Biochem. Biophys. Res. Commun.* **2016**, *480*, 669–674. [\[CrossRef\]](#)
13. Ducreux, S.; Gregory, P.; Schwaller, B. Inverse regulation of the cytosolic Ca<sup>2+</sup> buffer parvalbumin and mitochondrial volume in muscle cells via Sirt1/PGC-1 $\alpha$  axis. *PLoS ONE* **2012**, *7*, e44837. [\[CrossRef\]](#)
14. Chen, Z.; Tao, S.; Li, X.; Yao, Q. Resistin destroys mitochondrial biosynthesis by inhibiting the PGC-1 $\alpha$ /NRF1/TFAM signaling pathway. *Biochem. Biophys. Res. Commun.* **2018**, *504*, 13–18. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Luo, P.; Wang, L.; Luo, L.; Wang, L.; Yang, K.; Shu, G.; Wang, S.; Zhu, X.; Gao, P.; Jiang, Q. Ca<sup>2+</sup>-Calcineurin-NFAT pathway mediates the effect of thymol on oxidative metabolism and fiber-type switch in skeletal muscle. *Food Funct.* **2019**, *10*, 5166–5173. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Ostojic, S.M. Benefits and drawbacks of guanidinoacetic acid as a possible treatment to replenish cerebral creatine in AGAT deficiency. *Nutr. Neurosci.* **2019**, *22*, 302–305. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Edison, E.E.; Brosnan, M.E.; Meyer, C.; Brosnan, J.T. Creatine synthesis: Production of guanidinoacetate by the rat and human kidney in vivo. *Am. J. Physiol. Ren. Physiol.* **2007**, *293*, 1799–1804. [\[CrossRef\]](#)



18. Zhao, W.; Li, J.L.; Xing, T.; Zhang, L.; Gao, F. Effects of guanidinoacetic acid and complex antioxidant supplementation on growth performance, meat quality, and antioxidant function of broiler chickens. *J. Sci. Food Agric.* **2020**, *101*, 3961–3968. [\[CrossRef\]](#)
19. Volek, J.S.; Duncan, N.D.; Mazzetti, S.A.; Staron, R.S.; Putukian, M.; Gómez, A.L.; Pearson, D.R.; Fink, W.J.; Kraemer, W.J. Performance and muscle fiber adaptations to creatine supplementation and heavy resistance training. *Med. Sci. Sports Exerc.* **1999**, *31*, 1147–1156. [\[CrossRef\]](#)
20. Zhu, Z.; Gu, C.; Hu, S.; Li, B.; Zeng, X.; Yin, J. Dietary guanidinoacetic acid supplementation improved carcass characteristics, meat quality and muscle fibre traits in growing-finishing gilts. *J. Anim. Physiol. Anim. Nutr.* **2020**, *104*, 1454–1461. [\[CrossRef\]](#)
21. Wang, Y.; Liu, R.; Tian, X.; Fan, X.; Shi, Y.; Zhang, W.; Hou, Q.; Zhou, G. Comparison of Activity, Expression, and S-Nitrosylation of Calcium Transfer Proteins between Pale, Soft, and Exudative and Red, Firm, and Non-exudative Pork during Post-Mortem Aging. *J. Agric. Food Chem.* **2019**, *67*, 3242–3248. [\[CrossRef\]](#)
22. Serrano, A.L.; Petrie, J.L.; Rivero, J.L.; Hermanson, J.W. Myosin isoforms and muscle fiber characteristics in equine gluteus medius muscle. *Anat. Rec.* **1996**, *244*, 444–451. [\[CrossRef\]](#)
23. Bao, G.; Liu, X.; Wang, J.; Hu, J.; Shi, B.; Li, S.; Luo, Y. Effects of Slaughter Age on Myosin Heavy Chain Isoforms, Muscle Fibers, Fatty Acids, and Meat Quality in Longissimus Thoracis Muscle of Tibetan Sheep. *Front. Vet. Sci.* **2021**, *8*, 689589. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Reiser, P.J. Current understanding of conventional and novel co-expression patterns of mammalian sarcomeric myosin heavy chains and light chains. *Arch. Biochem. Biophys.* **2019**, *662*, 129–133. [\[CrossRef\]](#)
25. Orzuna-Orzuna, J.F.; Dorantes-Iturbide, G.; Lara-Bueno, A.; Mendoza-Martínez, G.D.; Miranda-Romero, L.A.; López-Ordaz, R.; Hernández-García, P.A. Productive Performance, Carcass Traits, and Meat Quality in Finishing Lambs Supplemented with a Polyherbal Mixture. *Agriculture* **2021**, *11*, 942. [\[CrossRef\]](#)
26. Muroya, S.; Nakajima, I.; Chikuni, K. Amino acid sequences of multiple fast and slow troponin T isoforms expressed in adult bovine skeletal muscles. *J. Anim. Sci.* **2003**, *81*, 1185–1192. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Selsby, J.T.; Morine, K.J.; Pendrak, K.; Barton, E.R.; Sweeney, H.L. Rescue of dystrophic skeletal muscle by PGC-1 $\alpha$  involves a fast to slow fiber type shift in the mdx mouse. *PLoS ONE* **2012**, *7*, e30063. [\[CrossRef\]](#)
28. He, X.; Duan, Y.; Yao, K.; Li, F.; Hou, Y.; Wu, G.; Yin, Y.  $\beta$ -Hydroxy- $\beta$ -methylbutyrate, mitochondrial biogenesis, and skeletal muscle health. *Amino Acids* **2016**, *48*, 653–664. [\[CrossRef\]](#)
29. Russell, A.P.; Feilchenfeldt, J.; Schreiber, S.; Praz, M.; Crettenand, A.; Gobelet, C.; Meier, C.A.; Bell, D.R.; Kralli, A.; Giacobino, J.P.; et al. Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle. *Diabetes* **2003**, *52*, 2874–2881. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Jäger, S.; Handschin, C.; St-Pierre, J.; Spiegelman, B.M. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1 $\alpha$ . *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 12017–12022. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Choi, Y.K.; Park, J.H.; Baek, Y.Y.; Won, M.H.; Jeoung, D.; Lee, H.; Ha, K.S.; Kwon, Y.G.; Kim, Y.M. Carbon monoxide stimulates astrocytic mitochondrial biogenesis via L-type Ca<sup>2+</sup> channel-mediated PGC-1 $\alpha$ /ERR $\alpha$  activation. *Biochem. Biophys. Res. Commun.* **2016**, *479*, 297–304. [\[CrossRef\]](#)
32. Brookes, P.S.; Yoon, Y.; Robotham, J.L.; Anders, M.W.; Sheu, S.S. Calcium, ATP, and ROS: A mitochondrial love-hate triangle. *Am. J. Physiol. Cell Physiol.* **2004**, *287*, C817–C833. [\[CrossRef\]](#) [\[PubMed\]](#)
33. He, D.T.; Gai, X.R.; Yang, L.B.; Li, J.T.; Lai, W.Q.; Sun, X.L.; Zhang, L.Y. Effects of guanidinoacetic acid on growth performance, creatine and energy metabolism, and carcass characteristics in growing-finishing pigs. *J. Anim. Sci.* **2018**, *96*, 3264–3273. [\[CrossRef\]](#)
34. Papa, S.; Martino, P.L.; Capitanio, G.; Gaballo, A.; De Rasmio, D.; Signorile, A.; Petruzzella, V. The oxidative phosphorylation system in mammalian mitochondria. *Adv. Exp. Med. Biol.* **2012**, *942*, 3–37. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Venhoff, N.; Lebrecht, D.; Pfeifer, D.; Venhoff, A.C.; Bissé, E.; Kirschner, J.; Walker, U.A. Muscle-fiber transdifferentiation in an experimental model of respiratory chain myopathy. *Arthritis Res. Ther.* **2012**, *14*, R233. [\[CrossRef\]](#)
36. Rasbach, K.A.; Gupta, R.K.; Ruas, J.L.; Wu, J.; Naseri, E.; Estall, J.L.; Spiegelman, B.M. PGC-1 $\alpha$  regulates a HIF-2 $\alpha$ -dependent switch in skeletal muscle fiber types. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 21866–21871. [\[CrossRef\]](#)
37. Lin, J.; Wu, H.; Tarr, P.T.; Zhang, C.Y.; Wu, Z.; Boss, O.; Michael, L.F.; Puigserver, P.; Isotani, E.; Olson, E.N.; et al. Transcriptional co-activator PGC-1 $\alpha$  drives the formation of slow-twitch muscle fibres. *Nature* **2002**, *418*, 797–801. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Casas, F.; Pesseme, L.; Grandemange, S.; Seyer, P.; Gueguen, N.; Baris, O.; Lepourry, L.; Cabello, G.; Wrutniak-Cabello, C. Overexpression of the mitochondrial T3 receptor p43 induces a shift in skeletal muscle fiber types. *PLoS ONE* **2008**, *3*, e2501. [\[CrossRef\]](#)
39. Teodoro, J.S.; Palmeira, C.M.; Rolo, A.P. Mitochondrial Membrane Potential ( $\Delta\Psi$ ) Fluctuations Associated with the Metabolic States of Mitochondria. *Methods Mol. Biol.* **2018**, *1782*, 109–119. [\[CrossRef\]](#)
40. Bagkos, G.; Koufopoulos, K.; Piperi, C. A new model for mitochondrial membrane potential production and storage. *Med. Hypotheses* **2014**, *83*, 175–181. [\[CrossRef\]](#)
41. Guo, R.; Gu, J.; Zong, S.; Wu, M.; Yang, M. Structure and mechanism of mitochondrial electron transport chain. *Biomed. J.* **2018**, *41*, 9–20. [\[CrossRef\]](#)
42. Kang, I.; Chu, C.T.; Kaufman, B.A. The mitochondrial transcription factor TFAM in neurodegeneration: Emerging evidence and mechanisms. *FEBS Lett.* **2018**, *592*, 793–811. [\[CrossRef\]](#)
43. Mulder, H. Transcribing  $\beta$ -cell mitochondria in health and disease. *Mol. Metab.* **2017**, *6*, 1040–1051. [\[CrossRef\]](#)



44. Wenz, T. Regulation of mitochondrial biosynthesis and PGC-1 $\alpha$  under cellular stress. *Mitochondrion* **2013**, *13*, 134–142. [[CrossRef](#)]
45. Liu, W.; Chen, G.; Li, F.; Tang, C.; Yin, D. Calcineurin-NFAT Signaling and Neurotrophins Control Transformation of Myosin Heavy Chain Isoforms in Rat Soleus Muscle in Response to Aerobic Treadmill Training. *J. Sports Sci. Med.* **2014**, *13*, 934–944.
46. Parsons, S.A.; Wilkins, B.J.; Bueno, O.F.; Molkentin, J.D. Altered skeletal muscle phenotypes in calcineurin Aalpha and Abeta gene-targeted mice. *Mol. Cell. Biol.* **2003**, *23*, 4331–4343. [[CrossRef](#)] [[PubMed](#)]
47. Roberts-Wilson, T.K.; Reddy, R.N.; Bailey, J.L.; Zheng, B.; Ordas, R.; Gooch, J.L.; Price, S.R. Calcineurin signaling and PGC-1alpha expression are suppressed during muscle atrophy due to diabetes. *Biochim. Biophys. Acta* **2010**, *1803*, 960–967. [[CrossRef](#)] [[PubMed](#)]
48. Wu, H.; Rothermel, B.; Kanatous, S.; Rosenberg, P.; Naya, F.J.; Shelton, J.M.; Hutcheson, K.A.; DiMaio, J.M.; Olson, E.N.; Bassel-Duby, R.; et al. Activation of MEF2 by muscle activity is mediated through a calcineurin-dependent pathway. *EMBO J.* **2001**, *20*, 6414–6423. [[CrossRef](#)] [[PubMed](#)]
49. Zammit, P.S. Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. *Semin. Cell Dev. Biol.* **2017**, *72*, 19–32. [[CrossRef](#)]
50. Lu, Y.; Zou, T.; Wang, Z.; Yang, J.; Li, L.; Guo, X.; He, Q.; Chen, L.; You, J. Dietary guanidinoacetic acid improves the growth performance and skeletal muscle development of finishing pigs through changing myogenic gene expression and myofibre characteristics. *J. Anim. Physiol. Anim. Nutr.* **2020**, *104*, 1875–1883. [[CrossRef](#)] [[PubMed](#)]