Preventive effect of fermented whey protein mediated by *Lactobacillus gasseri* IM13 via the PI3K/AKT/FOXO pathway in muscle atrophy

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**ABSTRACT**

This study investigated the preventive effects of whey protein fermented with *Lactobacillus gasseri* IM13 (F-WP) against dexamethasone (DEX)-induced muscle atrophy. C2C12 muscle cells were treated with F-WP followed by DEX treatment. DEX treatment inhibited myotube formation and the expression of myogenic regulatory factors; however, pretreatment with F-WP attenuated DEX-induced damage. F-WP significantly activated the phosphorylation of the IGF-1/PI3K/AKT pathway and improved muscle homeostasis suppressed by DEX. Moreover, F-WP alleviated the phosphorylation of mTOR, S6K1, and 4E-BP1 and enhanced muscle protein synthesis. Muscle-specific ubiquitin ligases and autophagy lysosomes, which were activated by the dephosphorylation of FOXO3a by DEX treatment, were significantly attenuated by F-WP pretreatment of myotubes. For peptidomic analysis, F-WP was fractionated using preparative HPLC (prep-HPLC), and the amino acid sequences of 11 peptides were identified using MALDI-TOF/MS/MS. In conclusion, fermentation of whey protein by the specific probiotic strain IM13 produced bioactive peptides with high antioxidant and anti-sarcopenic effects, which markedly enhanced myogenesis and muscle protein synthesis while diminishing muscle protein degradation compared with intact whey protein.

Key Words: Whey protein, *Lactobacillus gasseri*, Fermentation, Muscle atrophy, Peptide

**INTRODUCTION**

Skeletal muscle atrophy is described as the loss of muscle mass owing to cellular shrinkage and can be triggered by pathogenic factors, such as malnutrition, hormonal changes, inflammation, intestinal dysbiosis, muscle mitochondrial dysfunction, oxidative stress, and physical inactivity (Zhu et al., 2023). Although the mechanisms involved in muscle atrophy are multifactorial, muscle atrophy primarily results from a combination of increased protein degradation and reduced protein synthesis (Sartori et al., 2021). Muscle homeostasis mainly depends on the activity of the phosphoinositide 3-kinase (PI3K)–protein kinase B (AKT)–mammalian target of rapamycin (mTOR) signaling pathway (Cohen et al., 2015). The decrease in PI3K–AKT signaling under conditions of muscle atrophy triggers a chain reaction, a reduction in mTOR activity, which in turn causes a decline in muscle protein synthesis, is coupled with an increase in forkhead box protein O (FOXO) activity, which accelerates muscle protein degradation (Yoshida and Delafontaine, 2020).

Dexamethasone (DEX), commonly used to induce muscle atrophy (Figure 1), inhibits insulin-like growth factor-1 (IGF-1) and IGF-1 binding protein 3 (IGFBP3), thereby suppressing PI3K–AKT–mTOR signaling (Mishra et al., 2022). Consequently, DEX inhibits the phosphorylation of the downstream targets of mTOR, such as ribosomal protein S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1), which are crucial for initiating mRNA translation and protein synthesis (Sartori et al., 2021). Conversely, DEX can cause oxidative stress in skeletal muscle cells and induce muscle atrophy by stimulating ubiquitin ligase casitas B-lineage lymphoma proto-oncogene-b (Cbl-b) and decreasing IGF-1 levels (Uchida et al., 2018, Chen et al., 2020). In addition, DEX directly targets regulated in development and DNA damage response 1 (REDD1) and Kruppel-Like Factor 15 (KLF15) via the glucocorticoid receptor and inhibits mTOR activity (Shimizu et al., 2011). KLF15 increases the level of transcription of branched-chain aminotransferase 2 (BCAT2) and stimulates the breakdown of branched chain amino acids (BCAAs), which are critical for skeletal muscle (Mann et al., 2021). DEX also activates the glucocorticoid receptor, thereby promoting the expression of FOXO, particularly FOXO3a, and stimu-
lating the transcription of atrogenes via the FOXO-mediated ubiquitin-proteasome pathway (UPP) and autophagy lysosomal pathway (ALP) (Zhang et al., 2020). Further, DEX stimulates muscle proteolysis by promoting the expression of atrogin-1/muscle atrophy F-box (MAFbx) and muscle RING-finger protein-1 (MuRF1), which are muscle-specific E3 ubiquitin ligases. MAFbx targets myoblast determination protein 1 (MyoD) and myogenin, transcription factors that activate muscle-specific genes, which are essential for skeletal myotube formation, while MuRF1 interacts with myosin heavy chain (MyHC), a myofibrillar protein (Bodine and Baehr, 2014). Moreover, KLF15 is reportedly involved in muscle protein degradation through the transcriptional regulation of atrogin-1/MAFbx and MuRF1 (Shimizu et al., 2011). In the ALP, microtubule-associated protein 1A/1B-light chain 3 (LC3) and BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) initiate autophagy, a cellular recycling process that contributes to muscle atrophy by breaking down cellular components and proteins, while cathepsin-L degrades proteins through lysosomes (Tailandier and Polge, 2019, Zhang et al., 2020).

Whey protein, which is rich in leucine, reportedly improves protein synthesis through the PI3K–AKT pathway and inhibits proteolysis by reducing the expression of atrogin-1/MAFbx and MuRF1 (Duan et al., 2018, Lee et al., 2022). It is also abundant in antioxidant bioactive peptides and exhibits enhanced beneficial properties when fermented with lactic acid bacteria (LAB) (Corrochano et al., 2018). This process generates an increased amount of bioactive peptides that significantly enhance the antioxidant activity of whey protein. Antioxidant activities have shown protective effects against DEX-induced muscle atrophy (Balboa et al., 2020, Karnia et al., 2021). Moreover, fermentation generates bioactive peptides with antimicrobial and immunomodulatory effects, reduces the levels of allergenic substances, and increases water solubility (Mann et al., 2019, Zhao and Ashaolu, 2020, Khan et al., 2021). Previous studies have demonstrated that the antioxidant activity of bioactive peptides in milk are associated with the composition and sequence of

Figure 1. The scheme of various molecules involved in muscle atrophy and myogenesis. Dexamethasone (DEX) induces muscle atrophy.

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their amino acids (Nielsen et al., 2017). However, the mechanisms by which fermented whey protein and the resultant peptides affect muscle protein synthesis and breakdown have not been thoroughly examined to date.

In this study, we investigated whether whey protein fermented with specific LAB strains could prevent muscle atrophy by regulating the PI3K/AKT/FOXO pathway in DEX-induced C2C12 skeletal muscle myotubes. First, we fermented whey protein with 5 LAB strains with probiotic potential and evaluated their proteolytic, antioxidative, and antiatrophic activities. The \textit{Lactobacillus gasseri} IM13 (IM13) strain was selected based on its proteolytic activity and functionality. Next, we evaluated the effects of whey protein fermented with IM13 (F-WP) on myotube differentiation, muscle protein synthesis, and muscle degradation in DEX-treated C2C12 skeletal muscle myotubes. Finally, F-WP was fractionated by preparative HPLC, and peptide identification and characterization were performed using MALDI-TOF/MS/MS.

**MATERIALS AND METHODS**

**Fermentation of whey protein**

Whey protein isolate (WP; Carbery Food Ingredients Limited, Ballineen, Ireland) was mixed with glucose at a ratio of 10:1 (wt/wt), dissolved in deionized water to create a 5% (wt/vol) solution, and then sterilized. Five LAB strains, isolated from infant feces, that had probiotic potentials (\textit{L. gasseri} IR13, IM13 and \textit{Lacticaseibacillus rhamnosus} IM14, IM18, IM19) were inoculated into WP mixture and incubated at 37°C for 48 h. Following fermentation, the solutions were centrifuged at 28000 × g for 10 min at 4°C, and the resulting supernatants were lyophilized and stored at –80°C.

**Measurement of antioxidant activity**

The reducing power and radical-scavenging activities of fermented WP were determined using ferric-reducing antioxidant power (FRAP), 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2-diphenyl-1-picrylhydrazyl (DPPH) assays as described by Oh et al. (2014).

**Determination of proteolytic activity**

The 5 selected \textit{Lactobacillus} strains were evaluated for their proteolytic activity toward WP according to the \textit{o}-Phthalaldehyde (OPA) method described previously (Church et al., 1983). The OPA reagent (180 μL) was mixed with the sample, standard (leucine), and blank (deionized water) (10 μL) and allowed to react at room temperature for 2 min. The absorbance was estimated at 340 nm using Epoch 2 microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). The proteolytic activity was expressed as the leucine concentration (mM).

**Cell culture and treatment**

Murine skeletal muscle cell line C2C12 (American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco’s modified Eagle’s medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in a CO2 incubator at 37°C under humidified air. When the myoblasts reached 80–90% confluence, the medium was replaced with differentiation medium containing Dulbecco’s modified Eagle’s medium supplemented with 2% horse serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin to differentiate myoblasts into myotubes. WP and F-WP (10, 25, and 50 μg/mL) were administered to C2C12 myotubes for 48 h after 4 d of differentiation. The protein concentrations of the samples were adjusted using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). After complete differentiation, 100 μM DEX (Sigma-Aldrich, St. Louis, MO, USA) dissolved in a serum-free medium was administered to C2C12 myotubes to induce muscle atrophy.

**Jenner–Giemsa staining and measurement of myotube diameter**

Jenner-Giemsa staining was performed after DEX treatment on myotubes to measure changes in myotube formation. The myotubes were washed with cold phosphate-buffered saline (pH 7.4) 3 times, fixed using 100% methanol for 5 min, and air-dried for 10 min. Staining was performed using the Jenner–Giemsa method as described earlier (Velića and Bunce, 2011). Photographs of stained myotubes were captured using an EVOS XL Core Imaging System (Thermo Fisher Scientific, Waltham, MA, USA) at 4 × and 20 × magnifications. The diameter of the myotubes was quantified based on 50 myotubes per group from 10 random fields using the ImageJ software (version 4.16; National Institutes of Health, Bethesda, MD, USA).

**Myotube viability**

The effect of DEX on myotube viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-
nyltetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA) assay. C2C12 cells were seeded in 96-well plates at a density of $3 \times 10^3$ cells/well. After treatment with DEX, the medium was replaced with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylnyltetrazolium bromide reagent, and the cells were incubated at 37°C for 2 h in the dark. Next, formazan crystals were dissolved in dimethyl sulfoxide (Samchun Chemicals Co., Seoul, Korea), and the absorbance was measured at a wavelength of 540 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA) and described as a percentage of the untreated normal group.

**Creatine kinase activity assay**

To determine the degree of muscle damage, creatine kinase activity was evaluated using a creatine kinase activity kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer’s instructions. The creatine kinase reagent was added to the supernatant of the myotube lysate in a microplate. The absorbance was measured at a wavelength of 340 nm twice at 5 min intervals. The results were normalized to the protein concentration and expressed as units/L. Each assay was performed 3 times.

**Western blotting**

C2C12 myotubes were washed 3 times with cold phosphate-buffered saline and lysed using RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing a PhosSTOP EASY pack protease inhibition cocktail (Roche Diagnostics, Rotkreuz, Switzerland) and phosphatase inhibition cocktail (Roche Diagnostics, Rotkreuz, Switzerland) on ice. The cell extracts were centrifuged at 18,000 × g for 20 min at 4°C, and the total protein concentration was quantified using a bicinchoninic acid protein assay kit. Equal amounts of protein (30 µg) were separated on 6–12% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk in washing buffer (0.1% Tween-20 in TBS: TBST) for 1 h at 23°C and incubated with primary antibodies, which were diluted with 5% BSA to 1:1000 overnight at 4°C. The antibodies used in this study are listed in Supplementary Table 1. Next, the membranes were washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (7074S; Cell Signaling Technology, Inc., Beverly, MA, USA) or goat anti-mouse IgG (GTX213111–01; GeneTex, Irvine, CA, USA) for 1 h. After washing, the blots were visualized using an enhanced chemiluminescence substrate (Bio-Rad, Hercules, CA, USA), and images were captured using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA). The bands were quantified using the ImageJ software (version 4.16; National Institutes of Health, Bethesda, MD, USA).

**RNA isolation and quantitative real-time PCR**

Total cellular RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The amount of isolated RNA was estimated using a spectrophotometer with a Take3 Microvolume plate (Bio-Tek, Winooski, VT, USA) before cDNA synthesis using the BioFACT 2X Reverse Transcription Pre-Mix (BioFACT, Daejeon, Korea). Quantitative real-time PCR (qRT-PCR) was performed with GoTaq qPCR Master Mix (Promega Co., Madison, WI, USA) using the QuantStudio 3 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). The relative expression level of the examined genes was analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Sequences of the PCR primers used are listed in Supplementary Table 2.

**Peptide identification using MALDI-TOF/MS and MS/MS analysis**

F-WP was separated by preparative HPLC (prep-HPLC) using a C18 column to identify the peptide sequence. The sample was extracted using solid-phase extraction according to previous studies (Cavaliere et al., 2021). Fractionation of hydrolyzed peptides by fermentation was then performed using prep-HPLC with an Xbridge BEH C18 OBD Prep Column, 5 µm 19 × 250 mm (Waters, Milford, MA, USA), according to a method described previously (Gürbüz and Heinonen, 2015). Briefly, separation was performed with ddH2O/formic acid (99.9/0.1, vol/vol) as mobile phase A and MeOH/formic acid (99.9/0.1, vol/vol) as mobile phase B. The gradient elution started at 5% B for 15 min and increased to 35% constantly over 120 min. Finally, mobile phase B was decreased to 75% for washing step and maintained for 5 min. The chromatography was performed at a flow rate of 6 mL/min. Sequentially, each prep-HPLC fraction was analyzed by MALDI-TOF/MS/MS using the method described previously (Oh et al., 2017). Briefly, each fraction was mixed with a matrix solution (α-cyano-4-hydroxycinnamic acid), and the mixture was spotted onto a MALDI target. MALDI-TOF/MS experiments were performed using a Bruker Autoflex instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Laser-desorbed positive ions were analyzed after acceleration at 19 kV in the reflector mode for peptide digestion. For each displayed mass spectrum, at least
2000 laser shots were collected from several positions on the spots.

**Statistical analysis**

All results are expressed as the mean ± standard deviation. Statistical analysis was performed using the SPSS software (version 25.0; IBM, Chicago, IL, USA). Differences between groups were compared using one-way ANOVA and Duncan’s multiple comparison test. Statistical significance was set at \( P < 0.05 \), unless otherwise stated.

**RESULTS**

**Fermentation properties of whey protein by LAB strains**

To evaluate fermentation properties, the fermented WP from each of the 5 strains was assessed for antioxidant, proteolytic, and anti-atrophic activities (Figure 2). Antioxidant activities were determined by FRAP assay for reductive capability, and ABTS and DPPH assays for radical-scavenging activities (Figure 2A–C). All fermented WP exhibited significantly increased antioxidant activities compared with non-fermented WP \( (P < 0.05) \). WP fermented by IM13 exhibited a FRAP value of 91.8 \( \mu \text{M} \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \) (Figure 2A), 15.8% ABTS (Figure 2B), and 26.6% DPPH scavenging activity (Figure 2C). WP fermented by IM13 exhibited significantly higher reducing power and radical scavenging activities than that fermented by other strains \( (P < 0.05) \). Moreover, the degree of hydrolysis of the fermented WP by IM13 was 69.6%, which was significantly higher than the value obtained with other strains \( (P < 0.05) \). To evaluate the anti-atrophic effects in DEX-induced muscle cells, the mRNA expression of atrogin-1/MAFbx and MuRF1 was measured after sample administration to muscle cells. WP fermented by IM13, IM14, IM18, and IM19 showed significant decreases in the mRNA expression of atrogin-1/MAFbx and MuRF1 compared with normal WP \( (P < 0.05) \). In addition, IM13 significantly increased antioxidant, proteolytic, and anti-atrophic activities during fermentation of WP. Especially, IM13 showed the highest microbial, proteolytic, and antioxidant activities at 45 h of WP fermentation (Supplementary Figure S1).

**Effect of WP and F-WP on myotube formation in DEX-induced muscle atrophy**

To evaluate the effects of WP and F-WP on the structural integrity of myotubes, Jenner-Giemsa staining was performed to estimate changes in myotube diameter and number of nuclei (Figure 3A). DEX decreased myotube diameter (Figure 3B) and the number of nuclei (Figure 3C); however, pretreatment with WP and F-WP significantly attenuated the DEX-induced changes \( (P < 0.005 \text{ and } P > 0.05, \text{ respectively}) \). In addition, myotube viability and creatine kinase activity were evaluated to determine the preventive effect on damages by DEX (Figure 2D-E). Pretreatment with WP and F-WP significantly attenuated decreases in myotube viability (Figure 3D; \( P < 0.001 \)) and increases in creatine kinase activity induced by DEX (Figure 3E; \( P > 0.05 \)). Pretreatment with F-WP significantly inhibited the DEX-induced increase in myotube diameter, the number of nuclei, and cell viability compared with that with WP in a dose-dependent manner \( (P < 0.05) \). F-WP caused a dose-dependent decrease in creatine kinase activity; however, there was no significant difference compared with WP. Moreover, the mRNA expression of myogenesis-related genes in myotubes was evaluated by qRT-PCR (Figure 3F-I). Exposure to DEX significantly inhibited the mRNA expression of MyoD, myogenin, MyHC-IIX, and MyHC-IIA \( (P < 0.001) \). However, WP showed preventive effects on MyHC-IIX, IIA (25 and 50 \( \mu \text{g/mL} \)), MyoD, and myogenin (50 \( \mu \text{g/mL} \)). There was no significant difference between the WP-treated group and negative control group (NC) at 10 \( \mu \text{g/mL} \). F-WP markedly prevent the DEX-induced decrease in the expression of MyoD and MyHC IIX in a dose-dependent manner \( (P < 0.05 \text{ and } P < 0.001, \text{ respectively}) \). Moreover, F-WP showed increased expression of MyoD (50 \( \mu \text{g/mL} \)) and MyHC-IIX (25 and 50 \( \mu \text{g/mL} \)). In particular, F-WP showed a significantly greater effect in restoring MyoD, myogenin, and MyHC IIA expression than WP at 50 \( \mu \text{g/mL} \) \( (P < 0.001) \).

**Effects of WP and F-WP on muscle protein synthesis through the PI3K–AKT pathway in DEX-induced muscle atrophy**

To evaluate the effects of F-WP on muscle protein synthesis through the PI3K–AKT pathway, the mRNA expression of IGF-1 and IGFBP3 was examined (Figure 4A). DEX treatment inhibited IGF-1 and IGFBP3 mRNA expression \( (P < 0.001) \). However, pretreatment with F-WP significantly alleviated the changes induced by DEX treatment in a dose-dependent manner \( (P < 0.05) \). At 50 \( \mu \text{g/mL} \) treatment, F-WP showed a significantly greater increase in the mRNA expression of IGF-1 and IGFBP3 (Figure 4B–C). Moreover, F-WP positively stimulated IGF-1 expression in a dose-dependent manner (Figure 4D; \( P < 0.001 \)).
Figure 2. Antioxidant, proteolytic, and anti-atrophic activities of fermented whey protein

WP was fermented with 5 LAB strains and then antioxidant activities were determined using (A) FRAP assay, and (B) ABTS scavenging activity and (C) DPPH scavenging activity assays. (D) Proteolytic activity of the LAB strain was determined by the OPA method and expressed as the degree of hydrolysis compared with the released peptide from normal WP. Differentiated C2C12 myotubes were exposed to fermented WP for 48 h and then subjected to 100 µM DEX for an additional 24 h. The levels of mRNA expression were assessed via quantitative RT-PCR. (E) Relative mRNA expression of atrogin-1/MAFbx and (F) MuRF1. The expression levels were normalized to those of GAPDH as the reference gene and are presented as fold-change against the control group. Mean ± SD from 3 separate experiments. The different letters (a-d) denote significant differences at P < 0.05.

WP, normal whey protein; IR13, WP fermented by Lactobacillus gasseri IR13; IM13, WP fermented by L. gasseri IM13; IM14, WP fermented by Lacticaseibacillus rhamnosus IM14; IM18, WP fermented by L. rhamnosus IM18; IM19, WP fermented by L. rhamnosus IM19; CON, control group; DEX, dexamethasone; NC, DEX only treatment group.
IGF-1 and IGFBP3 compared with WP ($P < 0.005$ and $P < 0.05$, respectively). Further, the phosphorylation level of PI3K and AKT was estimated by Western blotting (Figure 4B). DEX treatment significantly blocked the phosphorylation of PI3K and AKT ($P < 0.05$). At concentrations of 25 and 50 µg/mL, pretreatment with F-WP significantly increased the phosphorylation of PI3K and AKT ($P < 0.05$). We next assessed the protein levels of FOXO3a, mTOR, 4E-BP1, and S6K1 and those of the phosphorylated forms (Figure 4C). DEX treatment blocked the phosphorylation of FOXO3a, mTOR, 4E-
**Figure 4.** Muscle protein synthesis through the IGF-1/PI3K/AKT pathway in dexamethasone (DEX)-induced muscle atrophy. Differentiated C2C12 myotubes were conditioned with various concentrations (10, 25, and 50 µg/mL) of WP and F-WP for 48 h, followed by 100 µM DEX application for an additional 24 h. (A) The mRNA expression of IGF-1 and IGFBP-3. The expression levels were normalized to those of GAPDH as the housekeeping gene. (B) The protein expression of PI3K and AKT. (C) The protein expression of FOXO3a, mTOR, 4E-BP1, and S6K1. Phosphorylation of proteins related to muscle protein synthesis via the PI3K/AKT pathway was determined by Western blot analysis. The protein expression was represented as the ratio of its phosphorylated form. Results are from 3 independent experiments and expressed as the mean ± S.D. ***P < 0.001, **P < 0.005, *P < 0.05 represent significance relative to the DEX-only NC group; ***P < 0.001, **P < 0.05, *P < 0.05 indicate significant inter-group differences. WP, normal whey protein; F-WP, WP fermented by *Lactobacillus gasseri* IM13; CON; control group; DEX, dexamethasone; NC, DEX only treatment group.
Figure 5. Muscle protein degradation through ubiquitin-proteasome and autophagy lysosomal system in dexamethasone (DEX)-induced muscle atrophy. Differentiated C2C12 myotubes were exposed to various concentrations (10, 25, and 50 µg/mL) of WP and F-WP for 48 h, and then subjected to 100 µM DEX for an additional 24 h. The mRNA expression levels related to muscle protein degradation were analyzed using quantitative RT-PCR, with normalization to GAPDH. (A) The mRNA expression of protein synthesis inhibitor (Cbl-b and REDD1) (B) genes in the ubiquitin-proteasome pathway (atrogin-1/MAFbx and MuRF1). (C) Genes in autophagy lysosomal pathway (LC3, cathepsin-L, and BNIP3). (D) BCAA degradation genes (KLF15 and BCAT2). Results are expressed as the mean ± SD from 3 separate experiments. ***P < 0.001, **P < 0.005, *P < 0.05 compared with NC group; ***P < 0.001, **P < 0.005, *P < 0.05 represent significant differences between marked groups. WP, normal whey protein; F-WP, WP fermented by Lactobacillus gasseri IM13; CON, control group; DEX, dexamethasone; NC, DEX only treatment group.
BP1, and S6K1. However, pretreatment with F-WP significantly attenuated the DEX-induced suppression of the phosphorylation of FOXO3a and S6K1 at 25 and 50 µg/mL and that of mTOR and 4E-BP1 at 10, 25, and 50 µg/mL. In particular, 50 µg/mL F-WP caused a significant increase in the phosphorylation of PI3K, AKT, FOXO3a, and mTOR compared with WP (P < 0.05). Moreover, 50 µg/mL F-WP restored these DEX-induced decreases to the levels of the control group.

WP and F-WP regulated muscle protein degradation through FOXO3a transcription in DEX-induced muscle atrophy

To understand how F-WP affects FOXO3a-related muscle protein degradation, qRT-PCR was performed (Figure 5). Treatment with DEX increased the mRNA expression of Cbl-b and REDD1 (P < 0.001); however, pretreatment with F-WP decreased the DEX-induced increase in a dose-dependent manner (P < 0.05; Figure 5A). DEX also increased the mRNA and protein expression of the UPP-related molecules atrogin-1/MAFbx and MuRF1 (Figure 5B and Supplementary Figure S2) as well as those of the ALP-related molecules LC3, cathepsin-L, and BNIP3 (Figure 5C and Supplementary Figure S2). F-WP significantly prevented the increase in the protein and mRNA expression of the UPP- and ALP-related molecules induced by DEX, especially at 25 and 50 µg/mL, compared with WP (P < 0.05). In addition, F-WP significantly attenuated the DEX-induced upregulation of KLF15 and BCAT2, which are related to BCAA degradation (P < 0.05; Figure 5D). The F-WP group showed a significant decrease in the expression of BCAT2 at 50 µg/mL compared with that of the WP group (P < 0.05), while the expression of KLF15 showed no significant difference.

Identification of peptides derived from fermentation of whey protein

To generate peptide profiles derived from the fermentation of whey protein with IM13, 8 different fractions were collected from the F-WPs using prep-HPLC. Peptidomic analysis of the generated fractions resulted in the identification of 11 peptides by MALDI-TOF/MS/MS in the m/z range of 700–3500 Da (Supplementary Figure S3). The peptides were originated from either β-casein (9) or κ-casein (2). Moreover, most of the peptides identified in this study have been previously described as bioactive peptides, such as antioxidant and antimicrobial peptides (Nielsen et al., 2018, Rosa et al., 2023). In particular, 4 peptides that require further bioactivity studies were newly identified in this study (Table 1).

DISCUSSION

Muscle atrophy is associated with muscle degradation that outpaces muscle protein synthesis, leading to sarcopenia (Sartori et al., 2021). DEX induces muscle atrophy by inhibiting myogenesis and muscle protein synthesis and stimulating muscle protein degradation (Zhang et al., 2020). WP supplements promote muscle growth and enhance exercise performance owing to their high leucine content, which improves protein synthesis and reduces protein degradation in muscles (Duan et al., 2018, Lee et al., 2022). The fermentation of WP with LAB produces bioactive peptides and amino acids with antioxidant activities (Mann et al., 2019, Dineshbhai et al., 2022).

In this study, we fermented WP with 5 LAB strains with probiotic potential and evaluated their preventive effects on DEX-induced muscle atrophy in C2C12 myotubes. We assessed the functional properties of the fermented WP by estimating its antioxidant, proteolytic, and antiatrophic activities. As fermentation progresses, the degree of hydrolysis increases, which could lead to enhanced antioxidant activity with increased leucine

Table 1. Peptide identification

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<th>Protein</th>
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<th>Position</th>
<th>Sequence</th>
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<td>β-casein</td>
<td>2992.7749</td>
<td>48 - 72</td>
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<td>(Rubak et al., 2022)</td>
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<td>158 - 169</td>
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<td>(Oh et al., 2016, Rosa et al., 2023)</td>
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<td>159 - 169</td>
<td>MHQPQPLPPT</td>
<td>(Fialho et al., 2018, Komatsu et al., 2023)</td>
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</table>
content, as reportedly previously (Mathur et al., 2020). Among the 5 _Lactobacillus_ strains, WP fermented by strain IM13 exhibited the highest antioxidant and proteolytic activities, as well as the highest leucine content. This leads to a significant decrease in the expression of atrogin-1/MAFbx and MuRF1. Therefore, WP fermented with IM13 (F-WP) was determined to have the potential for preventing muscle atrophy, and changes in muscle atrophy-related factors were evaluated in DEX-treated C2C12 myotubes pretreated with F-WP.

Myogenesis, the formation of muscle fibers from myogenic precursor cells, is a crucial process in the development and regeneration of skeletal muscles, and the dysregulation of myogenesis by DEX can contribute to muscle atrophy (Bentzinger et al., 2012). DEX can lead to a substantial reduction in viability, diameter, and nucleus number and detrimental effects on the expression of myogenic regulatory factors (Zammit, 2017, Shen et al., 2019). Pretreatment with F-WP prevented DEX-induced muscle atrophy, as evidenced by the restoration of viability, myotube diameter, and number of nuclei in myotubes. Pretreatment with F-WP also inhibited the DEX-induced decrease in creatine kinase activity, an essential enzyme for muscle energy metabolism and a marker of muscle integrity and damage (Hwang et al., 2015). Myogenesis is divided into 2 stages: myoblast proliferation and differentiation mediated by myogenic regulatory factors, such as MyoD, myogenin, and MyHC (Zammit, 2017). Elevated atrogin-1/MAFbx by DEX targets MyoD and myogenin, which results in the activation of polyubiquitination; MuRF1 exhibits intrinsic ubiquitin-conjugating activity, which induces proteasome-mediated degradation of MyHC by stimulating its ubiquitination (Bodine and Baehr, 2014).

Our results showed that, as atrogin-1/MAFbx expression increased, the expression levels of MyoD, myogenin, and MyHC decreased following DEX treatment. Moreover, pretreatment with F-WP significantly decreased the expression of atrogin-1/MAFbx, followed by an increase in MyoD, myogenin, and MyHC levels, which were significantly higher than those induced by normal WP. These findings suggest that F-WP effectively counteracts DEX-induced cellular shrinkage and contributes to the maintenance of structural integrity and myogenesis of myotubes.

Furthermore, DEX stimulates muscle atrophy by inhibiting the IGF-1 and PI3K/AKT pathways, which leads to the suppression of mTOR and activation of FOXO transcription factors, resulting in myotube shrinkage (Sartori et al., 2021, Mishra et al., 2022). Our study demonstrates that F-WP exerts a significant effect on the regulation of protein synthesis in DEX-induced muscle atrophy, particularly through the IGF-1 signaling pathway. IGF-1 is a well-established stimulator of muscle protein synthesis and is a crucial factor in muscle growth and maintenance (Yoshida and Delainey, 2020). In our study, F-WP effectively rescued the DEX-inhibited expression of IGF-1 and its binding protein IGFBP3. F-WP also markedly inhibited DEX-stimulated expression of Cbl-b and REDD1, which had detrimental effects on IGF-1 and its receptor (Frost et al., 2009, Nakao et al., 2009). These results suggest that F-WP restores the anabolic actions of IGF-1, which are primarily mediated by the PI3K/AKT/mTOR signaling pathway (Mishra et al., 2022). We observed that F-WP attenuated DEX-induced downregulation of key components of this pathway, such as PI3K, AKT, and mTOR. Additionally, F-WP treatment restored the downstream targets of mTOR, such as 4E-BP1 and S6K1, both of which are implicated in the initiation of mRNA translation and protein synthesis (Laplante and Sabatini, 2012, Sartori et al., 2021). These findings provide a mechanistic understanding of how F-WP's enhance muscle protein synthesis, especially via phosphorylation, and counteract muscle atrophy by modulating the IGF-1 signaling pathway and its downstream targets, including S6K1. Moreover, IM13 enhanced the preventive effect of WP on muscle atrophy via IGF-1 and the PI3K/AKT/mTOR pathway during fermentation.

Moreover, our study demonstrated that F-WP effectively ameliorated DEX-induced downregulation of AKT phosphorylation, thereby modulating FOXO-mediated transcription of key genes involved in muscle atrophy, such as atrogin-1/MAFbx and MuRF1, E3 ubiquitin ligases involved in the UPP, and BNIP3, a marker of ALP (Bodine and Baehr, 2014, Zhang et al., 2020). Cathepsin-L and LC3, ALP-related genes, exhibited similar mRNA expression levels. F-WP exerted enhanced preventive effects on UPP- and ALP-mediated muscle protein degradation compared with WP. F-WP also significantly decreased the expression of KLF15, a transcription factor involved in BCAA degradation, and transcriptional upregulation of atrogin-1/MAFbx and MuRF1 (Cid-Díaz et al., 2021). Furthermore, BCAT2, a downstream marker of KLF15, was decreased following pretreatment with F-WP. These findings suggest that F-WP not only enhances protein synthesis by modulating the IGF-1-PI3K/AKT/mTOR signaling pathway, but also ameliorates protein degradation by regulating the UPP and ALP through FOXO-mediated transcription of key genes, resulting in beneficial effects on muscle protein homeostasis.

The production of whey protein hydrolysates by microbial hydrolysis is an interesting approach for enhancing the bioactivity of whey. Eleven peptides were identified, 9 of which were derived from β-casein. Sev-
eral studies have determined the presence of β-casein fragments in whey protein (Cunsolo et al., 2007). Most peptides, including casein-derived \textsuperscript{158}WMHQPHQ-PLPPT\textsuperscript{169}, were previously demonstrated to have antioxidant and antimicrobial activities (Oh et al., 2016; Nielsen et al., 2018; Komatsu et al., 2023; Rosa et al., 2023).

Existing research on the DEX-induced C2C12 muscle atrophy model has primarily focused on specific aspects of the IGF-1-mediated PI3K–AKT–mTOR pathway, leaving a gap in the comprehensive understanding. However, our research addresses this gap by offering a comprehensive analysis of the entire IGF-1–PI3K–AKT signaling pathway, including its effects on both mTOR and FOXO regulation, along with the downstream regulators S6K1, 4E-BP1, UPP, and ALP, and the upstream regulators Cbl-b and REDD1 (Figure 6). This comprehensive approach allowed us to explore the novel effects of fermented whey proteins on muscle protein synthesis and degradation in the context of DEX-induced muscle atrophy.

In conclusion, the present study demonstrated that F-WP prevented DEX-induced muscle atrophy by enhancing myogenesis and protein synthesis via the IGF-1–PI3K–AKT–mTOR pathway and by reducing protein degradation through FOXO-mediated regulation of the UPP and ALP. Moreover, treatment with F-WP promoted autophagy and myogenesis by reducing the expression of cathepsin L and BNIP3 and upregulating the expression of MyoD and myogenin. The beneficial effects of F-WP are associated with its high leucine content, antioxidant activity, and bioactive peptides. Collectively, these findings suggest that F-WP demonstrates the potential to prevent muscle atrophy and promote overall muscle protein homeostasis.

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Figure 6. Results of dexamethasone (DEX) treatment and fermented whey protein (F-WP) administration in the muscle cell model. F-WP administration to DEX-induced C2C12 myotube has preventive effects on the enhancement of muscle protein synthesis and myogenesis, and reduction of muscle protein degradation through the ubiquitin-proteasome and autophagy lysosomal system, indicating the improvement in muscle homeostasis.


