Food Science and Biotechnology
https://doi.org/10.1007/s10068-024-01606-z

RESEARCH ARTICLE

Fermented antler extract attenuates muscle atrophy by regulating the PI3K/Akt pathway and inflammatory response in immobilization-treated C57BL/6J mice

Jihee Yoo[1] · Changhee Kim[2] · Hyerin Lee[1] · Bong Soo Ko[3] · Dong-Woo Lee[1,2] · Jae-Kwan Hwang[1,2]

Received: 26 February 2024 / Revised: 4 May 2024 / Accepted: 16 May 2024

Abstract

Muscle atrophy or muscle wasting, which is featured by reduced muscle function and mass, typically results from disuse, aging, and chronic diseases. The deer antler, which refers to the young and non-ossified antlers of various species of deer-related animals, is not fully calcified and comprises of densely growing hair. Here, we investigated whether *Bacillus subtilis*-fermented antler extract (FAE) inhibits immobilization-induced muscle atrophy in C57BL/6J mice. Oral administration of FAE increased grip strength, exercise performance, muscle mass, and volume in mice. FAE stimulated the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, enhancing the mammalian target of rapamycin pathway for muscle synthesis. FAE phosphorylated Forkhead box O3 and downregulated muscle RING finger-1 and atrogin-1 for proteolysis. FAE inhibited the mRNA expression of tumor necrosis factor alpha and interleukin-6 through nuclear factor kappa B. Consequently, FAE attenuated muscle atrophy by regulating the PI3K/Akt pathway and inflammation.

Keywords Antler · Fermentation · Immobilization · Muscle atrophy

Introduction

Skeletal muscle stands as the largest organ, accounting for approximately 40% of the body weight, making it pivotal and fundamental to maintaining human physiological health. Physiologically, skeletal muscles play two major roles in the body (Frontera and Ochala, 2015; Kim and Hwang, 2020). First, similar to the adipose and hepatic tissues, skeletal muscles metabolize fatty acids, glucose, and proteins. Thus, skeletal muscle is a target organ for the attenuation of metabolic diseases, such as obesity and diabetes. Second, by producing energy through nutrient catabolism, skeletal muscles are used to perform physical activities, such as locomotion, walking, swimming, and running (Kim and Hwang, 2020). Muscle atrophy or muscle wasting, which is featured by the loss of muscle strength and mass, is a crucial factor that impairs skeletal muscle function. The three major causes of muscle atrophy are aging, disuse (lack of physical activity), and diseases, such as cancer and metabolic diseases (Bowen et al., 2015). Thus, a biological and physiological understanding and elucidation of the development of muscle wasting, as well as knowing how the three causes aggravate muscle wasting, are important to alleviate the symptoms and improve muscle health and quality of human life.

✉ Dong-Woo Lee
leehicam@yonsei.ac.kr

✉ Jae-Kwan Hwang
jkhwang@yonsei.ac.kr

Jihee Yoo
yojihee05@gmail.com

Changhee Kim
changgml@gmail.com

Hyerin Lee
hshs7915@naver.com

Bong Soo Ko
bsko@biocare.co.kr

[1] Department of Bioindustrial Engineering, Yonsei University, Seoul, Republic of Korea

[2] Department of Biotechnology, Yonsei University, Seoul, Republic of Korea

[3] Research and Development Center, Biocare Corporation, Yangju, Republic of Korea

Published online: 13 June 2024

Muscle atrophy is closely related to the skeletal muscle protein content. Approximately 50–75% of all body proteins are stored in the skeletal muscle. Thus, the skeletal muscle is an established reservoir of proteins or amino acids (Frontera and Ochala, 2015). The balance between protein synthesis and degradation determines the skeletal muscle mass. Muscle wasting occurs when this balance is disrupted in abnormal states, particularly when the rate of protein catabolism exceeds that of protein anabolism (Bowen et al., 2015). Several molecular mechanisms have been suggested to regulate the homeostasis of the protein as a potential therapeutic strategy, including the phosphatidylinositol 3-kinase (PI3K) pathway or target molecules, such as myostatin and tumor necrosis factor-alpha (TNF-α) (Bowen et al., 2015; Huang et al., 2022; Kim and Hwang, 2020).

Despite the struggle to elucidate the molecular mechanisms and develop pharmacological agents, there have been no Food and Drug Administration-approved drugs for muscle atrophy owing to safety issues and low efficacy. Exercises, such as resistance and strength training, and/or dietary supplements, including proteins and amino acids, have been suggested as interventions to increase muscle weight or to prevent or/and inhibit the development of muscle wasting (Jang et al., 2023; Kadakia et al., 2023). Therefore, plant extracts, food-derived ingredients, and phytochemicals have received attention as safe interventions for treating muscle atrophy. In particular, molecular targets, which are related to protein turnover, have been the focus of research for their development against muscle atrophy and muscle loss (Kim and Hwang, 2020; Wang et al., 2021).

Deer antler, known as "nok-yong" (*Cervus elaphus,* antler), refers to the young and non-ossified antlers of various species of deer-related animals. These antlers are not fully calcified and comprise of densely grown hair. They regenerate cartilaginous tissue annually (Kim and Rhyu, 2000). Various therapeutic effects of antler extracts have been reported, including anti-osteoporotic (Kim et al., 2014a) and memory-improvement effects (Lee et al., 2009). The antler extract stimulated muscle differentiation, inhibited muscle atrophy in C2C12 myoblasts (Jo et al., 2021), and increased grip strength in mice (Huang et al., 2014). Fermentation has been actively considered as a treatment process for enhancing antler utilization (Kim et al., 1994, 2009). Antler fermentation has been reported to raise the extraction yield, efficacy, and content of active compounds (Kim et al., 2009). Previous studies have also reported that use of fermented antlers exhibit superior physiological activity and efficacy, compared to the use of non-fermented antlers (Choi et al., 2013; Kim et al., 2009). With respect to its efficacy in treating muscle atrophy, intake of *Lactobacillus curvatus*-fermented antler extract increased grip strength and muscle weight in middle-aged (Kim et al., 2021) and dexamethasone (DEX)-treated mice (Jeon et al., 2022). However, the inhibitory effect of *Bacillus subtilis*-fermented antler extract (FAE) on the development of muscle atrophy has not yet been demonstrated. In this study, we investigated whether FAE attenuates muscle atrophy in immobilization-treated C57BL/6J mice. In addition, we compared efficacy of non-fermented antler extract (NFAE) and FAE with respect to treating muscle atrophy.

Materials and methods

Sample preparation

The samples used in this study were obtained from Biocare (Yangju, Korea). Dried antlers (*Cervus elaphus*) were purchased from Provelco Cooperative Ltd. (Christchurch, New Zealand). After grinding the dried antler, it was extracted with water at 110 °C for 6 h. Subsequently, *B. subtilis* SST9960 (Biocare) was inoculated and fermented at 30 °C for 72 h. Sterilization was performed to stop the fermentation. The samples were then filtered, concentrated, and dried. The fermented extract was designated FAE, whereas the extract obtained without fermentation was designated NFAE.

Animal experiment

Eight-week-old C57BL/6J mice (Daehan Biolink, Chungcheong, Korea) were acclimatized to the environment for 1 week, and then used in the experiment. Mice were assigned to five groups. To induce muscle atrophy, the right leg of the mice was immobilized according to a previously described method (Caron et al., 2011; Kim et al., 2020). Immobilization was applied to all groups, except for the control group (CON). After 1 week, the staple was removed and NFAE at 300 mg/kg/day (NFAE300) and FAE at 300 mg/kg/day (FAE300) and 600 mg/kg/day (FAE600) were orally administered daily for the next 1 week. Mice in both the CON and only immobilization-treated group (IMM) were administered saline instead of samples. After oral administration, mice were sacrificed by exsanguination under anesthesia with 2,2,2-tribromoethanol (Sigma-Aldrich, St Louis, MO, USA). The tibialis anterior (TA), extensor digitorum longus (EDL), soleus, and gastrocnemius (GAS) muscles were weighed. TA muscle was frozen in liquid nitrogen and stored at -70 °C for further analysis to elucidate the underlying molecular mechanism.

The experimental environment was controlled and maintained at relative humidity of 55 ± 5% and temperature of 25 ± 2 °C. The day and night were adjusted in a 12 h cycle. The mice had free access to tap water and a standard diet (EEGJ30060; Cargill Agri Purina, Seongnam, Korea; 4.5% fat, 20% protein, 8% fiber, 12% moisture,

etc.). This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei Laboratory Animal Research Center (permit number: IACUC-A-201901-847-02).

Physical activity

Physical activity was evaluated using a treadmill device (LE8710MTS; Panlab, Barcelona, Spain) for running tests, and a Chatillon force measurement system (Columbus Instrument, Columbus, OH, USA) was used to measure grip strength. Each experiment was performed as previously described (Kim et al., 2020).

Micro-computed tomography imaging

Under anesthesia, the volumes of the mice were measured using positron emission tomography and single-photon emission computed tomography system (Siemens Inveon, Knoxville, TN, USA).

Histological analysis

Isolated TA muscles were fixed in 10% formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. Random areas of stained tissues were examined and photographed using a CK40 inverted microscope (Olympus, Tokyo, Japan) equipped with a T500 camera (magnification, 200X; eXcope, Daejeon, Korea). The cross-sectional areas (CSA) of the captured images were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

TA was lysed in a lysis buffer (ELPIS-Biotech, Daejeon, Korea) containing a protease inhibitor cocktail (Sigma-Aldrich). The protein concentration of the lysate was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by electrophoresis using sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes. The membranes, which were blocked with 5% skim milk for 1 h at room temperature, were incubated overnight with primary antibodies including α-tubulin, Forkhead box O3 (FoxO3), phospho (p)–FoxO3 (Thr32), mammalian target of rapamycin (mTOR), p–mTOR (Ser2448), 70-kDa ribosomal protein S6 kinase (p70S6K), p–p70S6K (Thr389), eukaryotic initiation factor 4E binding protein 1 (4E-BP1), p-4E-BP1 (Thr37/46), PI3K, p-PI3K (Tyr458), Akt, and p-Akt (Ser473) (Cell Signaling Technology, Beverly, MA, USA) and nuclear factor kappa B (NF-κB) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Bethyl Laboratories, Inc., Montgomery, TX, USA). Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Little Chalfont, UK) and captured using GeneSnap program and G:BOX EF imaging system (Syngene, Cambridge, UK). Protein band intensities on each blot were quantified by densitometric analysis using the ImageJ software (National Institutes of Health).

Reverse transcription-polymerase chain reaction

RNA was isolated from TA muscle tissue with the TRIzol reagent (Takara, Otsu, Japan). The cDNA was synthesized from RNA using the reverse transcription premix (ELPIS-Biotech). PCR amplification was conducted using the synthesized cDNA, primer pairs (Bioneer, Daejeon, Korea) and PCR premix (ELPIS-Biotech). The Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) was used for cDNA synthesis and amplification. For muscle RING finger-1 (MuRF-1), the primer sequences were 5′-CGTGAC CACAGAGGGTAAAGAA-3′ (forward) and 5′-GCACCA ACGTAGAAAAGTGTCC-3′ (reverse). For atrogin-1, the primer sequences used were 5′-ATCCTTATGCACGCT GGTCC-3′ (forward) and 5′-CTCTTCCACAGTAGCCGG TC-3′ (reverse). For TNF-α, the primer sequences used were 5′-CCGATGGGTTGTACCTTGTC-3′ (forward) and 5′-TGG AAGACTCCTCCCAGGTA-3′ (reverse). For interleukin-6 (IL-6), the primer sequences used were 5′-AGTTGCCTT CTTGGGACTGA-3′ (forward) and 5′-TCCACGATTTCC CAGAGAAC-3′ (reverse). For β-actin, the primer sequence used were 5′-GAAGGAGATTACTGCTCTGGCTC-3′ (forward) and 5′-CTCAGTAACAGTCCGCCTAGAA-3′ (reverse). The reaction mixtures were incubated for an initial denaturation at 94 °C for 10 min, followed by 30–35 cycles at 94 °C for 30 s for denaturation, at 56 °C for 1 min for annealing, and at 72 °C for 1 min for extension. PCR products were separated by agarose gel electrophoresis. Each band was visualized and captured using GeneSnap program and G:BOX EF imaging system (Syngene). PCR bands were quantified by densitometric analysis using the ImageJ software (National Institutes of Health).

Statistical analysis

Data are expressed as mean ± standard deviation (SD) and analyzed using analysis of variance (ANOVA) followed by Duncan's test (SPSS version 23.0; SPSS Inc., Chicago, IL, USA). Differences between groups were considered significant at $p < 0.05$.

Results and discussion

Effects of NFAE and FAE on physical activity

Physical activity was evaluated concerning grip strength, distance, and running time. The IMM group exhibited significantly lower grip strength in the fore/hindlimb than the CON group. However, the NFAE300, FAE300, and FAE600 groups showed remarkably elevated grip strength in the fore/hindlimb by 10.53%, 21.29%, and 28.92%, respectively, compared with the IMM group. Additionally, compared to the NFAE300 group, the grip strengths of the FAE300 and FAE600 groups were significantly increased. The grip strength of the forelimbs in the sample-treated groups was significantly higher than that of the IMM group (Fig. 1A). The IMM group showed a significant decrease in both exercise distance and exercise time by 31.83% and 24.31%, respectively, compared to the CON group, while the FAE treatment remarkably increased immobilization-reduced exercise distance and time. However, the administration of NFAE at 300 mg/kg/day did not significantly influence exercise capacity. Compared with the NFAE group, the FAE-treated groups did not show significant differences (Fig. 1B, C).

Mitochondria are major organelles that produce energy for muscle activity. Mitochondrial dysfunction results in increased reactive oxygen species (ROS) levels and induces oxidative stress, muscle cell apoptosis, and muscle atrophy (Calvani et al., 2013). Thus, several studies have shown that regulating mitochondrial function and/or stimulating mitochondrial biogenesis is a potential target for preventing and inhibiting sarcopenia and disuse muscle atrophy (Calvani et al., 2013; Kim and Hwang, 2020). Peroxisome

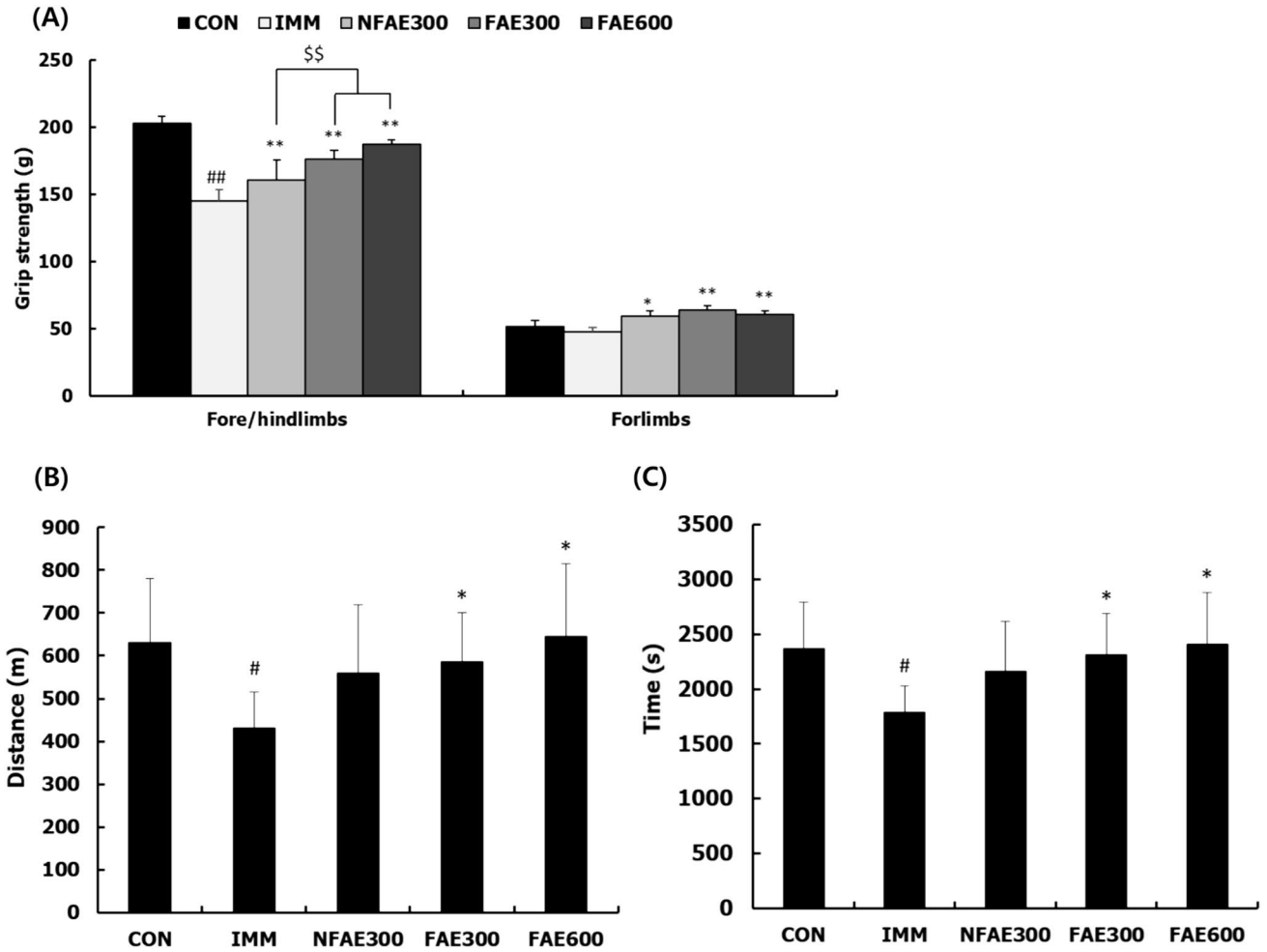

Fig. 1 Effects of non-fermented and fermented antler extracts on physical activities. Muscle atrophy was induced in the right leg of C57BL/6J mice by immobilization for 1 week. Following immobilization, saline, NFAE (300 mg/kg/day), FAE (300 and 600 mg/kg/day) was orally administered for 1 week. The grip strength of the fore/hindlimbs and forelimbs (**A**); running distance (**B**) and running time on the treadmill (**C**). Data are presented as mean ± SD. Group differences were assessed by Duncan's multiple range test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus CON group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus IMM group, $^{\$\$}p < 0.01$ versus NFAE300

proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) is a major target protein to regulate mitochondrial biogenesis (Kim and Hwang, 2020). Dihydromyricetin ameliorated DEX-induced muscle atrophy by activating PGC-1α and restoring mitochondrial function and mitochondrial biogenesis (Huang et al., 2018). Morin prevented DEX-induced muscle atrophy by decreasing the production of ROS and increasing the PGC-1α protein expression (Ulla et al., 2021). Mitochondrial biogenesis and its major regulator, PGC-1α, are closely related with exercise endurance (Kim et al., 2018). In this study, FAE significantly increased the running distance and time in immobilization-treated mice (Fig. 1B, C). Previous study presented that antler extract upregulated PGC-1α and sirtuin 1 in C2C12 myoblasts (Jung et al., 2021). Thus, improving the function of mitochondria in muscle through PGC-1α might be involved in the attenuating effect of FAE on muscle atrophy.

Effects of NFAE and FAE on muscle volume, CSA, and muscle mass

No significant differences in body weight were observed between the groups. However, compared to the CON group, body weight was slightly decreased by 1.38% in the IMM group; however, it was slightly increased by 1.21% in the FAE600 group, compared to the IMM group. Liver and spleen weights were not significantly different between the groups. Immobilization treatment significantly decreased the GAS, soleus, and TA muscle weights by 26.00%, 19.6%, and 28.90%, respectively, compared to those in the CON group. However, sample treatments did not affect the soleus muscle weight despite a slight increase. NFAE and FAE treatments remarkably increased GAS and TA muscle weights compared to those in the IMM group. A comparing between the NFAE and FAE groups showed that among the four muscles, only the GAS muscle weight significantly increased. EDL muscle weight was not significantly different between the groups (Table 1).

In this study, we treated immobilization to the right leg of mice to induce muscle atrophy of right leg, indicating that muscle atrophy was not occurred in other parts of entire body. Significant difference of the body weight among groups was not exhibited. In terms of muscle weights, compared to the CON group, immobilization remarkably reduced GAS, TA, and soleus muscle (Table 1). This is consistent with previous study that in the same model, immobilization treatment for 1 week did not affect the body weight but decreased muscle mass of immobilized leg (Caron et al., 2011).

Muscle volume was measured using microcomputed tomography. Representative images of the muscle volume are shown in Fig. 2A. Compared to the CON group, the IMM group exhibited a significant decrease by 17.95% in muscle volume. Compared to the IMM group, the NFAE300, FAE300, and FAE600 groups showed a significant increase in muscle volume. Additionally, compared to the NFAE300 group, FAE treatment significantly increased muscle volume (Fig. 2B). Representative images of the histological analysis of the CSA are shown in Fig. 2C. The CSA of the TA muscle significantly decreased by 34.64% in the IMM group, compared to that in the CON group. NFAE treatment at 300 mg/kg/day remarkably increased the immobilization-reduced CSA by 59.49%. FAE treatment at 300 and 600 mg/kg/day resulted in significant concentration-dependent increases in the immobilization-decreased CSA of 86.24% and 113.07%, respectively. The CSA of the TA muscle was significantly higher in the FAE300 and FAE600 groups than that in the NFAE300 group (Fig. 2D).

The major hallmarks of muscle atrophy are loss of muscle mass and strength (Kim et al., 2020). Aging (sarcopenia), disease (cachexia), and disuse (inactivity) are the major causes of muscle atrophy (Kim and Hwang, 2020). Although these three causes are distinct, they all contribute to muscle wasting through complex interactions. In particular, disuse increases the rate of muscle mass loss in patients with sarcopenia and other diseases because of the lack of physical activity and prolonged hospital stay (Bowen et al., 2015). In this study, we used an immobilization-treated mouse model to investigate the effects of FAE on muscle atrophy. Previous studies have shown that grip strength, muscle weight, and

Table 1 Body weight and liver, spleen, and muscle weights

Parameters	CON	IMM	NFAE300	FAE300	FAE600
Body weight (g)	24.24 ± 0.94	23.91 ± 1.10	23.81 ± 0.60	23.61 ± 0.49	24.2 ± 0.73
Liver (mg)	1145.1 ± 135.12	1162 ± 100.75	1109.9 ± 85.58	1087 ± 158.04	1060.9 ± 79.76
Spleen (mg)	55.2 ± 6.55	55.4 ± 8.4	59.5 ± 8.96	54.8 ± 11.55	56.7 ± 6.63
Gastrocnemius muscle (mg)	130.78 ± 10.64	$96.78 \pm 5.32^{\#\#}$	$105.99 \pm 8.33^{*}$	$120.41 \pm 8.40^{**,\$\$}$	$120.51 \pm 4.30^{**,\$\$}$
Soleus muscle (mg)	7.05 ± 0.99	$5.01 \pm 1.42^{\#}$	5.23 ± 1.31	6.66 ± 1.18	6.61 ± 0.67
Tibialis anterior muscle (mg)	50.50 ± 2.86	$40.59 \pm 7.50^{\#\#}$	$45.99 \pm 4.03^{*}$	$46.61 \pm 3.20^{*}$	$48.92 \pm 3.56^{**}$
Extensor digitorum longus muscle (mg)	18.73 ± 2.01	17.8 ± 2.91	17.58 ± 1.82	19.48 ± 2.77	18.4 ± 2.00

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus CON group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus IMM group, $^{\$\$}p < 0.01$ versus NFAE300

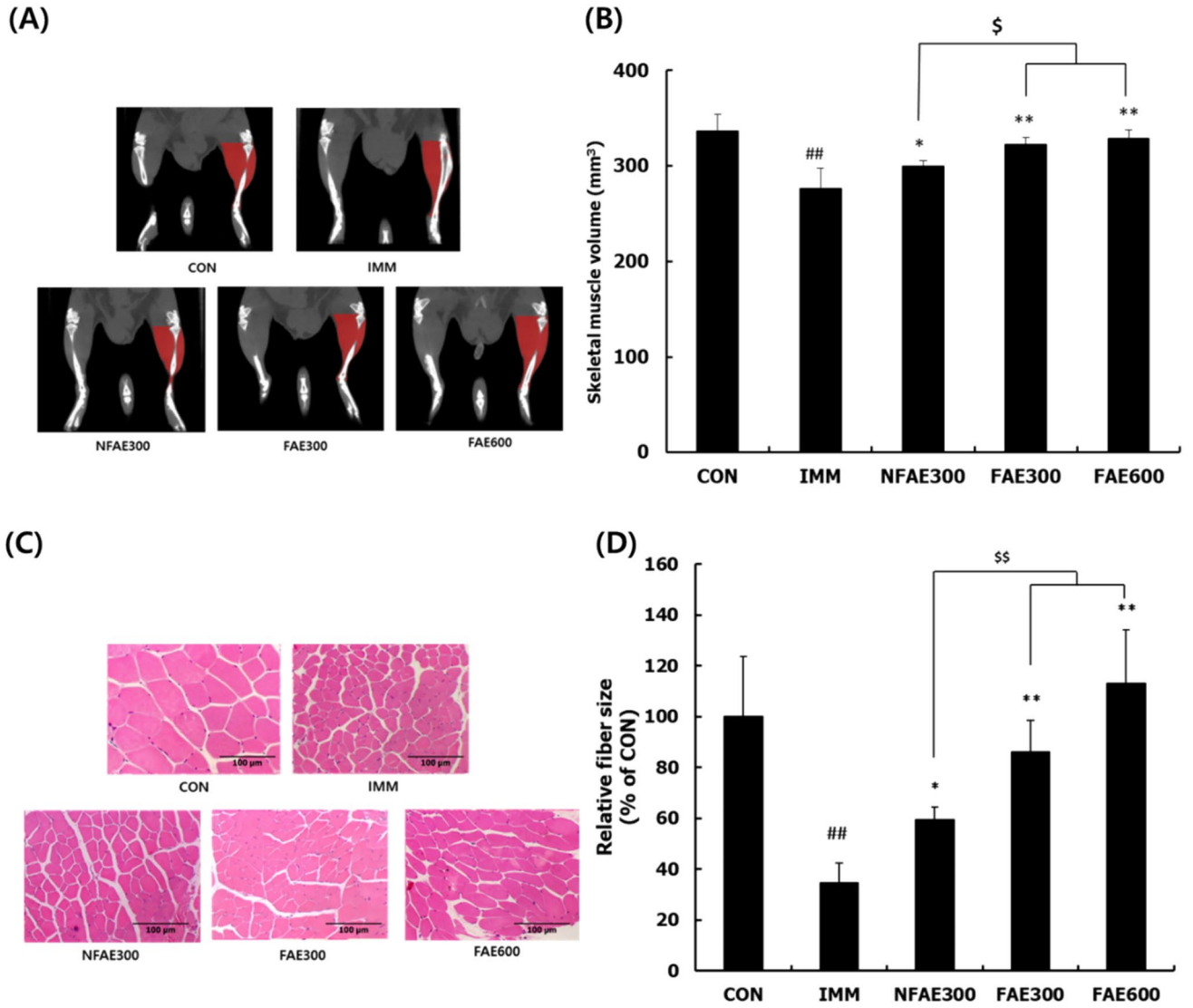

Fig. 2 Effects of non-fermented and fermented antler extracts on muscle volume, and cross-sectional area. Muscle atrophy was induced in the right leg of C57BL/6J mice by immobilization for 1 week. Following immobilization, saline, NFAE (300 mg/kg/day), FAE (300 and 600 mg/kg/day) was orally administered for 1 week. Representative micro-computed tomography images (**A**); quantified muscle volume of hindlimb (**B**); representative images of TA muscles stained with hematoxylin and eosin (magnification, X200) (**C**); quantified cross-sectional area of the TA muscle (**D**). Data are presented as mean ± SD. Group differences were assessed by Duncan's multiple range test. $^{\#\#}p < 0.01$ versus CON group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus IMM group, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ versus NFAE300

myofiber CSA reduced in immobilized mice (Caron et al., 2011; Kim et al., 2020). Immobilization consistently reduced grip strength, running time, distance, muscle weight, and CSA in this study. Both the NFAE and FAE treatments significantly increased the immobilization-reduced grip strength of the fore/hindlimbs, muscle volume, muscle mass, and CSA. Compared to the NFAE group, fermentation of the antler extract significantly enhanced the efficacy of NFAE (Figs. 1, 2, Table 1). These results suggest that antler extract can alleviate immobilization-induced muscle atrophy by increasing muscle mass and strength, and that the fermentation process further augments its effectiveness.

Effects of NFAE and FAE on protein synthesis-related and proteolysis-related pathways

In the TA muscle, the protein expression of p-PI3K and p-Akt was significantly lower in the IMM group than that in the CON group. However, treatments with NFAE and FAE significantly restored their expression. The p-PI3K protein expression in the NFAE300 group was significantly different, compared to FAE600 group but not FAE300 group. The FAE300 and FAE600 groups showed remarkably higher p-Akt expression levels than the NFAE group (Fig. 3A). Immobilization significantly decreased the protein

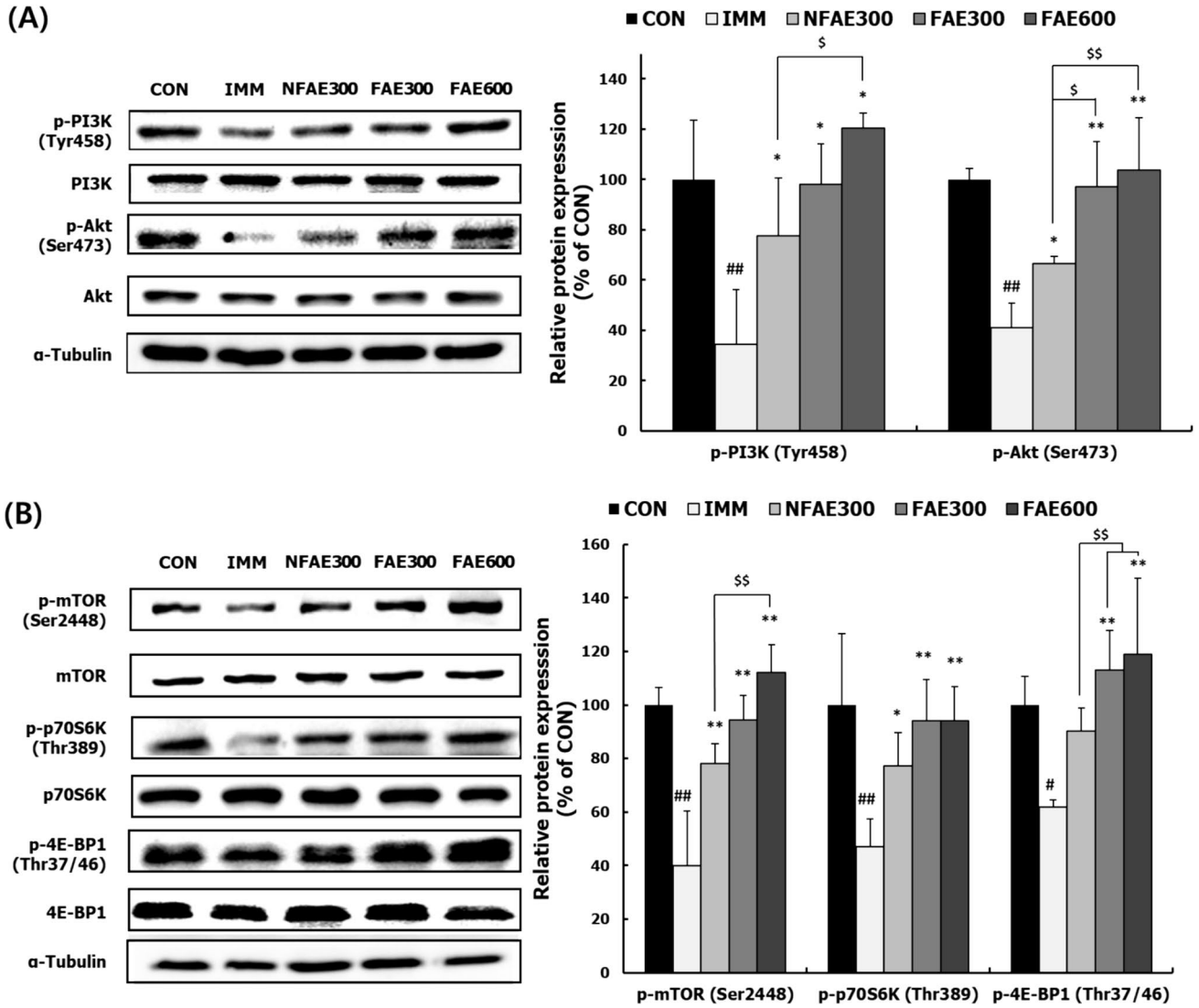

Fig. 3 Effects of non-fermented and fermented antler extracts on protein synthesis-related biomarkers. At the end of animal experiment, total proteins were isolated from the right TA muscle. p-PI3K, PI3K, p-Akt, and Akt protein expression was determined by Western blotting (**A**); p-mTOR, mTOR, p-4E-BP1, 4E-BP1, p-p70S6K, and p70S6K protein expression was determined by Western blotting (**B**). α-Tubulin was used as an internal control. Data are presented as mean ± SD. Group differences were assessed by Duncan's multiple range test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus CON group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus IMM group, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ versus NFAE300

expression of p-mTOR, p-p70S6K, and p-4E-BP1 compared to the CON group. However, FAE treatment significantly increased the immobilization-reduced protein expression of p-mTOR, p-p70S6K, and p-4E-BP1, and NFAE administration remarkably increased the protein expression of p-mTOR and p-p70S6K but not p-4E-BP1. In terms of p-p70S6K expression, there was no difference between the NFAE and FAE groups, whereas the protein expression of p-mTOR and p-4E-BP1 was significantly upregulated in the FAE600 group compared to that in the NFAE300 group (Fig. 3B).

The protein expression of p-FoxO3 and mRNA expression of MuRF-1 and atrogin-1 were assessed in the TA muscle tissue of mice. Compared to the CON group, the IMM group showed a significant decrease in p-FoxO3 protein expression. Although the average protein expression of p-FoxO3 was increased in both the NFAE300 and FAE300 groups, the difference was not significant. However, the protein expression of p-FoxO3 in the FAE600 group was significantly increased to a level similar to that in the CON group, compared to the IMM and NFAE300 groups (Fig. 4A). The mRNA expression of MuRF-1, which was significantly increased immobilization, was markedly reduced by the NFAE and FAE treatments. Although atrogin-1 mRNA expression did not differ significantly between the IMM and NFAE groups, FAE treatment markedly decreased its expression in a dose-dependent manner. Compared to

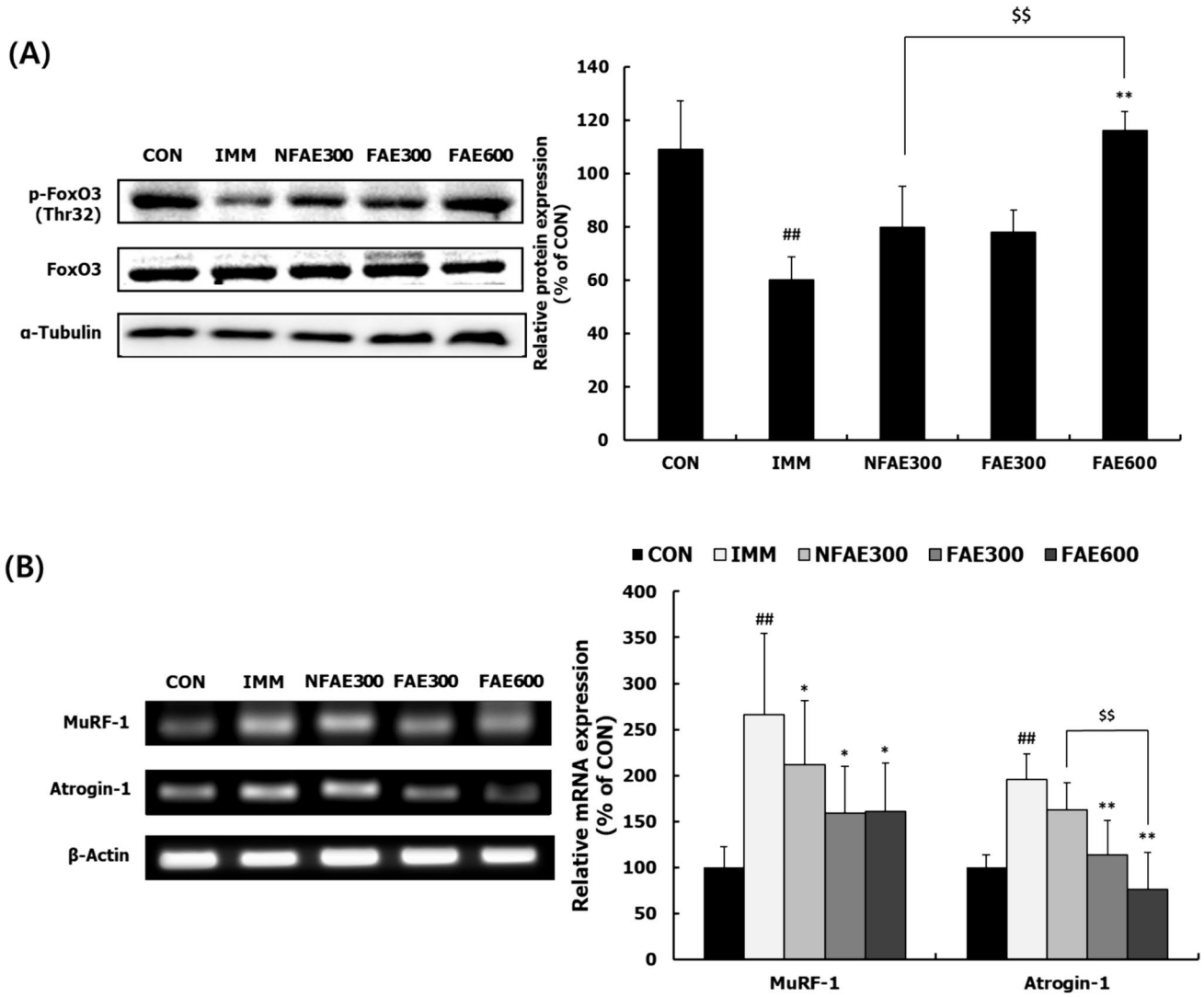

Fig. 4 Effects of non-fermented and fermented antler extracts on proteolysis-related biomarkers. At the end of animal experiment, total mRNA or proteins were isolated from the right TA muscle. p-FoxO3 and FoxO3 protein expression was determined by Western blotting (**A**); MuRF-1 and atrogin-1 mRNA expression was analyzed by RT-PCR (**B**). α-Tubulin and β-actin was used as an internal control. Data are presented as mean ± SD. Group differences were assessed by Duncan's multiple range test. $^{\#\#}p < 0.01$ versus CON group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus IMM group, $^{\$\$}p < 0.01$ versus NFAE300

the NFAE300 group, the FAE600 group, however, not the FAE300 group, showed a significant difference in atrogin-1 mRNA expression (Fig. 4B).

Several studies have suggested the molecular mechanisms for the development of agents that alleviate muscle atrophy (Huang et al., 2022; Kim and Hwang, 2020). The PI3K/Akt signaling is a molecular cascade that simultaneously regulates protein synthesis and degradation. When Tyr458 of PI3K and Ser473 of Akt are phosphorylated, the PI3K/Akt pathway is activated, leading to increased phosphorylation of mTOR at Ser2448 (Shao et al., 2020). Phosphorylated mTOR not only increases the phosphorylation of 4E-BP1 at the Thr37/46 site, thereby freeing eukaryotic translation initiation factor 4B (eIF4B) and enhancing the initiation of translation, but also activates ribosomal protein S6 or a part of the translational machinery by phosphorylating sites at Thr389 on p70S6K; thus, protein anabolism is stimulated (Figueiredo et al., 2017; Kim and Hwang, 2020). When FoxO3 acts as a transcription factor, it is translocated from the cytoplasm to the nucleus, where atrogin-1 and MuRF-1 are upregulated. Both atrogin-1 and MuRF-1 stimulate the attachment of ubiquitin to target proteins, and polyubiquitin-attached proteins are degraded into amino acids via the 26S proteasome. However, activated Akt, which is phosphorylated at Ser473, blocks FoxO3 translocation to the nucleus by phosphorylating Thr32 of FoxO3 (Ballesteros-Álvarez and Andersen, 2021). As phosphorylated FoxO3 cannot enter the nucleus, proteolysis-related processes are blocked (Bowen

et al., 2015; Kim et al., 2020). Several animal models of disuse-related muscle atrophy have commonly shown a reduced PI3K/Akt pathway in immobilization-treated muscle tissue (Jeon and Choung, 2021; Kim et al., 2020). Consistently, in immobilization-treated hindlimb muscles, p-PI3K and p-Akt were downregulated compared to those in non-treated mice. Furthermore, immobilization significantly reduced the protein levels of p-mTOR, p-p70S6K, p-4E-BP1, and p-FoxO3, and increased the mRNA expression of MuRF1 and atrogin-1. However, FAE treatment increased the protein levels of p-PI3K, p-Akt, p-mTOR, p-p70S6K, p-4E-BP1, and p-FoxO3 in the immobilized muscles. Additionally, it decreased the mRNA expression of MuRF-1 and atrogin-1 (Figs. 3, 4). These results indicated that immobilization inactivates the PI3K/Akt pathway, resulting in reduced muscle mass. However, FAE reversed the immobilization-induced inactivation of the PI3K/Akt pathway, thereby delaying the development of muscle atrophy.

Effects of NFAE and FAE on inflammatory cytokine expression

In this study, the protein expression of NF-κB and the mRNA expression of IL-6 and TNF-α were assessed in TA muscle tissue using western blotting and RT-PCR, respectively. The results showed a significant increase in NF-κB expression in the IMM group, compared to the CON group. The NFAE300 group did not show a significant inhibition of NF-κB protein expression, however, its expression significantly decreased in the FAE300 and FAE600 groups in a dose-dependent manner, showing a level similar to that of the CON group. Additionally, a significant difference was observed between the FAE300 and FAE600 groups and the NFAE300 group (Fig. 5A). The changes in IL-6 and TNF-α mRNA expression were consistent with NF-κB protein expression. Immobilization significantly increased IL-6 and TNF-α mRNA expression, compared to the CON group. However, the NFAE300, FAE300, and FAE600 groups showed a significant decrease in their mRNA expression. Significant differences were also observed between the NFAE and FAE groups (Fig. 5B).

Inflammatory cytokines are one of the main factors that cause muscle atrophy (Huang et al., 2022). When inflammatory cytokines, produced by other tissues and circulated through blood stream, attach to receptors located in the muscle cells, they not only trigger cellular signaling-related proteolysis but also upregulate the expression of inflammatory cytokines by activating NF-κB. Inflammatory cytokines, which are expressed in muscle cells, function as paracrine and/or autocrine factors, indicating that their stimulatory effects on inflammatory cytokines gradually increase (Kim and Hwang, 2020). Curcumin, which is an well-known anti-inflammatory compound, was presented to mitigate cancer-induced muscle atrophy by targeting NF-κB and decreasing IL-6, myostatin, and TNF-α (Zhang et al., 2022). Apigenin also inhibited denervation-induced muscle atrophy by reducing the expression of NF-κB, IL-6, and TNF-α (Choi et al., 2018). In this study, NFAE and FAE treatments reduced the mRNA expression of IL-6 and TNF-α which were upregulated by immobilization treatment. NFAE300 group did not show a significant effect on NF-κB expression; however, FAE treatment significantly increased the protein level of NF-κB, compared to the IMM and NFAE300 groups. Thus, FAE treatment reduced the inflammatory response by inactivating the NF-κB pathway (Fig. 5). NF-κB works as a transcriptional factor to regulate the expression of inflammatory cytokines as well as MuRF-1 and atrogin-1 (Huang et al., 2022). In this study, MuRF-1 and atrogin-1 mRNA expression levels were significantly downregulated in the NFAE300 group compared to those in the IMM group, however, no significant changes in p-FoxO3 were found between the two groups (Fig. 4). NF-κB protein expression was significantly upregulated by FAE at 300 mg/kg/day in comparison to the IMM group (Fig. 5A). Thus, the reduced mRNA expression of MuRF-1 and atrogin-1 in the FAE300 group might result from the increased NF-κB protein expression rather than p-FoxO3 protein expression.

Antler extracts contain several chemical compounds, such as sialic acid, gangliosides, uronic acid, and glycosaminoglycan (Kim et al., 2009). Sialic acid was shown to exert an anti-inflammatory effect by inactivating the NF-κB pathway (Li et al., 2023). The anti-inflammatory effects of several types of gangliosides, including gangliosides GM1 and GM3, have also been revealed (Galleguillos et al., 2022; Kim et al., 2014b). Sialic acid also decreased MuRF-1 and atrogin-1 mRNA expression in DEX-treated C2C12 myoblasts (Jeon et al., 2022). Glycosaminoglycan stimulated muscle reinnervation and increases insulin-like growth factor-1 (IGF-1) levels in the muscle, a protein that stimulates the PI3K/Akt pathway (Gorio et al., 2001). Therefore, the effects of fermented and non-fermented antler extracts on atrophic muscles may originate from a mixture of compounds with anti-inflammatory activities and regulation of muscle atrophy-related pathways.

Fermentation is a straightforward food processing method that enhances nutrient utilization and causes positive changes in the composition and flavor of food products (Adebo and Gabriela Medina-Meza, 2020). Fermentation has been found to increase the content of bioactive compounds compared to that before fermentation (Kim et al., 2009). Deer antler contains a range of active compounds, including hyaluronic acid, collagen, minerals, and sugars, with commonly identified constituents, such as sialic acid and gangliosides. After the antler extract was obtained, the fermentation process increased the yield of biologically active compounds and their physiological activities

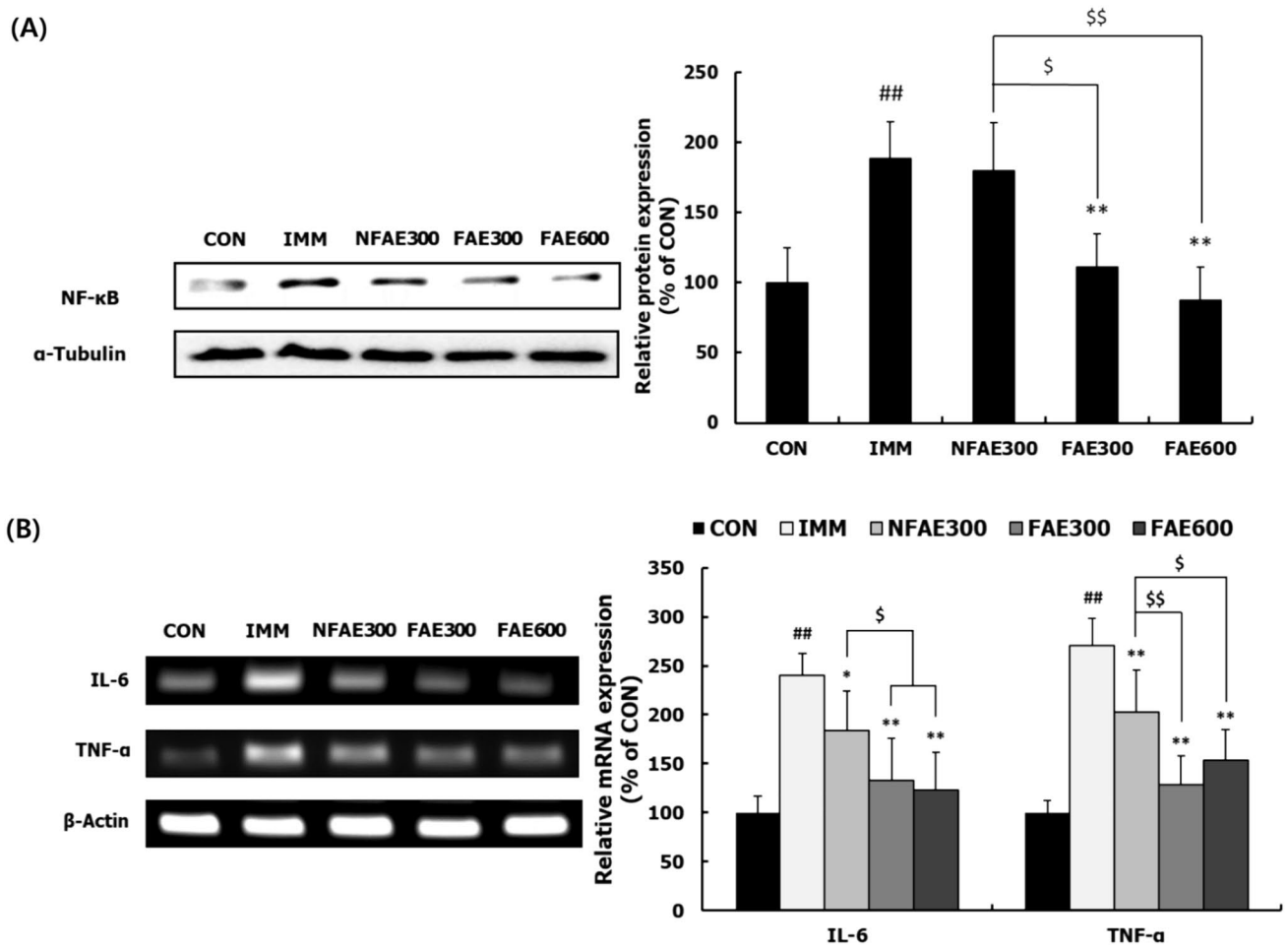

Fig. 5 Effects of non-fermented and fermented antler extracts on inflammatory cytokines. At the end of animal experiment, total mRNA or proteins were isolated from the right TA muscle. NF-κB was determined by Western blotting (**A**); IL-6 and TNF- α mRNA expression was analyzed by RT-PCR (**B**). α-Tubulin and β-actin was used as an internal control. Data are presented as mean ± SD. Group differences were assessed by Duncan's multiple range test. $^{\#\#}p < 0.01$ versus CON group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus IMM group, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ versus NFAE300

(Jeon et al., 2022; Kim et al., 2009). The results of extract fermentation varied widely according to the fermenting strain. A previous study demonstrated that among several strains, including *Bacillus* spp., *Lactobacillus* spp., and *Inonotus* spp., *B. subtilis* was most effective for fermentation with respect to yield and antioxidant activity (Kim et al., 2009). Thus, *B. subtilis* was used to ferment the antler extract to increase its effect on muscle atrophy. Grip strength of the fore/hind limbs, weight of the GAS muscle, and muscle volume were higher in the FAE300 group than in the NFAE300 group (Figs. 1, 2). With respect to the PI3K/Akt pathway, there was no significant difference between the NFAE300 and FAE300 groups. However, the anti-inflammatory effect of FAE at 300 mg/kg/day was more significant than that of NFAE (Figs. 3, 5). These results indicated that the greater effect of FAE treatment in reversing immobilization-induced muscle atrophy compared to NFAE treatment was due to its anti-inflammatory effect. This may have resulted from the increased content of bioactive compounds produced by fermentation. However, further studies are needed to identify the compounds that increased.

Oral administration of FAE resulted in greater improvements in exercise performance, grip strength, muscle mass, and CSA in the immobilized C57BL/6J mice. FAE stimulated the PI3K/Akt pathway, leading to the subsequent activation of the mTOR/p70S6K/4E-PB1 pathway for protein synthesis and reduction in MuRF-1 and atrogin-1 levels. Furthermore, FAE conferred anti-inflammatory effect by deactivating the NF-κB pathway. Compared to NFAE treatment, FAE treatment was more effective in attenuating muscle atrophy at the same dose. Thus, FAE is useful in preventing and attenuating muscle atrophy. However, further clinical studies are required to clarify its efficacy and safety.

Acknowledgements This study was financially supported by Biocare Corporation.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

References

Adebo OA, Gabriela Medina-Meza I. Impact of fermentation on the phenolic compounds and antioxidant activity of whole cereal grains: A mini review. Molecules. 25(4): 927 (2020)

Ballesteros-Álvarez J, Andersen JK. mTORC2: The other mTOR in autophagy regulation. Aging Cell 20: e13431 (2021)

Bowen TS, Schuler G, Adams V. Skeletal muscle wasting in cachexia and sarcopenia: molecular pathophysiology and impact of exercise training. Journal of Cachexia, Sarcopenia and Muscle. 6(3): 197-207 (2015)

Calvani R, Joseph AM, Adhihetty PJ, Miccheli A, Bossola M, Leeuwenburgh C, Bernabei R, Marzetti E. Mitochondrial pathways in sarcopenia of aging and disuse muscle atrophy. Biological Chemistry. 394(3): 393-414 (2013)

Caron AZ, Haroun S, Leblanc E, Trensz F, Guindi C, Amrani A, Grenier G. The proteasome inhibitor MG132 reduces immobilization-induced skeletal muscle atrophy in mice. BMC Musculoskeletal Disorders. 12: 185 (2011)

Choi SW, Moon SH, Yang HJ, Kwon DY, Son YJ, Yu R, Kim YS, Kim SI, Chae EJ, Park SJ, Kim SH. Antiresorptive activity of *Bacillus*-fermented antler extracts: inhibition of osteoclast differentiation. Evidence-Based Complementary and Alternative Medicine. 2013: 748687 (2013)

Choi WH, Jang YJ, Son HJ, Ahn J, Jung CH, Ha TY. Apigenin inhibits sciatic nerve denervation–induced muscle atrophy. Muscle and Nerve. 58(2): 314-318 (2018)

Figueiredo VC, Markworth JF, Cameron-Smith D. Considerations on mTOR regulation at serine 2448: implications for muscle metabolism studies. Cellular and Molecular Life Sciences. 74: 2537-2545 (2017)

Frontera WR, Ochala J. Skeletal muscle: a brief review of structure and function. Calcified Tissue International. 96(3): 183-195 (2015)

Galleguillos D, Wang Q, Steinberg N, Zaidi A, Shrivastava G, Dhami K, Daskhan GC, Schmidt EN, Dworsky-Fried Z, Giuliani F, Churchward M, Power C, Todd K, Taylor A, Macauley MS, Sipione S. Anti-inflammatory role of GM1 and other gangliosides on microglia. Journal of Neuroinflammation. 19(1): 9 (2022)

Gorio A, Citterio C, Muller EE, Di Giulio AM. Glycosaminoglycan-promoted muscle reinnervation and insulin-like growth factor-I levels are affected by anti-growth hormone-releasing hormone exposure. Journal of Neuroscience Research. 66(6): 1112-1117 (2001)

Huang CC, Chen YM, Kan NW, Chao HL, Ho CS, Hsu MC. *Cornu cervi pantotrichum* supplementation improves exercise performance and protects against physical fatigue in mice. Molecules. 19(4): 4669-4680 (2014)

Huang L, Li M, Deng C, Qiu J, Wang K, Chang M, Zhou S, Gu Y, Shen Y, Wang W, Huang Z, Sun H. Potential therapeutic strategies for skeletal muscle atrophy. Antioxidants. 12(1): 44 (2022)

Huang Y, Chen K, Ren Q, Yi L, Zhu J, Zhang Q, Mi M. Dihydromyricetin attenuates dexamethasone-induced muscle atrophy by improving mitochondrial function via the PGC-1α pathway. Cellular Physiology and Biochemistry. 49(2): 758-779 (2018)

Jang JY, Kim D, Kim ND. Pathogenesis, intervention, and current status of drug development for sarcopenia: A review. Biomedicines. 11(6): 1635 (2023)

Jeon H, Kim YT, Jang WY, Kim JY, Heo K, Shim JJ, Lee JL, Yang DC, Kang SC. Effects of *Lactobacillus curvatus* HY7602-fermented antlers in dexamethasone-induced muscle atrophy. Fermentation. 8(9): 454 (2022)

Jeon SH, Choung SY. Oyster hydrolysates attenuate muscle atrophy via regulating protein turnover and mitochondria biogenesis in C2C12 cell and immobilized mice. Nutrients. 13(12): 4385 (2021)

Jo K, Jang WY, Yun BS, Kim JS, Lee HS, Chang YB, Suh HJ. Effect of Deer Antler extract on muscle differentiation and 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR)-induced muscle atrophy in C2C12 cells. Food Science of Animal Resources. 41(4): 623-625 (2021)

Jung S, Kim SH, Jeung W, Ra J, Heo K, Shim JJ, Lee JL. Fermented antler improves endurance during exercise performance by increasing mitochondrial biogenesis and muscle strength in mice. Applied Sciences. 11(12): 5386 (2021)

Kadakia KC, Hamilton-Reeves JM, Baracos VE. Current therapeutic targets in cancer cachexia: A pathophysiologic approach. American Society of Clinical Oncology Educational Book. 43: e389942 (2023)

Kim C, Hwang JK. Flavonoids: nutraceutical potential for counteracting muscle atrophy. Food Science and Biotechnology. 29(12): 1619-1640 (2020)

Kim C, Kim MB, Hwang JK. Red bean extract inhibits immobilization-induced muscle atrophy in C57BL/6N mice. Journal of Medicinal Food. 23(1): 29-36 (2020)

Kim DH, Han SB, Park JS, Han MJ. Fermentation of antler and its biological activity. Korean Journal of Pharmacognosy. 25(3): 233-237 (1994)

Kim HY, Rhyu MR. Sectional composition of minerals in domestic deer antler. Korean Journal of Food Science and Technology. 32(1): 31-36 (2000)

Kim MK, Jung EY, Lee HS, Shin KS, Kim YK, Ra KS, Park CS, Woo MJ, Lee SH, Kim JS, Suh HJ. Isolation of strain for the preparation of the fermented antler and its physiological activities. Journal of the Korean Society of Food Science and Nutrition. 38(9): 1237-1242 (2009)

Kim JH, Jung TY, Seo J, Lee S, Kim MG, Leem KH, Lim SC. Involvement of MAPK signaling pathway in the osteogenic gene expressions of *Cervi Pantotrichum Cornu* in MG-63 human osteoblast-like cells. Life Sciences. 94(1): 45-53 (2014)

Kim SJ, Chung TW, Choi HJ, Jin UH, Ha KT, Lee YC, Kim CH. Monosialic ganglioside GM3 specifically suppresses the monocyte adhesion to endothelial cells for inflammation. The International Journal of Biochemistry and Cell Biology. 46: 32-38 (2014)

Kim MB, Kim T, Kim C, Hwang JK. Standardized *Kaempferia parviflora* extract enhances exercise performance through activation of mitochondrial biogenesis. Journal of Medicinal Food. 21(1): 30-38 (2018)

Kim YT, Jeon H, Kim SH, Heo K, Shim JJ, Lee JL, Yang DC, Kang SC. Fermented antler recovers stamina, muscle strength and muscle mass in middle-aged mice. Applied Sciences. 12(1): 106 (2021)

Lee MR, Sun BS, Gu LJ, Wang C, Fang ZM, Wang Z, Mo EK, Ly SY, Sung CK. Effects of the deer antler extract on scopolamine-induced memory impairment and its related enzyme activities. Journal of the Korean Society of Food Science and Nutrition. 38(4): 409-414 (2009)

Li D, Xie T, Guo T, Hu Z, Li M, Tang Y, Wu Q, Luo F, Lin Q, Wang H. Sialic acid exerts anti-inflammatory effect through inhibiting MAPK-NF-κB/AP-1 pathway and apoptosis in ulcerative colitis. Journal of Functional Foods. 101: 105416 (2023)

Shao Q, Zhang Z, Cao R, Zang H, Pei W, Sun T. CPA4 promotes EMT in pancreatic cancer via stimulating PI3K-AKT-mTOR signaling. Onco Targets and Therapy. 13: 8567-8580 (2020)

Ulla A, Uchida T, Miki Y, Sugiura K, Higashitani A, Kobayashi T, Ohno A, Nakao R, Hirasaka K, Sakakibara I, Nikawa T. Morin attenuates dexamethasone-mediated oxidative stress and atrophy in mouse C2C12 skeletal myotubes. Archives of Biochemistry and Biophysics. 704: 108873 (2021)

Wang Y, Liu Q, Quan H, Kang SG, Huang K, Tong T. Nutraceuticals in the prevention and treatment of the muscle atrophy. Nutrients. 13: 1914 (2021)

Zhang J, Zheng J, Chen H, Li X, Ye C, Zhang F, Zhang Z, Yao Q, Guo Y. Curcumin targeting NF-κB/ubiquitin-proteasome-system axis ameliorates muscle atrophy in triple-negative breast cancer cachexia mice. Mediators of Inflammation. 2022: 2567150 (2022)