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Hispidin-enriched *Sanghuangporus sanghuang* mycelia SS-MN4 ameliorate disuse atrophy while improving muscle endurance

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Abstract

Background Disuse atrophy is a frequent cause of muscle atrophy, which can occur in individuals of any age who have been inactive for a prolonged period or immobilization. Additionally, acute diseases such as COVID-19 can cause frequent sequelae and exacerbate muscle wasting, leading to additional fatigue symptoms. It is necessary to investigate potent functional nutrients for muscle reinforcement in both disuse atrophy and fatigue to ensure better physical performance.

Methods The effects of *Sanghuangporus sanghuang* SS-MN4 mycelia were tested on two groups of 6-week-old male mice—one with disuse atrophy and the other with fatigue. The disuse atrophy group was divided into three sub-groups: a control group, a group that underwent hind limb casting for 7 days and then recovered for 7 days and a group that was administered with SS-MN4 orally for 14 days, underwent hind limb casting for 7 days and then recovered for 7 days. The fatigue group was divided into two sub-groups: a control group that received no SS-MN4 intervention and an experimental group that was administered with SS-MN4 orally for 39 days and tested for exhaustive swimming and running on Day 31 and Day 33, respectively. RNA sequencing (RNA-seq) and western blot analysis were conducted on C2C12 cell lines to identify the therapeutic effects of SS-MN4 treatment.

Results In a disuse atrophy model induced by hind limb casting, supplementing with 250 mg/kg of SS-MN4 for 14 days led to 111.2% gastrocnemius muscle mass recovery and an 89.1% improvement in motor function on a treadmill ($P < 0.05$). In a fatigue animal model, equivalent SS-MN4 dosage improved swimming (178.7%) and running (162.4%) activities ($P < 0.05$) and reduced blood urea nitrogen levels by 18% ($P < 0.05$). SS-MN4 treatment also increased liver and muscle glycogen storage by 34.36% and 55.6%, respectively, suggesting a higher energy reserve for exercise. RNA-seq and western blot studies from the C2C12 myotube showed that SS-MN4 extract upregulates Myh4 and helps sustain myotube integrity against dexamethasone damage.

Conclusions Supplementation of SS-MN4 (250-mg/kg body weight) with hispidin as active compound revealed a potential usage as a muscle nutritional supplement enhancing muscle recovery, fast-twitch fibre regrowth and fatigue resistance.

Keywords C2C12; disuse atrophy; fatigue; hispidin; *Sanghuangporus sanghuang* mycelia

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Introduction

Generally, skeletal muscle mass and strength gradually decline up to ~3% annually after the age of 60. Risk factors such as genetics, nutrients, reduced physical activity and environment can greatly influence muscle maintenance.[1] The cause of muscle atrophy varies depending on the underlying condition, but physical inactivity is a significant factor.[2] Disuse atrophy not only causes a decline in muscle strength with increased risk of developing sarcopenia but also affects the quality of life. Studies have shown that individuals diagnosed with the low skeletal muscle mass are at increased risk of chronic diseases and cognitive dysfunction.[3] Moreover, as an individual's muscle grip weakens and mobility deteriorates, lower muscle mass leads to a lower metabolic rate to utilize glucose, resulting in fat accumulation and abnormal blood sugar metabolism. As the disease progresses, sarcopenic obesity significantly increases the risk of cardiovascular disease and mortality.[4] Healthcare related to sarcopenia was estimated at $18.5 billion in the United States in 2000, accounting for 1.5% of healthcare expenditure.[5]

The current treatment for patients with muscle disuse atrophy involves the use of neuromuscular electrical stimulation and protein-based nutritional supplements to slow down the rate of muscle loss.[6] In the previous proposed mechanisms for the decline of muscle mass including, changes in hormone levels,[7] and loss of myocytes via apoptosis.[8] Muscle apoptosis is caused by complex and interdependent pathophysiological mechanisms, including ageing, acute diseases, lack of physical activity, nerve muscle damage, oxidative stress, inflammation and mitochondrial dysfunction.[9] For instance, during the recent outbreak of severe acute respiratory syndrome coronavirus 2 (COVID-19) pandemic, physical lockdown and lack of nutrient uptakes resulted in a significant deterioration in the quality of individual skeletal muscle. Additionally, between 53% and 63% of post-acute sequelae COVID-19 (long COVID) patients were diagnosed with muscle weakness.[10] A new hypothesis suggests that patients who have recovered from COVID-19 may develop sarcopenia due to fatigue symptoms and muscle disuse, posing additional risks.[11] Thus, there is a pressing need for muscle nutritional reinforcement in those populations to ensure better physical performance and improved quality of life.

Natural phytochemical extracts, which are often enriched from vegetables, grains, nuts, fruits and herbs, can provide various health benefits by simultaneously acting on the antioxidant and anti-inflammation activity at the cellular level.[12] Due to ageing, the digestive system becomes less efficient, and an animal protein-based diet can burden the digestive tracks in the elderly population.[13] Therefore, the development of natural supplements with the bioactive compounds can provide a non-pharmaceutical strategy for muscle maintenance. *Sanghuangporus sanghuang*, a traditional medicinally valuable mushroom used as an herbal ingredient to alleviate sickness in humans for more than hundreds of years, has been identified its multiple functions in immunomodulatory activities, antiviral, antioxidant, antidiabetic, anti-inflammatory, antimicrobial and antitumor-related diseases in modern pharmacological studies.[14] Among the phytochemical constituents of *S. sanghuang*—hispidin, the yellow polyphenol pigment, displayed excellent antioxidant and anti-obesity properties.[15] In our previous study, we observed that hispidin-enriched *S. sanghuang* can upregulate nuclear factor erythroid 2-related factor 2 (Nrf2) in a dosage-dependent manner.[16] Deficiency of Nrf2 expression has been reported as a vital marker associated with sarcopenia development in vivo.[17] Clinical study also showed that the Nrf2–antioxidant response element (ARE) pathway is impaired in older adults (63 ± 1 years) and the benefit of exercising is less effective when compared with the young adult (23 ± 1 years).[18] Based on the above assumption, a specific fermented batch from hispidin-enriched *S. sanghuang* strain (SS-MN4; muscle nutrient-4) was investigated based on its intrinsic effects of Nrf2 gene upregulation, which can provide an alternative healthcare supplement for delaying muscle atrophy while decreasing muscle fatigue effects. The aim of this study will systematically investigate the effectiveness of SS-MN4 in muscle improvement using two different mouse models (disuse atrophy and fatigue) and uncover its molecular mechanism using in vitro C2C12 cell line (*Scheme 1*).

Materials and methods

S. sanghuang *mycelia SS-MN4 fermentation, extraction and compound isolation*

S. sanghuang mycelia SS-MN4 enriched with hispidin were cultivated according to the previous study.[19] In brief, 1-cm^3 mycelia agar block was collected and transferred to the 1-L broth containing glucose (1%), yeast extract (0.3%) and $MgSO_4$ (0.05%) with pH adjustment to 5. The 1-L flask is placed on a shaker with a rotational speed at 120 rpm and incubated at 25°C for 7 days. The solution is then transferred to 500-L and 20-t fermenter for 10-day scale-up production. The finalized SS-MN4 biomass was collected, lyophilized and grinded into powder form on the 10th day. The powder was later quantified using high-performance liquid chromatography (HPLC) with hispidin content at ~3 mg/g. To obtain SS-MN4 ethanoic extract, SS-MN4 powder (50 g) was dissolved in 1 L of absolute alcohol extracted using an ultrasonic bath. The extracted solution is then filtered by No. 4 Whatman filter paper and concentrated using a rotary evaporator (R-220; BÜCHI Labortechnik AG, Flawil, Switzerland). Dried extract matter was resuspended in either phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO) to obtain a concentrated

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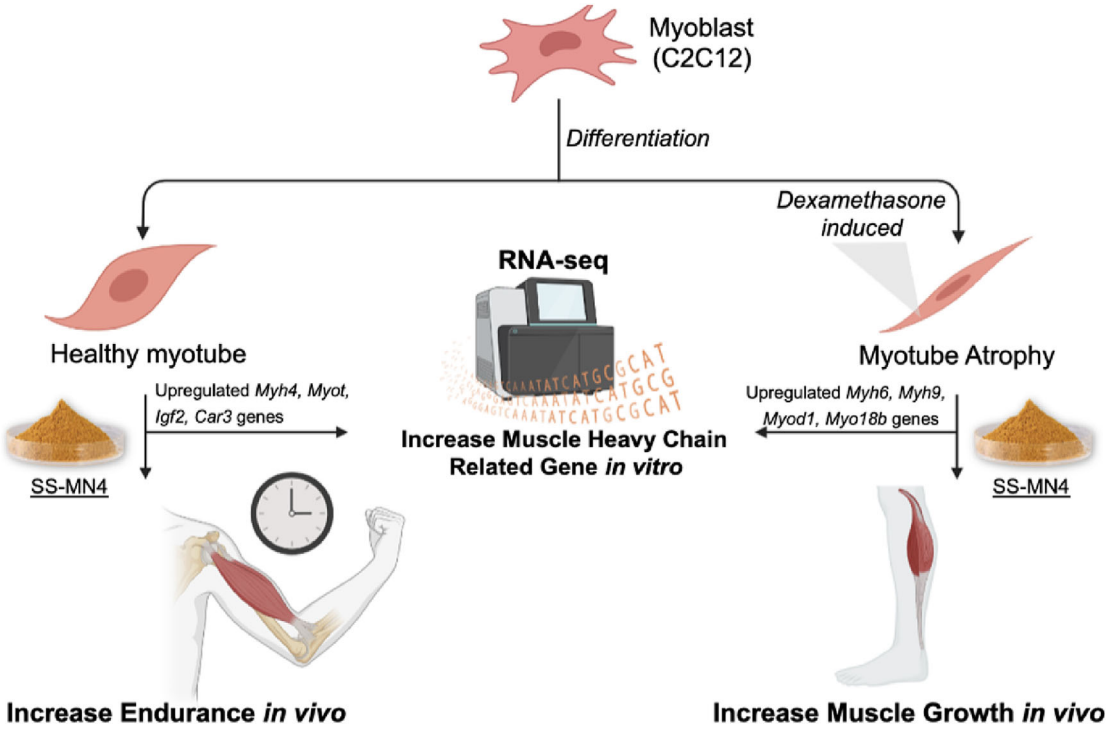

Scheme 1 Schematic diagram of SS-MN4 as muscle nutrient for two different mouse models (fatigue and disuse atrophy).

sample at 100 mg/mL. The extracted sample was sterilized through 0.22-μm syringe for in vitro and in vivo studies. For hispidin purification, the extraction protocol was conducted according to previous publication with slight modification.[20] In brief, the extract was partitioned using hexane–90% MeOH (2:1) mixture and centrifuged at 3600 × *g* for 5 min. This procedure is repeated two times, and the hispidin was concentrated using the rotary evaporator. To confirm the hispidin compound, ultra-high-performance liquid chromatography (UHPLC) analysis (Agilent 1290 system, Agilent Technologies, Santa Clara, CA, USA) was performed using Kinetex Core-Shell C18 analytical column (3 × 100 mm, particle size of 1.7 μm; Phenomenex, CA, USA). The parameter for mobile-phase gradient is set at 40°C with 0.1% formic acid (A) and acetonitrile (B). The gradient elution profile is set as follows: 0–10 min, 15–40% (B); 10–10.1 min, 40–100% (B); 10.1–12 min, 100–100% (B); 12–12.5 min, 100–15% (B); and 12.5–15 min, 15–15% (B). The retention time (RT) for hispidin peak is at 3.36 min measured with 380-nm UV wavelength at a flow rate of 1.0 mL/min.

Experimental animals and performance tests

For disuse atrophy animal model, 6-week-old C57BL/6J male mice only were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and approved by the Institute Animal Care and Use Committee of Medical and Pharmaceutical Industry Technology and Development Center, New Taipei City, Taiwan (IACUC-1100006). All animals were housed 2 weeks in the controlled environment with an ambient temperature of 23 ± 1°C and 60 ± 5% relative humidity, with a 12:12-h light regime before experiment. All diet and purified water were available ad libitum. The mice at 10 weeks old were handled with humane care and were divided into three groups (n = 7 per group) for treatment as follows: (a) sham-controlled mice, (b) mice with 7-day hind limb casting (to simulate rapid muscle atrophy) with 7-day muscle recovery and (c) mice with 7-day hind limb casting and 7-day recovery together with orally administered SS-MN4 treatment (250 mg/kg/day) for a total of 14 days. The mice body weight and food intake were recorded every day before sacrifice. To analyse the muscle performance, all mice were tested on the rodent treadmill with mouse lane assembly and an electronic grid (Ugo Basile, Italy) to analyse the number of shocks. The treadmill speed is set at 18–20 m/min with no incline for 30 min. The Grip Strength Meter System (GSM, Ugo Basile, Italy) was used to measure the hind limb grip strength for each mouse.

For fatigue animal model, 6-week Bltw:CD1 (ICR) male mice only were obtained from the BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan) and approved by the Institute Animal Care and Use Committee of National Taiwan Sport University (IACUC-11010). All animals were housed 2 weeks in the

controlled environment with an ambient temperature of 22 ± 2°C and 65 ± 5% relative humidity, with a 12:12-h light regime before experiment. All diet and purified water were available ad libitum. As for fatigue experiment, the mice were divided into two groups (n = 6 per group) for treatment as follows: (a) vehicle with no SS-MN4 intervention and (b) orally administered SS-MN4 treatment (250 mg/kg/day) for a total of 39 days with exhaustive swimming test on Day 31 and exhaustive running test on Day 33. The mice body weight was recorded on Days 0, 7, 14, 21, 28, 37 and 39 before sacrifice. The diet and water intake were recorded everyday throughout the study. The endurance performance of mice followed the similar method from previous publication with slight modification.[21] In brief, a mouse treadmill (MK680C, Muromachi Kikai Co., Ltd., Japan) was used to detect the exhaustion time. The climbing slope is set at 5°, 10-m/min initial running speed with increment of 2 m/min after 5 min of running, with an electric shock grid current fixed to 2 Hz, 1.22 mA behind the treadmill. To define run to exhaustion, the fatigue characteristic is that the body falls into the electric shock area for 5 s and shows no willingness to run. In regard to the swimming challenge, the swim time of each mouse was recorded in water that was maintained at a temperature of 30°C until the point of exhaustion. The mice were swim grouped and attached to a lead fish sinker that weighed 5% of their body weight. Exhaustion was determined by observing the loss of coordinated movements and the inability to swim to the surface to breathe within a 7-s time frame; 0.2 mL of blood was also collected after swim challenging, the blood was collected at three time points and the blood lactate and blood urea nitrogen (BUN) level analysis using Hitachi 7060 autoanalyser (Hitachi, Tokyo, Japan). The mouse liver and hind limb calf muscles were washed with normal saline and then wiped dry and weighed. The tissue was cut at the same position of the tissue and aliquoted and stored at −80°C for subsequent analysis of tissue hepatic glucose content. The tissue samples were homogenized using five times the weight volume (w/v) using tissue homogenizer (Next Advance, Cambridge, MA, USA). The tissue homogenate was dispensed into microcentrifuge tubes, centrifuged at 4°C and 12 000 × g for 15 min, and the upper extract was taken out for direct analysis of liver glycogen.

C2C12 differentiation and dexamethasone-induced damage

For maintenance, mouse skeletal myoblast-derived cells C2C12 (ATCC CRL-1772; Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA), in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Before differentiation, 1.5 × 10^5 cells were seeded in 6-cm dish and induced differentiation when the cell density has reached 80% confluence. During differentiation, C2C12 medium was replaced with 2% horse serum (HS)–DMEM with or without SS-MN4 extract at different doses (25–100 μg/mL). C2C12 myoblasts were incubated 3 days to allow myotube transformation. Dexamethasone (DEX) (D4902, Sigma-Aldrich, USA) damage was freshly prepared (50 μM) and given on the third day for 24 h for myotube damage.

C2C12 haematoxylin and eosin staining

For haematoxylin and eosin (H&E) staining of myotube treated with SS-MN4, C2C12 myoblasts were first differentiated as described in the procedures above. Before staining, the SS-MN4-treated myotubes were fixed for 30 min using PBS containing 4% formaldehyde solution (33220, Sigma-Aldrich, USA). Next, the cell is stained with Gill's haematoxylin followed by 1% eosin. The myotube image is taken using ZEISS Axio inverted microscope (Vert.A1, Jena, Germany). The methodology previously established for quantifying myotube diameters was utilized.[22] Specifically, 10 images were randomly captured from 6-cm plates with at least three replicates, measuring no fewer than 150 myotubes. To ensure an unbiased analysis, the origin of the cultures and the treatment group were blinded to the investigator. The collected images were then analysed using ImageJ (US National Institutes of Health, USA) for statistical calculations.

RNA-seq library preparation

Stranded RNA library was constructed by Swift RNA Library Kit (Swift Biosciences). Briefly, mRNA was first purified from 3 μg of high-quality total RNA by NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490). Purified mRNA was reverse transcribed to cDNA. A tailing and ligation were performed to add adapter. Finally, a few cycles of PCR were performed to enrich the library. The quality and quantity of library were confirmed by gel electrophoresis, Qubit HS DNA assay and quantitative PCR (qPCR) measurement. Validated library was applied for cluster generation and sequencing on the NovaSeq 6000 system.

RNA-seq analysis

The sequence data were processed with nf-core/rnaseq pipeline (https://nf-co.re/rnaseq) Version 3.9 for best practice of reproducibility. Briefly, the fastq quality control was performed using FastQC, and filtered reads were trimmed with Trim Galore software. The trimmed fastq files were then aligned to GRCm38 mouse genome using STAR and quantified with Salmon to generate gene-by-sample count matrix.

Differential analysis of gene expression was performed using DESeq2 package with $\log_2$ fold change ≥ 1 or ≤ -1, and false discovery rate (FDR) <0.05. clusterProfiler package was used to perform pathway enrichment and visualization of differentially expressed gene between sample groups.

RNA extraction and real-time PCR analysis

Differentiated C2C12 myotubes' total RNA was isolated according to the manufacturer's recommendation protocol using the GeneJET RNA Purification Kit (Thermo Scientific, Lithuania) and quantified by BioDrop (Cambridge, UK). Total RNA (2 μg) was used to synthesize single-strand cDNA according to the manufacturer's protocol by using iScript cDNA Synthesis Kit (Bio-Rad, USA). The desired cDNA fragments were amplified by real-time qPCR using the muscle associated primers. Reactions were carried out in 20-μL reaction volumes containing 1-μL cDNA, 5× iTaq Universal SYBR Supermix (Bio-Rad, USA) and the relevant primers. For this study, the primer is organized in *Table S1*.

Western blot analysis

Total cell lysates were quickly prepared in lysis buffer (radioimmunoprecipitation assay [RIPA], Thermo Scientific, USA) at 4°C containing freshly added protease inhibitor cocktail (GenDEPOT, USA). Protein extracts were quantified and mixed with sodium dodecyl sulfate (SDS) sample buffer and boiled at 90°C 5 min for protein denature. Each well was loaded with 50 μg of protein separated by SDS–polyacrylamide gel electrophoresis (PAGE) on 8% or 10% gels and then transferred to nitrocellulose membranes (MilliporeSigma, Burlington, MA, USA). Blots were incubated with primary antibodies against AMPK, myosin heavy chain (MYHC) and β-actin (Cell Signaling Technology, Danvers, MA, USA) or α-tubulin. Protein expression was analysed using detection kit (WesternBright, Advansta, USA) and measured by Bio-Rad imaging system (ChemiDoc XRS+, USA).

Statistical analysis

For cell study, data were analysed using one-way analysis of variance (ANOVA) method, followed by Sidak's multiple comparison test. For animal study, data were analysed using a one-way ANOVA method, followed by Duncan's multiple range test. For all figure data analyses, *P* value below 0.05 is considered statistically significant. The graph was drawn using software GraphPad Prism 9.

Results

SS-MN4 ameliorate disuse atrophy in vivo

In order to determine if SS-MN4 supplementation can ameliorate disuse atrophy, a disuse atrophy model was introduced by casting an animal's hind limb for 1 week to induce rapid muscle atrophy. After casting for 7 days, the vehicle group (casting only) showed a significant decrease in both body weight (*Figure 1A*) and grip strength (*Figure 1B*). In contrast, 250 mg/kg of SS-MN4 supplementation with casting did not show significant changes in body weight loss when compared to the sham group. Despite that all groups showed a rebound in body weight on Day 14 after cast removal, the vehicle group still exhibited a significant grip strength decline compared with Day 0 (~73.6%). On the other hand, the SS-MN4 supplementation group demonstrates a substantial recovery in muscle strength compared with Day 0 (~89.3%). To further analyse the muscle performance, all mice were tested on the rodent treadmill with an electronic grid to analyse the number of shocks. A lower number of shocks correlate with better muscle performance. On Day 14, the treatment group demonstrates that the SS-MN4 nutrient supplement can significantly lower the number of shocks and the treadmill performance is comparable to the control (*Figure 1C*). Gastrocnemius muscle mass and soleus muscle mass were further obtained to explore the physiological state of the muscle recovery. The result showed that treadmill improvement may contribute to the significant difference in gastrocnemius muscle mass (*Figure 1D*) whereas soleus muscle mass did not show significant recovery (*Figure 1E*). According to the H&E tissue findings and cross-sectional area analysis (*Figure S1*), the gastrocnemius tissue in the group that received SS-MN4 treatment showed a larger cross-sectional area (1548.79 ± 177.8 μm^2) compared to the vehicle group (1108.04 ± 177.8 μm^2). This provides direct evidence that the improved treadmill performance was due to the overall recovery of the gastrocnemius tissue. Throughout the experiment, all groups showed similar food consumption amount indicating that the muscle atrophy or supplementation of SS-MN4 did not alter the appetite (data not shown). Biomarker for muscle atrophy symptom like myostatin (MSTN) in serum was also investigated, and the current result revealed that SS-MN4 can result in suppression of circulating MSTN, which may aid to improve muscle mass (*Figure 1F*).

SS-MN4 ameliorate fatigue symptom in vivo

In contrast with the short-term effect of SS-MN4 supplementation, the long-term benefits of SS-MN4 supplementation for the healthy animal models were further examined. The

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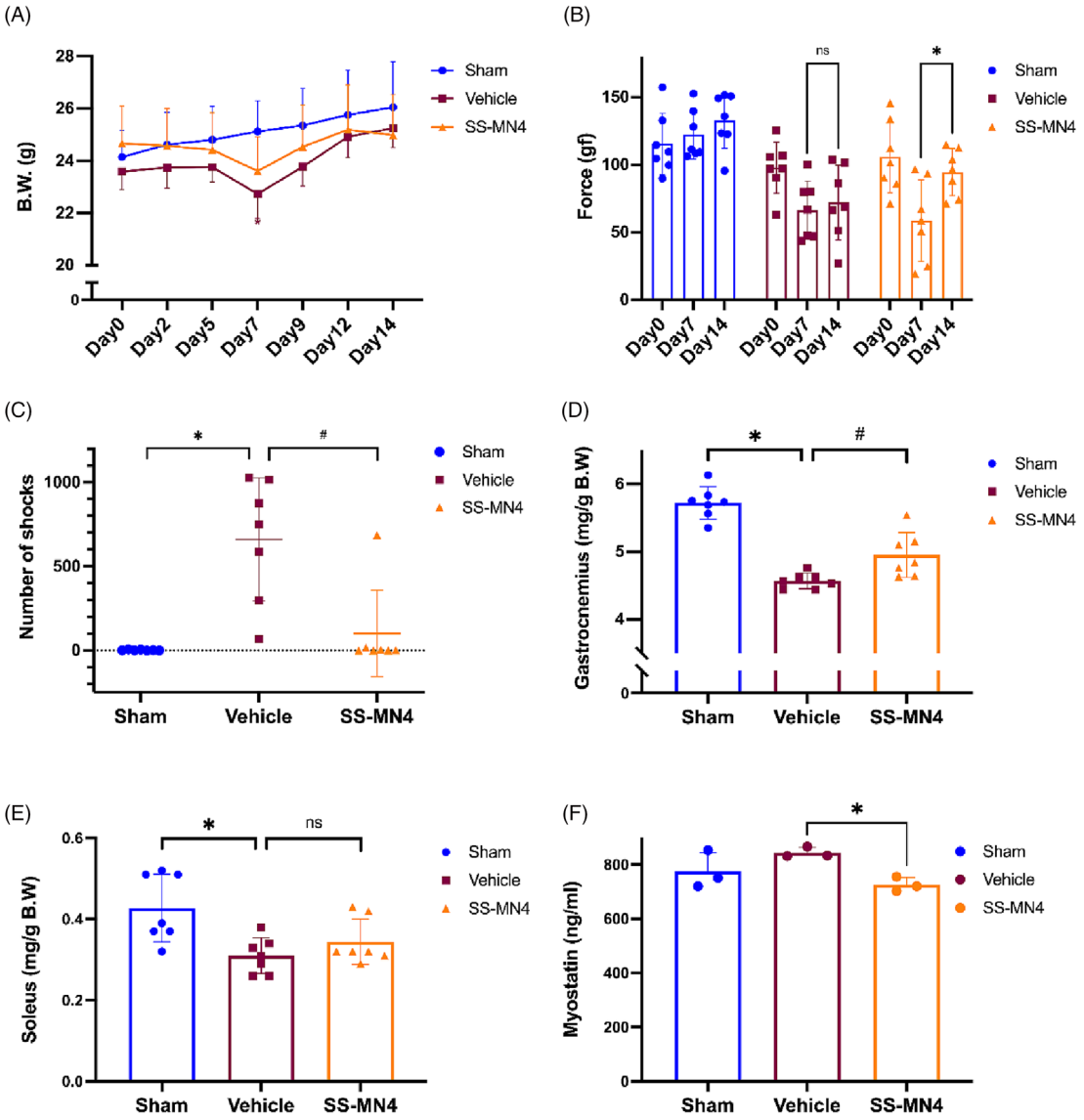

Figure 1 Effects of SS-MN4 nutrient supplement on disuse atrophy model. (A) Body weight, (B) grip strength, (C) treadmill performance, (D) gastrocnemius muscle mass, (E) soleus muscle mass and (F) serum circulating myostatin (MSTN). Vehicle group (casting only) showed a significant decrease in both body weight and grip strength; 14-day supplementation of SS-MN4 can significantly ameliorate the muscle loss effect and is comparable to the control. Treadmill improvement may contribute to the significant difference in gastrocnemius muscle mass rather than soleus muscle mass. Suppression of serum circulating MSTN may aid in improving muscle mass. Data were expressed as mean ± SD (n = 6). *$P < 0.05$ and $^{\#}P < 0.05$ indicate significant differences compared with the selected group.

result showed that after 39 days of SS-MN4 intervention (250 mg/kg/day), there is no difference in body weight (*Figure 2A*). The SS-MN4 treatment group showed significant improvement in both swimming (~178.7%) and running (~162.4%) compared with the vehicle (*Figure 2B*). Lactate acid analysis was investigated through mice blood sampling after swimming challenge. The result showed that the SS-MN4 group can significantly lower the lactate production after exercise (t = 15 min, $P < 0.05$) and showed a higher clearance of lactate at time 30 min (*Figure 2C*). Post-exercise BUN results showed a significant reduction in BUN value by 18% ($P < 0.05$) when compared with the control (*Figure 2D*). Liver and hind leg muscle tissue were later obtained to explore two primary glycogen storage organs, suggesting that significant improvement in fatigue symptoms with SS-MN4 treatment may contribute to a higher energy reservoir (liver increased by 34.36% and muscle increased by 55.6%) for a breakdown during the exercise challenge (*Figure 2E*).

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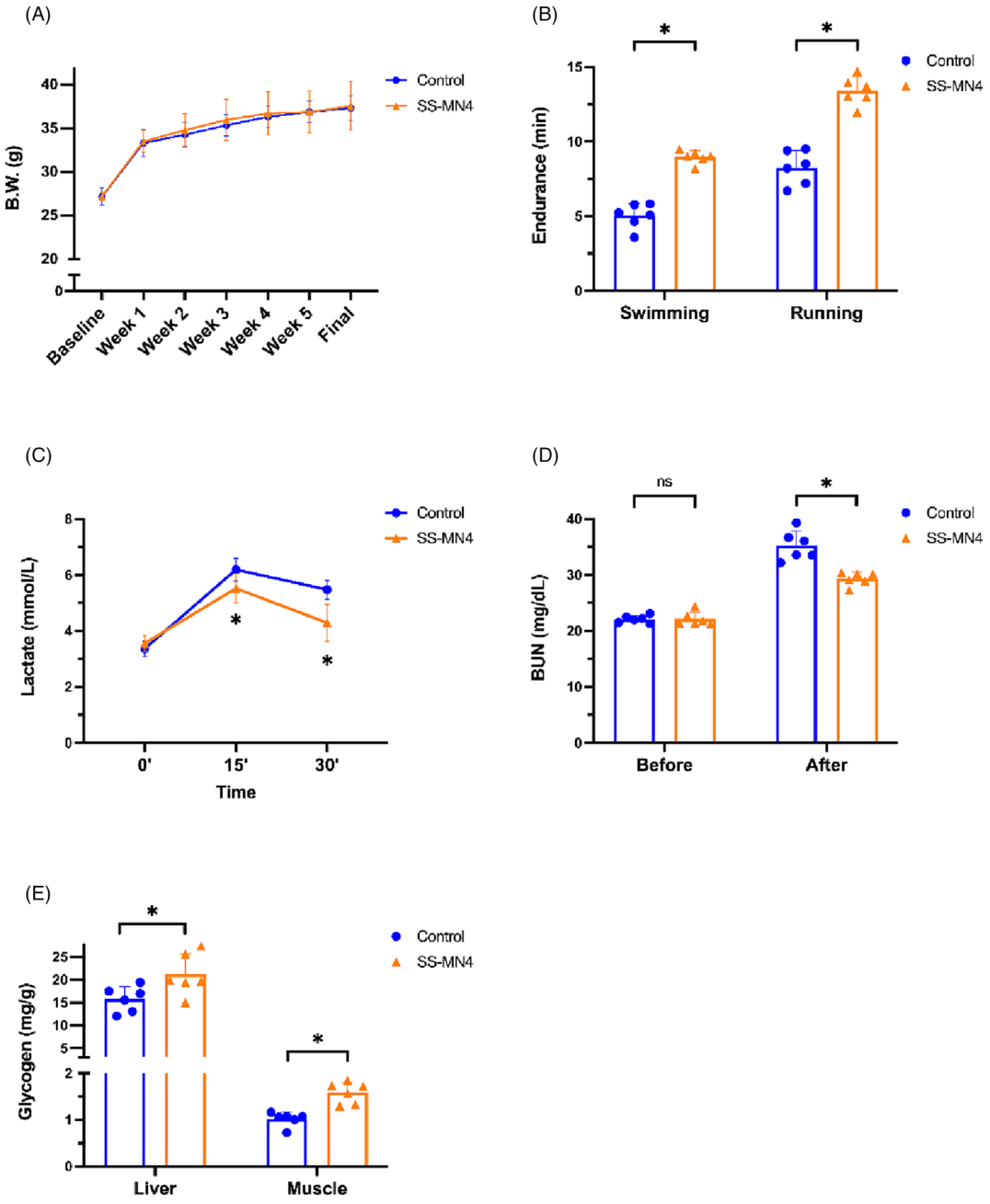

Figure 2 Effects of SS-MN4 nutrient supplement on in vivo endurance model. After 39 days of SS-MN4 supplementation at 250 mg/kg/day, fatigue symptoms were improved. No significant difference was observed in body weight change after long-term consumption (A). The SS-MN4 treatment group exhibited a significant improvement in both swimming (~178.7%) and running (~162.4%) compared to the control group (B). The SS-MN4 supplement significantly reduced lactate production after exercise (C), and post-exercise blood urea nitrogen (BUN) levels showed a significant reduction in BUN value compared to the control (D). The improvement in fatigue symptoms with long-term SS-MN4 treatment might contribute to higher energy reserves in organs (E). Data were expressed as mean ± SD (n = 6). *$P < 0.05$ indicates significant differences compared with the control group.

C2C12 myotube revealed SS-MN4 protection against dexamethasone damage

DEX was commonly used as an atrophic compound to cause damage to the differentiated C2C12 myotube. SS-MN4 ethanolic extract, obtained from the *S. sanghuang* mycelia, was pre-supplemented 24 h before DEX damage to the myotube to analyse its protective effect (*Figure 3A*). Through H&E staining result, DEX at 50 μM showed significant myotube atrophy compared with control whereas SS-MN4 supplementation at a concentration of 25 μg/mL showed significant myotube protection (*Figure 3B*).

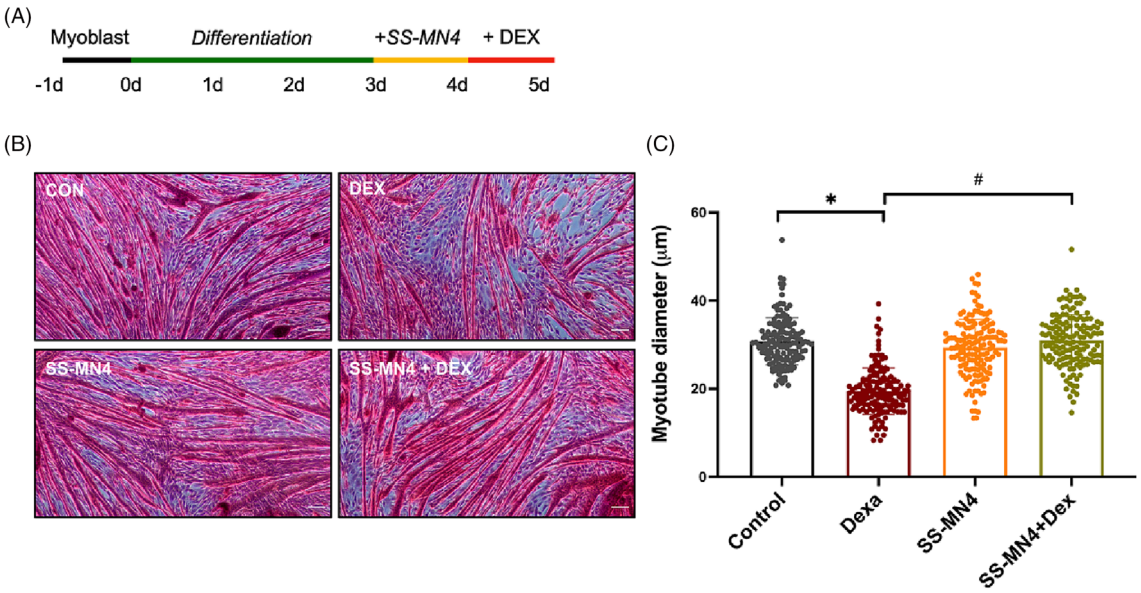

Figure 3 Protection of C2C12 myotubes by SS-MN4 against dexamethasone damage. Pre-supplementation with SS-MN4 24 h before dexamethasone damage (A) showed significant myotube protection at a concentration of 25 μg/mL against dexamethasone damage at 50 μM, as demonstrated by H&E staining (B). Quantification of myotube diameter (n = 150) for each group was calculated to determine the overall protective efficiency (C). Data were expressed as mean ± SD (n = 6). $^{*}P < 0.05$ and $^{\#}P < 0.05$ indicate significant differences compared with the selected group.

Quantification of myotube diameter (n = 150) for each group was calculated to understand the overall protective efficiency. The result showed that SS-MN4 at 25 μg/mL can significantly sustain the damage from the DEX treatment (*Figure 3C*).

SS-MN4 RNA-seq bioinformatics reveals signalling in myogenesis pathway

To further understand the underlying molecular mechanisms of SS-MN4 acting on C2C12 muscle cells, we assayed gene expression with high-throughput sequencing technology. First, we assayed SS-MN4 action on healthy muscle cells (untreated vs. SS-MN4); 163 genes were differentially expressed including *Myh4*, *Myot*, *Igf2* and *Car3* (*Figure 4A* and *Table S2*). Genes that are upregulated are involved in muscle cell differentiation and muscle cell development (*Figure 4B*). In the damaged muscle model (untreated vs. DEX), a total of 407 and 639 differentially expressed genes were observed between DEX and the control group (*Table S3*). Pathway analysis on the DEX-upregulated gene showed negative regulation of locomotion, cell motility and cellular component movement, which recapitulated the muscle damage process (*Figure 4C*). SS-MN4 supplementation treated the DEX-damaged group and identified 664 upregulated and 264 downregulated genes (*Table S4*). Specifically, MYHC-related genes (*Myh9* and *Myh6*) and myogenesis-related genes (*Myod1* and *Myo18b*) were upregulated (*Figure 4D*). Pathway analysis on upregulated genes revealed involvement in the biological process of muscle tissue development, muscle cell differentiation and response to growth factors (*Figure 4E*).

SS-MN4 prevent myotube atrophy through upregulation of myosin heavy chain-related gene and MYH4 protein

To correlate with the RNA-seq result, the effect of SS-MN4 ethanoic extract on C2C12 RNA expression for muscle growth was analysed using real-time PCR. Results revealed that 25 μg/mL of SS-MN4 extract can increase *Myogenin* expression while the *Murf* gene expression was suppressed (*Figure 5A*). When further identifying the relative MYHC genes, SS-MN4 at concentration of 25–100 μg/mL significantly increased the expression of the fast skeletal *Myh4* and *Myh1* genes, as compared to the slow fibre gene *Myh7* (*Figure 5B*). This correlates with the mechanistic study of gastrocnemius tissue immunohistochemistry (IHC) staining in the group that received SS-MN4 treatment showed a higher ratio (97.67 ± 1.53%) of anti-fast myosin positive staining compared to the control group (79.67 ± 3.22%) (*Figure S2*). In association with gene expression, a western blot result also demonstrated that SS-MN4 ethanoic extract and its major active compound, hispidin, showed a significant improvement (25 μg/mL) in MYH4 protein expression and reduced the damage caused by DEX (*Figure 5C*). Interestingly, SS-MN4 exhibits higher MYH4 expression compared to hispidin alone. This suggests that there may be other active compounds worth investigating in future studies.

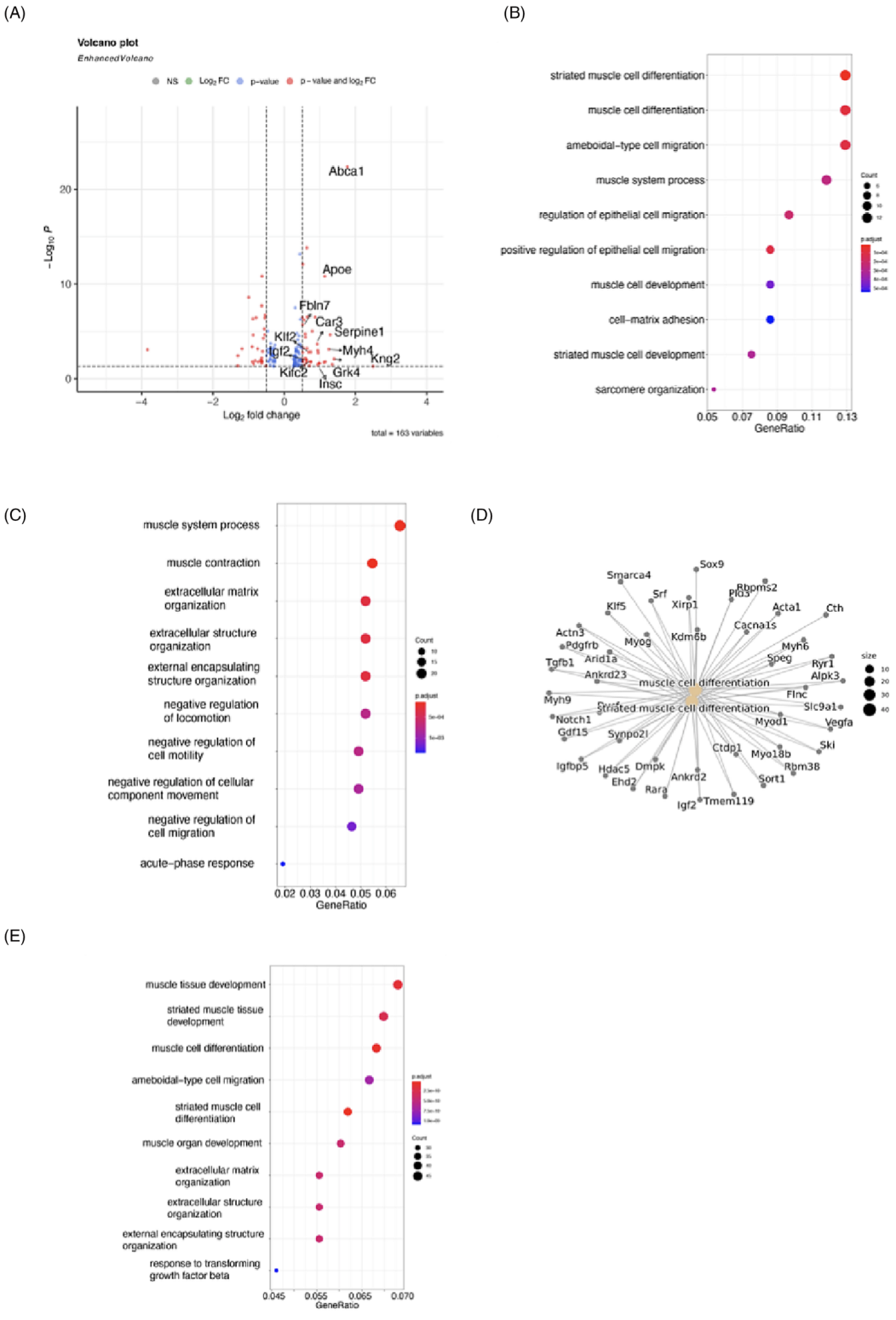

Figure 4 Pathway analysis of treatment effect of SS-MN4 using bulk RNA sequencing. Genes involved in the action of SS-MN4 action on healthy muscle cells (untreated vs. SS-MN4) (A). Genes that are upregulated are involved in muscle cell differentiation and muscle cell development (B). Pathway analysis on dexamethasone (DEX)-upregulated gene showed negative regulation of locomotion, cell motility and cellular component movement, which recapitulated the muscle damage process (C). SS-MN4 supplementation treated the DEX-damaged group and identified myosin heavy chain-related genes (*Myh9* and *Myh6*) and myogenesis-related genes (*Myod1* and *Myo18b*) were upregulated (D). Pathway analysis on upregulated gene revealed involvement in the biological process of muscle tissue development, muscle cell differentiation and response to growth factors (E).

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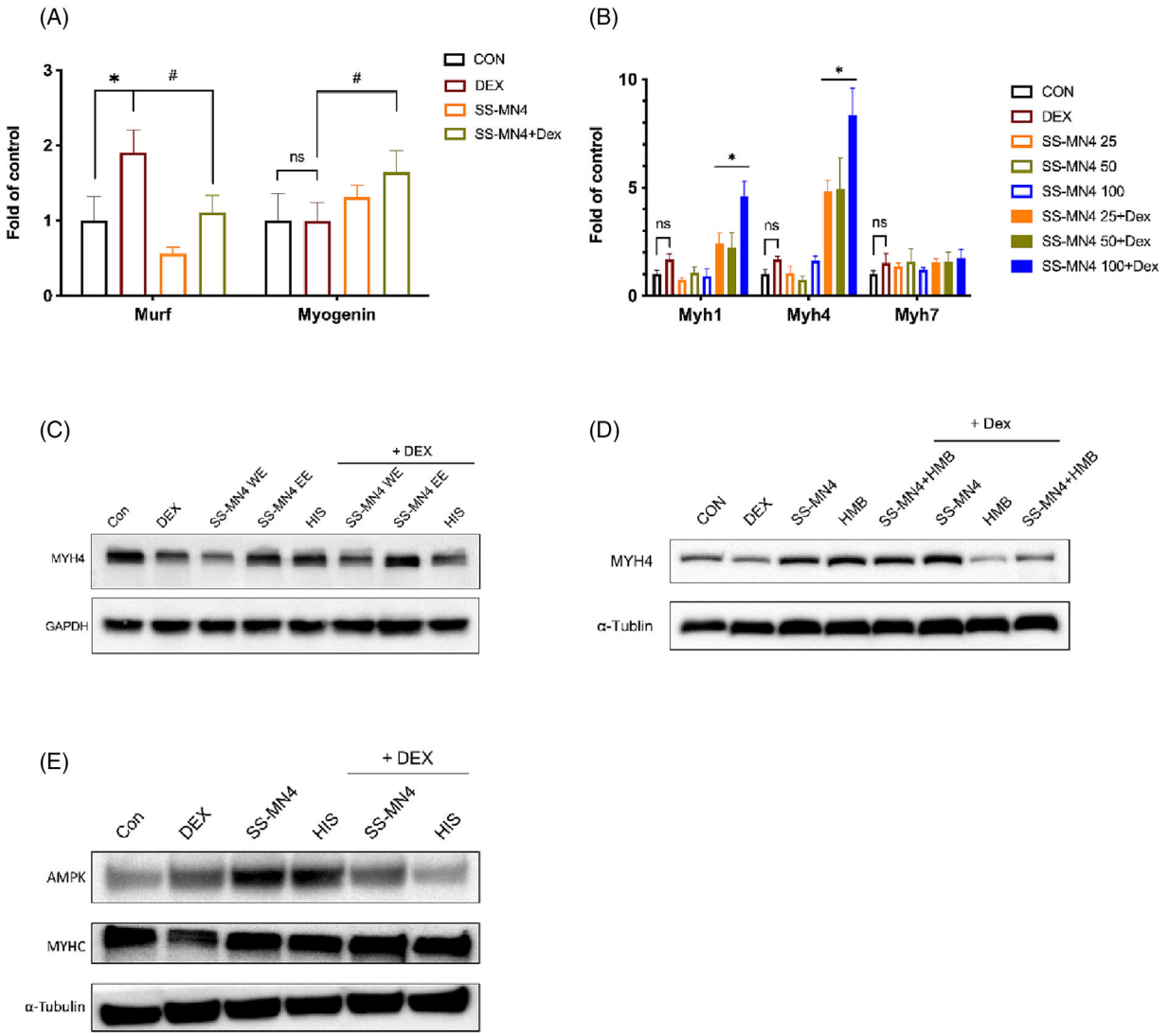

Figure 5 Supplementation with SS-MN4 can prevent myotube atrophy by upregulating myosin heavy chain-related genes and proteins. Real-time analysis showed that 25 μg/mL of SS-MN4 extract increased *Myogenin* expression while suppressing the *Murf* gene expression (A). Fast skeletal *Myh4* and *Myh1* genes were significantly enhanced compared to the slow fibre gene *Myh7* (B). Western blot results demonstrated that SS-MN4 ethanolic extract and its major active compound, hispidin, significantly improved MYH4 protein expression at a concentration of 25 μg/mL, thus protecting against dexamethasone (DEX) damage (C). At a concentration of 25 μg/mL, SS-MN4 resulted in significant MYH4 protein protection compared with β-hydroxy-β-methylbutyrate (HMB) at 100 μg/mL (D). Both SS-MN4 and hispidin increased AMPK and total myosin heavy chain (MYHC) expression when there was no DEX damage (E). $^{*}P < 0.05$ and $^{\#}P < 0.05$ indicate significant differences compared with the selected group.

In this study, β-hydroxy-β-methylbutyrate (HMB) supplementation was also used as a common effective compound for muscle anabolic growth. Indeed, the result showed that HMB at 100-μg/mL concentration reveals a significant protein overexpression comparable with the SS-MN4 at 25 μg/mL (*Figure 5D*). DEX resulted in decreased expression of MYH4 protein. In contrast to the HMB group exposed to DEX damage, which showed comparable levels of MYH4 protein compared to DEX-only groups, the SS-MN4 group was able to maintain high levels of MYH4 protein. Therefore, it is suggested that SS-MN4 alone or in combination with HMB may aid in restoring MYH4 protein expression. Moreover, as AMPK is a protein crucial for maintaining cellular energy balance, the results show that both SS-MN4 and hispidin enhance the expression of AMPK and total MYHC without damage caused by DEX (*Figure 5E*). This may explain why long-term use of SS-MN4 can increase overall glycogen levels in animal tissue. However, when there is DEX-induced damage, SS-MN4 extract and hispidin may activate different pathways to increase the overall MYHC protein level.

Discussion

The skeletal muscle is the body's largest metabolic organ. Through proper nutritional interventions and exercise, individuals can improve muscle physical function, such as fatigue resistance and increased muscle mass.[23] However, in some scenarios, such as acute illness, hospitalization and ageing, sufficient physical activity may not be attainable, which can increase the risk of developing sarcopenia.[24] Furthermore, studies have shown that low skeletal mass and its complications are associated with adverse outcomes in postoperative recovery and longer hospital stays, especially in those over 65 years old.[25] Therefore, it is crucial to identify potent functional nutrients that can help reinforce muscles, not only in the context of ageing but also in the aftermath of acute illness.

In this study, a potent muscle nutrient candidate from the *S. sanghuang* mycelia SS-MN4 was studied based on the two animal models. The study chose cast immobilization as the disuse atrophy model because it is a commonly used method that induces rapid atrophy due to muscle inactivity.[26] We demonstrated that hind limb casting can simulate rapid muscle mass decline, which is consistent with other murine immobilization studies indicating a plateau at Day 7 for gastrocnemius weight loss.[27] The 7-day period for casting also decreases the chance of muscle inflammation affecting the study of supplementation. This is due to the fact that muscle inflammation is not usually heightened during the first phase of muscle atrophy induced by cast immobilization.[28] Under no alteration in total food consumption between each group, 250 mg/kg/day of SS-MN4 was able to attenuate the disuse atrophy effect from the immobilization. We analysed the amino acid composition of SS-MN4 and found that the leucine content (data not shown) does not play a major role due to clinical trials suggest that up to ~5-g leucine per meal in the daily meals is necessary for myofibrillar protein synthesis.[29] Gastrocnemius mass decline is considered a key functional indicator for the early onset of sarcopenia symptom,[30] and our supplementation of SS-MN4 has shown improvement in gastrocnemius mass, which can contribute to the recovery of grip force and endurance test. This consumption dosage is equivalent to ~1.22 g/day for a 60-kg human adult, which is a practical dosage for future clinical trial translation.[31] Circulation myokine MSTN was also investigated as a biomarker for muscle wasting symptoms, and current result revealed that suppression of circulating MSTN may aid in muscle mass recovery.[26]

Physical exercise can help to reduce fatigue symptoms and increase muscle performance with greater force. To evaluate whether the long-term effect of SS-MN4 supplementation can help to decrease muscle fatigue, a similar animal study was conducted following Huang's group to evaluate the exercise performance.[21] Our result indicates that supplementation of the 250 mg/kg/day of SS-MN4 for a total of 39 days showed significant improvement in both swimming and running activities. Lactate and BUN values are two well-known biomarkers for muscle fatigue,[32] and our results reveal a clear reduction in fatigue biomarkers, which indicates that the muscular network may become more efficiently maintained in energy expenditure. To be more precise, long-term consumption showed the benefits of SS-MN4 in increasing the liver and muscle glycogen storage capacity, which primarily led to a better long-term exercise performance. A recent study has shown that overexpression of protein targeting to glycogen (PTG) in animal is a key regulator for endurance improvement because it can increase liver glycogen levels.[33] We hypothesized that SS-MN4 supplementation may generate a similar mechanism, which increased the PTG expression in vivo. Nonetheless, the untargeted metabolomics approach of SS-MN4 for hepatic glycogen network will be investigated in a separate study.

RNA-seq bulk analysis showed that SS-MN4 can potentially reverse the muscle atrophy in the atrophic model induced by DEX via recruiting muscle differentiation and development pathway. A recent transgenic mouse study identified that fast muscles like quadriceps are predominantly composed of *Myh4* or *Myh1* gene.[34] We confirmed that *Myogenin*, *Myh1* and *Myh4* are indeed overexpressed in the SS-MN4 treatment, which may correlate with the recovery of the gastrocnemius muscle in vivo. DEX is a well-studied synthetic corticosteroid that induced fast-twitch muscle type IIb fibre atrophy.[35] Our western blot result is consistent with the gene expression, which revealed that SS-MN4 treatment can help to increase MYH4 protein expression. A muscle topology study showed that fast-twitch fibres feature more power generation whereas slow-twitch fibres are more related to fatigue resistance.[36] We analysed slow-twitch fibre gene expression results like *Myh7*, but it is not significantly expressed in C2C12 under SS-MN4 treatment.

Hispidin, one of the main active compounds in SS-MN4, has been shown to have beneficial effects in multiple diseases, including cancer, metabolic syndrome, cardiovascular disease, neurodegenerative disease and viral diseases. However, its effect on muscle-related research is rarely discussed. In the current study, we demonstrated that SS-MN4, which contains hispidin, is comparable to the common supplement HMB. For the first time, the results revealed that both supplements demonstrate a similar increase in total MYHC protein level (data not shown) under normal treatment, but SS-MN4 demonstrates an advantage in MYH4 protein protection against DEX damage. Interestingly, SS-MN4 exhibits higher MYH4 expression compared to hispidin alone. This suggests that there may be other active compounds worth investigating in future studies.

We further analysed the AMPK protein, which regulates skeletal muscle mass and cellular energy homeostasis, in both the SS-MN4 treatment and its active compound. Current results indicate that both SS-MN4 and hispidin increase the

expression of AMPK and total MYHC when there is no DEX damage. We suspect that SS-MN4 and hispidin can increase glycogen metabolism, which leads to the activation of AMPK. The benefit of long-term consumption of SS-MN4 can increase overall glycogen storage levels in animal tissue and thus further decrease the fatigue symptom. In contrast, when there is muscle damage due to DEX, SS-MN4 extract and hispidin may trigger different pathways via recovery of the mitochondria damage from DEX to increase the MYHC protein.[37] This finding also correlates with a recent study, which suggests that AMPK activation is not required to induce hypertrophy.[38] Recent report demonstrated that low concentrations of DEX (1 μM) can promote myogenic differentiation (MYH1/2) via a direct enhancement of kinesin-1 motility.[39] While the concentration of DEX used in this study differed from that of our study, it is important to acknowledge that the initial muscle composition and DEX concentration are crucial factors in determining the impact on muscle mass and function.

Nevertheless, a clinical study has shown that 4 weeks of HMB supplementation with high-intensity interval training (HIIT) can improve endurance performance.[40] Considering that SS-MN4 (~3.08 mg/g of hispidin, RT of 2.396 min, *Figure S3*) has demonstrated its long-term beneficial effects in endurance, future translation of this supplement to sports athletes troubled with injury is recommended due to its intrinsic property of muscle recovery. There are still some limitations to our current findings of SS-MN4 effects due to our animal models. This study did not consider age as a factor that is a general cause of muscle wasting. Future age-induced muscle wasting models will need to be analysed to see whether SS-MN4 is affected by age.

In summary, supplementing SS-MN4 with hispidin at a dose of 250-mg/kg body weight (BW) can alter the metabolic profile of muscle cells. This alteration can result in increased glycogen storage, maintenance of C2C12 myotube integrity and increased gene expression that favours muscle development. Ultimately, these advantages can help ease disuse atrophy symptoms and enhance muscle endurance, making it a hopeful supplement for those who might be at risk of developing sarcopenia early on.

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Conflict of interest statement

Grape King Bio Ltd. provided support in the form of salaries for I-C Li, T-Y Lu, T-W Lin, H-T Chu, Y-L Lien, T-J Li and C-C Chen and research materials but did not have any additional role in the study design, data collection and analysis, decision to publish or preparation of the manuscript. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

References

1. Therakomen V, Petchlorlian A, Lakananurak N. Prevalence and risk factors of primary sarcopenia in community-dwelling outpatient elderly: a cross-sectional study. *Sci Rep* 2020;**10**:19551.
2. Brook MS, Stokes T, Gorissen SHM, Bass JJ, McGlory C, Cegielski J, et al. Declines in muscle protein synthesis account for short-term muscle disuse atrophy in humans in the absence of increased muscle protein breakdown. *J Cachexia Sarcopenia Muscle* 2022;**13**:2005–2016.
3. Oudbier SJ, Goh J, Looijaard S, Reijnierse EM, Meskers CGM, Maier AB. Pathophysiological mechanisms explaining the association between low skeletal muscle mass and cognitive function. *J Gerontol A Biol Sci Med Sci* 2022;**77**:1959–1968.
4. Atkins JL, Wannamathee SG. Sarcopenic obesity in ageing: cardiovascular outcomes and mortality. *Br J Nutr* 2020;**124**:1102–1113.
5. Janssen I, Shepard DS, Katzmarzyk PT, Roubenoff R. The healthcare costs of sarcopenia in the United States. *J Am Geriatr Soc* 2004;**52**:80–85.
6. Dirks ML, Wall BT, van Loon LJC. Interventional strategies to combat muscle disuse atrophy in humans: focus on neuromuscular electrical stimulation and dietary protein. *J Appl Physiol* 1985;**2018**:850–861.
7. Bian A, Ma Y, Zhou X, Guo Y, Wang W, Zhang Y, et al. Association between sarcopenia and levels of growth hormone and insulin-like growth factor-1 in the elderly. *BMC Musculoskelet Disord* 2020;**21**:214.
8. Cheema N, Herbst A, McKenzie D, Aiken JM. Apoptosis and necrosis mediate skeletal muscle fiber loss in age-induced mitochondrial enzymatic abnormalities. *Aging Cell* 2015;**14**:1085–1093.
9. Shou J, Chen PJ, Xiao WH. Mechanism of increased risk of insulin resistance in aging skeletal muscle. *Diabetol Metab Syndr* 2020;**12**:14.
10. Soares MN, Eggelbusch M, Naddaf E, Gerrits KHL, van der Schaaf M, van den

Borst B, et al. Skeletal muscle alterations in patients with acute Covid-19 and post-acute sequelae of Covid-19. *J Cachexia Sarcopenia Muscle* 2022;**13**:11–22.
11. Martone AM, Tosato M, Ciciarello F, Galluzzo V, Zazzara MB, Pais C, et al. Sarcopenia as potential biological substrate of long COVID-19 syndrome: prevalence, clinical features, and risk factors. *J Cachexia Sarcopenia Muscle* 2022;**13**:1974–1982.
12. Samtiya M, Aluko RE, Dhewa T, Moreno-Rojas JM. Potential health benefits of plant food-derived bioactive components: an overview. *Foods* 2021;**10**:839.
13. Matison AP, Milte CM, Shaw JE, Magliano DJ, Daly RM, Torres SJ. Association between dietary protein intake and changes in health-related quality of life in older adults: findings from the AusDiab 12-year prospective study. *BMC Geriatr* 2022;**22**: 211.
14. Chen W, Tan H, Liu Q, Zheng X, Zhang H, Liu Y, et al. A review: the bioactivities and pharmacological applications of *Phellinus linteus*. *Molecules* 2019;**24**:1888.
15. Park IH, Chung SK, Lee KB, Yoo YC, Kim SK, Kim GS, et al. An antioxidant hispidin from the mycelial cultures of *Phellinus linteus*. *Arch Pharm Res* 2004;**27**:615–618.
16. Li IC, Chang FC, Kuo CC, Chu HT, Li TJ, Chen CC. Pilot study: nutritional and preclinical safety investigation of fermented hispidin-enriched *Sanghuangporus sanghuang* mycelia: a promising functional food material to improve sleep. *Front Nutr* 2021;**8**:788965.
17. Huang DD, Yan XL, Fan SD, Chen XY, Yan JY, Dong QT, et al. Nrf2 deficiency promotes the increasing trend of autophagy during aging in skeletal muscle: a potential mechanism for the development of sarcopenia. *Aging (Albany NY)* 2020;**12**:5977–5991.
18. Done AJ, Gage MJ, Nieto NC, Traustadottir T. Exercise-induced Nrf2-signaling is impaired in aging. *Free Radic Biol Med* 2016; **96**:130–138.
19. Li IC, Chen CC, Sheu S-J, Huang IH, Chen C-C. Optimized production and safety evaluation of hispidin-enriched *Sanghuangporus sanghuang* mycelia. *Food Sci Nutr* 2020;**8**:1864–1873.
20. Guo J, Liu X, Li Y, Ji H, Liu C, Zhou L, et al. Screening for proteins related to the biosynthesis of hispidin and its derivatives in *Phellinus igniarius* using iTRAQ proteomic analysis. *BMC Microbiol* 2021;**21**:81.
21. Chen YM, Wei L, Chiu YS, Hsu YJ, Tsai TY, Wang MF, et al. *Lactobacillus plantarum* TWK10 supplementation improves exercise performance and increases muscle mass in mice. *Nutrients*.
22. Castillero E, Alamdari N, Lecker SH, Hasselgren PO. Suppression of atrogin-1 and MuRF1 prevents dexamethasone-induced atrophy of cultured myotubes. *Metabolism* 2013;**62**:1495–1502.
23. Hargreaves M, Spriet LL. Skeletal muscle energy metabolism during exercise. *Nat Metab* 2020;**2**:817–828.
24. Welch C, Hassan-Smith ZK, Greig CA, Lord JM, Jackson TA. Acute sarcopenia secondary to hospitalisation—an emerging condition affecting older adults. *Aging Dis* 2018;**9**:151–164.
25. Sousa AS, Guerra RS, Fonseca I, Pichel F, Amaral TF. Sarcopenia and length of hospital stay. *Eur J Clin Nutr* 2016;**70**:595–601.
26. Xie WQ, He M, Yu DJ, Wu YX, Wang XH, Lv S, et al. Mouse models of sarcopenia: classification and evaluation. *J Cachexia Sarcopenia Muscle* 2021;**12**:538–554.
27. Lang SM, Kazi AA, Hong-Brown L, Lang CH. Delayed recovery of skeletal muscle mass following hindlimb immobilization in mTOR heterozygous mice. *PLoS ONE* 2012;**7**: e38910.
28. Kawanishi N, Funakoshi T, Machida S. Time-course study of macrophage infiltration and inflammation in cast immobilization-induced atrophied muscle of mice. *Muscle Nerve* 2018;**57**:1006–1013.
29. Murphy CH, Saddler NI, Devries MC, McGlory C, Baker SK, Phillips SM. Leucine supplementation enhances integrative myofibrillar protein synthesis in free-living older men consuming lower- and higher-protein diets: a parallel-group crossover study. *Am J Clin Nutr* 2016;**104**:1594–1606.
30. Sayed RK, de Leonardis EC, Guerrero-Martinez JA, Rahim I, Mokhtar DM, Saleh AM, et al. Identification of morphological markers of sarcopenia at early stage of aging in skeletal muscle of mice. *Exp Gerontol* 2016;**83**:22–30.
31. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm* 2016;**7**:27–31.
32. Wan JJ, Qin Z, Wang PY, Sun Y, Liu X. Muscle fatigue: general understanding and treatment. *Exp Mol Med* 2017;**49**:e384.
33. Lopez-Soldado I, Guinovart JJ, Duran J. Increased liver glycogen levels enhance exercise capacity in mice. *J Biol Chem* 2021; **297**:100976.
34. Dos Santos M, Backer S, Aurade F, Wong MM, Wurmser M, Pierre R, et al. A fast *Myosin* super enhancer dictates muscle fiber phenotype through competitive interactions with *Myosin* genes. *Nat Commun* 2022;**13**:1039.
35. Gupta A, Gupta Y. Glucocorticoid-induced myopathy: pathophysiology, diagnosis, and treatment. *Indian J Endocrinol Metab* 2013;**17**:913–916.
36. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. *Physiol Rev* 2011;**91**:1447–1531.
37. Liu J, Peng Y, Wang X, Fan Y, Qin C, Shi L, et al. Mitochondrial dysfunction launches dexamethasone-induced skeletal muscle atrophy via AMPK/FOXO3 signaling. *Mol Pharm* 2016;**13**:73–84.
38. Kido K, Egawa T, Fujiyoshi H, Suzuki H, Kawanaka K, Hayashi T. AMPK is indispensable for overload-induced muscle glucose uptake and glycogenesis but dispensable for inducing hypertrophy in mice. *FASEB J* 2021;**35**:e21459.
39. Lin JW, Huang YM, Chen YQ, Chuang TY, Lan TY, Liu YW, et al. Dexamethasone accelerates muscle regeneration by modulating kinesin-1-mediated focal adhesion signals. *Cell Death Discov* 2021;**7**:35.
40. Miramonti AA, Stout JR, Fukuda DH, Robinson EH, Wang R, La Monica MB, et al. Effects of 4 weeks of high-intensity interval training and β-hydroxy-β-methylbutyric free acid supplementation on the onset of neuromuscular fatigue. *J Strength Cond Res* 2016;**30**:626–634.