Research Article

Functional interference (crosstalk) between gut microbiome, proteolysis, apoptosis and muscle hypertrophy: Role of resistance training and supplement

Mona Nouri[1], Hamid Arazi[2,3]*

Abstract

Primary objective of this study was to examine the interplay between grip strength, a functional marker of hypertrophy, and its connection to the Firmicutes to Bacteroidetes ratio in the gut microbiome. Twenty-five male Wistar rats were divided into five groups using a computerized randomizer: old and young control groups (OC, YC), old resistance training group (OR), old supplement group (OS), and old resistance training combined with supplement group (ORS). Rats in the OR and ORS cohorts underwent eight weeks of ladder-climbing resistance training three times a week, while those in the OS group were given supplements 5 times per week after the intervention. Muscle samples were collected from all rats two days' post-intervention. FOXO1, BAX, and cytochrome C, were assessed using PCR-real time. Analysis of the data was carried out using one-way ANOVA and post-hoc Tukey testing. The results revealed a decrease in FOXO1 and apoptotic gene expression post-intervention, with a more pronounced reduction observed in the ORS group compared to the other groups ($p<0.05$). Notably, supplementation alone did not impact FOXO1 expression, akin to the effect of exercise on cytochrome C. A moderate negative correlation was documented between the F/B ratio and grip strength ($p= 0.003$; $r= -0.54$). Additionally, positive and moderate correlations were observed between FOXO1, BAX, cytochrome C, and the F/B ratio ($p<0.05$). These findings emphasize a functional association between the gut microbiome and muscle through their metabolites, indicating mutual regulation. Furthermore, it is suggested that exercise and supplements may further enhance these interconnected mechanisms.

Key Words: **F/B ratio, BAX, FOXO1, Resistance training, Aging, Muscle hypertrophy**

1. Department of Exercise Physiology, University Campus, University of Guilan, Rasht, Guilan, Iran. 2. Department of Exercise Physiology, Faculty of Sport Sciences, University of Guilan, Rasht, Guilan, Iran. 3. Department of Exercise Physiology, Faculty of Sport Sciences, Ferdowsi University of Mashhad, Mashhad, Iran.

*Author for correspondence: hamidarazi@yahoo.com

P.O. Box: 41635-1438, Rasht, Iran. Tel: +98 911-1399207 Fax: +98 13-33690675

M N: 0009-0003-6793-5871; H A: 0000-0002-1594-6515

Received 15 January 2024; Accepted 19 March 2024

Introduction

In recent years, gut microbiome interaction with other bodily organs gain huge attention especially in relation to skeletal muscle. Many researchers revealed that gut microbiota is in close relation with different concepts of skeletal muscle function including mass, metabolism (Chen et al., 2023) and performance (Mohr et al., 2020). Healthy gut microbiota normally contains 1,100–2,000 bacterial taxa belonging to 10 phyla, with 99% of the species dominated by the Firmicutes and Bacteroidetes phyla (Chen et al., 2023). Therefore, Firmicutes to Bacteroidetes ratio (F/B ratio) become an index that varies in different physiological and pathophysiologic condition and its balance used as an important tool for normal intestinal or other body part function assessment. For instance, it was shown that F/B ratio increase in obesity while decrease in inflammatory bowel disease (Stojanov et al., 2020). The composition of the gut microbiota undergoes various changes between population due to their genetic, host environment, life style and nutrition. Microbiota play key role in anti- or pro-inflammatory action depends on their metabolites such as short chain fatty acids (SCFA) or lipopolysaccharides (LPS) respectively (Wang et al., 2020). Studies have been shown that LPS can trigger inflammatory and apoptotic cascades by affecting Toll like receptors and other pro-inflammatory cytokines such as interleukin-1β and interleukin -6 (Tucureanu et al., 2018). In a study by Lahiri et al, its identified that physiological atrophy as same as what seems in aging and known as "Sarcopenia" is associated with gut microbiota and Forkhead-box family members belonging to the 'O' category (FOXO) 3 was higher in tibialis anterior muscle of Germ free mice (GF) compare to pathogen free mice (PF). In this study it also observed that myosin heavy chain genes MyHCIIa, MyHCIIb, and MyHCIIx was reduced in GF mice (Lahiri et al., 2019).

It established that preservation of muscle mass depends on balance between muscle protein synthesis (MPS) and breakdown (MPB) (Park et al., 2017). Imbalance or dysregulation of this process may cause loss of muscle mass

Journal of Exercise & Organ Cross Talk

and strength and can induce muscle atrophy. As previously mentioned, sarcopenia is a clinical condition that progress with aging and cause gradual decrease in muscle function and strength which recently classified as disease in International Classification of Diseases, Tenth Revision, Clinical Modification (ICD-10-CM). Underlying mechanism of sarcopenia is multifaceted and it seems that Mammalian Forkhead-box family members belonging to the 'O' category (FoxO) is one of key gene in this phenomenon that has multifunctional role and acts as stimulator of proteolysis, apoptosis, muscle proliferation and myoblast differentiation (Xu et al., 2017). Studies have shown that FoxO1 regulates skeletal muscle atrophy through both apoptotic and proteolytic pathways in various tissues (Alikhani et al., 2007; McLoughlin et al., 2009). It was observed that overexpression of FOXO1 in transgenic mice caused decrease in several structural genes and loss of size in type I and II fiber and number of I fiber significantly decreased in this mice model (Kamei et al., 2004). BCL2 associated X gene (BAX) is a pro-apoptosis gene that is cause of mitochondrial permeability transition pore (mPTP) opening and the release of cytochrome C from mitochondria to cytosol which consequently causes DNA fragmentation in situation where there is imbalance between pro-apoptotic protein (Bax) and antiapoptotic protein (Yoo et al., 2018). Since one of the proven causes of sarcopenia is the accumulation of dysfunctional mitochondria and BAX induce release of cytochrome C, and given that gut microbiota can stimulate expression of FOXO1 and consequently BAX by action of FOXO, investigating the relation between F/B ratio and activity of FOXO and BAX guaranteed.

On the other hand, by considering frontiers in therapeutic approaches in the field of reducing the rate of weakening of muscle mass with aging, the role of exercise activity, especially resistance exercise, and nutritional supplements such as probiotics, vitamin D and leucine have proven. In consistent with these context, in a study it has been suggested that probiotics such as Lacticaseibacillus and Bifidobacterium had immunomodulatory features and it beside their ability to enrich gut microbial phyla (O'Brien et al., 2022). Indeed, by considering vitamin D deficiency in majority of older adult and ability of vitamin D both in strengthening antioxidants defense against free radical (that can cause mitochondrial dysfunction) (Mastali et al., 2023) and improve of muscle protein synthesis and stimulates muscle hypertrophy through increase protein synthesis, translational efficiency, ribosomal expansion by their receptor VDR (Bass et al., 2020), it converts to essential component especially for this generation. Also it suggested that vitamin D can insert antioxidant properties by stabilizing mitochondrial function (de Las Heras et al., 2020). Considering the proven role of protein supplements and amino acids, especially leucine, in targeting the anabolic process in muscle in elderly adult (Devries et al., 2018; Tezze et al., 2023); it seems that the combination of these three elements (probiotics, especially lactobacillus and bifidobacterium, vitamin D and leucine) in the form of a supplement has a beneficial effect on this process. However, no one study has investigated the relationship between the mentioned factors in proteolysis, apoptosis and its relationship with the ratio of Firmicutes to Bacteroidetes. Also, due to the evidence of the effects of FOXO1 and BAX on muscle mass, the role of resistance training and the mentioned supplement, which are a combination of effective substances in the field of muscle volume and strength, have not been investigated yet. Therefore, the present study seeks to answer the hypothesis that whether exercising separately or in combination with the mentioned supplement can affect the activity of the mentioned FOXO1, BAX and cytochrome C genes and also the possible relationship between them with the F/B ratio and whether the functional index of "muscle strength" in this study "grip strength" is affected by such interventions is the subject of this research.

Materials and Methods

Study design and participants

The research was carried out in compliance with the ARRIVE and PREPARE guidelines, adhering strictly to the principles of the 3Rs and conducted within a laboratory environment. For the determination of sample size, the resource equation approach was employed, specifically for a one-way ANOVA, as outlined below:

The calculation of the between-subject error degrees of freedom (DF) for a one-way ANOVA (which represents the within-subject DF) was computed as follows: $DF = N - k = kn - k = k(n - 1)$, where N denotes the total number of subjects, k signifies the number of groups, and n represents the number of subjects per group. By manipulating the formula, the value of n can be derived as: $n = DF/k + 1$

Subsequent to establishing an acceptable range for the DF, the DFs within the equations were substituted with the minimum (10) and maximum (20) DFs in order to determine the minimum and maximum number of animals per group: minimum $n= 10/k+1$ and maximum $n = 20/k+1$. This computation yielded a range of 3 to 5 samples per group as required by our study (Arifin & Zahiruddin, 2017). At the onset of the experiment, twenty-five male Wistar rats (5 per group) belonging to two age categories (Young: 8-12 weeks and Old: 18-24 months) were procured from the RAZI institute to partake in the study. Subsequently, the rats were randomly allocated into five experimental groups, comprising of an Old Control Group (OC; n = 5), a Young Control Group (YC; n = 5), an Old Resistance Training Group (OR; n = 5) which underwent training utilizing the ladder climbing protocol, an Old Resistance Training Plus Supplement Group (ORS; n = 5), and an Old Supplement Group (OS; n = 5) which received the suppl-

-ement without undergoing any training regimen.

Animal care and feeding

At the commencement of the experiment, the rats were accommodated in communal home cages alongside members of the same group (five animals per cage, with an additional two animals in a separate reserve cage). They were provided with ad libitum access to water and standard rodent chow, containing the following nutritional components per 100 grams of chow: Carbohydrates 48.8g, Protein 21g, Fat 3g, Calcium 0.8g, Phosphorus 0.4g, Fiber 5g, Moisture 13%, Ash 8g, with a total energy content of 306.2 kcal/100g (Abdul Kadir et al., 2015). The rats were subjected to a 12-hour light/dark cycle at a temperature range of 20–22 °C. The average weight of the animals was recorded as 283.6±8.79 grams in the Young Control group and 342.17±26.65 grams in the Old group. Furthermore, the rats were housed in rodent rooms equipped with direct-exhaust IVC systems and air ventilation operating at rates of 5 to 6 air changes per hour (ACH). They were situated in standard laboratory environments, utilizing large Euro standard type IV cages (Barker et al., 2017) for housing purposes.

Exercise protocol: Familiarization

Rats were introduced to a resistance training (RT) regimen by climbing a vertical ladder (1.1 m tall, 0.18 m wide, with a 2-cm grid, and an 80º incline) over a three-day period with 48-hour rest intervals, initially without any added weight. The load, represented by a weight clip, was attached to the rats' tails using a self-adhesive foam strip or glue, with the weight calculated as a percentage of each rat's weight (e.g., 50% representing 50% of the rat's weight divided by 1000 and then multiplied by the desired weight). Rats were positioned at the base of the ladder and familiarized with climbing. The familiarization process varied among individuals, but three days were deemed sufficient to establish the baseline for the protocol with a total of 9 rats, 3 in each experimental group. Rats were not prodded to climb but initiated climbing voluntarily, with gentle assistance given by pulling when needed. Rats failing to climb or requiring excessive stimuli were excluded from the study the following day. Some rats displayed a jumping behavior during climbing, while others climbed in a stepwise manner, though these behaviors were not criteria for the study. Rats rested for 2 minutes at the top of the ladder, and the process continued until they climbed the ladder three times consecutively without external stimuli.

Maximum carrying capacity assessment

Following the familiarization phase, each rat underwent an assessment to determine its maximum carrying capacity (MCC) through 4-9 ladder climbs with progressively heavier loads. The initial climb was set at 75% of the animal's body weight, with an additional 30g added after successful completion of each climb. The rat's MCC for a training session was determined by the maximum load it could successfully carry along the full length of the ladder, with failure marked when a rat could not progress after three consecutive stimuli to the tail.

Resistance training protocol

Resistance training (RT) was conducted on Saturdays, Mondays, and Wednesdays, three times a week, over an 8-week period. Each climb involved 8-12 dynamic movements with loads ranging between 65-85% of the rat's maximum carrying capacity. Each RT session comprised 5-8 movements per climb, and the rats completed a total of 7 ladder climbs per session lasting about 15 minutes each. Training intensity was adjusted according to the rat's ability to carry the load, with intensity levels ranging from 50-83% over the 8-week program, with 2-minute rest periods between climbs. The RT protocol was adapted from a study by de Farias Junior, G. C. et al (de Farias Junior et al., 2020) but modified to span an 8-week duration. Notably, rats were unable to carry 75% of their body weight during the initial session or MCC estimation session, necessitating load adjustments for successful climbs. Additionally, none of the experimental groups could manage to carry 100% of their body weight in the final session, leading to no further weight increments. Weight progression was implemented every two to three weeks, and the training sessions were conducted in the morning between 10-12 A.M.

Supplementation protocol

The supplement composition, preparation, and storage procedures were as follows: Lactiplantibacillus plantarum (LP) (PTCC: 1058) and Bifidobacterium bifidum (PTCC: 1644) were sourced from the Iranian Research Organization for Science and Technology (IROST). The bacteria were grown in a MRS agar medium (Merck, Germany) at 37°C for 24 hours. Prior to supplementation, the cells underwent centrifugation at 3000× g for 10 minutes and were washed twice with phosphate-buffered saline (PBS). The resulting pellets were resuspended in PBS with a pH of 7.2 (Chen et al., 2016).

The human dosage of Lactobacillus plantarum (LP) was set at 2 × 10^10 colony-forming units (CFU) per day, a dosage level chosen based on a study by Lee et al (Lee et al., 2021). However, for our study, this dosage was adjusted for rats to 2.06 × 10^8 CFU per kilogram. This rat dosage was determined through a conversion from the human equivalent dose (HED) based on body surface area, using the formula provided by the US Food and Drug Administration. Accounting for the differences in body surface area between rats and humans, a conversion coefficient of 6.2 was applied.

Bifidobacterium bifidum (PTCC: 1644) was also procured from the IROST. To activate the bacteria, they were inoculated in an

Erlenmeyer flask containing peptone water and incubated at 37°C for 24 hours, as recommended by the manufacturer. The activated probiotic was cultured on MRS agar plates under anaerobic conditions using the surface plate method for 24-48 hours. For Bifidobacterium bifidum, a dosage of 5 × 10^6 CFU per rat per day was administered, following the dosage used in a study by Mohabbat et al (Mohabbat & Arazi, 2024). Vitamin D (Sun Vit®, Iran Hormone) was purchased in ampoule form from Milad Poya Company. The vitamin D was mixed with sesame oil at a ratio of 621.7 IU= 0.94 mg vitamin D + 1 ml sesame oil. This mixture was then combined with a suspension of L-leucine prepared according to rat volumes at 0.135 g L-leucine per kilogram of body weight. Finally, the probiotics were added to this mixture and administered orally through gavage. The supplement was stored at a temperature of 4˚C in a standard refrigerator, being taken out ten minutes before use. Administration was carried out via gavage through the mouth using a 16-18 ga stainless steel feeding sterilized tube (Mohabbat & Arazi, 2024).

Grip strength test

The modified forelimb grip strength test was utilized to assess grip strength. In this test, the rodent grips a bar connected to a monitoring device with its forelimbs, while the examiner pulls the tail of the rodent horizontally. The maximal value recorded during three trials represents the forelimb grip strength (Mesquita et al., 2021).

Extraction and sampling of gastrocnemius muscle tissue

The hindlimbs of the rats were shaved prior to muscle tissue extraction. A plexiglass platform was utilized to immobilize the rats during the procedure. An incision was made along the leg, from the heel to the vertebral column, followed by careful dissection of the skin and removal of the gastrocnemius muscle. The muscle samples were immediately frozen at -80°C and stored for 1 weeks before further processing. It should be noted that FOXO1, BAX and cytochrome C genes DNA was extracted from gastrocnemius muscle tissue.

Analysis of gene expression

RNA was extracted from 50 mg of gastrocnemius muscle tissue from each rat (n=5/group) using TRIZOL reagent and a homogenizer. The extracted RNA was dissolved in nuclease-free water and stored at -80°C. The concentration and purity of RNA were evaluated using a Nanodrop 2000 spectrophotometer. The purity was confirmed by assessing the 260/280 and 260/230 nm ratios, with an optimal range of 1.9-2.0.

Reverse transcription for qRT-PCR

Table 1. Primer sequences of target genes.

Gene	Primer sequence 5'-3'
r-FOXO1	F: GAGGACGGGCTGCTAAGAAA R: AGTCTCCCACTGATGGTGCT
r-BAX	F: CGGCGAATTGGAGATGAACTG R: GCAAAGTAGAAGAGGGCAA
r-Cytochrome C	F: TTGTTGGACAGCCCCGATTT R: ATAGGTTTGAGGCGACACCC
Firmicutes	F: GGAGYATGTGGTTTAATTCGAAGCA R: AGCTGACGACAACCATGCAC
Bacteroidetes	F: GGARCATGTGGTTTAATTCGATGAT R: AGCTGACGACAACCATGCAG
r-GAPDH	F: AGGTCGGTGTGAACGGATTTG R: TGTAGACCATGTAGTTGAGGTCA

Prior to reverse transcription, genomic DNA was removed from 1 µg of total RNA using DNase I. The purified RNA was reverse-transcribed into cDNA using MMLV reverse transcriptase and oligo (dT) primers, followed by storage at -20°C. cDNA synthesis was performed on samples from 25 rats (5 per group). The relative expression level of the target gene was determined using the $2^{-\Delta\Delta CT}$ method, where the Ct values of the target and housekeeping genes were used to calculate expression levels. The sequences of the primers used are provided in table 1.

Fecal sample collection

Following the final administration of exercise and supplements to the rats at the end of the 8th week, the rats underwent a 24-hour fasting period with access to water. Subsequently, after perianal disinfection, the rats were stimulated to defecate through abdominal massage. Sterile forceps were then used to collect 2-4 capsules or 1-2 ml of feces, which were stored in sterile cryopreservation tubes at -80°C for subsequent fecal DNA extraction and detection of bacterial flora (Chi et al., 2021). Finally, this stool samples used to extract DNA for firmicutes and Bacteroidetes bacteria. Also, total bacteria DNA selected but were excluded from the study and haven't been reported here.

DNA extraction and analysis of gut microbiota

Bacterial DNA extraction from rat fecal samples was conducted using the QIAamp DNA Stool Mini Kit from Qiagen (Germany). The extracted DNA samples were diluted to a concentration of 1 ng/µl and then amplified using the commonly used PCR primers 515F/806R targeting the V4 variable region of the 16S rRNA gene. High-fidelity DNA polymerase from New England Biolabs, in conjunction with the Phusion® High-Fidelity PCR Master Mix kit, was employed for this amplification. The resulting PCR products underwent purification, pooling, and were utilized for the construction of DNA libraries with the TruSeq® DNA PCR-Free Sample Preparation Kit from Illumina. Subsequent 16S rRNA sequencing was performed on the Illumina MiSeq platform (2 × 250 bp paired-end) (Dong et al., 2023).

Statistical analysis

The normal distribution of data was assessed using the Shapiro-Wilk test. Differences between study groups were analyzed using one-way ANOVA, with the post-hoc Tukey test utilized for intergroup comparisons across different variables. The correlation between variables was evaluated through the Pearson correlation test. Data analysis was carried out using GraphPad Prism version 9.5.1 along with and SPSS version 27. GraphPad Prism was also employed for graph design, with statistical significance set at $p < 0.05$.

Results

Result of one-way ANOVA revealed that there were statistically significant (p=0.006) differences between groups. Multiple comparisons Post hoc tukey test was indicated 8 weeks of resistance training plus supplement decreased FOXO1 gene expression drastically to the level of YC group which no difference was observed between ORS and YC groups (p=0.999). Decline of FOXO1 gene expression also was observed in OR compare to OC group (p=0.029). Supplementation alone didn't have any influence on gene expression level in comparison with OC group (p=0.188). The level of FOXO1 gene expression dramatically was high in OC compare to intervention groups and YC group (figure 1a). In relation to BAX gene our finding indicated that all intervention, both supplement (p=0.003) and exercise (p=0.001) had large depressive effect on gene expression level compare to OC group. Combination of exercise and supplement showed strong impact to decline BAX gene expression equally to the YC group compare to OC group (p<.001) but no differences were shown between intervention (figure 1b).

Cytochrome C showed that resistance training alone didn't have any effect and was as same as OC group (p=0.079). Weak impact was observed in OS (p=0.038) and ORS (p=0.017) groups compare to OC group that decreased level of gene expression of cytochrome C. There were no significant differences between interventions in gene expression level in cytochrome C as shown in figure 2a.

Lowest level of firmicutes to Bacteroidetes ratio was observed in YC and ORS groups compare to OC group (p=<.001). OS group showed lower level of F/B ratio in comparison with OC group (p=0.011) but resistance training alone didn't have any impact on F/B ratio (p=0.209) and was similar to OC group. It seems that supplement insert synergistic effect on resistance training and combination of supplement with resistance training was more effective than each of them separately (p=<.001) (figure 2b). Finally, it seems that supplement or exercise suppress gene expression of firmicutes rather than Bacteroidetes.

Result of one-way ANOVA in pretest of grip strength between groups was significant at p<.001 but tukey postdoc test revealed that it differences just exist between YC group with other groups (p=0.001) (figure3a). In contrast with pretest, result of posttest indicated that both exercise plus supplement (p=0.001) and exercise alone (p=0.001) increased grip strength interestingly but supplement separately didn't change this factor (p=0.883). Indeed, ORS showed drastically increase in grip strength even equal to YC group (p=0.873). These results indicate that combination of supplement with exercise was more effective than one alone but more influence was observed in OR than OS (p=0.001) (figure 3b).

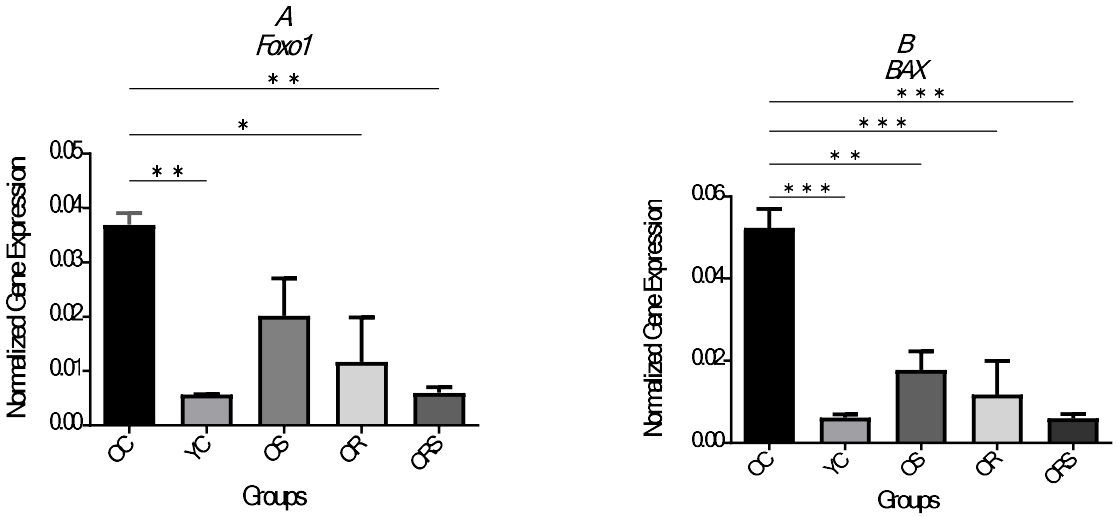

Figure 1. Post intervention normalized gene expression of FOXO1; a and BAX; b. OC refers to the old control group, YC represents the young control group, OS denotes the old group receiving supplements, OR signifies the old group undergoing resistance training, and ORS indicates the old group receiving both supplements and resistance training. Asterisks denote significant differences as follows: * (p=0.033), ** (p=0.002), and *** (p=0.001).

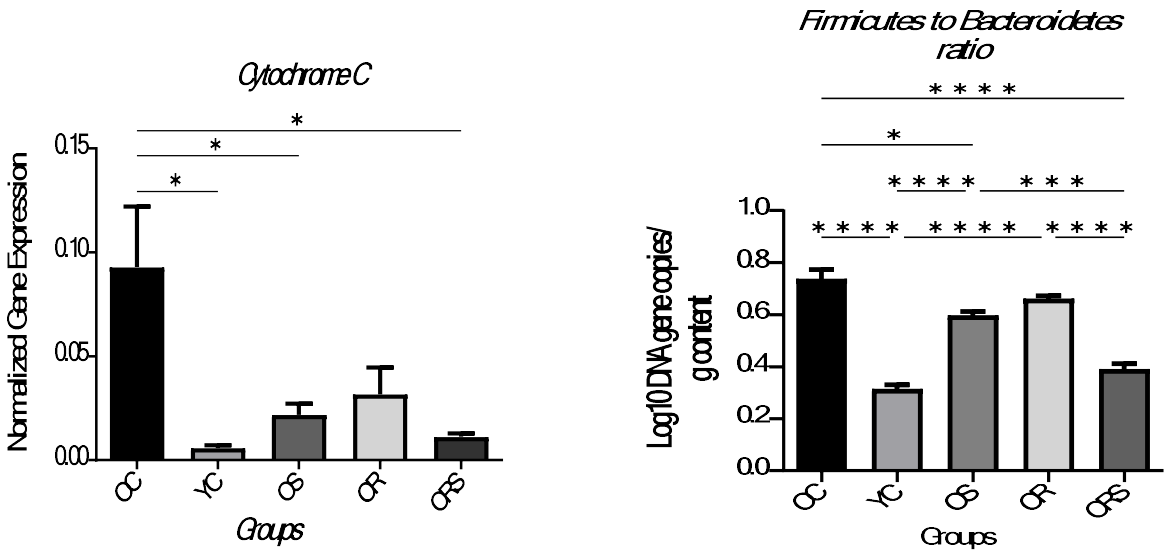

Figure 2. Post intervention normalized gene expression level of Cytochrome C; a and F/B ratio; b. OC represents old control group, YC stands for young control group, OS denotes for old supplement group, OR representing resistance training group and ORS stands for old resistance training plus supplement. Asterisks indicate significant differences at* (p=0.033), *** (p=0.001) and **** (p=<.001).

Pearson correlation test showed that there was moderate negative correlation (r=-0.5483), CI (95%; -0.7453 to -0.2631) between F/B ratio and grip strength in posttest. This test also demonstrated that there were average positive correlation between F/B ratio and FOXO1(r=0.6628), CI (95%; 0.2279 to 0.8772), BAX (r=0.6854), CI (95%; 0.2669 to 0.8864) and cytochrome C (r=0.7275), CI (95%; 0.3431 to 0.9032).

Discussion

The first aim of this study was to evaluate crosstalk between grip strength as functional index of muscle hypertrophy in rodents with F/B ratio as representative of gut microbiome attributes. The second aim was investigation the role of resistance training and supplement in F/B ratio, apoptosis and proteolysis factors modulation. We found that FOXO1 gene expression was decrease by administration of resistance training and supplement to the level of young control group. in consistence with our finding in a study by Fu et al it was observed that 4 weeks of resistance training both in normal and hypoxic condition could decrease FOXO1 gene expression and also it was shown that myotube diameter increased (Fu et al., 2024). Supplementation alone didn't impact FOXO1, in contrast with this finding Mulet and colleagues, indicated that some strains of probiotics are capable to downregulate FOXO1 and NF-κB but it should be note that the nature of stimulus must be considered because that they inserted Hydrogen Peroxide as incentive and its mechanism of action to FOXO1 different due to their oxidative action (Mulet et al., 2017). Another possible cause for this Dissimilarity may depends on mixture of our supplement. In a study it was established that vitamin D can attenuates FOXO1-target gene expression and this study was conducted on C2C12 muscle cells. In the mentioned study it also suggests that vitamin D can suppress atrogin 1 and cathepsin L gene expression (Hirose et al., 2018). This result showed that combination of exercise and mix of used supplement was able to decrease FOXO1 gene expression. In relation to apoptosis factors activity, it was interesting that all interventions have the capacity to subdue BAX gene expression. But, it is very critical to note that in majority of research on apoptosis pathway BAX was assessed with BCL-2 gene and its ration was considered as apoptosis activity which show one limitation of present research. Another limitation of present study was that assessment of more relevant atrophy pathway genes such as MAFbx/atrogin-1, MuRF1, etc. had not taken place and also in genes related to apoptosis it may be more effective to evaluate caspase genes activity along with BAX. But, this issue hasn't been proposed in our study.

In contrast with efficiency of resistance training alone in BAX expression, it was demonstrated that cytochrome C didn't get any affect from this stimulus, but combination of exercise with supplement as shown in ORS group decrease level of this gene expression. Supplement alone also have capacity to reduce gene expression, this effect may insert by vitamin D on mitochondria, as mentioned before, vitamin D can improve mitochondria health by stabilizing enzymatic action in mitochondria that is one of main source of apoptosis (Reddy et al., 2022). Vitamin D also can suppress BCL-2 activity and have positive impact on apoptosis and caspase genes in higher dosage. It should be interpreted by considering sample and also type of intervention as dosage employed in different studies wasn't equal. In consistence with this result and finding of Hirose et al study, it was suggested that

vitamin D3 can suppress related muscle atrophy genes expression consists of FOXO, MAFbx, and MuRF1. Indeed, vitamin D can improve gene expression of myosin heavy chain (MyHC) proteins, MyoD, and MyoG, and increased the phosphorylation of AMPK and AKT. It seems that these functions mediated from VDR (Talib et al., 2023).

Regarding resistance training, Pena et al reported that 8 weeks of resistance training caused decrease in caspase and BCL-2 activity in muscle of elderly adult. It also demonstrated that vitamin D can suppress phosphorylation of ULK1 which is one of key inhibitor of mTOR activation (Mejías-Peña et al., 2017). Overall, it is obvious that combination of vitamin D and probiotics can interfere with raising in inflammatory response and oxidant concentration which are as main stimulator of proteolysis and apoptosis (Storz, 2011) and blend of resistance training and leucine amino acids act as fortifier of mTOR activation as integratory signal center related to Hypertrophy. However, it would have been better to have an additional analysis on VDR as a vitamin D receptor as its functional arm, but this remained unclear in the present study.

Balance between Firmicutes to Bacteroidetes is very important to maintain body function and disruption in this ratio was associated with different disease for instance it was observed that increase in mentioned index is related to obesity, gestational diabetes mellitus, benign prostatic hyperplasia, and increased left atrial diameter (An et al., 2023). Our findings in relation to F/B ratio revealed that both supplement and resistance training plus supplement was capable to decrease F/B ratio. Conversely, in a study on human subjects it was observed that resistance training and balance diet induce marked increase after 6 weeks. Typical exercise used in this study was aerobic and the intensity and duration of exercise was high which can cause of difference between present study (Huang et al., 2020). More intense exercise can trigger inflammatory response and disrupt intestinal barrier which aggravate pathogen entrance to blood. Another possible reason may depend on their obese and overweight participants, as noted before, F/B ratio increased in obesity. As reported in the study of Barzak et al, decrease in training volume is align with decrease in F/B ratio (Barzak et al., 2022). Our study used resistance training which left for half an hour but study of Huang and their colleagues was continued for 5 hours a day for 6 sessions per weeks which was more intense. Grip strength was markedly increased post intervention in training intervention groups but supplement alone didn't affect grip strength. In consistence with our finding, Aamann and their colleagues showed that 5 days of resistance training for 4 weeks increased muscle mass (larger gastrocnemius circumference and weight) (Aamann et al., 2019). It seems that increased grip strength as hypertrophy markers only stemming from resistance training due to synergistic effect that have seen in ORS group and lake of improvement in grip strength in OS group. In agreement with this finding, Rips et al, observed that 7 months vitamin D supplementation didn't affect grip strength in physically active subject (Rips et al., 2022).

Functional crosstalk between F/B ratio and muscle hypertrophy which considered as increase in grip strength in present study manifested by balancing F/B ratio to the level that was seen in

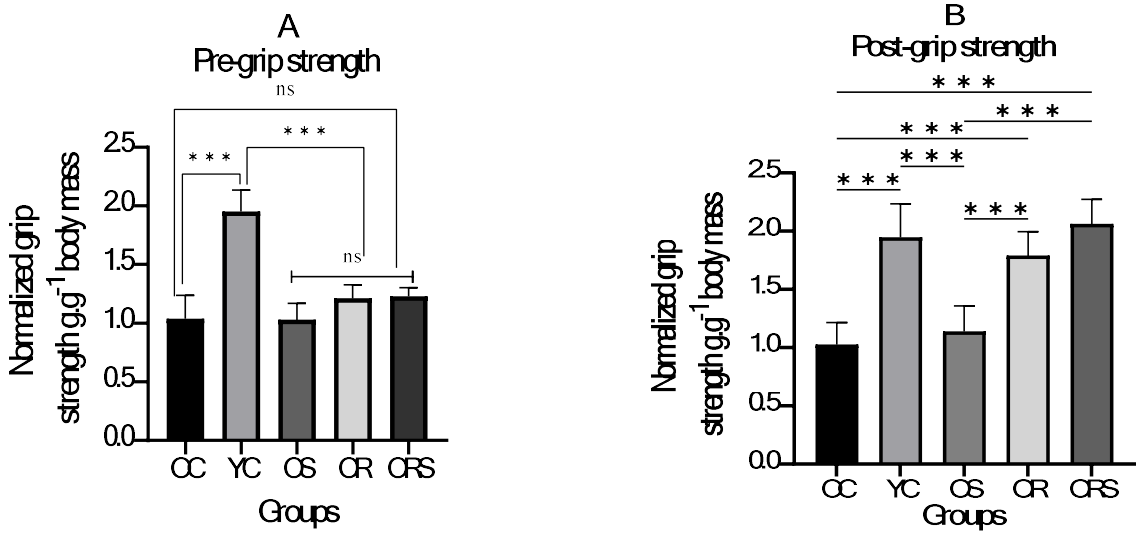

Figure 3. Displays the pre and posttest values of relative grip strength in different study groups. Part A presents the pretest grip strength among various study groups, while Part B shows the post-intervention values of relative grip strength in grams of body weight and grams of carried overload. The abbreviations used in the figure are as follows: OC for the old control group, YC for the young control group, OS for the old supplement group, OR for the old resistance training group, and ORS for the old resistance training plus supplement group. *** Significant differences at (p=0.001), and ns stands for non-significant.

YC group. aging is accompanied by gradual increase in F/B ratio which stated that casus obesity and other mentioned disease (An et al., 2023). Another key note to consider for existence of this crosstalk is that proteolysis factor as foxo1 and healthy mitochondrial function markers (decline of cytochrome C and BAX) was observed. We postulated that potential intestine–muscle axis signaling pathway may exist as Gut-muscle axis. Synergistic intestinal microbiota and vitamin D, probiotics, and leucine interaction toward muscle protein synthesis and mitochondrial function improvement may be manifested by the influence of mTOR and FOXO signaling on oxidative stress and immunological function modulation. As previously research established, firmicutes as lactic acid fermenting strain can induce inflammatory response and trigger FOXO and apoptotic factors. On the other hands, Resistance training has been shown to have anti-inflammatory effects in various tissues and organs throughout the body, including the gut. For instance, it was shown that resistance training might decrease the zonulin level and increase mucin production and thereby reduce inflammation in the gut. In the other side, given that short chain fatty acids (SCFA) compose main source of nutrition for intestinal epithelial cells and by this observation that regular exercise increases the diversity of the intestinal microbiota and the number of beneficial bacteria, particularly those that produce SCFA it seems natural that there is a two-way communication between the muscle and the gut microbiome (Wagner et al., 2024). As the results of correlation evaluation showed, decrease in F/B ratio was associated with in-crease in grip strength. A possible explanation for this finding may in relation to enrichment of gut microbiome content by physical activity and decrease in pro-inflammatory cytokines as before discussed. Also, it observed that decrease in F/B ratio correlated with decrease in FOXO1, BAX and cytochrome C and it seems normal when considering increase in this ratio with aging. Finally, further research on the impact of combination of probiotics, vitamin D and leucine along with resistance training on muscle hypertrophy signaling pathway include mTOR and its downstream cascade seems to be crucial as our studied factors indirectly affect skeletal muscles hypertrophy and exercise capacities.

Conclusion

Our research findings suggest that exercise can influence the breakdown of proteins and cell death in older individuals, thus reducing the rate at which muscle function declines with age. Furthermore, our study revealed that the increased F/B ratio, a marker of inflammation in aging individuals, can be improved by a combination of exercise and supplements. Specifically, probiotics, a key component of the supplement used, were found to enhance grip strength as additive to exercise and alone. However, the synergistic effects of combining exercise with probiotics were evident in various mentioned factors. Our research indicates that a combination of resistance training with Vitamin D, probiotics, and leucine may help shift the activity levels of genes and intestinal microbiome towards those typically seen

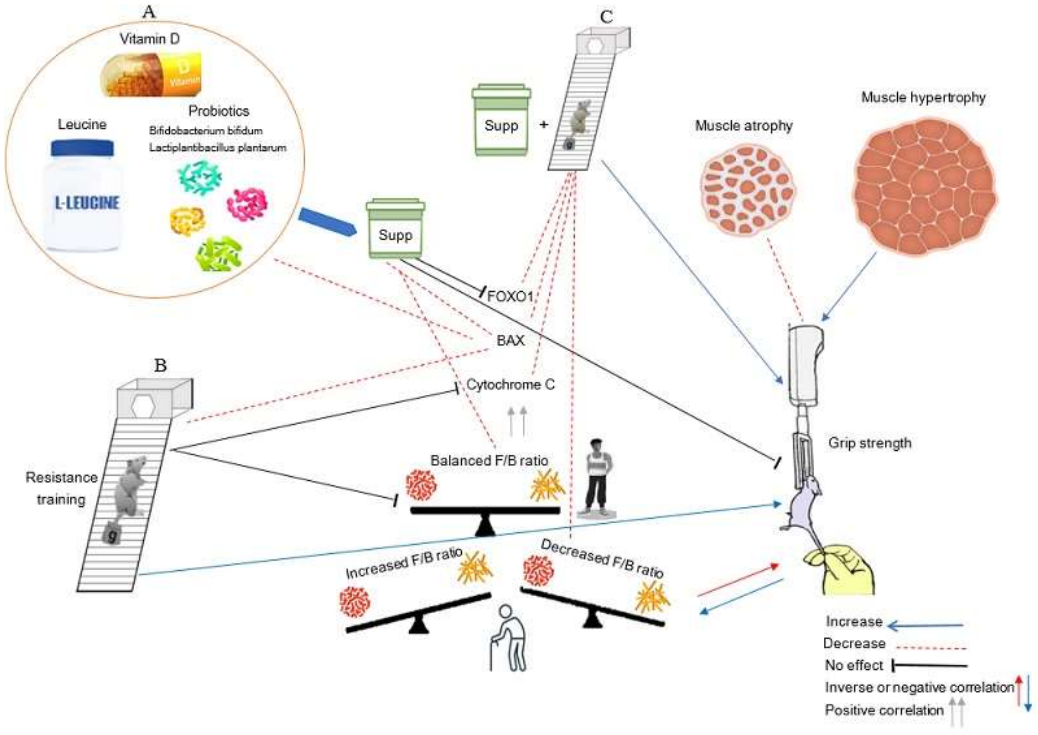

Figure 4. Schematic conclusion of the study outcomes beside its intervention. A represents supplement, B shows resistance training, and C displays resistance training plus supplement intervention respectively.

in younger individuals (figure 4). While some factors were not directly comparable, owing to limited existing studies in this arena, caution should be exercised in interpreting these results and making generalizations. More controlled human studies are required to further investigate this promising field because of our limitation in assessment of microbiome profile by microbiomics approach or using shotgun sequencing of the total microbial community for providing whole microbiota properties and its relation to measured genes.

What is already known on this subject?

Recent studies have shown that the F/B ratio can be influenced by various factors, including lifestyle choices, medication usage, physical activity, and nutrition. However, the connection between this ratio and proteolysis and apoptosis was previously unclear, and previous assumptions were primarily based on the anti-inflammatory properties of the gut microbiome on other organs.

What this study adds?

The current study revealed that the F/B ratio is associated with muscle function and proteolysis. Furthermore, it was demonstrated that the administration of resistance training and nutraceutical components can restore the gut microbiota composition to a state similar to that of younger individuals. Additionally, a positive correlation between apoptosis and the F/B ratio, which was previously unknown, was observed.

Organ Cross-Talk Tips:

- The F/B ratio, serving as a critical functional indicator of the gut microbiome, is linked to key programmed cell death markers like cytochrome c and Bax.
- Our finding aligns with the concept of the gut-muscle axis, and this relationship can be enhanced through resistance training and probiotic supplementation enriched by vitamin D and leucine in older individuals.

Acknowledgements

The authors extend their gratitude to all individuals who contributed to the execution and progression of this research, with special thanks to the Iranian Research Organization for Science and Technology for their provision of supplemental materials for this study.

Funding

None.

Compliance with ethical standards

Conflict of interest To ensure that all procedures were conducted in accordance.

Ethical approval All phases of the housing and utilization of rats in the experimental protocol were conducted in compliance with the guidelines and regulations set forth by the Animal Ethics Committee of Shahid Beheshti University, Tehran, Iran (ethical code: IR.SBU.REC.1402.121).

Informed consent Animal study.

Author contributions

Conceptualization: H.A.; Methodology: H.A., M.N.; Software: M.N., M.M; Validation: M.N., Formal analysis; Investigation: M.N., H.A.; Resources: H.A.; Data curation: H.A., M.M.; Writing - original draft: M.N.; Writing – review & editing: H.A., M.M; Visualization: H.A.; Supervision: H.A. Project administration: M.N., M.M.; Funding acquisition: H.A.

References

Aamann, L., Ochoa-Sanchez, R., Oliveira, M., Tremblay, M., Bémeur, C., Dam, G.,...Rose, C. F. (2019). Progressive resistance training prevents loss of muscle mass and strength in bile duct-ligated rats. Liver International, 39(4), 676-683. doi: https://doi.org/10.1111/liv.13997

Abdul Kadir, N. A. A., Rahmat, A., & Jaafar, H. Z. (2015). Protective effects of Tamarillo (Cyphomandra betacea) extract against high fat diet induced obesity in Sprague-Dawley rats. Journal of obesity, 2015(1), 846041. doi: https://doi.org/10.1155/2015/846041

Alikhani, M., MacLellan, C. M., Raptis, M., Vora, S., Trackman, P. C., & Graves, D. T. (2007). Advanced glycation end products induce apoptosis in fibroblasts through activation of ROS, MAP kinases, and the FOXO1 transcription factor. American Journal of Physiology-Cell Physiology, 292(2), C850-C856. doi: https://doi.org/10.1152/ajpcell.00356.2006

An, J., Kwon, H., & Kim, Y. J. (2023). The firmicutes/bacteroidetes ratio as a risk factor of breast cancer. Journal of Clinical Medicine, 12(6), 2216. doi: https://doi.org/10.3390/jcm12062216

Arifin, W. N., & Zahiruddin, W. M. (2017). Sample size calculation in animal studies using resource equation approach. The Malaysian journal of medical sciences: MJMS, 24(5), 101. doi: https://doi.org/10.21315/mjms2017.24.5.11

Barker, T. H., George, R. P., Howarth, G. S., & Whittaker, A. L. (2017). Assessment of housing density, space allocation and social hierarchy of laboratory rats on behavioural measures of welfare. PloS one, 12(9), e0185135. doi: https://doi.org/10.1371/journal.pone.0185135

Barzak, B., Hankus, K., Parmar, S., & Wozniak, S. (2022). The effect of physical activity on gut microbiota. A review. Medical Journal of Cell Biology, 10(4), 138-143. doi: https://doi.org/10.2478/acb-2022-0021

Bass, J. J., Nakhuda, A., Deane, C. S., Brook, M. S., Wilkinson, D. J., Phillips, B. E.,…Andersen, D. (2020). Overexpression of the vitamin D receptor (VDR) induces skeletal muscle hypertrophy. Molecular metabolism, 42, 101059. doi: https://doi.org/10.1016/j.molmet.2020.101059

Chen, S., Zhang, P., Duan, H., Wang, J., Qiu, Y., Cui, Z.,…Xie, L. (2023). Gut microbiota in muscular atrophy development, progression and treatment: New therapeutic targets and opportunities. The Innovation. doi: https://doi.org/10.1016/j.xinn.2023.100479

Chen, Y.-M., Wei, L., Chiu, Y.-S., Hsu, Y.-J., Tsai, T.-Y., Wang, M.-F., & Huang, C.-C. (2016). Lactobacillus plantarum TWK10 supplementation improves exercise performance and increases muscle mass in mice. Nutrients, 8(4), 205. doi: https://doi.org/10.3390/nu8040205

Chi, T., Zhao, Q., & Wang, P. (2021). Fecal 16S rRNA gene sequencing analysis of changes in the gut microbiota of rats with low-dose aspirin-related intestinal injury. BioMed Research International, 2021(1), 8848686. doi: https://doi.org/10.1155/2021/8848686

de Farias Junior, G. C., de Sousa Neto, I. V., Guzzoni, V., Pisani, G. D., Royer, C., de Lima, C. L.,…Durigan, J. L. Q. (2020). Remodeling process in bone of aged rats in response to resistance training. Life Sciences, 256, 118008. doi: https://doi.org/10.1016/j.lfs.2020.118008

de Las Heras, N., Martín Giménez, V. M., Ferder, L., Manucha, W., & Lahera, V. (2020). Implications of oxidative stress and potential role of mitochondrial dysfunction in COVID-19: therapeutic effects of vitamin D. Antioxidants, 9(9), 897. doi: https://doi.org/10.3390/antiox9090897

Devries, M. C., McGlory, C., Bolster, D. R., Kamil, A., Rahn, M., Harkness, L.,…Phillips, S. M. (2018). Leucine, not total protein, content of a supplement is the primary determinant of muscle protein anabolic responses in healthy older women. The Journal of nutrition, 148(7), 1088-1095. doi: https://doi.org/10.1093/jn/nxy091

Dong, H., Tang, X., Ye, J., & Xiao, W. (2023). 16S rRNA gene sequencing reveals the effect of fluoxetine on gut microbiota in chronic unpredictable stress-induced depressive-like rats. Annals of General Psychiatry, 22(1), 27. doi: https://doi.org/10.1186/s12991-023-00458-x

Fu, P., Zhu, R., Gao, W., & Gong, L. (2024). Effects of resistance training on alleviating hypoxia-induced muscle atrophy: Focus on acetylation of FoxO1. Journal of Cellular and Molecular Medicine, 28(3), e18096. doi: https://doi.org/10.1111/jcmm.18096

Hirose, Y., Onishi, T., Miura, S., Hatazawa, Y., & Kamei, Y. (2018). Vitamin D attenuates FOXO1-target atrophy gene expression in C2C12 muscle cells. Journal of Nutritional Science and Vitaminology, 64(3), 229-232. doi: https://doi.org/10.3177/jnsv.64.229

Huang, J., Liao, J., Fang, Y., Deng, H., Yin, H., Shen, B., & Hu, M. (2020). Six-week exercise training with dietary restriction improves central hemodynamics associated with altered gut microbiota in adolescents with obesity. Frontiers in endocrinology, 11, 569085. doi: https://doi.org/10.3389/fendo.2020.569085

Kamei, Y., Miura, S., Suzuki, M., Kai, Y., Mizukami, J., Taniguchi, T.,…Aburatani, H. (2004). Skeletal muscle Foxo1 (Fkhr) transgenic mice have less skeletal muscle mass, down-regulated type I (Slow twitch/red muscle) fiber genes, and impaired glycemic control*[Boxs]. Journal of Biological Chemistry, 279(39), 41114-41123. doi: https://doi.org/10.1074/jbc.M400674200.

Lahiri, S., Kim, H., Garcia-Perez, I., Reza, M. M., Martin, K. A., Kundu, P.,…Zhang, H. (2019). The gut microbiota influences skeletal muscle mass and function in mice. Science translational medicine, 11(502), eaan5662. doi: https://doi.org/10.1126/scitranslmed.aan5662

Lee, M.-C., Tu, Y.-T., Lee, C.-C., Tsai, S.-C., Hsu, H.-Y., Tsai, T.-Y.,…Huang, C.-C. (2021). Lactobacillus plantarum TWK10 improves muscle mass and functional performance in frail older adults: A randomized, double-blind clinical trial. Microorganisms, 9(7), 1466. doi: https://doi.org/10.3390/microorganisms9071466

Mastali, V. P., Hoseini, R., & Azizi, M. (2023). The effect of short-term vitamin D on the antioxidant capacity following exhaustive aerobic exercise. African Health Sciences, 23(1), 584-591. doi: https://doi.org/10.4314/ahs.v23i1.61

McLoughlin, T. J., Smith, S. M., DeLong, A. D., Wang, H., Unterman, T. G., & Esser, K. A. (2009). FoxO1 induces apoptosis in skeletal myotubes in a DNA-binding-dependent manner. American Journal of Physiology-Cell Physiology, 297(3), C548-C555. doi: https://doi.org/10.1152/ajpcell.00502.2008.

Mejías-Peña, Y., Estébanez, B., Rodriguez-Miguelez, P., Fernandez-Gonzalo, R., Almar, M., de Paz, J. A.,…Cuevas, M. J. (2017). Impact of resistance training on the autophagy-inflammation-apoptosis crosstalk in elderly subjects. Aging (Albany NY), 9(2), 408. doi: https://doi.org/10.18632/aging.101167

Mesquita, P. H., Lamb, D. A., Godwin, J. S., Osburn, S. C., Ruple, B. A., Moore, J. H.,…Young, K. C. (2021). Effects of resistance training on the redox status of skeletal muscle in older adults. Antioxidants, 10(3), 350. doi: https://doi.org/10.3390/antiox10030350

Mohabbat, M., & Arazi, H. (2024). Effect of resistance training plus enriched probiotic supplement on sestrin2, oxidative stress, and mitophagy markers in elderly male Wistar rats. Scientific Reports, 14(1), 7744. doi: https://doi.org/10.1038/s41598-024-58462-4

Mohr, A. E., Jäger, R., Carpenter, K. C., Kerksick, C. M., Purpura, M., Townsend, J. R.,…Pyne, D. B. (2020). The athletic gut microbiota. Journal of the International Society of Sports Nutrition, 17, 1-33. doi: https://doi.org/10.1186/s12970-020-00353-w.

Mulet, A., Perelmuter, K., Bollati-Fogolin, M., Crispo, M., & Grompone, G. (2017). Forkhead box protein O1 is linked to anti-inflammatory probiotic bacteria acting through nuclear factor-κB pathway. J Microb Biochem Technol, 9, 074-081. doi: https://doi.org/10.4172/1948-5948.1000347

O'Brien, M. T., O'Sullivan, O., Claesson, M. J., & Cotter, P. D. (2022). The athlete gut microbiome and its relevance to health and performance: a review. Sports Medicine, 52(Suppl 1), 119-128. doi: https://doi.org/10.1007/s40279-022-01785-x.

Park, S. S., Kwon, E.-S., & Kwon, K.-S. (2017). Molecular mechanisms and therapeutic interventions in sarcopenia. Osteoporosis and sarcop-

-enia, 3(3), 117-122. doi: https://doi.org/10.1016/j.afos.2017.08.098

Reddy, A. M., Iqbal, M., Chopra, H., Urmi, S., Junapudi, S., Bibi, S.,…Abdel-Daim, M. M. (2022). Pivotal role of vitamin D in mitochondrial health, cardiac function, and human reproduction. EXCLI journal, 21, 967. doi: https://doi.org/10.17179/excli2022-4935

Rips, L., Toom, A., Kuik, R., Varblane, A., Mölder, H., Tammaru, M.,…Gapeyeva, H. (2022). Seven-month wintertime supplementation of 1200 IU vitamin D has no effect on hand grip strength in young, physically active males: a randomized, controlled study. Journal of the International Society of Sports Nutrition, 19(1), 437-454. doi: https://doi.org/10.1080/15502783.2022.2100718

Stojanov, S., Berlec, A., & Štrukelj, B. (2020). The influence of probiotics on the firmicutes/bacteroidetes ratio in the treatment of obesity and inflammatory bowel disease. Microorganisms, 8(11), 1715. doi: https://doi.org/10.3390/microorganisms8111715.

Storz, P. (2011). Forkhead homeobox type O transcription factors in the responses to oxidative stress. Antioxidants & redox signaling, 14(4), 593-605. doi: 10.1089/ars.2010.3405

Talib, N. F., Zhu, Z., & Kim, K.-S. (2023). Vitamin D3 Exerts Beneficial Effects on C2C12 Myotubes through Activation of the Vitamin D Receptor (VDR)/Sirtuins (SIRT) 1/3 Axis. Nutrients, 15(22), 4714. doi: https://doi.org/10.3390/nu15224714.

Tezze, C., Sandri, M., & Tessari, P. (2023). Anabolic Resistance in the Pathogenesis of Sarcopenia in the Elderly: Role of Nutrition and Exercise in Young and Old People. Nutrients, 15(18), 4073. doi: https://doi.org/10.3390/nu15184073.

Tucureanu, M. M., Rebleanu, D., Constantinescu, C. A., Deleanu, M., Voicu, G., Butoi, E.,…Manduteanu, I. (2018). Lipopolysaccharide-induced inflammation in monocytes/macrophages is blocked by liposomal delivery of Gi-protein inhibitor. International journal of nanomedicine, 63-76. doi: https://doi.org/10.2147/IJN.S150918

Wagner, A., Kapounková, K., & Struhár, I. (2024). The relationship between the gut microbiome and resistance training: a rapid review. BMC Sports Science, Medicine and Rehabilitation, 16(1), 4. doi: https://doi.org/10.1186/s13102-023-00791-4

Wang, J., Chen, W.-D., & Wang, Y.-D. (2020). The relationship between gut microbiota and inflammatory diseases: the role of macrophages. Frontiers in Microbiology, 11, 535016. doi: https://doi.org/10.3389/fmicb.2020.01065

Xu, M., Chen, X., Chen, D., Yu, B., & Huang, Z. (2017). FoxO1: a novel insight into its molecular mechanisms in the regulation of skeletal muscle differentiation and fiber type specification. Oncotarget, 8(6), 10662. doi: https://doi.org/10.18632/oncotarget.12891.

Yoo, S.-Z., No, M.-H., Heo, J.-W., Park, D.-H., Kang, J.-H., Kim, S. H., & Kwak, H.-B. (2018). Role of exercise in age-related sarcopenia. Journal of exercise rehabilitation, 14(4), 551. doi: https://doi.org/10.12965/jer.1836268.134.