

Apigenin as a media supplement to promote muscle satellite cell differentiation for cultured meat production: an in silico and in vitro study

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ABSTRACT

Muscle satellite cells (MSCs) are essential for cultured meat (CM) production, and natural compound supplementation can enhance their proliferation and differentiation. Thus, this study used in silico analysis to identify natural compounds inhibiting myostatin (MSTN; a negative regulator of muscle development) and promoting MSC differentiation. Apigenin showed stable binding to MSTN (−6.5 kcal/mol) and was confirmed to directly interact with MSTN by cellular thermal shift assay, indicating its potential as a promising compound. Next, myoblasts (C2C12) and MSCs derived from bovine, chicken, and porcine species were cultured in media supplemented with apigenin. This led to increased expression of myosin heavy chain and downregulation of MSTN. Furthermore, the expression levels of SMAD2 and SMAD3, as well as their phosphorylated forms, which are downstream components of MSTN signaling, were also reduced. Notably, chicken MSCs exhibited the most pronounced response to apigenin supplementation, with differentiation reaching 235% and 135% of the control (control set as 100%) at 0.1 nM and 1 nM, respectively. Similar trends of enhanced MSC differentiation were observed in other species at 0.1 nM, with C2C12 cells increasing by 118% and bovine MSCs by 174%. Additionally, apigenin supplementation inhibited the production of intracellular reactive oxygen species (ROS). Therefore, apigenin shows potential as a natural additive or media supplement for CM production by promoting MSC differentiation, inhibiting MSTN, and suppressing ROS activity. Thus, this study suggests a potential strategy for improving the efficiency and quality of CM production through the supplementation of natural compounds.

1. Introduction

In contrast to conventional livestock slaughtering, cultured meat (CM) is produced in vitro through cell culture techniques using the myogenesis of muscle satellite (stem) cells (MSCs) (Y. Zhang et al., 2025). Consequently, CM offers a sustainable and efficient alternative to traditional meat. Moreover, by leveraging the self-renewal and

differentiation capabilities of MSCs, the production of CM can reduce environmental impact and address future proteinaceous food supply issues to meet the rising global protein demand. Myogenesis is vital for skeletal muscle (SM) growth, regeneration, and function. Therefore, myogenesis is essential in CM, as meat primarily consists of SM, which comprises approximately 90% muscle fibers and 10% connective and fat tissue (Syed Sayeed Ahmad et al., 2025; Post, 2012). Additionally, CM

Abbreviations: MSCs, Muscle satellite cells; CM, cultured meat; MSTN, Myostatin; ROS, Reactive oxygen species; SM, skeletal muscle; 3D, three-dimensional; PDB, Protein Data Bank; DS, Discovery Studio; MDs, Molecular dynamics simulations; RMSDs, Root mean square deviations; RMSFs, root mean square fluctuations; Rg, Radii of gyration; DMEM, Dulbecco's modified Eagle medium; FBS, Fetal bovine serum; PS, Penicillin–streptomycin; FGF2, Fibroblast growth factor 2; CETSA, Cellular thermal shift assay; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; SDS–PAGE, Sulfate–polyacrylamide gel electrophoresis; PVDF, Polyvinylidene fluoride; BSA, Bovine serum albumin; TBS, Tris-buffered saline; MYH, Myosin heavy chain; MYOG, Myogenin; ANOVA, Analysis of variance; TGF-β, Transforming Growth Factor beta; p-SMAD2, Phosphorylated-SMAD-2; p-SMAD3, Phosphorylated-SMAD-3; p-m-TOR, Phosphorylated-mTOR.

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production relies on several other key components, such as the activation of MSCs, a nutrient-rich growth medium (S. S. Ahmad et al., 2023), membrane proteins (IgLON4 and IgLON5) (Lim et al., 2021; Lim et al., 2022), and scaffolds to provide structural support (Ahmad, Ahmad, et al., 2024). Together, these elements enable the efficient and high-quality cultivation of CM.

MSCs are the key cell type for CM production, as sufficient proliferation and effective differentiation of MSCs represent some of the most critical factors in the manufacturing process. Furthermore, the composition of the culture medium used to grow these cells plays a significant role, as the medium directly impacts both the production cost and safety of the CM (Messmer et al., 2022; Pajcin et al., 2022). Currently, animal serum is commonly used to culture MSCs, which not only increases production costs but also raises concerns regarding food safety and ethical issues due to the original animal source (Stout et al., 2022). Consequently, extensive research is underway to discover components that can supplement the medium and promote MSC proliferation and differentiation while reducing or replacing the use of serum. Currently, plant-derived natural compounds have gained attention as effective supplements in culture media to promote MSC differentiation and mature myotube formation (Ahmad, Lee, et al., 2024; Ahmad, Lim, et al., 2024; Lim et al., 2025; Yan et al., 2022), potentially through mechanisms such as the regulation of myostatin (MSTN). Thus, using natural compounds would potentially a safe and cost-effective approach and may help address ethical concerns associated with animal-derived components (Li et al., 2022; Yamanaka et al., 2023; Yu et al., 2024).

However, experimentally screening a large number of individual natural compounds is both time-consuming and costly, making the process inefficient. Therefore, strategies utilizing in silico analyses have been widely adopted. These approaches first select target proteins, predict the natural compounds to which the proteins are likely to bind, and then analyze the biological activity of these compounds to identify efficient and promising candidates (Chigozie et al., 2025; Yao, 2012). In this regard, MSTN, a well-known protein that regulates MSC proliferation and differentiation, can also act as a key inhibitor of muscle development (negative regulator). Various studies have sought to inhibit MSTN to increase muscle mass (Ahmad, Lee, et al., 2024; E. J. Lee et al., 2022; Wagner et al., 2005). *Mstn*^{−/−} knockout mice have been reported to possess increased SM mass (J. Lin et al., 2002), and blocking MSTN activity further increased muscle mass (S. J. Lee, 2021). Notably, natural compounds such as quercetin and laxogenin have been identified as MSTN inhibitors, and the supplementation of these compounds has been shown to promote MSC differentiation for mature myotube formation and CM production in multiple livestock species, including bovine, porcine, and chicken (Ahmad, Lim, et al., 2024; Lim et al., 2025). However, these studies have largely focused on individual candidate compounds, and a systematic strategy for identifying MSTN-targeting natural products from plant-derived metabolite libraries remains limited. In particular, *Aloe vera*-derived natural compounds have not yet been comprehensively explored in the context of MSTN-mediated myogenic regulation.

Therefore, this study aimed to prepare an in-house library for natural compounds derived from *Aloe vera*. Around 40 compounds were collected and prepared for in silico analysis targeting the muscle growth inhibitor MSTN. Subsequently, we identified apigenin as a potential inhibitor of MSTN. Further, biological activity on MSCs derived from bovine, porcine, and chicken was evaluated, thereby proposing the possible use of apigenin as a culture media supplement for the production of CM. Unlike previous studies focusing on single-compound supplementation, this study integrates a library-based in silico screening approach followed by cross-species experimental validation, thereby enabling a more systematic identification of MSTN modulators. These in silico studies can serve as the initial basis for the subsequent screening of natural compounds. The identified and selected candidates were then advanced to the in vitro analysis, which can provide more reliable and widely accepted data. This progression strengthens the scientific validity

and supports further experimental investigations in a more accurate and credible format.

2. Materials and methods

2.1. In silico analysis

The three-dimensional (3D) crystal structure of the selected target protein, MSTN, was obtained from the RCSB Protein Data Bank (PDB ID: 3HH2; <https://www.rcsb.org/>) (Cash et al., 2009). Protein was prepared by removing any heteroatoms, deleting all noncatalytic water molecules and ligands, and adding H atoms, before assigning bond orders for molecular docking. The active site was defined with coordinates X = −29.37, Y = −20.90, and Z = 21.61 to evaluate the binding affinities of the selected natural compounds. Around 40 natural compounds derived from *Aloe vera* were prepared, and the individual structures of these compounds were downloaded from the PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) database in .sdf format. Further, the structure was created using Discovery Studio 2022 (DS). Thereafter, PyRx (Dallakyan & Olson, 2015) was adopted to perform the molecular docking. Additionally, molecular dynamics simulations (MDSs) were performed using GROMACS 2019.6 (Van Der Spoel et al., 2005) to check the stability of the selected complex obtained by docking analysis. The topology files of the compounds were prepared using SwissParam (Bugnon et al., 2023), and the target topology file was generated utilizing the CHARMM27 force field (Smith et al., 2015). Here, a dodecahedron box of extended TIP3P water was used to immerse the complexes. The simulation box was neutralized using the gmX genion module, and the bond lengths were constrained. NVT and NPT ensembles were used to complete the equilibration process in two steps. Finally, MDS was executed and the outcomes of the complex were analyzed in the form of root mean square deviations (RMSDs), root mean square fluctuations (RMSFs), radii of gyration (Rg), and numbers of H bonds. Furthermore, pkCSM (Pires et al., 2015) was used to predict the toxicity of the small molecule.

2.2. Animal ethical considerations

This study was approved by the Institutional Animal Care and Use Committee of Yeungnam University (approval number: AEC2022–022). All procedures involving the isolation and culture of MSCs from bovine, porcine, and chicken tissues were conducted in accordance with institutional and national guidelines for the care and use of animals, ensuring ethical standards and minimizing animal use.

2.3. C2C12 cell culture

C2C12 cells (Korean Cell Line Bank, Seoul, Korea) were cultured in growth medium consisting of Dulbecco's modified Eagle medium (DMEM) (Cytiva, Marlborough, MA, USA) supplemented with 10% fetal bovine serum (FBS; Cytiva) and 1% penicillin–streptomycin (PS; Cytiva), in a humidified incubator at 37 °C with 5% CO₂. When the cells reached a confluency of approximately 90%, the growth medium was replaced with differentiation medium consisting of DMEM supplemented with 2% FBS and 1% PS.

2.4. Bovine, chicken, and porcine MSC isolation and culture

Cranial thigh muscle tissues were obtained from bovine (34 months of age), porcine (3 days postnatal), and chicken embryos (embryonic day 16). The samples were finely minced and enzymatically dissociated using 1% pronase (Roche, Mannheim, Germany) for 1 h at 37 °C. The resulting cell suspension was centrifuged at 1000 ×g for 3 min, and the supernatant fraction containing dissociated cells was subsequently passed through a 100 µm cell strainer (Millipore, Darmstadt, Germany) to remove residual tissue debris. The filtered suspension was centrifuged again at 1000g for 5 min, and the resulting cell pellet was resuspended in

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 5 ng/mL fibroblast growth factor 2 (FGF2; Miltenyi Biotec GmbH, Auburn, CA, USA). The cells were then seeded onto collagen-coated culture plates (Corning, Brooklyn, NY, USA) and maintained at 37 °C in a humidified atmosphere containing 5% CO₂. The culture medium was refreshed daily throughout the incubation period. Bovine, chicken, and porcine MSCs were cultured in growth media (MSC: Ham's F10 (Cytiva) + 20% FBS + 1% PS + 5 ng/mL FGF2 in a humidified 5% CO₂ incubator at 37 °C. When the cells reached a confluency of approximately 100%, the growth medium was replaced with differentiation medium consisting of DMEM supplemented with 2% FBS and 1% PS.

2.5. Apigenin supplementation in media

Apigenin (Sigma-Aldrich, St. Louis, MO, USA; empirical formula: C₁₅H₁₀O₅; purity ≥95%; molecular weight: 270.24) was first dissolved in dimethyl sulfoxide (DMSO, ≥99.9%, Sigma-Aldrich, St. Louis, MO, USA) to prepare a 10 mM stock solution. The stock solution was subsequently serially diluted to obtain the desired working concentrations. Prior to addition to the culture medium, each apigenin solution was thoroughly vortexed and briefly centrifuged to ensure complete dissolution and to minimize potential precipitation.

To assess the impact of apigenin, C2C12 cells or MSCs were cultured in growth or differentiation media supplemented with varying concentrations of apigenin (0, 0.1, 1, 10, 100, or 1000 nM) for 4 days. Apigenin-containing media were freshly prepared and replaced every 2 days throughout the culture period.

2.6. Cellular thermal shift assay (CETSA)

To investigate the interaction between MSTN and apigenin, C2C12 cells were incubated with either DMSO or 0.1 nM apigenin at 37 °C in a CO₂-controlled incubator for 2 h. Following treatment, cells were harvested, washed three times with phosphate-buffered saline (PBS), and resuspended in PBS containing a protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The cells were then subjected to heat shock at 40, 46, 52, 58, and 64 °C for 3 min in a PCR machine (Applied Biosystems, Foster City, CA, USA). After heat exposure, the samples underwent three freeze–thaw cycles using liquid nitrogen. The resulting lysates were centrifuged, and the supernatants were collected for Western blot analysis.

2.7. MTS assay

To analyze cell proliferation, cells were incubated in growth medium supplemented with CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA) at 37 °C in a humidified incubator with 5% CO₂ for 1 h. The absorbance was measured at 490 nm using a VersaMax microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.8. Creatine kinase activity

MSCs were cultured in differentiation medium supplemented with apigenin for 4 days. After incubation, the cells were washed with PBS, harvested, and centrifuged at 10,000 ×g for 15 min. The resulting supernatants were analyzed using a creatine kinase assay kit (BioAssay Systems, Hayward, CA, USA), and the absorbance was measured at 340 nm using a VersaMax™ microplate reader. The obtained values were then used to calculate enzyme activity according to the following equation.

$$\text{CK (U/L)} = \frac{\text{O.D40min} - \text{O.D20min}}{\text{O.D CALIBRATOR} - \text{O.DH2O}} \times 150$$

2.9. RNA isolation, cDNA synthesis, and real-time RT-PCR

Total RNA was extracted, and real-time RT-PCR was conducted following a previously described protocol (E. J. Lee et al., 2018). The sequences of the primers used in the analysis are listed in Supplementary Table 1.

2.10. Western blot

Protein samples (40 µg from C2C12 cells or 100 µg from MSCs) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. These membranes were incubated in blocking solution and then incubated with primary antibodies diluted in 1% skim milk or bovine serum albumin (BSA) in Tris-buffered saline (TBS) at 4 °C overnight. The used primary antibodies were myosin heavy chain (MYH, C2C12: 1:500; MSCs: 1:250; Santa Cruz Biotechnology, Dallas, TX, USA), myogenin (MYOG, 1:1000; Abcam Limited, Cambridge, UK), MSTN (1:1000; Cloud-Clone Corp., Houston, TX, USA), SMAD2 (1:1000; Cell Signaling Technology, Danvers, MA, USA), phosphorylated-SMAD2 (p-SMAD2, 1:1000, Cell Signaling Technology), SMAD3 (Cell Signaling Technology), phosphorylated-SMAD3 (p-SMAD3, 1:1000, Cell signaling Technology), mTOR (1:1000, Cell Signaling Technology), phosphorylated-mTOR (p-mTOR, 1:1000, Cell Signaling Technology) and β-actin (1:1000; Santa Cruz Biotechnology). After washing, membranes were incubated with HRP-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit; GeneTex, Irvine, CA, USA) at room temperature for 1 h. Protein bands were visualized using the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Full-length Western blot images are presented in Supplementary Fig. 1.

2.11. Immunocytochemistry

Immunocytochemistry was conducted based on a previously described protocol (E. J. Lee et al., 2018). Briefly, cells were fixed with formaldehyde and permeabilized using 0.2% Triton X-100 (Sigma-Aldrich) for 5 min. After washing with PBS, nonspecific binding was blocked by incubating the cells in 1% normal goat serum for 30 min. Cells were then incubated with a primary antibody against MYH (1:50 dilution) at 4 °C overnight. The following day, Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (1:100; Thermo Fisher Scientific) was applied at room temperature for 1 h. Nuclei were counterstained with DAPI (Sigma-Aldrich), and fluorescence images were acquired using a fluorescence microscope.

2.12. Reactive oxygen species activity (ROS) analysis

C2C12 cells or MSCs were incubated in differentiation media with or without apigenin for 4 days, followed by incubation with 2',7'-dichlorofluorescein (Sigma-Aldrich) at 37 °C for 1 h. After washing with PBS, fluorescence was measured using a VersaMax™ microplate reader (excitation 498 nm, emission 522 nm).

2.13. Statistical analysis

For gene expression analysis, normalized mean values were compared using a Dunnett's post hoc test, following one-way analysis of variance (ANOVA) conducted in SAS software (version 9.0, SAS Institute, Cary, NC, USA). GAPDH was used as an internal control. Statistical significance was defined as a value of $p < 0.05$. The data are presented as the mean ± standard deviation (SD) ($n > 3$). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.0001$.

3. Results

3.1. *In silico* analysis

Apigenin was found to be non-toxic in terms of Ames toxicity, non-hepatotoxic, non-sensitizing to the skin, and had 93.25% human intestinal absorption. *In silico* screening and MDS (100 ns) were conducted to investigate the interaction between apigenin and MSTN, as well as the stability of the complex. Apigenin can bind with the MSTN and possesses a free energy of binding of -6.5 kcal/mol. Six H bonds (PHE2:N-UNL1:O, GLY3:N-UNL1:O, ARG17:NH₂-UNL1:O, SER80:OG-UNL1:O, ARG105:NE-UNL1:O, and ARG105:NH₂-UNL1:O) were observed in the complex structure formation of apigenin-MSTN during the docking study. In addition to the H bond, a hydrophobic (UNL1:H-ASP104:O) and electrostatic interaction (ASP1:N-UNL1:O) were also observed. The total amino acid residues, including Asp1, Phe2, Gly3, Asp5, Arg17, Lys78, Ser80, Asp104, and Arg105, were present in the apigenin-MSTN complex (Fig. 1A–C).

Furthermore, MDS was performed for the selected apigenin-MSTN complex up to 100 ns to evaluate the stability of the complex. The complex was found to be stable with less fluctuation. Here, the RMSD of the backbone and C α is shown in black and red, respectively. The RMSD values of the backbone and C α were obtained as 0.28 ± 0.04 and 0.29 ± 0.04 nm, respectively (Fig. 2A). Further investigation of the RMSD for the compound was conducted to evaluate the dynamic motion, revealing that apigenin binds effectively to the MSTN (Fig. 2B) and demonstrates improved structural stability after 13 ns throughout the MDS. Further, the H bonds play an essential role in stabilizing the protein-ligand complexes. A maximum of 4H bonds were observed in the complex, but only 2H bonds remained stable throughout the simulation period (Fig. 2C), thereby enhancing the stability of the complex. Here, less fluctuation was observed around the binding amino acid residues, such as Asp1, Phe2, Gly3, Asp5, Arg17, Lys78, Ser80, Asp104, and Arg105 (Fig. 2D). This finding reveals that apigenin exhibits enhanced stability within the binding pocket, where selected residues presented reduced fluctuation. The average Rg of the apigenin-MSTN complex was 1.83 ± 0.03 nm following a 100 ns simulation time (Fig. 2E).

3.2. Binding analysis between apigenin and MSTN

In silico analysis predicted a potential interaction between apigenin and MSTN. Therefore, to experimentally validate this interaction, this study employed a CETSA (Martinez Molina et al., 2013), which was based on the principle that ligand binding increases the thermal stability of target proteins. Following treatment of C2C12 cells with 0.1 nM apigenin and subsequent heat exposure, the MSTN signal was more prominent in apigenin-treated cells compared to the control group (Fig. 3A and B), suggesting that apigenin binding enhanced the thermal

stability of MSTN. These results further indicate that apigenin directly binds to MSTN and stabilizes its structure.

3.3. Effect of apigenin on C2C12 cell proliferation and differentiation

To assess the effect of apigenin on the proliferation and differentiation of C2C12 cells, various concentrations of apigenin, including 0, 0.1, 1, 10, 100, and 1000 nM, were applied, and biological responses were analyzed. Supplementation with 1 nM apigenin promoted a statistically significant $\sim 5\%$ increase in cell proliferation compared to the untreated group (Fig. 4A). Additionally, creatine kinase activity was significantly elevated with 0.1 nM (118%) and 1 nM (109%) apigenin during differentiation (Fig. 4B and Supplementary Fig. 2A). Apigenin treatment upregulated both mRNA and protein expression levels of MYH, while downregulating MSTN expression (Fig. 4C–E). In parallel, apigenin reduced the mRNA and protein expression levels of SMAD2 and SMAD3. Consistently, the levels of p-SMAD2 and p-SMAD3 were also decreased. Furthermore, apigenin treatment increased the protein expression of mTOR and p-mTOR (Supplementary Figs. 3A and 4A). ROS activity was also significantly suppressed by 0.1 nM apigenin (Fig. 4F and Supplementary Fig. 5A). These findings suggest that apigenin promotes C2C12 cell proliferation and differentiation while suppressing oxidative stress.

3.4. Effect of apigenin on bovine MSC proliferation and differentiation

To investigate the effect of apigenin on bovine MSCs, dose-dependent treatments were conducted, followed by analysis of cellular responses. At 1 nM, apigenin supplementation resulted in a significant $\sim 5\%$ increase in proliferation compared to the control (Fig. 5A and Supplementary Fig. 2B). Furthermore, apigenin markedly enhanced creatine kinase activity at 0.1 nM (174%), 1 nM (111%), and 10 nM (136%) during differentiation. Consistently, MYH expression at both mRNA and protein levels was increased, whereas MSTN expression was decreased (Fig. 5B–E). In parallel, SMAD2 and SMAD3 expression was downregulated at both transcript and protein levels. In agreement with these findings, the levels of p-SMAD2 and p-SMAD3 were also reduced. In addition, apigenin treatment enhanced mTOR signaling, as evidenced by increased levels of mTOR and p-mTOR (Supplementary Figs. 3B and 4B). The ROS levels were significantly reduced in the 0.1 nM and 1 nM supplemented groups (Fig. 5F and Supplementary Fig. 5B). These results indicate that apigenin enhances both proliferation and differentiation of bovine MSCs while mitigating oxidative stress.

3.5. Effect of apigenin on chicken MSC proliferation and differentiation

The influence of apigenin on chicken MSCs was assessed through dose-dependent treatment and subsequent analysis. A treatment of 1 nM induced a statistically significant $\sim 6\%$ increase in proliferation (Fig. 6A

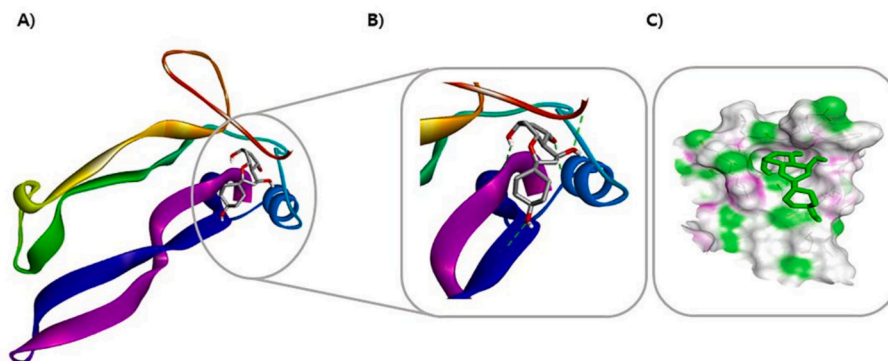


Fig. 1. The interaction of apigenin with MSTN. A) The complex structure of apigenin and MSTN. B) Interaction between H bonds in apigenin and MSTN. C) The positioning of apigenin within MSTN.

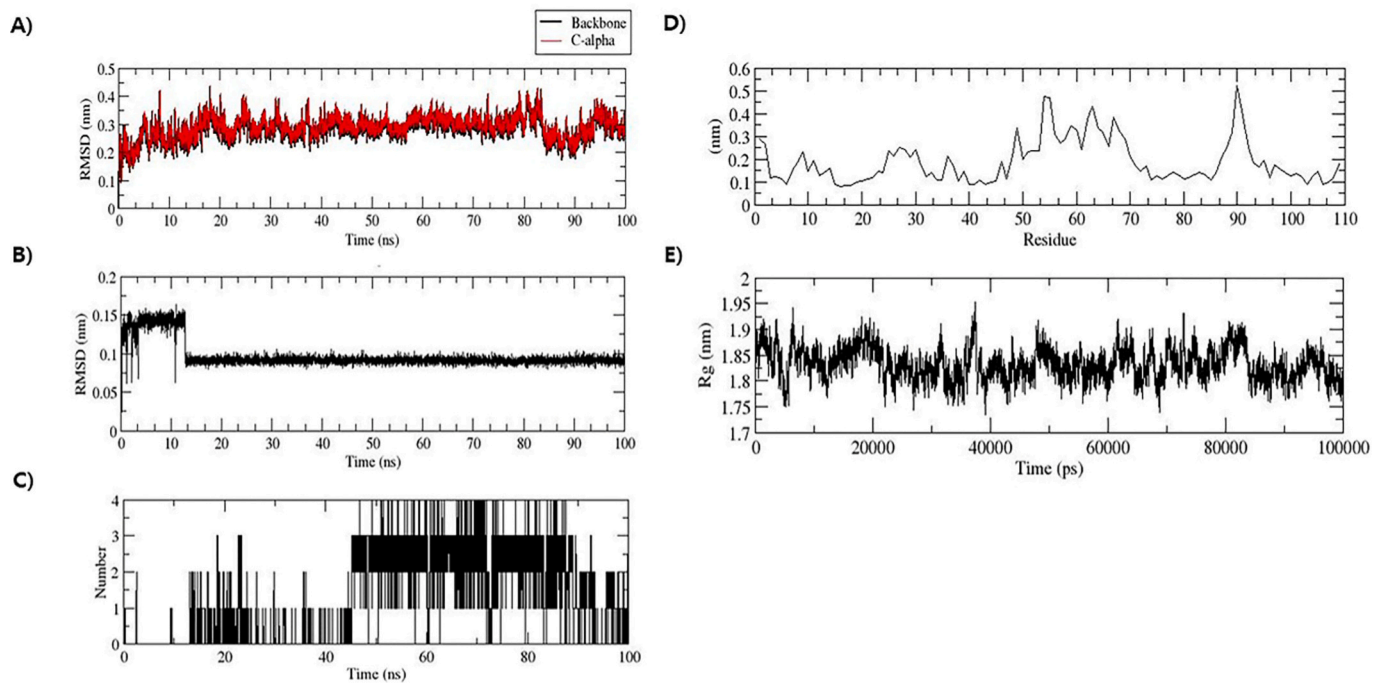


Fig. 2. Molecular dynamics simulation analysis of the apigenin–MSTN complex. A) Backbone RMSD (black) and C α RMSD (red) in MSTN—indicate protein structural stability throughout the simulation. B) RMSD of apigenin—reflects ligand conformational stability within the binding site. C) Time-dependent hydrogen bond formation between apigenin and MSTN residues—demonstrates sustained interactions. D) RMSF of MSTN residues in the complex—shows minimal fluctuations near the binding pocket. E) Rg of the complex—indicates stable and compact structural conformation during the simulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

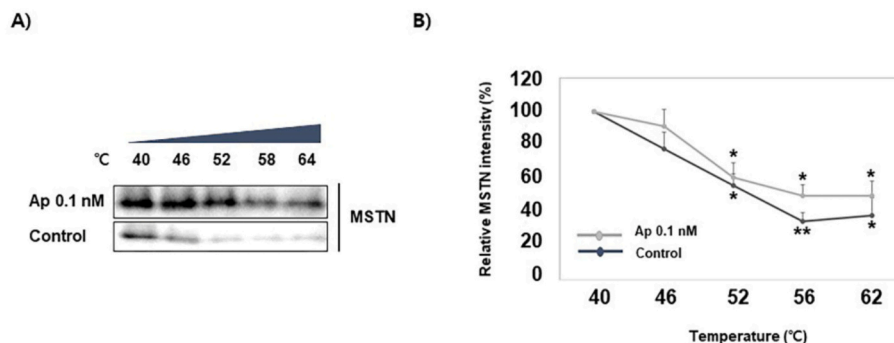


Fig. 3. Apigenin and MSTN protein binding. To demonstrate the direct binding between MSTN and apigenin, C2C12 cells were treated with apigenin (0.1 nM) or DMSO (control), followed by heat treatment at 40, 46, 52, 58, and 64 °C. MSTN protein levels were detected by Western blot analysis. MSTN thermal stability curves were generated for both the apigenin-treated and control groups. Significant differences compared to the control (non-treated cells) were determined by one-way ANOVA followed by Dunnett's post hoc test: * $p < 0.05$, ** $p < 0.01$ (mean $\pm$ SD, $n = 3$).

and Supplementary Fig. 2C). Creatine kinase activity was also significantly elevated at 0.1 nM (235%), 1 nM (135%), 10 nM (126%), and 100 nM (111%), while the activity was shown to decrease at 1000 nM. Consistently, apigenin treatment increased MYH expression at both mRNA and protein levels, while reducing MSTN expression (Fig. 6B–E). In parallel, SMAD2 and SMAD3 expression was downregulated at both transcript and protein levels, accompanied by decreased levels of p-SMAD2 and p-SMAD3. Furthermore, mTOR signaling was enhanced, as evidenced by increased expression of mTOR and p-mTOR (Supplementary Figs. 3C and 4C). In addition, ROS levels were significantly reduced at 0.1 nM and 1 nM apigenin (Fig. 6F and Supplementary Fig. 5C). Collectively, these findings suggest that apigenin supplementation significantly promotes chicken MSC proliferation and differentiation, and alleviates oxidative stress. Here, apigenin supplementation was found to be more effective for chicken MSC differentiation.

3.6. Effect of apigenin on porcine MSC proliferation and differentiation

To evaluate the effect of apigenin on porcine MSCs, cells were treated with increasing concentrations of apigenin and analyzed accordingly. At 10 nM, apigenin slightly reduced cell proliferation to approximately 90% of the control level, corresponding to an approximately 10% decrease in proliferation, indicating a modest inhibitory effect (Fig. 7A and Supplementary Fig. 2D). However, apigenin treatment increased MYH expression at both mRNA and protein levels, while reducing MSTN expression (Fig. 7B–E). In parallel, SMAD2 and SMAD3 expression was downregulated at both transcript and protein levels, accompanied by decreased levels of p-SMAD2 and p-SMAD3. Furthermore, mTOR signaling was enhanced, as evidenced by increased expression of mTOR and p-mTOR (Supplementary Figs. 3D and 4D). In addition, ROS levels were significantly reduced at 1 nM and 10 nM apigenin (Fig. 7F and Supplementary Fig. 5D). These data indicate that, despite the observed

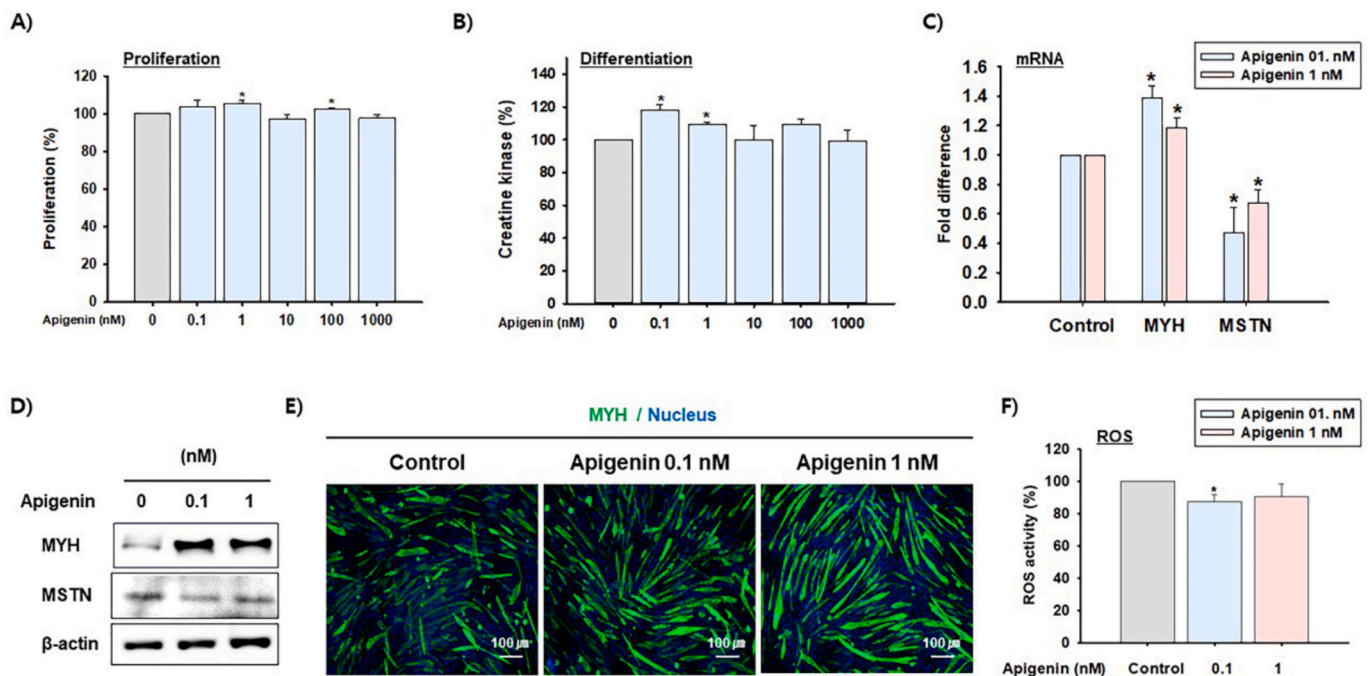


Fig. 4. The effects of apigenin on the proliferation and differentiation of C2C12 cells. Cells were incubated with proliferation medium supplemented with apigenin at concentrations of 0, 0.1, 1, 10, 100, or 1000 nM for 4 days. A) Cell proliferation was assessed using the MTS assay ($n = 7$). Once the cells reached a confluency of 100%, the medium was switched to differentiation medium containing the same apigenin concentrations and incubated for an additional 4 days. B) Cell differentiation was evaluated by measuring creatine kinase activity ($n = 5$). C–D) mRNA and protein expression levels of MYH and MSTN were analyzed in cells treated with 0, 0.1, and 1 nM apigenin using real-time RT-PCR and Western blotting, respectively ($n = 5$). E) MYH protein expression was confirmed by immunocytochemistry in cells treated with 0, 0.1, and 1 nM apigenin. F) ROS activity was measured in cells exposed to the same concentrations ($n = 6$). Untreated cells were used as the control. Significant differences compared to the control (non-treated cells) were determined by one-way ANOVA followed by Dunnett's post hoc test: $*p < 0.05$ (mean $\pm$ SD). green: MYH, blue: nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

anti-proliferative effect at higher doses, administering apigenin enhances porcine MSC differentiation and reduces oxidative stress.

4. Discussion

Structure-based virtual screening against MSTN was performed for the natural compounds obtained from *Aloe vera*. However, the collection of the compounds was not limited to the most abundant constituents, but its aimed to include all the possible phytochemicals reported in *Aloe vera*. A total of 40 *Aloe vera*-derived natural compounds were subjected to molecular docking against MSTN. Based on binding energy, interaction profiles, and docking stability, the top five compounds with the most favorable binding energies was present in supplementary Table 2. Among these candidates, apigenin exhibited the most promising profile, with a binding energy of -6.5 kcal/mol, along with favorable interaction patterns within the MSTN binding pocket. Importantly, apigenin was further prioritized due to its consistent performance in subsequent in vitro assays, where it demonstrated significant biological activity compared to other shortlisted compounds. To further evaluate the interaction between MSTN and its receptor activin receptor type IIB (ActR2B), protein-protein docking was performed using ZDOCK (<https://zdock.wenglab.org/>), yielding a global energy score of 1702.51, indicative of a stable MSTN–ActR2B complex. Subsequently, the MSTN–apigenin complex was docked with ActR2B. Notably, the resulting ZDOCK global energy score was reduced (global energy score: 1643.27) compared to MSTN alone, implying a weakened interaction between MSTN and its receptor in the presence of apigenin (Supplementary Fig. 6). This reduction in binding affinity suggests that apigenin may interfere with MSTN–ActR2B signaling. These kinds of inhibition may contribute in the SM formation.

Among the list of compounds, apigenin was found to be more suitable in terms of its binding affinity and physicochemical properties.

Apigenin is a dietary flavonoid found in many plants, including *Aloe vera*, *Camellia sinensis*, celery, celeriac, basil, and various vegetables. Apigenin possesses strong antioxidant and anti-inflammatory properties (Malik et al., 2017). Overall, apigenin was found to bind strongly with MSTN, with fluctuations ranging from 0.25 to 0.3 nm, which is well within the acceptable range of 0.3 nm (Khan et al., 2019), as analyzed using MDS. This suggests that the complex exhibits increased stability. A lower and constant RMSD value indicates a more stable and reliable binding interaction between the protein and ligand throughout the simulation period. The RMSF value was used to assess the flexibility of the amino acid residues in the complex upon ligand binding to the target (Al-Nema et al., 2020). Indeed, a lower RMSF value indicates stability, while higher values demonstrate flexibility and instability. The Rg is a main factor directly related to protein compactness, indicating how tightly the protein structure is folded. It is reported that a correctly folded protein maintains a stable Rg; thus, a lower Rg designates a better compactness level (S. S. Ahmad et al., 2021). Here, during 100 ns MDS, the complex displayed a consistently stable trajectory with minor variations, thereby indicating a more compact arrangement. Overall, the selected compound apigenin represents a promising candidate for further investigation for use in human consumption and as a media supplement in cell culture. Moreover, as apigenin is derived from natural resources, no significant toxicity or skin irritation was predicted by the in silico analysis. In this study, MSTN inhibition appears to be a potentially useful strategy for promoting myotube formation and maturation in CM production. The utilization of natural compounds as media supplements, due to their antioxidant and anti-inflammatory properties, may represent an effective strategy in enhancing SM development during cellular growth and differentiation. Consistent with this study, other natural compounds, such as quercetin (Ahmad, Lim, et al., 2024), laxogenin (Lim et al., 2025), and naringenin (Yan et al., 2022), have also been described to enhance the differentiation of MSCs and

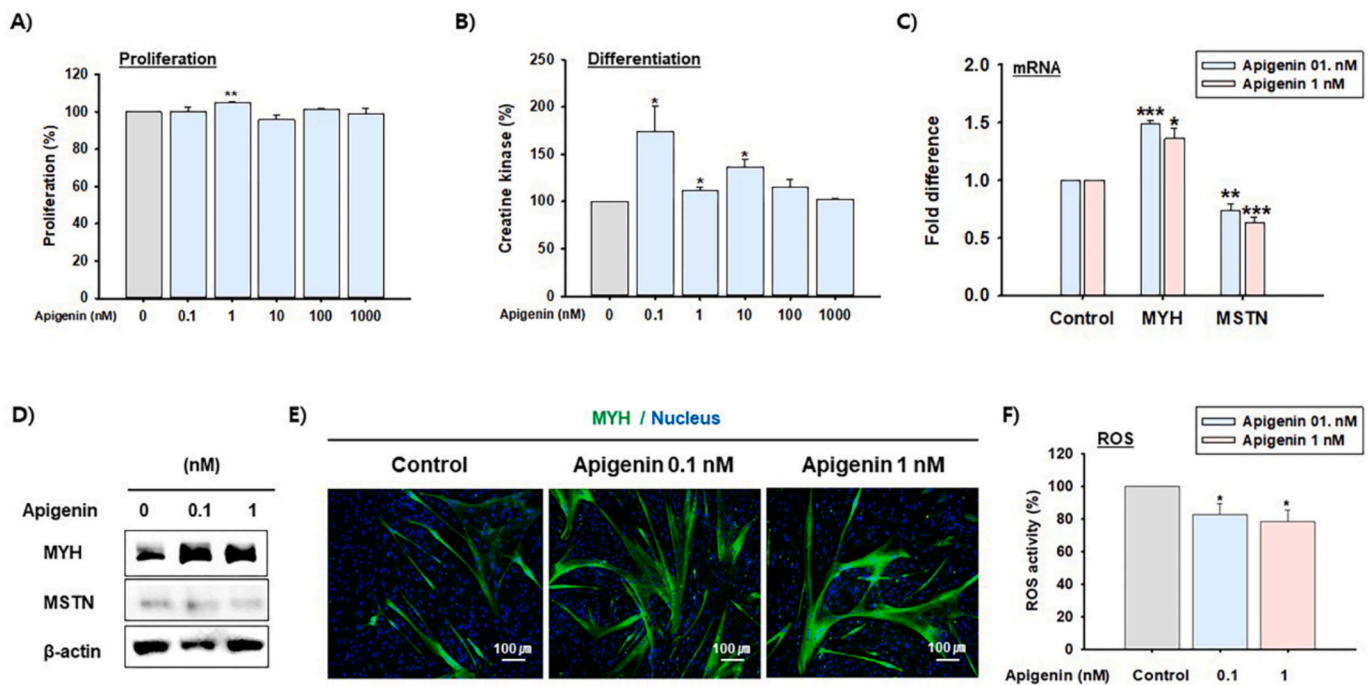


Fig. 5. The effects of apigenin on the proliferation and differentiation of bovine MSCs. Cells were incubated with proliferation medium supplemented with apigenin at concentrations of 0, 0.1, 1, 10, 100, or 1000 nM for 4 days. A) Cell proliferation was assessed using the MTS assay ($n = 4$). Once the cells reached a confluency of 100%, the medium was switched to differentiation medium containing the same apigenin concentrations and incubated for an additional 4 days. B) Cell differentiation was evaluated by measuring creatine kinase activity ($n = 6$). C–D) mRNA and protein expression levels of MYH and MSTN were analyzed in cells treated with 0, 0.1, and 1 nM apigenin using real-time RT-PCR and Western blotting, respectively ($n = 5$). E) MYH protein expression was confirmed by immunocytochemistry in cells treated with 0, 0.1, and 1 nM apigenin. F) ROS activity was measured in cells exposed to the same concentrations ($n = 5$). Untreated cells were used as the control. Significant differences compared to the control (non-treated cells) were determined by one-way ANOVA followed by Dunnett's post hoc test: * $p < 0.05$ (mean $\pm$ SD). green: MYH, blue: nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

promote myotube formation and maturation, making these natural compounds potential candidates for culture media supplementation.

Importantly, the attenuation of myogenic differentiation observed at the highest concentration of apigenin (1000 nM) suggests a potential concentration-dependent inhibitory effect. Although MTS analysis did not indicate a substantial reduction in overall cell viability, the absence of comprehensive apoptosis or cell death pathway analyses across all experimental conditions limits a definitive exclusion of subtle cytostatic or stress-related effects. Therefore, the reduced differentiation efficiency at higher concentrations may reflect not only non-cytotoxic modulation of signaling pathways but also possible activation of cellular stress responses that are not captured by viability assays alone. In particular, flavonoids at elevated concentrations have been reported to influence mitochondrial function and redox-sensitive signaling cascades, which may indirectly impair myogenic differentiation capacity without inducing overt cytotoxicity (Chen et al., 2020; C. Kim & Hwang, 2020). These findings highlight that apigenin exerts a narrow concentration-dependent effect on MSC biology, emphasizing the importance of defining an optimal therapeutic window for its application. From an industrial perspective, such a narrow effective range underscores the necessity for precise dose optimization when considering apigenin as a functional media supplement for CM production.

Following the *in silico* analysis, which suggested a potential interaction between apigenin and the MSTN protein, we confirmed that these molecules directly bind within cells using a CETSA. This technique enables the direct detection of protein–ligand interactions in intact cells by monitoring shifts in the thermal stability of target proteins upon ligand binding (Jafari et al., 2014; Tu et al., 2023). Based on these findings, we evaluated the effects of apigenin on the proliferation and differentiation of MSCs across various species. Apigenin-supplemented media promoted proliferation in bovine and chicken MSCs. Previous studies have

reported that apigenin inhibits cell proliferation in several cancer cell lines, including lung, colon, and bladder cancers (Shi et al., 2015; Xu et al., 2016; Zhou et al., 2017). However, at specific concentrations, apigenin has also been shown to promote the proliferation, osteogenic differentiation, and expression of osteogenesis-related markers in human osteoblasts (Pan et al., 2021; X. Zhang et al., 2015). In this study, supplementation of cultured media with apigenin at concentrations of 0.1, 1, and 10 nM enhanced MSC differentiation, as evidenced by a dose-dependent increase in creatine kinase activity and the mRNA and protein expression of MYH, both well-established indicators of myogenic differentiation. Notably, creatine kinase activity remains low in undifferentiated MSCs and increases as differentiation progresses, making this metric a reliable quantitative marker for myogenic differentiation (Al-Khalili et al., 2004). It has also been reported that higher expressions of MYH promote improved differentiation, meaning the conversion of MSCs to mature muscle tissues is more efficient (Y. A. Kim et al., 2024). Additionally, increased MYH expression usually correlates with enhanced myotube formation and sarcomere organization (E. J. Lee et al., 2025). Therefore, several studies have reported that using natural compounds in CM research has increased MYH expression and improved the maturity of the muscle tissue *in vitro* (Ahmad, Lim, et al., 2024; Lim et al., 2025; Yan et al., 2022). Meanwhile, the texture, mechanical properties, nutrients, and protein content are increasingly governed by how well MYHs are expressed and organized (Yan et al., 2022). Overall, supplementation of apigenin at 1 nM enhanced the proliferation of C2C12 cells and bovine MSCs by ~5% and of chicken MSCs by ~6%. Additionally, differentiation levels of 118%, 174%, and 235% were observed in C2C12 cells and bovine and chicken MSCs at 0.1 nM compared to the control. Further analysis showed that apigenin-supplemented media at concentrations of 0.1 nM (235%), 1 nM (135%), 10 nM (126%), and 100 nM (111%) promoted enhanced

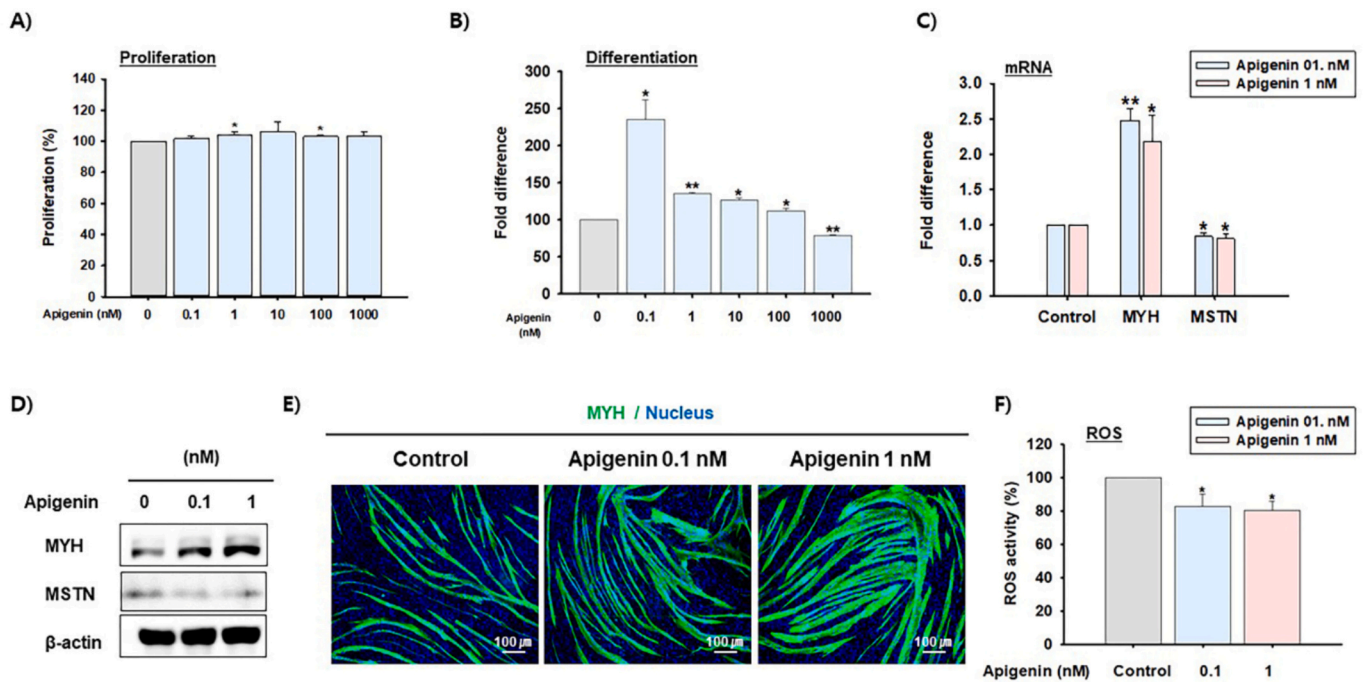


Fig. 6. The effects of apigenin on the proliferation and differentiation of chicken MSCs. Cells were incubated with proliferation medium supplemented with apigenin at concentrations of 0, 0.1, 1, 10, 100, or 1000 nM for 4 days. A) Cell proliferation was assessed using the MTS assay ($n = 4$). Once the cells reached a confluency of 100%, the medium was switched to differentiation medium containing the same apigenin concentrations and incubated for an additional 4 days. B) Cell differentiation was evaluated by measuring creatine kinase activity ($n = 9$). C–D) mRNA and protein expression levels of MYH and MSTN were analyzed in cells treated with 0, 0.1, and 1 nM apigenin using real-time RT-PCR and Western blotting, respectively ($n = 4$). E) MYH protein expression was confirmed by immunocytochemistry in cells treated with 0, 0.1, and 1 nM apigenin. F) ROS activity was measured in cells exposed to the same concentrations ($n = 5$). Untreated cells were used as the control. Significant differences compared to the control (non-treated cells) were determined by one-way ANOVA followed by Dunnett's post hoc test: * $p < 0.05$, ** $p < 0.01$ (mean $\pm$ SD). green: MYH, blue: nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chicken MSC differentiation. Therefore, lower concentrations of apigenin, such as 0.1 nM and 1 nM, may significant potential to enhance MSC differentiation in these selected species, particularly for chicken-derived MSCs. These results indicate that the magnitude of apigenin-induced myogenic differentiation differs across species, with chicken MSCs exhibiting the most pronounced response. Such interspecies variation may be attributed to differences in MSTN signaling sensitivity, as MSTN is a key negative regulator of muscle differentiation and its activity directly regulates myogenesis across livestock species (Ahmad, Lim, et al., 2024). In addition, alterations in MSTN signaling have been linked to metabolic and antioxidant pathways in muscle, suggesting that species-dependent MSTN activity may influence cellular responsiveness to bioactive compounds (Zhu et al., 2022). Another possible explanation is the variation in baseline oxidative status among MSCs derived from different species. Apigenin is a well-known flavonoid with strong antioxidant activity, capable of reducing ROS and enhancing antioxidant enzyme activity in muscle cells (Wang et al., 2020). Since oxidative stress negatively affects MSC function and differentiation potential, cells with higher basal oxidative stress may exhibit a greater response to antioxidant treatment (Haramizu et al., 2017). In addition, differences in cell isolation procedures, donor variability, and tissue origin could influence MSC heterogeneity, which in turn may affect their responsiveness to bioactive compounds such as flavonoids (Tierney & Sacco, 2016). Notably, the reduced proliferative response observed in porcine MSCs at higher apigenin concentrations suggests a species-specific sensitivity to flavonoids, possibly reflecting differences in metabolic processing or intracellular signaling thresholds. Collectively, these findings highlight the importance of considering species-dependent cellular contexts when evaluating the biological effects of apigenin. Apigenin treatment also suppressed the mRNA and protein expression of MSTN, a member of the Transforming Growth Factor beta (TGF- β)

superfamily, which is predominantly secreted in SM, and acts as a negative regulator of muscle growth and differentiation. Meanwhile, disrupting the expression of MSTN in mice has been shown to result in dramatic increases in muscle mass (Elkina et al., 2011; McPherron et al., 1997). Therefore, the downregulation of MSTN may contribute to enhance MSC growth and differentiation, contributing to increased muscle mass. Our findings suggest that apigenin not only binds directly to MSTN but also inhibits the expression of MSTN, thereby potentially promoting MSC differentiation.

In addition, apigenin reduced intracellular levels of ROS, which are highly reactive byproducts of oxygen metabolism and play roles in intracellular signaling and defense mechanisms. However, excessive ROS can lead to cellular damage (Schieber & Chandel, 2014). Moderate ROS levels in MSCs can promote activation and differentiation, whereas elevated ROS levels suppress key myogenic transcription factors such as MyoD and MYH2, and induce apoptosis and functional decline (Atashi et al., 2015; Bhattacharya & Scime, 2020; Lian et al., 2022). Thus, reducing excessive ROS is critical for maintaining the viability, proliferative capacity, and differentiation potential of MSCs during large-scale and long-term culture for CM production. Our findings suggest that apigenin may serve as a potential medium supplement for promoting MSC differentiation and supporting a favorable cellular environment while mitigating ROS-related cellular stress.

Currently, consumers are looking for meat products that are more nutritious, healthy, functional, and safe. This growing demand has caused the meat industry to explore new technologies. One popular approach is the addition of natural ingredients/compounds/supplements, such as healthy fats, minerals, vitamins, antioxidants, dietary fiber, probiotics, bioactive peptides, and natural compounds. These ingredients not only improve the meat products but also offer health benefits to consumers (Abdallah et al., 2020; Olmedilla-Alonso et al.,

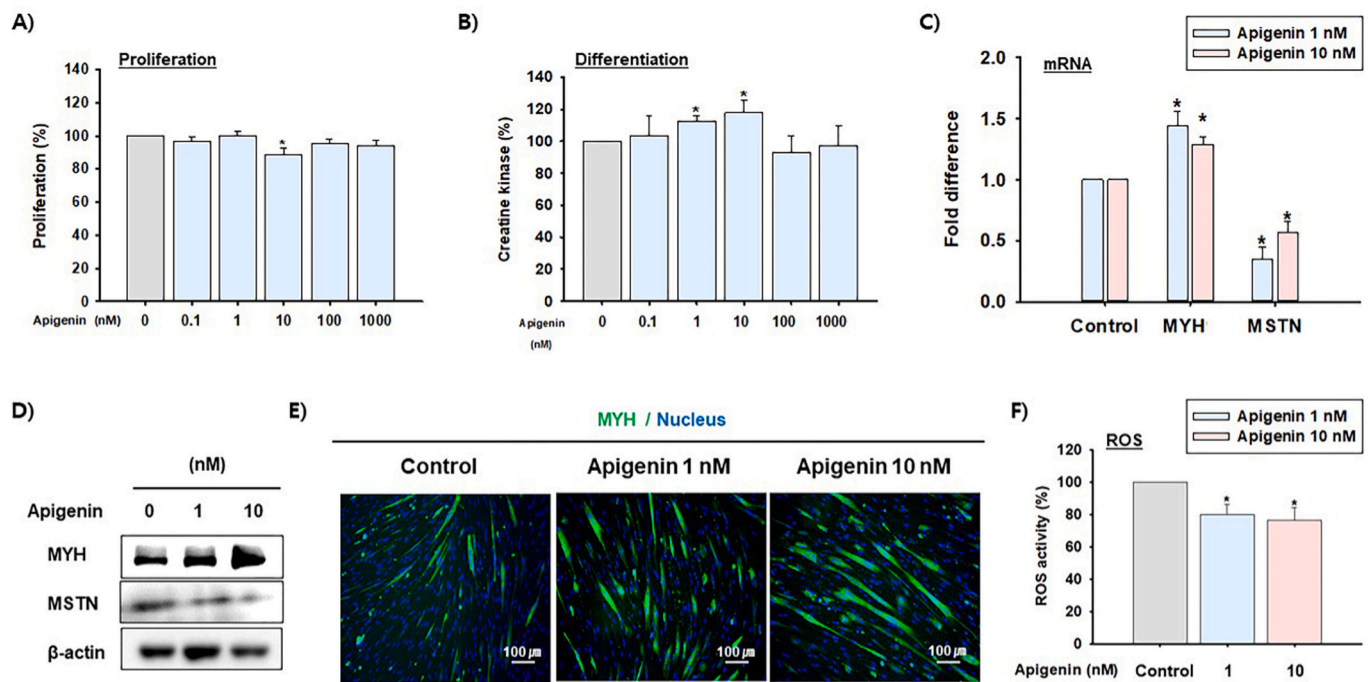


Fig. 7. The effects of apigenin on the proliferation and differentiation of porcine MSCs. Cells were incubated with proliferation medium supplemented with apigenin at concentrations of 0, 0.1, 1, 10, 100, or 1000 nM for 4 days. A) Cell proliferation was assessed using the MTS assay ($n = 8$). Once the cells reached a confluency of 100%, the medium was switched to differentiation medium containing the same Apigenin concentrations and incubated for an additional 4 days. B) Cell differentiation was evaluated by measuring creatine kinase activity ($n = 6$). C–D) mRNA and protein expression levels of MYH and MSTN were analyzed in cells treated with 0, 1, and 10 nM apigenin using real-time RT-PCR and Western blotting, respectively ($n = 4$). E) MYH protein expression was confirmed by immunocytochemistry in cells treated with 0, 1, and 10 nM apigenin. F) ROS activity was measured in cells exposed to the same concentrations ($n = 5$). Untreated cells were used as the control. Significant differences compared to the control (non-treated cells) were determined by one-way ANOVA followed by Dunnett's post hoc test: $*p < 0.05$, (mean $\pm$ SD). green: MYH, blue: nucleus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2013; Pogorzelska-Nowicka et al., 2018; Toldrá & Reig, 2011; W. Zhang et al., 2010). Flavonols have shown anti-obesity effects (Ono et al., 2006; Qi & Zhou, 2013), antioxidant (Ngoc et al., 2019), anti-diabetic (D. Lin et al., 2016), and potential anti-tumor activity (Chakroun et al., 2016). Certain natural phenolic compounds have been found to exhibit an inhibitory function against the invasion and metastasis of cancer cells (Kchaou et al., 2016; Weng & Yen, 2012). The addition of natural compounds to meat products enhances nutritional quality and safety, offering health benefits, such as antioxidant, anti-obesity, anti-diabetic, and anti-tumor effects. This positions natural compounds as valuable functional ingredients in the development of modern meat products such as CM.

Importantly, all experiments in this study were conducted under serum-containing culture conditions. Therefore, the potential of apigenin to reduce or replace serum components in culture media remains to be validated. Future studies using serum-free or serum-reduced systems are required to confirm its practical applicability in cultured meat production.

Future studies should validate the effects of apigenin in vivo and further elucidate the intracellular signaling pathways involved in MSC proliferation and differentiation. Moreover, establishing the optimal concentration and supplementation conditions for apigenin in the context of CM production is essential, along with comprehensive safety assessments to support the potential future industrial application of apigenin.

Ethics declarations

This study received ethical approval for all animal procedures from the Institutional Animal Care and Use Committee of Yeungnam University (approval number: AEC2022-022).

Animal ethical considerations

This study was approved by the Institutional Animal Care and Use Committee of Yeungnam University (approval number: AEC2022-022). All procedures involving the isolation and culture of muscle satellite cells (MSCs) from bovine, porcine, and chicken tissues were conducted in accordance with institutional and national guidelines for the care and use of animals, ensuring ethical standards and minimizing animal use.

CRediT authorship contribution statement

Eun Ju Lee: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Syed Sayeed Ahmad:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Shahid Parwez:** Writing – review & editing. **Puneet Kumar Singh:** Writing – review & editing. **Jeong Ho Lim:** Writing – review & editing. **Jihoe Kim:** Writing – review & editing. **Sun Jin Hur:** Writing – review & editing. **Inho Choi:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2026.107348>.

Data availability

Data will be made available on request.

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