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Research Article

Gut-muscle crosstalk: The effect of endurance training and probiotic supplementation on intestinal villus structure, postbiotics and VO_{2max} in old male rats

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Abstract

The aim of this study was the effect of endurance exercise and probiotic supplement enriched with amino acid leucine and vitamin D on the gut-muscle axis in aged male rats. For this purpose, 25 male Wistar rats (5 rats in each group) in two age groups of 8 to 12 weeks (young) and 18 to 24 months (elderly) were randomly divided into 5 equal groups of old control (OC), young control (YC), endurance exercise (OE), supplement group (OS) and endurance exercise plus supplement (OES) were divided. The results showed that 8 weeks of endurance training (three times a week) and supplemental oral gavage (5 times a week) caused a significant change in postbiotics (decrease of indoxyl sulfate (IXS) and increase of Short-chain fatty acids (SCFAs)). The role of OS in reducing IXS was more prominent than OE and OES variables; of course, the synergistic effect of OES (P=0.000), caused a greater improvement in the amount of SCFA. Also, Administering the supplement alone and at rest (without exercise) could not cause a significant increase in VO_{2max} (P=0.449). But, the effect of exercise on increasing VO_{2max} index was more effective than OS and even OES. Eventually, the independent variables made a significant difference on the Villus height (VH) (except for the OS group) and number of goblet cells (GC) compared to the OC group (P<0.05).

Key Words: **Gut-Muscle axis, Postbiotic metabolites, Endurance training, Probiotic supplementation, VO_{2max}, SCFA**

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Introduction

Aging is a major risk factor or cause of many diseases, or that many diseases are "age-related" and "aging-related" (Le and Janani, 2022). There are several factors that contribute to ageing. These factors may be biological, life style, social, psychological, spiritual, and cognitive and the diseases in the old age. These factors may not only lead to ageing but also to several diseases in the ageing process (Tieland et al., 2018). Sarcopenia is a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life and death (Santilli et al., 2014). Several factors can influence the development of sarcopenia in the older adults, including hormone and cytokine imbalance, age-associated systemic inflammation (inflammaging), gut microbiota dysbiosis, metabolic disorders (Bilski et al., 2022) poor nutrition, physical inactivity and mitochondrial dysfunction (de Marco et al., 2021).

Studies have also indicated that age-related gut microbiota (GM) alterations and gastrointestinal (GI) dysfunctions exist, such as bacterial overgrowth, increased intestinal permeability, and decreased absorption of nutrients in the gastrointestinal tract (Chen, L et al., 2021). Villus height (VH), Goblet cells (GCs) and crypt depth are known to play a vital role in nutrient absorption and digestion (Liu, S et al., 2023). Studies in rats have shown age-related losses in villous and enterocyte heights (Drozdowski & Thomson., 2006). Better-feed digestion and nutrient absorption capability may also be indirectly related to the immunostimulatory and antioxidative effects of Bacillus strains, which can significantly improve the intestinal morphology of the intestinal tract. Moreover, the immune-modulatory action enhances the mucosal barrier integrity, and the intestinal mucosal structure increases the villus height and the villus height to crypt depth ratio (Liu, S et al., 2023).

A crosstalk between microbiota and exercise is bidirectional, so that, the microbiota can be altered by exercise, due to increased oxidative stress, intestinal permeability, electrolyte imbalance, glycogen depletion, etc. In addition, the Microbiota affects athletic performance during exercise due to its ability to take up energy, modulate the immune system, regulate gastrointestinal (GI) tract health and reverse the inflammatory response after exercise (Díaz-Jiménez et al., 2021).

The mechanisms underlying improved aerobic performance involve adaptations in multiple physiological systems, including skeletal muscles. For example, muscle adaptations are characterized by greater mitochondrial volume and increased expression of mitochondrial enzymes involved in Beta oxidation, the tricarboxylic acid (TCA) cycle, and the electron transport system (Soares et al., 2019). Exercise has a distinctive impact on the microbiota. Recent evidence suggested that Physical activity, particularly of an aerobic exercise (Smith et al., 2022), improving the diversity and abundance of certain bacterial genera may have positive effects on the gut and neurology (Cataldi et al., 2022). It is reported that microbial diversity is increased in elite rugby players compared with matched controls (Boisseau et al., 2022). Also, in a study evaluating the physical endurance of mice using swimming as an index of exercise capacity, the germ-free mouse model showed lower exercise capacity and decreased endurance when compared to the mice harboring a single bacterial species in their guts (Yun et al., 2022). Moreover, Probiotic supplementation could also modulate microbiota diversity and abundance. For example, it is reported that lactobacillus plantarum PS128 supplementation might also have functional activities and an anti-inflammation effect through modulation of the microbiota (Huang et al., 2020). Moderate exercise alters microbiota composition and increases Lactobacilli and Bifidobacteria, promoting epithelial barrier integrity and producing SCFAs (postbiotics). Although the exact definition of postbiotics is debated, postbiotics include any substance released or produced by the metabolic activity of the microbiota that has beneficial effects on the host, directly or indirectly. Postbiotics, although not containing live bacteria, present benefits to host health through mechanisms similar to those involved in probiotics (Hijová, 2023). Specifically, Kerksick et al (2024) showed in the participants that supplemented with the postbiotic, endurance performance, grip strength, and muscle mass were all increased when compared to placebo. In addition to these changes, lactate and ammonia levels (metabolic indicators of stressful exercise) were reduced in the postbiotic group when compared to placebo. Aerobic exercise increases SCFAs and thereby alters luminal pH in the colon (Endres and kristina., 2023). Therefore, Exercise alters gut microbiota in a variety of ways, including gut transit duration and SCFA generation through AMPK activation. Besides, some of these beneficial benefits of exercise (moderate-intensity) on the host may be mediated by decreased intestinal permeability, which prevents pathogens from penetrating the intestinal barrier and consequently decreases systemic inflamm-ation (Mokarrami et al., 2024). Butyrate, a SCFA, upregulates the expression of tight junction proteins such as occludin and claudin-1 on epithelial cells via the AMP-activated protein kinase (AMPK) pathway and upregulation of genes that encode for these proteins. Furthermore, via histone deacetylase (HDAC) inhibition, butyrate promotes synaptopodin expression, which is involved in tight junction formation and maintenance (Iyer & Corr., 2021). Moreover, butyrate is able to strengthen the mucus layer of the gut epithelium by increasing the expression of Mucin (Fusco et al., 2023). SCFAs can induce anti-inflammatory responses against infection through various pathways. Because both increased gut permeability and muscle wasting are related to “inflammation”, SCFAs, a known potent anti-inflammatory, could act as a central mediator between the gut and muscles (Van Krimpen et al., 2021). They can activate glucoprotein (GPR) receptor as ligands, and modulate a pro-inflammatory factor and immune reaction through nuclear factor kappa-B (NF-κB) and AMPK pathways effectively (Zou Y et al., 2020). Besides, exercise promotes energy, harvesting the gut microbiota ferments complex dietary polysaccharides into SCFAs, SCFAs are the primary energy source for the intestinal epithelial cells, participating in the maintenance of intestinal mucosa integrity, which also improves glucose and lipid metabolism, controls energy expenditure as well as regulates the immune system and inflammatory responses (Zhang, L et al., 2022) which may help enhance muscle renewal and adaptability, improve exercise performance, and delay fatigue symptoms (Huang et al., 2019). Other studies have also shown that high levels of plasma and fecal acetate and propionate (Other SCFAs) are associated with endurance-exercise improvement (Wu, G et al., 2022). Endurance athletes exposed to high-intensity exercise often experience GI symptoms (diarrhea, nausea, abdominal cramps, and bloating symptoms) frequently associated with alterations in intestinal permeability, decreased gut barrier function, Release of LPS and indoxyl sulfate (negative intestinal metabolites) into the blood and systemic responses (endotoxemia and cytokinemia) (Łagowska et al., 2022). On the other side, intense training might overproduce ROS due to increased muscle effort. Excessive ROS production could cause a decrement in muscle force generation during repeated contractions and lead to muscle inflammatory diseases (Santibañez-Gutierrez et al., 2022). In this context, probiotics could be capable of decreasing ROS through an enhance antioxidant activity (Ofori-Attah et al., 2024). The potential mechanisms by which the microbiome modulates muscle mainly relate to cellular metabolism, inflammation, neuromuscular junctions, and mitochondrial function. Probiotics are living microorganisms that can benefit the health of the host when consumed in sufficient amounts (Li et al., 2023). Bifidobacterium and Lactobacillus strains are the most used probiotic bacteria (Zhang et al., 2023). They appears to have a significant role in increasing muscle mass (Toda et al., 2020), muscle strength and endurance (Przewłócka et al., 2020) in aged rats and older individuals. Scientific investigations have demonstrated that older individuals can derive advantages from these positive impacts. Consequently, interventions targeting the gut–muscle axis possess the potential to postpone the onset of muscle deterioration and age-related dysfunction (sarcopenia) (Olteanu et al., 2024).

The main beneficial effects of probiotics relate to decrease gastrointestinal (GI) symptoms, improve energy metabolism, modulating immune response, absorbing nutrients, the down-regulation of oxidative stress (Li et al., 2023) and improves intestinal permeability (Chaiyasut et al., 2022). The probiotic supplement used in our research is enriched with vitamin D and leucine amino acid. In previous research, it has been proven that supplementing soccer players with vitamin D improves their aerobic capacity as well as speed and explosive power (Michalczyk et al., 2020). On the other hand, L-Leucine is known to activate Ribosomal protein S6 kinase beta-1 (S6K1) and 4E binding protein 1 (4E-BP1), the critical downstream targets of Mammalian target of rapamycin (mTOR) signaling for protein synthesis, in skeletal muscle. It is also reported that Increases in plasma leucine and total branched-chain amino acids (BCAAs) concentrations have been associated with improved endurance performance and upper-body power (Kårlund et al., 2019).

The factors affecting microbiota diversity and composition are multifactorial, and the mechanisms of microbiota-mediated alterations of host cell function are crucial for homeostasis and the prevention of pathogenesis. However, the underlying mechanisms of which are largely unknown. Eventually, our hypothesis is that endurance exercise and probiotic supplement enriched with vitamin D and leucine amino acid may affect. Muscular endurance and Vo_{2max} by mediating intestinal microbiota metabolites and reducing inflammation. However, the combination of exercise and supplementation may have different effects on intestinal structural elements and skeletal muscles (Gut- muscles axis) that need to be evaluated.

Materials and Methods

Study design

In the first step, the study plan was approved by the scientific review board of Guilan University. Then the research protocol was approved by the Animal Research Ethics Committee of University of Guilan (IR.GUILAN.REC.1402.085). Therefore, the research has been carried out under the instructions of ARRIVE and PREPARE and the principles of 3Rs. This study investigated the effects of Endurance exercise with or without supplementation for 8 weeks in aged male Wistar rats. A number of 25 rats in two age groups were purchased from Razi Institute of Iran and were exposed to a 12-hour light/dark cycle with a temperature of 20-22° C and a humidity of about 40% with free access to food and water. During the period of aerobic training, the rats were regularly weighed every week. 2 days after the end of the training period, muscle endurance was evaluated and all animals were placed in a co2 chamber for safe and painless anesthesia and then sacrificed for data collection (intestinal jejunum tissue, blood sample and soleus muscle tissue). Became the collected samples were used for analysis by PCR-Real Time techniques.

Participants

25 male Wistar rats (5 rats in each group) in two age groups of 8 to 12 weeks (young) and 18 to 24 months (elderly) were purchased from Razi Institute. The resource equation method was used to calculate the sample size. Finally, based on the men-

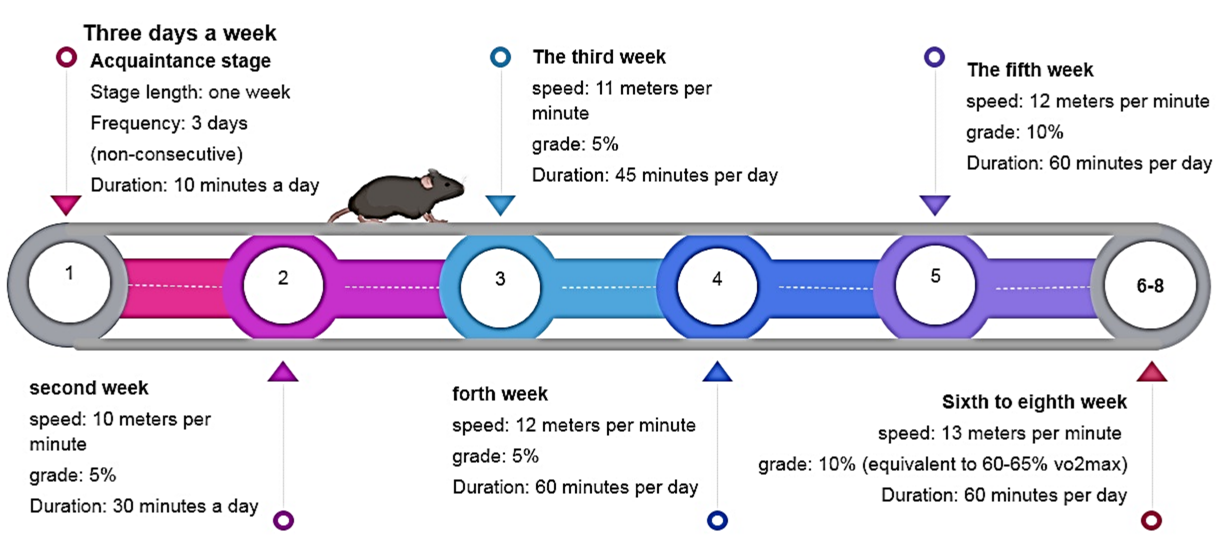

Figure 1. Eight-week protocol of Endurance training, at different grades and speeds. Legend: VO_{2max}: Maximal oxygen consumption.

-tioned equation, 5 animals were considered in each group. At the beginning of the experiment, the rats were randomly divided into five experimental groups, including an old control group (OC) group that continued the normal life cycle, a young control group (YC) group that was as active as the old control group, the old endurance training (OE) group, who were active using a treadmill protocol (three days a week), the old supplement (OS) group, who had no Endurance exercise and the combination endurance training and supplementation (OES) group. The supplement group received oral gavage (1 ml combination of vitamin D, lactobacillus and bifidobacterium probiotics, and leucine amino acid) 1-2 hours after training for 5 times a week.

Animal housing

At the beginning of the experiment, rats were housed in collective home cages with members of the same group (five animals per cage) with water and standard rodent food (carbohydrate 48.8 g, protein 21 g, fat 3 g, calcium 0.8 g, phosphorus 0.4 g, fiber 5 g, Moisture 13%, ash 8g and total energy 306.2 per 100g of food (kcal/100g)) were placed freely (Abdul et al., 2015). The average weight of animals in the OC group (359.1 ± 14.18), YC group (283.6 ± 8.797), OS group (364.3 ± 12.39), and OE group (297 ± 12.44)) and the OES group was (313.6 ± 5.653). Lactobacillus plantarum supplement; 2x1010 (CFU/kg/day) (Lee, Mon-Chien, et al., 2021) and Bifidobacterium bifidum. 5×106 (CFU/rat/day) (Khailova et al., 2009), Vitamin D; IU/Kg was 621.7 (Bass et al., 2020) and leucine amino acid was 0.135 gr/kg/day (Nicastro et al., 2012).

Endurance protocol

During the familiarization phase, the animals were acquainted with the training tool and its method, and then they ran for eight weeks (three sessions per week) according to the training protocol. When the rats were placed at the end of the treadmill to avoid running, a piece of wood was used to push the rats forward and implement the endurance training protocol in order to prevent the electric shock of the device. Also, in order to equate different speeds and grades of the rotating belt with the percentage of maximum oxygen consumption (VO_{2max}), the protocol of Qin et al., (2020) was used.

VO_{2max} measurements

The VO_{2max} measurement was conducted in the same way as had been described by Soares et al., (2019). VO2 was measured by open-circuit indirect calorimetry. For this measurement, the motor-driven treadmill chamber was coupled to a gas analyzer (Panlab, Harvard Apparatus), and the air flow inside the chamber was maintained at 2.0 L•min−1. The gas sensors were calibrated with primary gas standards containing known concentrations of O2 and CO2. The air leaving the chamber was automatically sampled and passed through the gas analyzer to determine the O2 and CO2 content of the air. The exercise started at a speed of 10 m.min−1 with increments of 1 m.min−1 every 3 min until the animals were fatigued. Fatigue was determined as the moment when the rat could not maintain the pace with the treadmill. The ambient temperature was controlled at 24 °C.

Histology and morphology of the small intestine

After euthanasia, Fragments of 2 cm of, jejunum were excised from all rat groups. Tissues were fixed in 4% paraformaldehyde for 48h and samples were then dehydrated with ethanol, cleared with xylene and embedded in paraffin. Semi-serial (5 µm-thick) sections were obtained for tissues and submitted to hematoxylin and eosin (HE) staining. In addition, Goblet cell distribution was observed from 5 randomly selected villus per jejunum using were also stained with periodic acid Schiff (PAS) or Sirius red. For morphometric analysis, the tissues were photographed using a digital camera coupled to An Leica DMI 6000B microscope. Morphometric measurements of the thickness of the layers or tunicas of all tissues, as well as the height of villi, were measured in images from sections stained with HE, using the straight tool of the Image J Software; pixel intensities were converted to micrometers (µm) by the calibration of this software with a slide containing a µm scale (Guimarães et al., 2021).

Chemicals and reagents

A blood sample was taken for IXS and used for assessment with enzyme-linked immunosorbent assay (ELISA) method based on the manufacturer's protocol. The assessment was done using a rat Indoxyl sulfate (IXS) ELISA kit (MyBioSource, Cat. No. MBS721886) and expressed as µg /ml respectively.

Analysis of short-chain fatty acids (SCFAs)

Table 1. Study variables in different groups (M±SD)

	old control (OC)	young control (YC)	supplement (OS)	endurance training (OE)	supplement Plus endurance training (OES)
SCFA	2.03±47.29	4.50±72.53	2.10±57.5	2.38±60.41	3.29±65.59
IXS	10.44±0.4707	0.7621±0.3193	2.095±0.4797	7.356±0.6255	3.788±0.8225
VH	123.0±14.37	259.5±20.35	159.4±18.44	171.2±12.80	201.0±11.97
GC	9.500±2.881	32.67±2.733	14.17±4.875	23.83±3.869	25.83±8.565
Vo_{2max}	29.89±2.095	42.36±2.6	32.55±1.566	58.23±4.408	52.43±3.138

SCFA: Short-chain fatty acid, IXS: Indoxyl Sulfate, VH: Villus height, GC: Goblet cell, VO_{2max}: Maximal oxygen consumption, M: mean, SD: standard deviation.

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The fresh cecal content (about 0.3 g) was accurately weighed, and then homogenized with PBS (9 mL/g) on ice, followed by centrifuging (3000 rpm) for 10 min at 4 °C. SCFA enzyme-linked immunosorbent assay kit was used to determine the contents of total SCFAs in supernatants according to the manufacturer's instructions. The absorbance was measured using a microplate reader at 450 nm, and the contents were calculated.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Analysis was completed using SPSS V27.0 software (SPSS, Chicago, IL). The one-way ANOVA was used to determine the difference among different groups, and multiple comparison Tukey post hoc tests were performed to identify the differences between the means within groups. Graph Pad Prism (Version 9.5.1) was applied to illustrate graphs and some statistical analyses. Data were considered significant at $P < 0.05$. Means and standard deviation of each variable are shown in Table 1.

Results

Short-chain fatty acids (SCFAs)

OE (P=0.002), OS (P=0.013) and OES (P=0.000) interventions caused a significant increase in SCFA metabolite levels compared to the OC group. However, no significant difference was observed between the effect of OS with OE (P=0.761) OS with OES (P=0.050) and OE with OES (P=0.290) groups in increasing the amount of SCFA. Of course, the effect of OES, compared to the separate effect of each of the OE and OS variables, caused a greater improvement in the amount of SCFA (p=0.000), so that no significant difference was observed between the effect of OES and the YC group (p=0.103). (Fig. A).

Indoxyl sulfate (IXS)

Administration of OS (P=0.000), OE (P=0.000) and OES (P=0.000) significantly decreased the amount of Indoxyl sulfate as a negative metabolite compared to the OC group. Of course, the effect of OS more effectively than the effect of OE (P=0.000) and even OES (P=0.001), reduced the amount of indoxyl sulfate compared to the YC group, so that no significant difference was observed between the OS group and the YC group (P=0.096). (Fig. B).

Maximal oxygen uptake (VO_{2max})

There was no significant difference between the OS and the OC (P=0.449). OE significantly increased VO_{2max} compared to the OC (P=0.000) and even the YC (P=0.000). Also, the effect of OE alone was more effective than the synergistic effect of OES (P=0.007), causing a significant increase in VO_{2max}. (Fig. E).

Villus height (VH)

As can be seen, the independent variables OS (P=0.005), OE (P=0.000) and OES (P=0.001) made a significant difference on the VH compared to the OC group. However, no significant difference was observed between the separate effect of each of the OE and OS variables (P=0.708). (Fig. C).

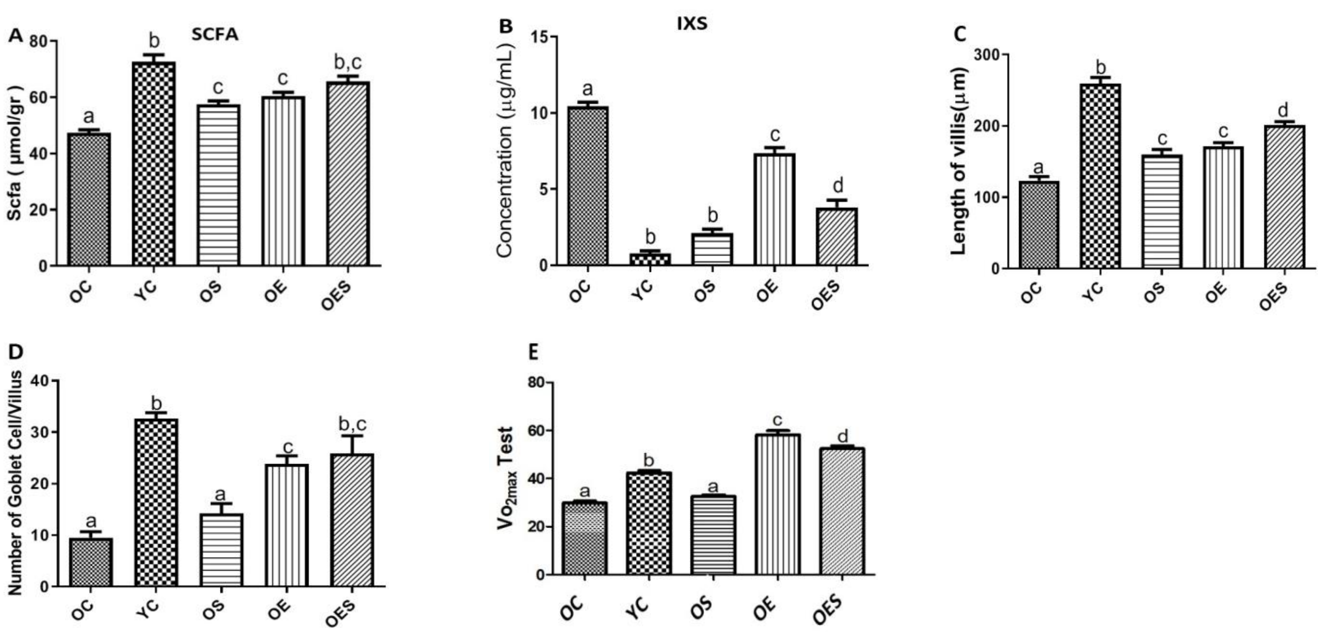

Figure 1. Differences of variables between the intervention and control groups. Graph A shows multiple comparisons between study groups in SCFA (Short-chain fatty acid), B in IXS (Indoxyl Sulfate), C in VH (Villus height), D in GCs (number of goblet cells) and E in VO_{2max} (Maximal oxygen consumption). OC stands for old control, YC for young control, OS for old plus supplement, OE for old plus endurance training, and OES for old plus supplement and endurance training.

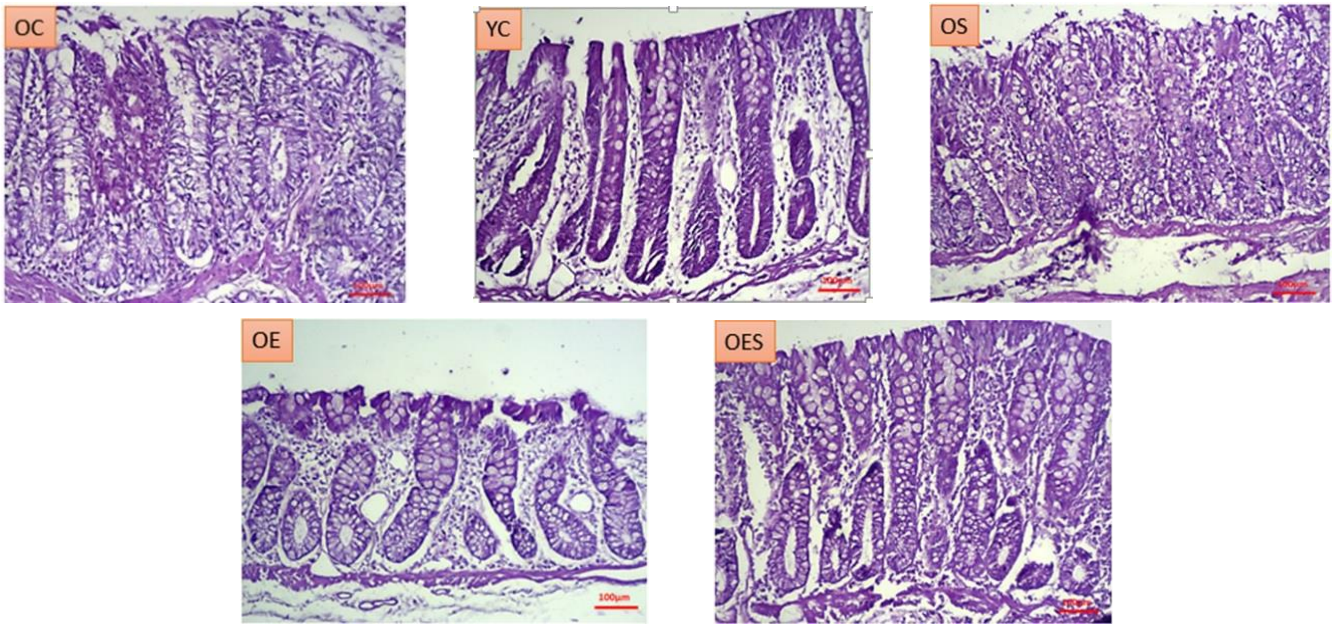

Figure 3. Periodic Acid Schiff (PAS) staining of rat jejunum. Histological sections from Old Control (OC), young control (YC) old plus supplement (OS) old plus endurance training (OE) and old and supplement and endurance training (OES). Scale bar = 100 μm.

Number of goblet cells (GC)

The noteworthy point here was that although the OS group did not cause a significant difference in the increase of GC compared to the OC group (P=0.512), the synergistic effect of OES (P=0.000) brought a higher improvement than the separate effect of OE (P=0.000) compared to OC; If there was no significant difference between OE and OES (P=0.958). Also, this difference between OES and YC was not significant (P=0.166). (Fig. 3).

Discussion

The present study aimed to assess and compare the effects of endurance exercise and probiotic supplement enriched with Vitamin D and leucine amino acid, after an 8-week program on the gut-muscles axis, including intestinal structural indicators (VH and GC), intestinal microbiota metabolites (SCFAs and IXS) and VO_{2max} index were concentrated in young vs. old male Wistar rats.

A more permeable gut barrier may allow for the increased passage of IXS and LPS from the intestinal lumen into the blood, where they negatively affect skeletal muscle (Lustgarten et al., 2019). Of note, probiotic supplementation could help to improve the intestinal barrier, and avoid endotoxemia and the process of inflammation (Han et al., 2023). In our research, the effect of probiotic supplementation (OS) in reducing the amount of IXS was more effective than exercise and even OES, so that there was no significant difference between the OS and the OY.

In skeletal muscle cells, circulating LPS could drive the activation of Toll-Like Receptors 4 and 5 (TLR-4 and TLR-5, respectively), and promote Nuclear Factor Kappa light chain enhancer of acti--vated B cell (NF-κB) pathway activation, reduce insulin sensitivity (reduction in CHO absorption), and enhance protein catabolism (Fang et al., 2022) and inflammatory cytokine production (Nardone et al., 2021). Also, the serum total and free concentrations of indoxyl sulfate (IXS) showed significant negative correlation with peak cardiac power (CPOmax) and VO_{2max} (Chinnappa et al., 2018). These changes in energy metabolism, could be considered a limiting factor for performance in endurance exercise.

Another mechanism of probiotics supplementation to improve aerobic capacity could be through the production of SCFAs, which are an extra energy source during exercise. In addition, some SCFAs could increase peroxisome proliferator activated receptor (PGC-1α) to generate more ATP for the energy required for exercise, and improve exercise performance, increase endurance and hence, the proportion of type I fibers (Santibañez-Gutierrez et al., 2022; Lee et al., 2020). In the current research, the synergistic effect of OES was more effective than the separate effect of each of the independent variables of exercise and supplementation, which caused a significant increase in SCFAs. So that no significant difference was observed between OES and the OY which is consistent with the results of other researches (Huang et al., 2020; Lee et al., 2024).

SCFAs are inhibitors of histone deacetylases (HDACs) which may explain the transcriptional effects of SCFAs on epithelial cells (Schilderink et al., 2013). Inhibition of histone deacetylase may contribute to increased mRNA expression of Peroxisome Proliferator-Activated Receptor-Gamma Coactivator 1-alpha (PGC-1α), and Peroxisome Proliferator-Activated Receptor delta

(PPAR-∂), and Carnitine Palmitoyltransferase-1b (CPT1b) which may play a role in stimulating mitochondrial function and subsequently improving endurance performance (Gao et al., 2009). It is reported that butyrate (is a SCFA) activated the UCP2-AMPK-ACC pathway by downregulating the peroxisome proliferator activated receptor gamma (PPARγ), which can reduce the lipogenesis (Peng et al., 2023). All the physiological mechanisms explained above, influence aerobic metabolism.

The term VO_{2max} refers to the maximum oxygen utilization during incremental exercise, and it usually represented aerobic capacity (Huang et al., 2020). Age-related declines in VO_{2max} are the consequence of decreases in maximal heart rate, stroke volume, maximal arterio-venous O2 difference, and diffusion capacity of the lungs, vascular conductance, skeletal muscle mitochondria density and enzyme activity, skeletal muscle mass and training volume. Age-related muscle loss (sarcopenia) also appears to play an important role in the decline in VO_{2max}; by the age of 50 about 10% of muscle, area is lost and this rate only increases in the decades that follow (Kim et al., 2016). There are few existent studies investigating the relationship between VO_{2max} and probiotic supplementation, yet several other studies have reported that supplementation with probiotics increased the maximum oxygen uptake of the subjects. Li et al (2023) have shown that after 8 weeks of Bifidobacterium lactis BL-99 supplementation combined with training, the VO_{2max} and sports performance of cross-country skiers was significantly upregulated and the correlation analysis showed a significant positive correlation between SCFAs and VO_{2max}. As well as, 66 male and female endurance runners who used a multi-strain probiotic showed a significant increase in maximal oxygen uptake (VO_{2max}) (Smarkusz-Zarzecka et al., 2020). The results also showed that Intake of probiotic yogurt resulted in a significant improved in VO_{2max} (due to the reduction of upper respiratory tract infections) and a non-significant decline in the records of 400-meter crawl swimming (Salarkia et al., 2013). Besides, several studies have also attempted to correlate the composition and metabolic capacity of the microbiota with cardiorespiratory fitness. Correlations between microbial composition and metabolic performance have also been noted, indicating an association between a high Firmicutes/Bacteroidetes ratio and VO_{2max} (Durk et al., 2019). Also, between microbial diversity, butyrate producers, and cardiorespiratory fitness (Bertuccioli et al., 2024). Moreover, in one study, differences in fecal microbiota were reported between athletes and sedentary controls. Athletes showed relative increases in different metabolic pathways, such as amino acid biosynthesis and carbohydrate metabolism, as well as increased production of SCFAs, such as acetate, propionate, and butyrate produced by the microbiota (Domínguez & García., 2022).

In the studies mentioned above, the improvement in VO_{2max} following the use of the supplement was achieved in combination with exercise and not by the use of the supplement alone. In our research, probiotic supplements were prescribed both separately and in combination with exercise, which better explains the effect of independent variables of exercise and probiotics. According to the present study, probiotic supplementation alone could not significantly improve the VO_{2max} index in elderly rats, which is in line with the results of Soares et al., (2019) research. It seems that probiotic supplementation does not cause changes in biomechanical factors (improvement of technique and elastic energy transfer during stretch–shortening cycles) and physiological adaptations (Increase in mitochondrial content) in skeletal muscle in resting conditions and without exercise. On the other hand, articles reported positive results regarding the administration of probiotics (determined), regulation of inflammation and oxidative stress, reduced prevalence of upper respiratory tract infections (URTIs) (Díaz-Jiménez, et al., 2021), energy harvesting, cerebral hormones and changes in neurological function (modulation of dopamine and serotonin neurotransmission) (Soares et al., 2019) which were associated with fatigue regulation for higher endurance performance (Huang et al., 2020). In this sense, considering that in our research, the muscular endurance of rats was not evaluated, there is a possibility that the supplement could improve muscular endurance through the mentioned mechanisms. so that In one study, Lactobacillus plantarum PS128 supplementation was associated with an improvement on endurance running performance through microbiota modulation and related metabolites, but not in maximal oxygen uptake (Huang et al., 2020). In explaining this, many studies have indicated that, in addition to exercise, supplementation of probiotics can increase intestinal SCFA levels. These can regulate the energy balance of the host and increase the use of nutrients (Huang et al., 2019). So that, the increase in Veilonella, by modulating the activity of the Cori Cycle, could provide an alternative and additional way to metabolize lactate, allowing further production of SCFAs capable of improving VO_{2max} with undoubted advantages on presentation (Bertuccioli et al., 2024). Modulation of microbiota might also enhance endurance in athletes and improvement of VO_{2max} might be due to the prevention of upper respiratory tract infection. Also, reduced anxiety and stress conditions due to probiotic supplements could improve athletes' brain function, resulting in high performance in competitions (Salleh et al., 2021). Of note, Probiotic supplementation has been shown to reduce oxidative stress by reducing ROS formation. In another related reaction, the production of catecholamines during exercise has been reported as another indicator of physical stress. Gut microbiota enriched with probiotics can increase catecholamine activity and thus minimize exercise-induced fatigue (Salleh et al., 2021). On the other hand, there is evidence that aerobic exercise can increase VO_{2max} by improving the gut barrier function, reducing in-

-flammation, and regulating energy metabolism. Physical activity has many potential mechanisms for lowering inflammation including changes to adipose, release of anti-inflammatory myokines, attenuating postprandial lipemic and glycemic responses, improving gut barrier function and microbial makeup (Miles et al., 2019). The gut barrier is indirectly strengthened by endurance exercise capacity. Lactobacillus and Bifidobacterium have been demonstrated repeatedly to improve intestinal barrier integrity through a greater capacity for butyrate synthesis (a type of SCFA). As a result, there is less migration of endotoxins and germs from the intestinal lumen (LPS and IXS) into the systemic circulation, which lowers inflammation and strengthens immunity (Ghaffar et al., 2024). Of note, the increase in SCFAs causes the intestinal pH value to drop slightly, creating an ideal climate for the proliferation of bifidobacteria (Schulze & Busse., 2024). Other studies have also shown that high levels of plasma and fecal acetate and propionate (Other SCFAs) are associated with endurance-exercise improvement (Wu, G et al., 2022). In addition, exercise promotes energy, harvesting the gut microbiota ferments complex dietary polysaccharides into SCFAs, which can be used as an energy source for liver and muscle cells and improves endurance performance by maintaining blood glucose for a long period of time (Huang et al., 2019). Therefore, exercise training probably to be more effective than nutritional strategies for the improvement in the VO_{2max} index, mainly due to mitochondrial biogenesis (Santibañez-Gutierrez et al., 2022). Moreover, This variation is not surprising considering that cardiorespiratory fitness and the VO_{2max} index depend on a range of factors, including heredity, age, gender, and body composition, as well as various physiological aspects, such as blood oxygen capacity, circulating blood volume, cardiac output, pulmonary blood flow, lung diffusion capacity, and lung ventilation (Brzeziański et al., 2022). Therefore, the synergistic effect of endurance training and probiotic supplements can possibly increase VO_{2max} through pathways such as improving the intestinal barrier, producing beneficial intestinal metabolites, reducing inflammation, and regulating energy metabolism.

In our research, the indices related to intestinal structure (VH and GC) were improved by endurance training (Figure 3); it seems that the effect of endurance activity on intestinal villus height is indirectly through SCFAs. For instance, Zhou et al (2020) reported that Intravenous, gastric, or distal ileal infusion of SCFAs markedly increased the villus height in the small intestine of conventional pigs. They concluded that SCFAs might be physiological stimuli that benefit intestinal growth by modulating intestinal morphology and proglucagon-derived peptide secretion. Among other researches that are inconsistent with the present research, the results of the research of Ducray et al., (2020). Did not observe any change in the GC after physical activity or high-intensity training. They stated that heat stress cou-sed by endurance training is one of the factors that decrease the number of Paneth cells. Probably, in our research, the intensity of endurance training was appropriate, which in addition to improving the intestinal structure (VH and GCs), brought about positive changes in intestinal metabolites (decrease in IXS and increase in SCFAs). They also reported that GCs were significantly increased in mice that received Bacillus subtilis BSB3 (BSB3) supplementation before running on a treadmill. On the other hand, the results of the present research are in line with the results of the research of Wang et al., (2020), who showed that Lactobacillus johnsonii BS15 probiotic improves the growth of jejunum and ileum by increasing the VH. VH and Crypt Depth (CD) are indicators of intestinal health and morphology. These indicators are closely related to the health of intestines. Therefore, the effect of probiotics is not limited to changes in gene expression, but can also be detected at the structural level, where they improve villus height and crypt depth, and hence affect absorptive and secretory processes in the small intestinal epithelium (Yosi t al., 2023). The epithelial lining of the intestinal tract is continuously renewed; longer villi are linked with activated cell mitosis (Shah et al., 2019). Various studies have reported increased villus height and improved nutrient absorption due to the consumption of probiotics (Yosi et al., 2023; Threadgold et al., 2021; Qaisrani et al., 2022; Hussein et al., 2020). In our research, the synergistic effect of supplementation with exercise (OES) was more effective than the separate effect of exercise (OE) and supplementation (OS) alone in improving the GC and VH. Also, despite the fact that supplement consumption had a lesser effect in improving the indicators related to the intestinal structure, the role of the OS in reducing the level of negative intestinal metabolites (IXS) was more prominent than OE and even the synergistic effect of OES.

Moreover, through the genomic pathway, vitamin D stimulates the proliferation and differentiation of muscle cells by modulating gene transcription in myoblasts, resulting in an increase in the synthesis of specific muscle proteins, such as myosin and calcium-binding protein (Iolascon et al. 2021). The mechanism by which vitamin D may improve aerobic performance remains unclear. It is possible that the CYP (cytochrome P450) enzymes involved in the activation of vitamin D to the biologically active 1, 25 (OH) 2D contain heme proteins that may affect the oxygen binding to hemoglobin (Brzeziański et al., 2022). Nevertheless, Some studies have shown that daily consumption of vitamin D supplement for 12 weeks had no effect on blood hemoglobin level and VO_{2max} index, and these findings are consistent with unchanged VO_{2max} (Gregory et al., 2013; Savolainen et al., 2022).

It is generally accepted that leucine supplementation can mitigate endurance exercise-induced skeletal muscle damage and fatigue, accentuate muscle protein synthesis and improve recovery and muscle performance (Dos Santos et al., 2016). A study showed that the supplementation of leucine in diet-induced obese mice significantly improved mitochondrial function in skeletal muscles through the activation of mTOR1 (Martínez-Arnau et al., 2020).However, the effect of leucine alone, the amino acid shown to stimulate muscle synthesis, has seldom been evaluated (Martínez-Arnau et al., 2020). Besides, in the absence of an adequate quantity of the other essential amino acids (EAAs), leucine alone may not be sufficient to support maximal rates of MPS (Waskiw-Ford et al., 2020).

As a conclusion, the present research showed that the synergistic effect of supplementation and exercise together leads to further improvement, especially in intestinal structure indices (increasing VH and GC) as well as SCFA production. Although the role of supplementation in reducing negative intestinal metabolites (IXS) was greater than exercise and even OES, the probiotic supplement alone and without combining with exercise could not cause a significant increase in VO_{2max} index compared to the control group. In addition, one of the limitations of our research was that we could not directly investigate the effect of gut microbiota on skeletal muscle, and this cross talk between gut and muscle was concluded by the changes of intestinal metabolites. Although a faecal/microbiota transplantation experiment may help address this limitation. Another limitation of the research was not evaluating the muscular endurance of the rats after applying the independent variables of exercise and supplementation. Maybe a test like the wire hanging test could help to solve this limitation. Finally, it should be noted that due to the small sample size, the results should be interpreted with caution.

Conclusion

A sedentary lifestyle and poor diet are important risk factors for sarcopenia. An integrated approach involving endurance training programs designed and adequate probiotic supplementation Probiotic enriched with vitamin D and amino acid leucine could through the gut-muscle axis (Improving intestinal barrier function and intestinal villus structure, increasing production of beneficial metabolites, reducing inflammation and increasing muscle function and aerobic capacity) be useful in counteracting sarcopenia.

Studies have shown that a combination of exercise and probiotics (OES) has a better effect on athletic performance than a single approach, such as exercise or probiotic supplements alone. especially in the current study that the probiotic supplement was enriched with the amino acid leucine and vitamin D And in particular, the significant effect of OES on intestinal structure (VH and GC) and SCFAs increase was more prominent than the separate effect of each exercise and supplement group. It is possible that probiotic supplementation can reduce the negative effects of intense exercise on the intestinal structure and the leakage of negative metabolites (IXS) into the blood. However, probiotic supplement alone could not increase Vo2max in resting conditions in elderly rats, and the role of exercise was evident in this context. On the other hand, the role of supplementation was more effective in reducing negative intestinal metabolites (IXS) than exercise and even OES. Although probiotics have an excellent overall safety record, they should be used with caution in certain patient groups. Finally, the properties of different probiotic species vary and can be strain-specific. Therefore, the effects of one probiotic strain should not be generalized to others without confirmation in separate studies. However, further research is needed to elucidate the specific mechanisms by which the gut microbiome and its metabolites influence muscle function and aerobic capacity.

What is already known on this subject?

Aging can cause inflammation by affecting gut function and gut microbiota composition, thereby leading to muscle dysfunction. Gut microbiota metabolites are key mediators influencing the gut-muscle axis. Probiotics, vitamin D, leucine amino acid and Endurance exercise, through changes in the intestinal microbiota, improving the intestinal epithelial barrier, inhibiting oxidative stress and reducing ROS production, lead to increasing the production of positive intestinal metabolites (SCFAs), reducing negative metabolites (IXS and LPS) and improve chronic and systemic inflammation, which will ultimately improve mitochondrial function and endurance exercise.

What this study adds?

The effect of OS in negative intestinal metabolites (IXS) was more effective than OE and OES; So that there was no significant difference with the OY control group.

The synergistic effect of OES in increasing positive intestinal metabolites (SCFAs), improving intestinal structure (GC and VH) was more effective than the separate effect of OS and OE; So that there was no significant difference with the OY control group.

The effect of exercise on increasing VO_{2max} index was more effective than OS and even OES; So that there was no significant difference with the OY control group.

Administering the supplement alone and at rest (without exercise) could not cause a significant increase in VO_{2max}.

Organ Cross-Talk Tips:

- Gut microbiota metabolites and "inflammation" are Key mediators communicating between the gut-muscle axis. It seems that supplements and exercise have an effect on the Cross-Talk between gut and Skeletal muscles through improving the intestinal structure and consequently reducing the leakage of negative metabolites (IXS and LPS) from the intestine into the blood and increasing positive intestinal metabolites (SCFAs).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Animals had free access to standard food and water. All stages of keeping and slaughtering rats were carried out according to the rules of the Animal Ethics Committee of Guilan University, rasht, Guilan, Iran (ethical code: IR.GUILAN.REC.1402.085).

Informed consent Not applicable

Author contributions

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

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