Gdański Uniwersytet Medyczny

Wydział Nauk o Zdrowiu z Instytutem Medycyny Morskiej i Tropikalnej

Rozprawa doktorska na stopień doktora w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauk o zdrowiu

Wpływ suplementacji probiotykami i witaminą D3 na wydolność fizyczną i funkcje błony śluzowej jelita sportowców

ang. Influence of the gut microbiome on athletic sport performance and intestinal epithelial cells permeability

mgr Katarzyna Przewłócka

Zakład Bioenergetyki i Fizjologii

Wysiłku Fizycznego

Promotor: dr hab. Jan J. Kaczor

Promotor pomocniczy: dr hab. Marcin Folwarski

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SPIS TREŚCI

1. WYKAZ PRAC WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

W skład rozprawy doktorskiej wchodzą następujące publikacje:

1. Przewłócka K., Folwarski M., Kaźmierczak-Siedlecka K., Skonieczna-Żydecka K., Kaczor J.J. *Gut-Muscle Axis Exists and May Affect Skeletal Muscle Adaptation to Training. Nutrients.* 2020;12(5):1451. doi: 10.3390/nu12051451. PMID: 32443396; PMCID: PMC7285193.

2. Przewłócka K., Kujach S., Sawicki P., Berezka P., Bytowska Z.K., Folwarski M., Kowalski K., Kaczor J.J. *Effects of Probiotics and Vitamin D_3 Supplementation on Sports Performance Markers in Male Mixed Martial Arts Athletes: A Randomized Trial. Sports Med Open*. 2023; 9(1):31. doi: 10.1186/s40798-023-00576-6. PMID: 37193828; PMCID: PMC10188824.

3. Przewłócka K., Folwarski M., Kaczmarczyk M., Skonieczna-Żydecka K., Palma J., Bytowska Z.K., Kujach S., and Kaczor J.J. *Combined probiotics with vitamin D_3 supplementation improved aerobic performance and gut microbiome composition in Mixed Martial Arts athletes. Frontiers in Nutrition*. 2023; doi: 10.3389/fnut.2023.1256226.

Tabela 1. Wskaźniki biometryczne publikacji

Rok publikacji	Czasopismo	Tytuł pracy	Typ badania	IF	MNisW
2020	Nutrients	Gut-Muscle Axis Exists and May Affect Skeletal Muscle Adaptation to Training	Przegląd (ang. *review)*	5,9	140
2023	Sports Medicine - Open	Effects of Probiotics and Vitamin D_3 Supplementation on Sports Performance Markers in Male Mixed Martial Arts Athletes: A Randomized Trial	Praca oryginalna (ang. *Original research*)	4,57	20
2023	Frontiers in Nutrition	Combined probiotics with vitamin D_3 supplementation improved aerobic performance and gut	Praca oryginalna (ang. *Original research*)	5.0	20

	microbiome composition in Mixed Martial Arts athletes		
Razem		15,47	180

2. WYKAZ STOSOWANYCH SKRÓTÓW

mTOR - mammalian target of rapamycin

NF-kB Nuclear Factor kappa B

FOXO - Forkhead box O

RED-S - relative energy deficiency in sport

SCFA - short chain fatty acid

CLA - conjugated linoleic acid

VO2max - maximal oxygen uptake

TNF-a - tumor necrosis factor a

SOD - *Superoxide dismutase*

KAT – catalase

GPx - Glutathione peroxidase

MAPK - mitogen-activated protein-kinase

IGF-1 - insulin-like growth factor 1

GALT - Gut-associated lymphoid tissue

PAMPs - pathogen associated molecular

TLR - Toll-like receptors

GABA - Gamma-aminobutyric acid

TREG - Regulatory T cells

IL-10 – Interleukin 1

TGF- β - *transforming growth factor β*

Iga-A - Immunoglobulin A

LPS – Lipopolysaccharides

MuRF1 - Muscle RING-finger protein-1

IKKβ - IκB kinase

IGFBP - IGF-binding protein

AMPK – AMP-activated protein kinase

PGC-1a – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

IL-1b – interleukin-1b

IL8 – interleukin-8

IL-6 – interleukin-6

DM2 – bone marrow differentiation factor 2

FABP2 - Fatty acid-binding protein 2

IL-1 – interleukin 1

IFN-γ - Interferon gamma

IDO - Tryptophan-2,3-Dioxygenase

MPO – *Myeloperoxidase*

AKT - Protein Kinase B

GLP-1 - Glucagon-like peptide-1

FXR - farnesoid X receptor

BA – bile acids

H2S - *Hydrogen sulfide*

IVAS - isovanillic acid 3-O-sulfate

PI3K- phosphatidylinositol 3-kinase

B2- *Riboflavin*

B12 – cobalamin

MMA – mixed martial arts

PCr – phosphocreatine

ATP - adenosine triphosphate

LA – lactate

PDH - pyruvate dehydrogenase

FADH2 – reduced form of *flavin adenine dinucleotide*

NADH - reduced form of nicotinamide adenine dinucleotide

Acetylo- CoA - acetylo coenzyme-A

FAO - The Food and Agriculture Organization of the United Nations

WHO - the World Health Organization

VDR - vitamin D receptor

25(OH)D3 - 25-hydroksy cholecalciferol

PRO+Vit D – probiotic group

VitD – vitamin D3 group

PRE – before intervention

POST – 4 weeks after intervention

SPIRIT - Standard Protocol Items: Recommendations for Interventional Trials

CFUs - colony forming units

BIA - bioelectrical impedance analysis

LBM - lean body mass

TFM - total fat mass

BM - body mass

WAnT - Wingate anaerobic test based

SIE - supramaximal sprints based on WAnT

TB – time point before exercise

T3 - 2-3 minutes after exercise

T15 - 15 minutes after exercise

T30 - 30 minutes after exercise

T60 – 60 minutes after exercise

LSD - least significant difference test

SD - standard deviation

FFM – fat free mass

W_{tot} – total work

MP – mean power

P_{max} – maximal power

P_{max} time – time to obtain maximal power

FI – fatigue index

BT0 – before supplementation, before exercise

BT30 - before supplementation, 30 minutes after exercise

AT0 – after supplementation, before exercise

AT30 – after supplementation, 30 minutes after exercise

CK – creatine kinase

LDH - lactate dehydrogenase

Słowa kluczowe w języku polskim:

Mikrobiota jelitowa, sportowcy, oś jelitowo-mięśniowa, MMA, trening, probiotyki, wydolność tlenowa, wydolność beztlenowa, utylizacja mleczanu, przepuszczalność jelitowa, stan zapalny

Słowa kluczowe w języku angielskim:

gut microbiota; athletes; gut-muscle axis, MMA, exercise, probiotics, anaerobic capacity, LA utilization, aerobic capacity, intestinal permeability, inflammation

3. STRESZCZENIE W JĘZYKU POLSKIM

Mikrobiom jelitowy stanowi złożony ekosystem zasiedlany przez zróżnicowane mikroorganizmy. Wydaje się, że utrzymanie optymalnego składu mirobiomu jelitowego jest niezbędne nie tylko dla zdrowia gospodarza, ale również może korzystnie wpływać na zdolności wysiłkowe sportowców. Wykazano, że niektóre drobnoustroje przyczyniają się do uszczelnienia bariery jelitowej, redukcji stanu zapalnego, usprawnienia tempa utylizacji mleczanu, a także mogą wpływać na metabolizm glukozy i funkcje mitochondriów, przez co mogą pośrednio wpływać na funkcjonowanie mięśni szkieletowych. Z kolei dysbioza jelitowa sprzyja obniżeniu fizjologicznej adaptacji do treningu poprzez nasilenie stanu zapalnego i wolnorodnikowego uszkodzenia makrocząsteczek, co w konsekwencji przyczynia się do gorszej regeneracji oraz atrofii mięśni szkieletowych. Wzajemna interakcja pomiędzy mięśniami szkieletowymi a mikrobiomem jelitowym nazywana jest osią jelitowo-mięśniową. W związku z tym, wydaje się że strategie celowane w mikrobiotę jelitową mogą potencjalnie korzystnie wpływać na proces adaptacji do treningu oraz na wyniki sportowe. Dodatkowo korzystne działanie specyficznych szczepów bakterii może być nasilone przez obecność witaminy D. Celem niniejszej rozprawy jest określenie, w jaki sposób mikrobiom jelitowy może wpływać na zdolności wysiłkowe sportowców oraz czy suplementacja probiotykami i witaminą D3 wpływa na skład i różnorodność mikrobiomu jelitowego sportowców, parametry przepuszczalności błony śluzowej jelita,

parametry wydolnościowe sportowców, tempo utylizacji mleczanu, parametry uszkodzenia mięśni szkieletowych oraz markery stanu zapalnego.

Badanie zaprojektowane zostało jako podwójnie zaślepiona próba kontrolowana placebo. Badanie uzyskało zgodę Niezależnej Komisji Bioetycznej przy Gdańskim Uniwersytecie Medycznym (No. NKNNB/643/2019-2020) oraz zostało zarejestrowane w Clinical Trials pod numerem NCT04759729. Do badania włączono 25 zawodników MMA płci męskiej. Zawodnicy spełniający kryteria włączenia zostali losowo przydzieleni do 2 grup, z których jedna otrzymała mieszankę probiotyczną i witaminę D3 (PRO+VitD), natomiast druga placebo i witaminę D3 (Vit D). Dodatkowo, celem wyrównania niedoborów, wszyscy zawodnicy otrzymali witaminę D3 Juvit D3 w dawce 3500 IU dziennie. Przed rozpoczęciem suplementacji przeprowadzony został test mocy beztlenowej oparty o protokół Wingate oraz test aerobowy przy użyciu ergometru rowerowego. Pobrano krew żylną, którą przeanalizowano pod kątem stężenia 25(OH)D3, aktywności kinazy kreatynowej oraz stężenia cytokin prozapalnych (IL-6, TNF- a) i przeciwzapalnych (Il-2, IL-15). Krew kapilarna pobrana została w 5 punktach czasowych (przed wykonaniem testu, 3,15, 30 i 60 minut po teście) celem oznaczenia mleczanu. Pobrana została również próbka kału, która następnie została poddana ocenie metagenomicznej przy użyciu metody sekwencjonowania nowej generacji. Zebrane próbki kału przeanalizowano również pod kątem obecności parametrów przepuszczalności jelit, takich jak kalprotektyna i zonulina oraz SCFA. Zawodnikom wykonano analizę składu ciała oraz zebrany został obszerny wywiad dotyczący żywienia, stosowanych suplementów oraz dotyczący planu treningowego. Zawodnicy poproszeni zostali o niewprowadzanie żadnych zmian w dotychczasowym planie żywieniowo-treningowym i zobligowani zostali do kontynuowania typowego treningu MMA. Po 4-tygodniowym okresie suplementacji cała procedura została powtórzona. Spośród 25 zawodników, 23 ukończyło protokół. Uzyskane wyniki zostały poddane analizie statystycznej z wykorzystaniem dwuczynnikowego testu ANOVA. Wyniki istotne statystycznie uznane były, jeżeli $p<0.05$.

W badaniu zaobserwowano, że 4-tygodniowa suplementacja probiotykami i witaminą D3 korzystnie wpłynęła na skład mikrobioty jelitowej i zmiany te korelowały z poprawą wydolności w trakcie testu aerobowego i anaerobowego. Wykazano znaczące różnice w czasie wysiłku do wyczerpania przed

suplementacją w porównaniu do wyników po suplementacji w grupie PRO+VITD. W grupie VIT D nie obserwowano statystycznie istotnych różnic. W teście mocy beztlenowej zaobserwowano poprawę średniej mocy w grupie PRO+VitD po okresie suplementacji w porównaniu z wynikiem przed suplementacją, podczas gdy w grupie Vit D nie widać było istotnych statystycznie zmian. Również zaobserwowano poprawę całkowitej pracy wykonanej podczas testu w grupie otrzymującej probiotyk, a efektu nie odnotowano w grupie Vit D. W badaniu wykazano również szybsze tempo utylizacji LA w grupie PRO + VitD w porównaniu z grupą Vit D. Pomimo suplementacji witaminą D3 w dawce 3500 IU na dobę, stężenie 25(OH)D3 w surowicy było nadal poniżej minimalnego poziomu (30 ng/ml), co sugeruje, że sportowcy uprawiający dyscypliny siłowo-szybkościowo-wytrzymałościowe powinni suplementować wyższe dawki. Wykazano, że poziom 25(OH)D3 był istotnie statystycznie wyższy 30 minut po wykonaniu sprintów, w porównaniu z wartościami przed ćwiczeniami. Jeszcze większy wzrost po wykonanym teście obserwowany był po 4-tygodniowym okresie suplementacji. Wyniki te sugerują, że mięśnie szkieletowe mogą zarówno magazynować, jak i uwalniać witaminę D3, natomiast trening fizyczny może w pewnym stopniu warunkować uwalnianie metabolitów witaminy D.

Wyniki uzyskane w niniejszym badaniu wskazują, że suplementacja probiotykami i witaminą D3 korzystnie wpływa na wyniki sportowe zawodników sportów walki poprzez poprawę wydolności zarówno tlenowej, jak i beztlenowej oraz usprawnienie tempa utylizacji mleczanu. Dodatkowo efekt jest widoczny nie poprzez wpływ na VO2 max, a poprzez korzystną modulację profilu mikrobiomu jelitowego, co potencjalnie prowadzi do ulepszonego wykorzystania LA.

4. STRESZCZENIE W JĘZYKU ANGIELSKIM

Title: The influence of probioticfs and vitamin D3 supplementation on sport performance and epithelial cells permeability in athletes

The human gut microbiome constitutes an intricate ecosystem hosting a variety of microorganisms. It appears, that preserving the ideal balance within this intestinal microbiome is essential, not just for the well-being of the host, but also for potentially enhancing the physical performance of athletes.

Certain microbes have demonstrated their ability to contribute to enhance the intestinal barrier, reducing inflammation, improving lactate utilization and potentially influencing glucose metabolism and mitochondrial function. Consequently, they can indirectly impact the skeletal muscles function. Conversely intestinal dysbiosis can disrupt physiological adaptation to training by increasing inflammation and causing oxidative stress via free radicals, negatively affecting recovery process and muscle atrophy. This interaction between skeletal muscles and the gut microbiome is referred to as the gut-muscle axis. Consequently, it appears that strategies aimed at regulating the intestinal microbiota may hold promise in positively influencing the adaptation to training and overall sports performance. Additionally, the advantageous effects of specific bacterial strains may be enhanced in the presence of vitamin D.

The study was designed as a double-blind placebo-controlled trial. The study was approved by the Independent Bioethics Commission at the Medical University of Gdańsk (No. NKNNB/643/2019-2020) and was registered in Clinical Trials under the number NCT04759729. The study included 25 male Mixed Martial Arts (MMA) athletes. Competitors, who met the inclusion criteria, were randomly assigned to 2 groups, one of which received probiotic mixture and vitamin D3 (PRO+VitD) and the other received placebo and vitamin D3 (Vit D). Additionally, to compensate for deficiencies, all athletes received vitamin D3 Juvit D3 at a dosage of 3500 IU per day. Before the supplementation period anaerobic power test based on the Wingate protocol and an aerobic test using a bicycle ergometer were carried out. Venous blood was collected and analyzed for 25(OH)D3, creatine kinase activity, pro-inflammatory (IL-6, TNF-a), and anti-inflammatory (Il-2, IL-15) cytokines. Capillary blood was collected at 5-time points (before the test, 3,15, 30, and 60 minutes after the test) to determine lactate. A faeces sample was also collected and subsequently subjected to metagenomic evaluation using a new generation sequencing method. The collected faeces samples were also analyzed for intestinal permeability parameters such as calprotectin and zonulin and SCFA. Athletes were analyzed body composition and an extensive interview was collected on nutrition, used supplements and training plan. Athletes were asked not to make any changes to the current nutrition and training plan and were required to continue their typical MMA training. After a 4-week supplementation period the whole procedure was

repeated. Of the 25 competitors, 23 completed the protocol. The results were statistically analyzed using a two-factor ANOVA test. Statistically significant results were considered if $p<0.05$.

Our results show that 4-week supplementation of probiotics and vitamin D3 positively affected the composition of the gut microbiota and these changes correlated with improved performance during aerobic and anaerobic testing. Significant differences were found in exercise time to exhaustion before supplementation compared to post-supplementation results ($p = 0.023$) in the PRO+VITD group. No statistically significant differences were observed in the VIT D group ($p = 0.685$). It was also no effect on VO2 max value in either group. In the anaerobic power test an improvement in mean power was observed in the PRO+VitD group after the supplementation period compared to the pre-supplementation value (7.73 ± 0.47 vs. 8.02 ± 0.45; $p<0.04$), while no statistically significant changes were observed in the VitD group (7.98 ± 0.65 vs. 7.95 ± 0.48; $p<0.99$). Also an improvement in the total work done during the test was observed in the group receiving the probiotic (232.00 ± 14.06 vs. 240.72 ± 13.38; $p<0.04$), an effect was not observed in the VitD group (239.34 ± 19.46 vs. 238.37 ± 14.35; $p<0.99$). The study also observed a faster rate of LA utilization in the PRO + VitD group (73.6 ±6.9%) compared to the Vit D group ($65.1 \pm 9.9\%$). Despite vitamin D3 supplementation at a dose of 3,500 IU per day, serum 25(OH)D3 concentrations were still below the minimum level (30 ng/ml), suggesting that athletes practicing engaged in strength-velocity-endurance sports should supplement with higher doses. However, it was observed that 25(OH)D3 concentrations were statistically significantly higher 30 minutes after the sprints compared to pre-exercise values ($p<0.05$). An even greater increase after the test was performed was observed after a 4-week supplementation period ($p<0.001$). These results suggest, that skeletal muscles can both store and release vitamin D3, while physical training can condition the release of vitamin D metabolites to some extent.

The results obtained in this study indicate, that probiotics can favorably influence the athletic performance of combat sports athletes by improving both aerobic and anaerobic capacity and improving the rate of lactate utilization. In addition the effect is seen not by affecting VO2 max, but by favorably modulating the gut microbiome profile, potentially leading to improved LA utilization.

5. WPROWADZENIE

5.1. Charakterystyka mikrobiomu jelitowego

Mikrobiom jelitowy stanowi złożony ekosystem, zasiedlany przez zróżnicowaną populację mikroorganizmów. Termin ten odnosi się również do materiału genetycznego wszystkich mikroorganizmów zasiedlających jelita, w tym genów, genomu oraz złożonych interakcji zachodzących pomiędzy nimi a organizmem gospodarza. Mianem mikrobioty jelitowej określa się z kolei społeczność mikroorganizmów zasiedlających jelita. Mimo iż terminy ,,mikrobiom" i ,,mikrobiota" często stosowane są wymiennie, znajomość subtelnych różnic pomiędzy nimi jest niezbędna w celu zrozumienia złożonych interakcji zachodzących w obrębie przewodu pokarmowego, a także wpływu mikroorganizmów na zdrowie gospodarza.

Szacuje się, że przewód pokarmowy człowieka zasiedlany jest przez około 10^{14} mikroorganizmów, co 10-krotnie przewyższa liczbę ludzkich komórek (1,2). Zgodnie z przeprowadzonym w pierwszej dekadzie XXI wieku projektem, dotyczącym metagenomiki ludzkiego przewodu pokarmowego (MetaHIT), w ludzkim jelicie egzystuje około 3,3 miliona genów drobnoustrojów, co 150-krotnie przewyższa liczbę wszystkich ludzkich genów (3). Najliczniejszą populację stanowią bakterie. Szacuje się, że w przewodzie pokarmowym zdrowego człowieka znajduje się ponad 1000 gatunków bakterii, należących głównie do gromad: *Firmicutes*, *Bacterioidetes*, *Actinobacteria* i *Proteobacteria* (2).

Wraz z jelitowym układem immunologicznym oraz błoną śluzową jelita mikrobiota jelitowa stanowi naturalny system obronny układu pokarmowego. Dzięki wykształconym przez mikroorganizmy mechanizmom możliwa jest dwukierunkowa komunikacja pomiędzy bakteriami jelitowymi a komórkami gospodarza, co tworzący interaktywny ekosystem. Ta wzajemna interakcja sprawia, że mikrobiota jelitowa może przyczyniać się do regulacji wielu procesów biologicznych i tym samym warunkować zdrowie gospodarza (4). W kontekście utrzymania homeostazy organizmu bioróżnorodność oraz bogactwo gatunkowe mikrobioty jelitowej odgrywają kluczowe znaczenie, natomiast dysbioza może przyczyniać się do rozwoju wielu chorób. Dane naukowe wskazują na istotny

wpływ bakterii jelitowych na stan odżywienia gospodarza, funkcje metaboliczne, dojrzewanie układu immunologicznego i komórek nabłonka jelit. Stanowią również obronę przed patogenami oraz mogą wpływać na funkcje mózgu oraz mięśni szkieletowych (5,6).

Mikrobiota jelitowa u każdego człowieka jest inna, a ponadto w trakcie życia może podlegać dynamicznym zmianom. Do czynników warunkujących jej skład zalicza się: sposób odżywiania, poziom aktywności fizycznej, genetykę, wiek, płeć, miejsce zamieszkania oraz stosowane leki. W kontekście modyfikacji różnorodności i liczebności poszczególnych szczepów bakteryjnych największe znaczenie przypisuje się diecie oraz aktywności fizycznej gospodarza (7). W świetle aktualnej wiedzy wydaje się, że mikrobiota jelitowa może być pośrednim czynnikiem wpływającym na proces adaptacji mięśni do treningu.

5.2. Wpływ aktywności fizycznej na mikrobiom jelitowy

Umiarkowana aktywność fizyczna, podobnie jak prawidłowa dieta, pozytywnie wpływa na skład mikrobioty jelitowej człowieka, przyczyniając się do większej bioróżnorodności oraz bogactwa gatunkowego. Prowadzi także do zwiększenia się liczby genów bakterii, które zaangażowane są w metabolizm węglowodanów i białek, a także powoduje wzmożoną produkcję krótkołańcuchowych kwasów tłuszczowych (SCFA) (8,9). Nadmierne obciążenia treningowe, na które narażona jest większość sportowców trenujących na poziomie wyczynowym, może negatywnie wpływać na skład mikrobioty jelitowej, przyczyniając się do dysbiozy oraz zaburzenia integralności nabłonka jelitowego.

Trening fizyczny związany jest ze wzrostem zapotrzebowania pracujących mięśni szkieletowych na tlen i składniki odżywcze. Fizjologiczną odpowiedzią na to jest zmiana dystrybucji przepływu krwi w organizmie – zwiększa się jej przepływ m.in. przez mięśnie szkieletowe i jednocześnie zmniejsza się przepływ trzewny. Długotrwałe zmniejszenie przepływu krwi przez jelita prowadzi do ich czasowego niedokrwienia, a w efekcie dysfunkcji błony śluzowej jelita i wzmożonej przepuszczalności bariery jelitowej, określanej w literaturze jako „przeciekające jelito" (10,11). W takich warunkach obserwuje się również negatywne zmiany w profilu drobnoustrojów jelitowych, przejawiające się wzrostem potencjalnie szkodliwych bakterii i jednoczesną redukcją taksonów produkujących mediatory

przeciwzapalne (12). Ponadto bakterie oraz ich toksyny mogą przenikać do krwioobiegu, aktywując zarówno lokalną, jak i ogólnoustrojową odpowiedź zapalną oraz nasilając stres oksydacyjny (13). Powszechnie wiadomo, że przewlekle utrzymujący się stan zapalny i stres oksydacyjny sprzyjają procesom katabolicznym, ograniczając tym samym procesy anaboliczne. Stan taki negatywnie wpływa na procesy regeneracji, jest czynnikiem ograniczającym funkcję mięśni i zdolności wysiłkowe (14). Utrzymanie odpowiedniej równowagi pomiędzy drobnoustrojami obecnymi w jelicie może mieć więc istotny wpływ na adaptację mięśni do treningu, regenerację i zdolności wysiłkowe sportowców.

5.3 Wpływ mikrobiomu jelitowego na zdolności wysiłkowe sportowców

Wykazano, że mikrobiota jelitowa może przyczyniać się do utrzymania integralności komórek nabłonkowych jelita oraz wpływać na stan odżywienia, modulację stanu zapalnego w organizmie, szlaki metabolizmu energetycznego, a także produkcję metabolitów o korzystnym działaniu na organizm gospodarza (6). Jednym z głównych mechanizmów, poprzez które mikrobiom jelitowy może wywierać efekt na funkcje mięśni szkieletowych, jest jego zdolność do modulowania procesu zapalnego oraz stresu oksydacyjnego poprzez szlaki metaboliczne obejmujące białka z rodziny mTOR, czynnik jądrowy NF-kB oraz białka FOXO (15). Ponadto niektóre gatunki bakterii mogą wspierać metabolizm mleczanu nagromadzonego w wyniku wykonywanych ćwiczeń. Doniesienia naukowe sugerują, że mleczan zgromadzony w surowicy może przenikać przez barierę jelitową i następnie, przy udziale bakterii jelitowych, ulegać konwersji do propionianu, który finalnie wykorzystywany jest do pozyskiwania dodatkowej energii w procesie glukoneogenezy (16). Również zaobserwowano zdolności poszczególnych szczepów bakteryjnych do niwelowania uszkodzenia mięśniowego spowodowanego wysiłkiem fizycznym, manifestującego się obniżaniem poziomu kinazy kreatynowej (CK) we krwi (17).

Korzystne działanie mikrobiomu jelitowego na zdolności wysiłkowe sportowców może być również związane z poprawą metabolizmu glukozy (18), funkcji mitochondriów (19), a także zdrowiem ośrodkowego układu nerwowego (20). Ponadto utrzymanie homeostazy w obrębie jelita sprzyja utrzymaniu integralności bariery jelitowej, zabezpieczając je tym samym przed nadmierną aktywacją układu immunologicznego. Wszystkie te mechanizmy mogą korzystnie wpływać

na fizjologiczną adaptację do treningu i przekładać się na poprawę zdolności wysiłkowych sportowców. Zjawisko to nazywane jest osią jelitowo-mięśniową i opiera się na założeniu, że niektóre drobnoustroje mogą wpływać na funkcjonowanie mięśni. Z drugiej strony dysbioza jelitowa sprzyja obniżeniu fizjologicznej adaptacji poprzez nasilenie stanu zapalnego, wolnorodnikowego uszkodzenia makrocząsteczek, co w konsekwencji przyczynia się do atrofii mięśni szkieletowych (14).

5.4 Znaczenie probiotyków w sporcie

W świetle aktualnej wiedzy wydaje się, że interwencje celowane w mikrobiotę jelitową mogą korzystnie wpływać na zdrowie gospodarza i przekładać się na poprawę wyników sportowych. Liczne badania naukowe wskazują na korzyści płynące z suplementacji probiotykami w populacji sportowców. Polegają one na poprawie homeostazy jelit. Zgodnie z definicją Organizacji Narodów Zjednoczonych ds. Żywienia i Rolnictwa (FAO) oraz Światowej Organizacji Zdrowia (WHO) mianem probiotyków określa się ,,żywe mikroorganizmy, które podawane w odpowiednich ilościach, przynoszą korzyści zdrowotne gospodarzowi" (21).

Wykazano, że suplementacja probiotykami poprzez wpływ na skład mikrobioty jelitowej może zmniejszać zarówno odpowiedź zapalną, jak i poprawiać potencjał antyoksydacyjny (22,23). W niektórych przypadkach zmiany te korelowały z poprawą wyników sportowych. Suplementacja szczepem *Lactobacillus plantarum* PS128 korzystnie wpłynęła na poprawę wydolności anaerobowej u triatlonistów (24). Podobny efekt zaobserwowano u rekreacyjnie trenujących mężczyzn po suplementacji szczepem *Bacillus coagulants* GBI-30 (17). Doniesienia naukowe sugerują, że niektóre bakterie mogą korzystnie wpływać na wydolność tlenową. Przeszczep szczepu *Veilonella atypica* u myszy korelował zarówno z wydłużeniem czasu ćwiczeń do odmowy, jak i wyższym poziomem wykorzystania mleczanu (16). Podobnie suplementacja mieszanką probiotyczną zawierającą gatunki *Lactobacillus* i *Bifidobacterium* poprawiła wydolność tlenową wyścigu triatlonowego, co korelowało z obniżonym poziomem endotoksemii (24). W innych badaniach obserwowano poprawę wydolności aerobowej poprzez wydłużenie dystansu osiągniętego w teście Coopera, a także wzrost całkowitej obfitości mikrobioty jelitowej w wyniku suplementacji szczepem

Bifidobacetrium longum OLP-01 (25). Wykazano również, że wieloszczepowe mieszanki probiotyczne, zawierające *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W22, *Levilactobacillus brevis* W63 i *Lactococcus Lactis* W58, przyczyniały się do obniżenia poziomu zonuliny w kale oraz obniżały parametry stanu zapalnego wywołanego wysiłkiem fizycznym (26). W innym badaniu ta sama mieszanka probiotyczna przyczyniła się do zmniejszenia szybkości degradacji tryptofanu spowodowanej treningiem oraz zredukowała liczbę infekcji górnych dróg oddechowych (27).

5.5 Synergistyczne oddziaływanie witaminy D3 i probiotyków

Odkrycie receptora witaminy D (VDR) w mięśniach szkieletowych dostarczyło dowodów naukowych potwierdzających pozytywny wpływ witaminy D3 na zdrowie mięśni szkieletowych. Obserwuje się, że deficyt, określany jako stężenie 25-hydroksykalcyferolu we krwi poniżej 30 ng/ml, powszechnie występuje w populacji Polski i obserwowany jest u 85% społeczeństwa (28). Doniesienia naukowe wskazują, że niedobór witaminy D3 koreluje z nasileniem stresu oksydacyjnego, co negatywnie wpływa zarówno na biogenezę i funkcję mitochondriów, jak i na metabolizm mięśni szkieletowych (29). W związku z tym zapobieganie niedoborom witaminy D3 w populacji sportowców może być korzystne nie tylko dla zdrowia, ale także dla procesów regeneracji i zdolności wysiłkowych zawodników.

W świetle aktualnej wiedzy wydaje się, że pozytywne działanie niektórych szczepów bakterii może być wzmocnione poprzez synergistyczne działanie witaminy D3. Potencjalne szlaki sygnalizacyjne osi jelitowo-mięśniowej w znacznym stopniu pokrywają się ze szlakami witaminy D3 i dotyczą w głównej mierze wpływu na syntezę białek mięśniowych i funkcję mitochondriów poprzez szlaki mTOR i FOXO, czyli zdolności do modulowania funkcji immunologicznych (30). Dodatkowo zarówno witamina D3, jak i bakterie jelitowe mogą wywierać wpływ na stres, funkcje mózgu i działać nuroprotekcyjnie, co również jest czynnikiem pośrednio wpływającym na mięśnie szkieletowe (29,31). W związku z tym możliwe jest, że suplementacja probiotyków wraz z witaminą D3 może mieć korzystny wpływ na funkcjonowanie mięśni u sportowców.

6. CELE PRACY I HIPOTEZA BADAWCZA

6.1. Cele pracy

Celem pracy było:

1. Określenie wpływu mikrobiomu jelitowego na zdolności wysiłkowe sportowców.
2. Ocena wpływu suplementacji probiotyków w połączeniu z witaminą D3 na skład i różnorodność mikrobiomu jelitowego sportowców oraz parametry przepuszczalności błony śluzowej jelita.
3. Ustalenie wpływu zmian składu mikrobiomu jelitowego na:

- parametry wydolnościowe sportowców,
- tempo utylizacji mleczanu po wysiłku fizycznym,
- parametry uszkodzenia mięśni szkieletowych po treningu w pobranej krwi,
- stężenie cytokin pro- i przeciwzapalnych w pobranej krwi.

Wszystkie wymienione cele zrealizowane zostały w ramach opublikowanego cyklu powiązanych ze sobą tematycznie artykułów naukowych.

6.2. Hipoteza badawcza

Połączona suplementacja probiotykami i witaminą D3 korzystnie wpływa na skład mikrobiomu jelitowego i może przekładać się na poprawę wyników sportowych.

7. OMÓWIENIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

Rozprawę doktorską stanowi cykl trzech, powiązanych ze spobą tematycznie, artykułów opublikowanych w recenzowanych czasopismach naukowych ze współczynnikiem oddziaływania (Imact factor; IF). Pierwszy artykuł

ma charakter pracy przeglądowej, dwa kolejne stanowią oryginalne badania eksperymentalne, przeprowadzone na zawodnikach uprawiajacych sporty walki.

Punktem wyjścia dla realizowanych badań był artykuł o charakterze przeglądowym:

Przewłócka K., Folwarski M., Kaźmierczak-Siedlecka K., Skonieczna-Żydecka K., Kaczor J.J. ***Gut-Muscle Axis Exists and May Affect Skeletal Muscle Adaptation to Training*.** Nutrients. 2020;12(5):1451. doi: 10.3390/nu12051451. PMID: 32443396; PMCID: PMC7285193.

Nadmierne obciążenia treningowe prowadzą do nasilenia stresu oksydacyjnego oraz stanu zapalnego, utrudniając tym samym fizjologiczną adaptację do treningu. Dodatkowo nieprawidłowa dieta oraz przeciążenia treningowe zakłócają równowagę jelitową, powodując rozregulowanie układu odpornościowego. Optymalizacja mikrobioty jelitowej może więc zabezpieczać przed nadmierną aktywacją układu immunologicznego, przyczyniając się do poprawy regeneracji po treningu, adaptacji mięśni do treningu i zdolności wysiłkowych sportowców. Związek pomiędzy mikrobiotą jelit a adaptacją mięśni do treningu wydaje się przejawiać poprzez zdolności bakterii do modulacji stanu zapalnego w organizmie, wpływ na procesy anaboliczne i kataboliczne, regulację biodostępności składników odżywczych, a także produkcję metabolitów o korzystnym działaniu na organizm gospodarza. Celem przeglądu było wyjaśnienie mechanizmów leżących u podstaw wzajemnej interakcji pomiędzy mikrobiomem jelitowym a mięśniami szkieletowymi, określonej mianem osi jelitowo-mięśniowej.

W pracy wykazano, że mikrobiota jelitowa może wpływać na zdrowie mięśni szkieletowych oraz zdolności wysiłkowe na drodze złożonych procesów, z których nie wszystkie zostały w pełni wyjaśnione. Jednym z najlepiej poznanych mechanizmów jest wpływ mikrobioty na układ odpornościowy. W obrębie błony śluzowej jelita zlokalizowane jest 70% całego układu immunologicznego, a fizjologiczną barierę pomiędzy mikroorganizmami i antygenami ze światła jelita stanowią komórki nabłonkowe. Enterocyty ograniczają mikroorganizmom i antygenom translokację do blaszki właściwej poprzez wzrost zawartości białek połączeń ścisłych, wydzielanie śluzu i peptydów przeciwdrobnoustrojowych. Utrzymanie integralności warstwy nabłonkowej błony śluzowej jest więc ważnym elementem zabezpieczającym przed nadmierną aktywacją komórek układu

immunologicznego (13). W warunkach homeostazy wzorce molekularne mikroorganizmów stymulują sekrecję cytokin, zaangażowanych w różnicowanie komórek dendrytycznych i makrofagów, aktywując w efekcie komórki T-regulatorowe (T_{reg}), których pobudzenie manifestuje się poprzez produkcję cytokin przeciwzapalnych. Zdrowa mikrobiota warunkuje również dojrzewanie limfocytów T i B, a także pomaga w utrzymaniu odpowiedniego poziomu immunoglobulin we krwi (13,32). Dysbioza z kolei może wyzwalać mediatory stanu zapalnego oraz wywierać negatywny wpływ na metabolizm gospodarza.

Dowody naukowe dotyczące zależności pomiędzy składem mikrobioty a funkcją mięśni zostały opisane w patogenezie sarkopenii u ludzi starszych, u których sarkopenia i ogólnoustrojowe osłabienie korelują z dysbiozą jelit, przyczyniającą się do zwiększonej przepuszczalności bariery jelitowej, poziomu lipopolisacharydów (LPS) we krwi, aktywacji układu immunologicznego i zmniejszenia wrażliwości na insulinę (33). W badania na modelach zwierzęcych zaobserwowano wyraźną korelację pomiędzy obniżeniem markerów atrofii mięśni a wzrostem masy i siły mięśni po suplementacji *Lactobacillus plantarum* (34,35). Mikrobiota jelitowa poprzez modulację stanu zapalnego może wpływać na procesy syntezy i katabolizmu białek mięśniowych, chroniąc jednocześnie przed niekorzystnym skutkami nadmiernego treningu. W badaniach wykazano, że suplementacja probiotykami w sporcie może przynieść korzystne efekty poprzez obniżanie parametrów stanu zapalnego we krwi i przyczynienie się do utrzymania fizjologicznej funkcji mięśni szkieletowych. Wykazano, że suplementacja szczepem *Streptococcus thermophilus* FP4 *i Bifidobacterium breve* BR03 przyczyniła się do obniżenia poziomu IL-6 po treningu, co ma wpływ na poprawę uczucia regeneracji po wysiłku (35). Podobnie w wyniku suplementacji *Bacillus subtilis* DE111 doszło do obniżenia poziomu prozapalnego TNF-a (36). W innym badaniu szczep *Lactobacillus plantarum* PS128 przyczynił się do obniżenia poziomu kinazy kreatynowej, TNF-a, IFN-γ, IL-6 i IL-8 oraz zwiększenia poziomu przeciwzapalnej IL-10 (24).

Jednym z czynników mogących ograniczyć zdolności wysiłkowe sportowców jest dostępność glikogenu. W świetle aktualnej wiedzy wydaje się, że mikrobiota jelitowa może również wpływać na transport glukozy poprzez swoje metabolity. Wykazano, że SCFA aktywuje receptory Gpr41 i Sglt1, zaangażowane w jelitowy transport glukozy (37). W badaniach na modelu zwierzęcym zaobserwowano, że

antybiotykoterapia korelowała z obniżeniem ekspresji genów dla GPR1 i SGLT1, co wiązało się z obniżeniem poziomu glikogenu mięśniowego (18). Obserwuje się, że mikrobiom jelitowy poprzez produkcję metabolitów fenolowych (białka IVAS) przyczynia się do poprawy zdolności komórek ludzkich do transportu, wchłaniania i metabolizowania glukozy poprzez aktywację transporterów glukozy GLUT-1 i GLUT 4 oraz aktywację kinazy PI3K i białek AKT (38).

Mikrobiota jelitowa poprzez obniżenie poziomu LPS we krwi i redukcję odpowiedzi zapalnej może pośrednio wpływać na funkcje mitochondriów. W warunkach dysbiozy dochodzi do aktywacji receptorów TLR, co w pośredni sposób przekłada się na pobudzenie kompleksów białkowych łańcucha oddechowego i w efekcie nadprodukcję reaktywnych form tlenu (RFT) przez mitochondria (39). Doniesienia naukowe wskazują, że niektóre bakterie patogenne mogą przyczyniać się do fragmentacji sieci mitochondrialnych (40), podczas gdy bakterie takie jak *Mycobacterium tuberculosis* i *Ehrlichia chaffeensis* są zdolne do hamowania generacji RFT poprzez wzrost aktywności endogennych systemów antyoksydacyjnych (39). Wpływ na funkcje mitochondriów wykazują również niektóre metabolity bakteryjne. Siarkowodór produkowany m.in. przez *Salmonellę* czy *Escherichię coli* wpływa na inhibicję oksydazy cytochromowej (39). Z kolei SCFA wykazują korzystny wpływ na regulację metabolizmu tlenowego poprzez wzrost stężenia białka PGC1- a (41). Powszechnie wiadomo, że SCFA są metabolitami odżywiającymi komórki nabłonka jelit, niezbędnymi do utrzymania integralności błony śluzowej jelita. Pozytywne działanie SCFA polega również na promowaniu zużycia tłuszczu jako głównego źródła energii i tym samym oszczędzaniu glikogenu mięśniowego oraz aktywowaniu acetylotransferazy histonów – enzymu mającego kluczowe znaczenie dla ekspresji genów (42).

Korzystny wpływ mikrobiomu jelitowego na zdrowie mięśni szkieletowych może przejawiać się również poprzez poprawę biodostępności niektórych aminokwasów (43), witaminy B2, B9, B12 i K (44) oraz metabolizm polifenoli (45). Również wykazano, że niektóre bakterie jelitowe są zdolne do konwertowania mleczanu w propionian, który następnie może być wykorzystywany jako dodatkowe źródło energii poprzez przemiany cyklu Cori. Istotną rolę w tym procesie przypisuje się bakterii z rodzaju *Veilonella atypica*, której podwyższoną liczebność obserwowano w jelicie sportowców wysokiego wyczynu. Dodatkowo przeszczep tej bakterii do jelit zwierząt laboratoryjnych przyczynił się do wydłużenia czasu trwania wysiłku do

odmowy(46). Można więc przypuszczać, że zdolność mikrobioty do modulacji procesów enzymatycznych i konwersji mleczanu do propionianu przyczynia się do poprawy wyników sportowych.

Aktualnie istnieją przekonujące dowody naukowe, potwierdzające istnienie dwukierunkowej komunikacji pomiędzy mikrobiom jelitowym a mózgiem, określanej mianem osi jelitowo-mózgowej. Wydaje się, że bakterie jelitowe mogą oddziaływać na ośrodkowy układ nerwowy poprzez swój potencjał do zmiany aktywności neuroprzekaźników i wpływać na układ nerwowym gospodarza w celu regulacji zdrowia psychicznego, a co za tym idzie, metabolizmu i zdolności wysiłkowej. Wykazano, że szczepy z rodzaju *Lactobacillus* produkują kwas aminomasłowy (GABA), inne bakterie z rodzaju Bacillus mycoides, *Bacillus subtilis* są zdolne do syntezy noradrenaliny, *Bacillus cereus, Bacillus mycoides, Bacillus subtilis* do produkcji dopaminy (np. *Bacillus cereus)*, a *Lactococcus lactis, Lactobacillus plantarum i Streptococcus thermophilus* do produkcji *serotoniny* (47–49). Wykazano również, że mikrobiom jelitowy poprzez regulację uwalniania neuroprzekaźników i hormonów wpływa na samopoczucie sportowców, ich nastrój, motywację i subiektywne poczucie regeneracji (20).

Poprzez niniejszy artykuł zrealizowałam pierwszy ze stawianych przez mnie celów, czyli określenie wpływu mikrobiomu jelitowego na zdolności wysiłkowe sportowców.

Po dokonaniu dogłębnego przeglądu artykułów naukowych zaobserwowałam, że w licznych, chociaż nie wszystkich, badaniach autorzy powiązali korzyści płynące z suplementacji probiotykami z poprawą zdolności wysiłkowych sportowców. Zaobserwowano, że zmiany profilu mikrobioty jelit, markerów stanu zapalnego czy też uszkodzenia mięśnia bezpośrednio przekładały się na wyniki sportowe i manifestowały się w głównej mierze poprzez wzrost wydolności beztlenowej osiąganej w teście Wingate (17,24), poprawę wydolności aerobowej (wydłużenie czasu trwania ćwiczeń do odmowy), poprawę czasu wyścigu(16,25,50) czy też poprawę siły mięśni (17,51). Jednak nie wszystkie przeprowadzone badania wykazały wpływ suplementacji probiotykiem na wydolność sportową

Kolejne dwie prace wchodzące w cykl publikacji oparte zostały o badanie oryginalne, zaprojektowane jako podwójnie zaślepiona próba kontrolowana

placebo. Badanie uzyskało zgodę Niezależnej Komisji Bioetycznej przy Gdańskim Uniwersytecie Medycznym (No. NKNNB/643/2019-2020) oraz zostało zarejestrowane w Clinical Trials pod numerem NCT04759729.

Do badania włączono 25 zawodników mieszanych sportów walki (MMA) płci męskiej, trenujących wyczynowo na terenie województwa pomorskiego. Zawodnicy spełniający kryteria włączenia zostali losowo przydzieleni do 2 grup, z których jedna otrzymała mieszankę probiotyczną i witaminę D3 (PRO+VitD), natomiast druga placebo i witaminę D3 (Vit D). Mieszankę probiotyczną stanowił produkt złożony z 5 szczepów (*Bifidobacterium lactis* W51*, Levilactobacillus brevis* W63, *Lactobacillus acidophilus* W22, *Bifidobacterium bifidum* W23 i *Lactococcus lactis* W58), a całkowita ilość komórek bakteryjnych wynosiła >2.5 × 109 jednostek tworzących kolonię (CFUs) na 1 gram ((≥ 500 millionów CFU w kapsułce). Zawodnicy przyjmowali 4 kapsułki dziennie. Jako placebo użyto identycznie wyglądającego produktu, zwierającego 40 mg maltodekstryny. Dodatkowo, celem wyrównania niedoborów, wszyscy zawodnicy otrzymali witaminę D3 Juvit D3 w dawce 3500 i.u. dziennie. Przed rozpoczęciem suplementacji przeprowadzony został test mocy beztlenowej, oparty o protokół Wingate, polegający na wykonaniu trzech 30-sekundowych sprintów przy użyciu ergometru rowerowego, oddzielonych 2-minutową przerwą. Przy pomocy ergometru rowerowego przeprowadzono również test aerobowy, którego celem była ocena pułapu tlenowego zawodników za pomocą analizatora gazów oddechowych. Krew z żyły łokciowej została pobrana 3-krotnie (na czczo - przed wykonaniem testu, 30 minut oraz 24 godziny po teście). Pobrana krew była wirowana w temperaturze 4°C przez 10 minut przy 2000 × g. Następnie uzyskana surowica i osocze zostały zamrożone i przechowywano je w temperaturze -80°C. Krew została przeanalizowana pod kątem stężenia 25(OH)D3 przy pomocy rozcieńczania izotopów metodą chromatografii cieczowej połączonej z tandemową spektrometrią mas (LC–MS/MS), aktywności kinazy kreatynowej metodą kinetyczną oraz stężenia cytokin prozapalnych (IL-6, TNF- α) i przeciwzapalnych (Il-2, IL-15) metodą testu immunoenzymatycznego (ELISA). Krew kapilarna pobrana została w 5 punktach czasowych (przed wykonaniem testu, 3,15, 30 i 60 minut po teście). Krew pobrano do probówek zawierających kwas polichlorowy, które następnie przechowywano w temperaturze -20 °C. Oznaczenia wykonane zostały z wykorzystaniem metody kinetycznej, bazującej

na szybkości reakcji. Pobrana została również próbka kału, która została rozporcjowana i następnie przechowywana w temperaturze -80°C w oczekiwaniu na dalsze oznaczenia. Próbki kału zostały poddane ocenie metagenomicznej przy użyciu metody sekwencjonowania nowej generacji. Skład mikrobiomu scharakteryzowano przede wszystkim poprzez różnorodność alfa i beta. Różnorodność alfa mierzono, używając wskaźników takich jak Chao1, ACE, Shannon i odwrócony Simpson. Różnorodność beta scharakteryzowana została poprzez dystans Bray-curtis. Zebrane próbki kału przeanalizowano również pod kątem obecności parametrów przepuszczalności jelit, takich jak kalprotektyna i zonulina oraz SCFA. Ocenę stężenia kalprotektyny i zonuliny w kale wykonano przy użyciu metody ELISA. Do oznaczenia zawartości SCFA w pobranych próbkach kału wykorzystano metodę chromatografii gazowej z systemem Agilent Technologies 1260 A GC z detektorem płomieniowo-jonizacyjnym (FID). Zawodnikom wykonano analizę składu ciała oraz zebrany został obszerny wywiad dotyczący żywienia, stosowanych suplementów oraz dotyczący planu treningowego. Zawodnicy poproszeni zostali o niewprowadzanie żadnych zmian w dotychczasowym planie żywieniowo-treningowym oraz o niewdrażanie żadnych nowych leków/suplementów. Zobligowani zostali do kontynuowania typowego treningu MMA. Po 4-tygodniowym okresie suplementacji cała procedura została powtórzona. Spośród 25 zawodników, 23 ukończyło protokół. Uzyskane wyniki zostały poddane analizie statystycznej z wykorzystaniem dwuczynnikowego testu ANOVA. Wyniki istotne statystycznie uznane były, jeżeli $p<0.05$.

Pierwsze wyniki przedstawione zostały w publikacji 2:

Przewłócka K., Kujach S., Sawicki P., Berezka P., Bytowska Z.K., Folwarski M., Kowalski K., Kaczor J.J. ***Effects of Probiotics and Vitamin D_3 Supplementation on Sports Performance Markers in Male Mixed Martial Arts Athletes: A Randomized Trial.*** Sports Med Open. 2023; 9(1):31. doi: 10.1186/s40798-023-00576-6. PMID: 37193828; PMCID: PMC10188824.

Głównym celem pracy było określenie, czy 4-tygodniowa suplementacja probiotykiem połączona z witamina D3 może korzystnie wpłynąć na wydolność beztlenową sportowców, tempo metabolizmu mleczanu oraz markery

uszkodzenia mięśnia. W badaniu wykazano, że 4-tygodniowa suplementacja probiotykami i witaminą D3 korzystnie wpłynęła na wydajność beztlenową zawodników podczas pierwszego sprintu. Zaobserwowano poprawę średniej mocy w grupie PRO+VitD po okresie suplementacji w porównaniu do wyniku przed suplementacją (7,73 ± 0,47 vs 8,02 ± 0,45; p<0,04), podczas gdy w grupie VitD nie zaobserwowano istotnych statystycznie zmian (7,98 ± 0,65 vs 7,95 ± 0,48; p<0,99). Również zaobserwowano poprawę całkowitej pracy wykonanej podczas testu w grupie otrzymującej probiotyk (232,00 ± 14,06 vs 240,72 ± 13,38; p<0,04), a efektu nie zaobserwowano w grupie VitD (239.34 ± 19,46 vs 238,37 ± 14,35; p<0,99).

W badaniu wykazano również poprawę tempa utylizacji mleczanu w grupie suplementowanej probiotykiem. Stężenie mleczanu (LA) we krwi w obu grupach przed suplementacją i wysiłkiem fizycznym oraz po SIE nie różniło się istotnie między grupami. Po czterech tygodniach suplementacji doszło do wzrostu stężenia LA w grupie Vit D (5,88 ± 1,55 mmol/L) w porównaniu z grupą PRO + VitD (4,73 ± 1,63 mmol/L) 60 minut po ćwiczeniach (p < 0,05). Ponadto wykazano również szybsze tempo utylizacji LA w grupie PRO + VitD (73,6 ± 6,9%) w porównaniu z grupą Vit D (65,1 ± 9,9%).

Badając wpływ suplementacji na markery uszkodzenia mięśnia szkieletowego, nie zaobserwowano żadnych istotnych statystycznie różnic pomiędzy grupami, co mogło wynikać z dużych odchyleń standardowych w obu grupach. W przeprowadzonym badaniu potrójny sprint nie zwiększał uszkodzeń mięśni mierzonych poprzez stężenie CK w osoczu sportowców. Możliwe, że tego rodzaju ćwiczenia, które skutkowały wysokim stężeniem LA, a także zgłaszanym wysokim zmęczeniem, nie były wystarczające do uszkodzenia tkanki mięśni szkieletowych u zawodników MMA.

Pomimo suplementacji witaminy D3 w dawce 3500 IU/dzień, stężenie 25(OH)D3 w surowicy było nadal poniżej minimalnego poziomu (30 ng/ml). Najprawdopodobniej dawki zalecane dla populacji zdrowych osób dorosłych są zbyt niskie, aby wywołać fizjologiczny efekt wśród sportowców. W badaniu zaobserwowano, że poziom 25(OH)D3 był istotnie statystycznie wyższy 30 minut po wykonaniu sprintów w porównaniu z wartościami przed ćwiczeniami (p<0,05). Jeszcze większy wzrost po wykonanym teście obserwowany był po 4-tygodniowym okresie suplementacji (p<0,001).

Wyniki przedstawione w niniejszej pracy odpowiadają na kolejne stawiane przeze mnie cele, pokazując, że suplementacja probiotykiem i witaminą D3 może korzystnie wpływać na wyniki sportowe zawodników sportów walki. Wykazałam, że 4-tygodniowy okres suplementacji przyczynił się do poprawy wydolności beztlenowej oraz usprawnienie tempa utylizacji mleczanu. Dodatkowo uzyskane wyniki wskazują, że dzienne dawki na poziomie 3500 IU witaminy D3 są zbyt niskie, aby wywołać fizjologiczny efekt w grupie sportowców. Wyniki przeprowadzonego badania potwierdzają że mięśnie szkieletowe mogą zarówno magazynować, jak i uwalniać witaminę D3, natomiast trening fizyczny może w pewnym stopniu warunkować uwalnianie metabolitów witaminy D3. Ze względu na prawdopodobne wyższe wykorzystywanie 25(OH)D3 przez mięśnie szkieletowe, osoby trenujące powinny stosować wyższe dawki witaminy D3.

Drugą część wyników przedstawiono w trzeciej publikacji:

Przewłócka K., Folwarski M., Kaczmarczyk M., Skonieczna-Żydecka K., Palma J., Bytowska Z.K., Kujach S., and Kaczor J.J. ***Combined probiotics with vitamin D_3 supplementation improved aerobic performance and gut microbiome composition in Mixed Martial Arts athletes.*** Frontiers in Nutrition. 2023***;*** doi: 10.3389/fnut.2023.1256226.

Celem badania było określenie, w jaki sposób suplementacja probiotykiem wpłynęła na skład mikrobioty jelitowej, przepuszczalność jelit oraz stężenie SCFA w kale oraz czy interwencja korzystnie wpłynęła na parametry stanu zapalnego we krwi i czy finalnie przyczyniła się do poprawy wydolności tlenowej zawodników MMA. Po 4-tygodniowym okresie suplementacji zaobserwowano istotne zmiany w składzie mikrobiomu jelitowego. Różnorodność beta mierzona metodą Bray-Curtis wykazała statystycznie istotne zmiany w składzie mikrobioty po interwencji w grupie PRO+VIT D ($p = 0,0005$), podczas gdy w grupie VIT D nie zaobserwowano żadnych znaczących różnic ($p = 0,145$). Wyniki wskazują również, że suplementacja probiotykiem znacząco wpłynęła na profil drobnoustrojów jelitowych i przyczyniła się do rozwoju bakterii mających potencjalnie korzystny wpływ na zdrowie gospodarza (m.in. *Bacteroides*, *Roseburia inulinivorans*, *Prevotella*, Lactobacillaceae). Dodatkowo stwierdzono znaczny wzrost klasy *Negativicutes* w grupie PRO+VIT D (Est = 1,98, $p = 0,006$), ale nie w grupie VIT D (Est = -0,25, $p = 0,738$). Niektóre bakterie z klasy *Negativicutes* zaangażowane są w

metabolizm mleczanu. Analiza zmian procentowych w wybranych stężeniach SCFA nie wykazała znaczących różnic pomiędzy grupami PRO+VIT D i VIT D. Zaobserwowano, że poziom propionianu po suplementacji obniżył się w obu grupach, jednak spadek ten był większy w grupie VIT D. W grupie PRO+VIT D obserwowano podobny trend, natomiast był on nieistotny statystycznie ($p = 0,061$).

Wyniki przeprowadzonego badania wskazują, że zmiany obserwowane w składzie mikrobiomu jelitowego korelowały z poprawą wydolności w trakcie testu aerobowego. Wykazano znaczące różnice w czasie wysiłku do wyczerpania przed suplementacją w porównaniu z wynikami po suplementacji ($p = 0,023$) w grupie PRO+VITD. W grupie VIT D nie zaobserwowano statystycznie istotnych różnic ($p = 0,685$). Nie stwierdzono różnic między grupami w maksymalnym poborze tlenu (VO2 max), maksymalnej mocy tlenowej (MAP) oraz maksymalnym współczynniku wymiany oddechowej (RER) podczas testu VO2 max. Dane te potwierdzają hipotezę, że przyjmowanie probiotyków może mieć pozytywny wpływ na wytrzymałość poprzez zmianę drobnoustrojów jelitowych, jednak nie wpływa to na wartość VO2 max. W poprzednim badaniu obserwowano znaczny wzrost tempa wykorzystania mleczanu wśród sportowców MMA po wysiłku, w wyniku suplementacji probiotykiem i witaminą D3. Wiadomo, że zdolność do metabolizowani LA ma ogromne znaczenie dla wyników sportowych, a nagromadzenie LA w tkankach mięśniowych przyczynia się do wystąpienia zmęczenia podczas treningu. Prawdopodobnie probiotyki mogą poprawić zdolności wytrzymałościowe nie poprzez wpływ na VO2 max, a poprzez korzystną modulację profilu mikrobiomu jelitowego, potencjalnie prowadząc do ulepszonego wykorzystania LA.

W badaniu nie zaobserwowano statystycznie istotnych różnic w stężeniach markerów stanu zapalnego w surowicy, takich jak IL-2, IL-6, IL-15 i TNF-a w wyniku interwencji. Po wysiłku widoczny był istotny statystycznie wzrost stężenia IL-6 w obu grupach, zarówno przed ($p < 0,001$ w obu grupach), jak i po suplementacji (PRO + VIT D $p=0,033$; VIT D $p=0,029$). Sparowana analiza nie wykazała jednak statystycznie istotnych zmian między grupami. Po 4 tygodniach suplementacji probiotykiem i witaminą D3 nastąpiło znacznie obniżenie stężenie kalprotektyny w kale – markera jelitowego stanu zapalnego. Różnica była istotna statystycznie i

wynosiła 69,50 ± 46,91 przed suplementacją i 34,79 ± 24,38 mmol/L po suplementacji.

W tej pracy zrealizowałam kolejny stawiany przez mnie cel, dotyczący wykazania wpływu suplementacji probiotyków w połączeniu z witaminą D3 na skład i różnorodność mikrobiomu jelitowego sportowców i parametry przepuszczalności błony śluzowej jelita na poziom markerów stanu zapalnego oraz wydolność fizyczną. Wyniki badania dowodzą, że połączona suplementacjia probiotykami i witaminą D3 może korzystnie wpływać na skład mikrobioty jelitowej. Obserwowany spadek stężenia kalprotektyny po suplementacji probiotycznej sugeruje, ze suplementacja probiotykiem mogła przyczynić się do uszczelnienia bariery jelitowej. Ponadto zaobserwowałam bezpośredni wpływ na wydolność fizyczną poprzez wydłużenie czasu trwania ćwiczeń do odmowy u zawodników MMA. Powyższe dane sugerują dwukierunkową drogę komunikacji między komórkami mięśniowymi a mikrobiotą jelitową, potwierdzając korzystne działanie połączonych probiotyków i witaminy D3 u sportowców wysokiego wyczynu.

8. PODSUMOWANIE CAŁOŚCI ROZPRAWY

Wyniki niniejszej rozprawy wskazują, że mikrobiota jelitowa korzystnie wpływa na prawidłowe funkcjonowanie mięśni szkieletowych oraz zdolności wysiłkowe na drodze złożonych procesów, z których nie wszystkie zostały w pełni wyjaśnione. W przeprowadzonym badaniu wykazałam że:

1. Połączona 4-tygodniowa suplementacja probiotykiem i witaminą D3 doprowadziła do korzystnych zmian profilu mikrobiomu jelitowego oraz przyczyniła się do uszczelnienia bariery jelitowej. W wyniku suplementacji doszło do wzrostu różnorodności beta oraz do rozwoju bakterii mających potencjalnie korzystny wpływ na zdrowie gospodarza, a także do obniżenia kalprotektyny – jelitowego markera stanu zapalnego.
2. Zmiany składu mikrobiomu jelitowego korelowały z poprawą wyników sportowych zarówno w teście wydolności beztlenowej jak i tlenowej. Korzystna modulacja profilu mikrobiomu jelitowego w wyniku połączonej

suplementacji, przyczyniła się do szybszego tempa utylizacji mleczanu po testach supramaksymalnych u zawodników MMA.

3. Uzyskane wyniki potwierdzają, że dochodzi do powysiłkowego wyrzutu 25(OH)D3 do krwi przed i po suplementacji. Wykazano, znacznie wyższe stężenie 25(OH)D3 we krwi 30 minut po intensywnym wysiłku w porównaniu do wartości przed wysiłkiem.

4. Mięśnie szkieletowe mogą zarówno magazynować jak i uwalniać 25(OH)D3, podkreślając rolę tej witaminy nie tylko dla prawidłowego funkcjonowania mięśni szkieletowych, lecz całego organizmu.

5. Osobom trenującym powinno zalecać się suplementację wyższymi dawkami witaminy D3, niż ogólne dawki zalecane dla populacji osób zdrowych, dorosłych.

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10. PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY DOKTORSKIEJ I OŚWIADCZENIA WSPÓŁAUTORÓW

nutrients

MDPI

Review

Gut-Muscle Axis Exists and May Affect Skeletal Muscle Adaptation to Training

Katarzyna Przewłócka [1], Marcin Folwarski [2], Karolina Kaźmierczak-Siedlecka [3], Karolina Skonieczna-Żydecka [4] and Jan Jacek Kaczor [1,*]

1 Department of Bioenergetics and Physiology of Exercise, Medical University of Gdańsk, 80-210 Gdańsk, Poland; k.przewlocka@gumed.edu.pl
2 Departmentof Clinical Nutrition and Dietetics, Medical University of Gdansk, 80-210 Gdańsk, Poland; marcinfol@gumed.edu.pl
3 Department of Surgical Oncology, Medical University of Gdansk, 80-210 Gdańsk, Poland; leokadia@gumed.edu.pl
4 Department of Human Nutrition and Metabolomics, Pomeranian Medical University, 70-204 Szczecin, Poland; karzyd@pum.edu.pl
* Correspondence: kaczorj@gumed.edu.pl; Tel.: +48-516-191-109

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Abstract: Excessive training may limit physiological muscle adaptation through chronic oxidative stress and inflammation. Improper diet and overtraining may also disrupt intestinal homeostasis and in consequence enhance inflammation. Altogether, these factors may lead to an imbalance in the gut ecosystem, causing dysregulation of the immune system. Therefore, it seems to be important to optimize the intestinal microbiota composition, which is able to modulate the immune system and reduce oxidative stress. Moreover, the optimal intestinal microbiota composition may have an impact on muscle protein synthesis and mitochondrial biogenesis and function, as well as muscle glycogen storage. Aproperly balanced microbiome may also reduce inflammatory markers and reactive oxygen species production, which may further attenuate macromolecules damage. Consequently, supplementation with probiotics may have some beneficial effect on aerobic and anaerobic performance. The phenomenon of gut-muscle axis should be continuously explored to function maintenance, not only in athletes.

Keywords: gut microbiota; athletes; muscle functions; gut-muscle axis

1. Introduction

The intestinal microbiota consists of microorganisms inhabiting the gastrointestinal tract, with the estimated number exceeding 10^{14} cells. The genome size of microbiota exceeds the human genome by 150 times, which encompasses around 10 times more bacterial cells than all human cells and over [1,2]. The biodiversity and overall composition of the gut microbiota plays a crucial role in maintaining normal homeostasis within the human body. Bacteria are the most abundant population of the gut microbiota, with more than 1000 different bacterial species being observed. The human gut microbiota consists mainly of four phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* [2]. An imbalance among these phyla may alter the microecological environment of the gastrointestinal tractand contribute to the development of various diseases. Intestinal bacteria are involved in many functions and have been shown toimpact the nutritional status of the host, metabolic functions, and maturation of the immune system, as well as epithelial cell maturation. Moreover, these bacteria protect against pathogens and may influence brain function [3,4]. Additionally, the composition of gut microbiota varies individually and may be modified by several factors, such as: genetic background

(but to a smaller extent), age, sex, place of residence, and drug administration [5,6]. However, diet and the level of physical activity are the main determinates for altering the biodiversity or changing the levels of a specific bacterial species within an established gut microbiota [7].

Moderate physical activity has a multidirectional and beneficial effect on the human body. Training stimulation causes physiological and metabolic adaptations. Main changes in the skeletal muscles include the increase in mitochondrial biogenesis and enhancing their function, concentration of the substrate transporting proteins, activity of the enzymes involved in metabolic pathways, and glycogen storage in the muscle [8]. As a result of regular exercise, muscle protein synthesis is intensified with changes that vary based on the intensity of the training. It is regulated by physical and chemical mechanisms [9]. In brief, the signalling pathways include short-term alterations to protein turnover and genes expression, as well as long-term changes to metabolism within the cells. Additionally, the activation of the mammalian target of rapamycin kinase (mTOR) plays a crucial role for increasing muscle protein synthesis, through its phosphorylation of initiating substrates and its promotion of translational signalling for anabolism [9]. However, it should be noted that excessive exercise may limit muscle building and cause a net loss of muscle mass via promoting inflammation and nutrient restriction, as well as oxidative and metabolic stress. In this scenario, excessive exercise may lead to the activation of the muscle atrophy pathways, increasing the levels of nuclear factor kappa B (NF-κB) or Forkhead box O (FOXO) in their phosphorylated forms [10,11].

Although multiple studies have illustrated that moderate physical activity has a beneficial effect on the gut microbiota, it is unclear if the gut microbiota influences muscle adaptation to extensive training. A recent study showed that excessive exercise among professional athletes may disturb the homeostasis of the gut microbiota [12]. Specifically, high volume training was associated with increased muscle requirements for oxygen and nutrients. Furthermore, long-term deterioration of the intestinal blood perfusion has been shown to cause temporary ischemia, leading to the dysfunction of the mucous membrane and increase of intestinal permeability [13]. As a result, substantial changes of the microbiota profile were observed via promoting a bloom of opportunistic pathogens and their associated toxins. Consequently, it could lead to the translocation of pathogens and bacterial toxins into the bloodstream, resulting in the activation of local and systemic inflammatory pathways [14]. From these studies, it is clear that the maintenance of a healthy microbiome, within the gut, does influence muscle adaptation to training. Specifically, the microbiota may have an indirect role through its modulation of inflammatory pathways and anabolic and catabolic processes, as well as the regulation of nutrient availability and metabolite production.

2. The Link between Diet, Physical Activity, and Microbiota

The composition and quality of diet significantly affect the exercise capacity of athletes. Adequate energy, macro- and micronutrients intake are essential to optimize protein synthesis, increase of energy reserves during exercise, improve regeneration after training, and reduce the risk of injury. Insufficient energy intake has multiple negative consequences, referred to as relative energy deficiency in sports (RED-S) [15]. It may impair sport performance through endocrine or immune system disorder, insufficient muscle glycogen storage and microbiome imbalance [16]. As such, the intake of carbohydrates, fats, and proteins, as well as the preservation of a healthy gut microbiome, are essential for maintaining an athlete's exercise capacity.

As the main indirect energy substrate for skeletal muslces, carbohydrates and their storage as glycogen, have a clear role in proper muscle function during both aerobic and anaerobic exercise. Specifically, an individual's ability to store carbohydrates as glycogen has been shown to affect mitochondria biogenesis and function as well as acting as a specific regulator for signalling pathway involved in training tolerance [17,18]. Intestinal bacteria also have a role for mainitaining exercise capacity through regulation of carbohydrates. They promote the colon fermentation of carbohydrates to produce short-chain fatty acids (SCFAs) from undigested fragments. SCFAs are characterized by a multiple of positive effects on the host organism, including the improvement of metabolic function

and enhancement of intestinal epithelial membrane [19,20]. Moreover, diets that reduce carbohydrate intake are linked to negative effects on exercise capactity, due to the association with increased fat consumption.

A low-carbohydrate diet with a high fat content impairs exercise economics and inhibits the growth of workout-induced aerobic fitness, in contrast to the high-carbohydrate diet [21]. Additionally, excessive fat intake may also significantly affect the composition of the intestinal microbiota limiting substrates to SCFAs production. Animal studies have shown an increase in the number of bacteria that induce pro-inflammatory cytokines synthesis elevates the content of plasma lipopolysaccharide (LPS) as well as enhancing NF-kB expression, linked to turning on the genes of pro-inflammatory character [22]. The high-fat diet also reduces the diversity of bacterial strains and the abundance of *Bacteroidetes*, promoting the growth of *Firmicutes* and *Proteobacteria* [23]. Furthermore, an increased amount of sulfate-reducing bacteria have also been demonstrated. These bacteria may produce sulfides, which lead to the reduction of disulfide bonds in the mucus and the breakdown of gel-forming polymer protein networks MUC2 secreted by goblet cells. These alterations play pivotal role in mucosal regeneration and mucus layer stability. An impaired mucosal barrier may exacerbate intestinal mucosa inflammation and promote inflammatory diseases [24]. All these observations were reported in the case of high-fat diet, containing mainly saturated fats and processed food. However, in the case of omega-3 acids and conjugated linoleic acid (CLA) unfavorable changes were not found. Their consumption increased butyrate synthesis and the *Bacteroidetes/Firmicutes* ratio [25].

Adequate protein intake is essential for maximizing muscle adaptation to the training processes, conducive to hypertrophy and muscular strength [26]. However, excessive protein intake causes an increase in the number of protein fermenting bacteria such as *Clostridium*, *Desulfovibrio*, *Peptostreptococcus*, *Acidaminococcus*, *Veillonella*, *Propionibacterium*, *Bacillus*, *Bacteroides*, *Staphylococcus*, and other species of the *Proteobacteria* family [27]. It was also associated with reducing the number of carbohydrate fermenting bacteria such as *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Prevotella*, *Ruminococcus*, *Roseburia*, and *Faecalibacterium* [28,29]. The fermentation of undigested protein residues in the colon, accompanied by the production of by-products, such as ammonia, biogenic amines, indole compounds, and phenols, have a potentially harmful effect on the intestine, metabolism, immunological, and neurological functions. These compounds may exacerbate the inflammatory response, increase tissue permeability, and intensify gastrointestinal symptoms [30]. It appears that protein overconsumption may be offset by higher carbohydrates intake, especially indigestible polysaccharides, which are the preferred substrate for intestinal bacteria [30].

Moderate training has a beneficial effect on the diversity of bacterial species inhabiting the gastrointestinal tract. The microbiome of various athletes has been correlated with high diversity and increased levels of bacterial genes involved in protein and carbohydrate metabolism and SCFAs production [31,32]. In addition, research conducted on cyclists showed that higher activity for carbohydrate metabolizing bacteria correlated with the frequency of exercise. Moreover, increasing the number of *Prevotella* was demonstrated to positively affect amino acid metabolic pathways, such as lysine biosynthesis, the metabolism of alanine, aspartate, and glutamate, D-glutamine and D-glutamate, as well as carbohydrate metabolism. In high-performance athletes, a larger share of methane-producing bacteria from the *Methanobrevibacter Smithii* family was also associated with an excessive production of energy and carbohydrate metabolism [33]. The study conducted by Durk et al. also found a positive link between the level of training expressed by maximal oxygen uptake (VO_{2max}) and the *Firmicutes/Bacteroidetes* ratio [34]. From an inflammatory standpoint, training-induced changes in intestinal microbiome composition seem to be beneficial to host health. Regular exercise may also support brain functions via enhancing the neuroprotective effect. As a result of training, an increase of gene expression of the kynurenine aminotransferases occurred, which are responsible for the conversion of the toxic metabolite tryptophan—kynurenine to the neuroprotective kynurenic acid. Inflammatory cytokines such as tumor necrosis factor α (TNF-α) have also been shown to promote the degradation of kynurenine to toxic quinolinic acid [35]. In addition, it seems that the optimal intestinal microbiota composition may

have a positive effect on the brain function and preventing depression, by modulating inflammation and affecting tryptophan metabolism. All of these may indirectly affect the quality of physical training [36].

As stated previously, excessive training may introduce microecological imbalances via intestinal ischemia, increased intestinal barrier permeability, and elevated oxidative stress. This leads to the exacerbation of inflammatory responses, and consequently, to increased catabolism along with muscle function deterioration. Adverse effects may also result from an increase ofa number of potentially harmful bacteria, such as *Peptostreptococcus*, *Staphylococcus*, *Peptoniphilus*, *Acidaminococcus*, and *Fusobacterium*, and a decrease of anti-inflammatory species including *Bacteroides*, *Faecalibacterium*, *Collinsella* and *Roseburia*. This was clearly shown in the study conducted by Karl et al. that analyzed stool samples of soldiers under prolonged physiological stress [37]. They showed an indirect relationship between intestinal microbiota composition, lifestyle, and skeletal muscle function. It supports the hypothesis of the gut-muscle axis and the necessity of targeted therapy for the microbiota athletes.

During physical training, there is an overproduction of reactive oxygen species (ROS), as a result of increased skeletal muscle effort. ROS generation causes lipid and protein peroxidation, muscle cell membranes components disruption, which all together consequently disturb muscle function [38]. Therefore, both training overload and lack of physical activity, as well as immobilization, raise oxidative stress [39,40]. On the other hand, regular training leads to the adaptation of antioxidant enzymes, increasing the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). It also reduces the damage caused by free radicals and increases the antioxidant potential and the activity of enzymes responsible for repairing damages caused by ROS [41]. These findings were supported by studies conducted by Maleki et al. They demonstrated that higher SOD and CAT activity, together with lower ROS levels occurred in the semen of participants performing recreational training compared to inactive participants or professional athletes [42]. Similar observations were made by Brinkmann et al., who reported that moderate intensity exercises induced higher SOD and GPx activity in the skeletal muscle [43]. In addition, ROS production has been shown to have a positive effect on aerobic potential by activating PGC-1α proteins. It leads to the increase of mitochondrial biogenesis and consequently the improvement of the aerobic capacity [44]. Previous studies have shown that ROS regulate muscle protein synthesis by affecting mitogen-activated protein-kinase (MAPK) activity, which supports the pro-anabolic insulin-like growth factor 1 (IGF-1) [45]. Recently, it has also been suggested that excessive supplementation of antioxidants can reduce cytochrome c oxidase and citrate synthase content, which impairs the electron transport chain (ETC) functions [44].

The intestinal microbiome may also contribute to oxidative stress reduction. Some bacterial strains have antioxidant properties through various mechanisms. These include the expression of antioxidant enzymes, modulation of inflammation caused by pro-inflammatory cytokines or presence of pathogens, and metabolism regulation through greater absorption of antioxidants [46]. Specifically, some studies have shown that bacterial species such as *Lactobacillus plantarum*, *Lactobacillus gasseri*, *Lactobacillus fermentum*, *Lactococcus Lactis* and *Streptococcus thermophilus* are able to increase SOD activity [47]. Additionally, *Lactobacillus*, *Lactococcus*, and *Bifidobacterium* genera have all been shown to elevate intestinal glutathione (GSH) levels, which plays a crucial role in scavenging of the hydroxyl radical (OH*) [47]. Similarly, animal studies have demonstrated that individuals whose microbiota was richer in *Escherichia coli* and *Enterococci*, while being poorer in *Lactobacilli*, had higher susceptibility to oxidative stress [48]. Martatelli et al. conducted a trial with athletes, showing that a *Lactobacillus rhamnosus* and *Lactobacillus paracasei* probiotic species supplementation increased plasma antioxidant levels and neutralized ROS generation as a response to high-intensity exercise. Probiotic supplementation was also associated with lower plasma reactive metabolite levels and higher plasma biological antioxidant potential, after afour-week intensive physical training period [46]. Overall, these findings clearly support the essential need to balance a proper diet, an adequate exercise regime, and a healthy microbiome to promote higher glycogen storage to increase mitochondrial function and muscle building. On the other hand, an inadequately balanced diet and an insufficient or excessive training

regime, as well as a dysfunctional microbiome, are all associated with increased inflammation, oxidative stress, a reduction in mitochondrial function, and the potential for muscle atrophy (Figure 1).

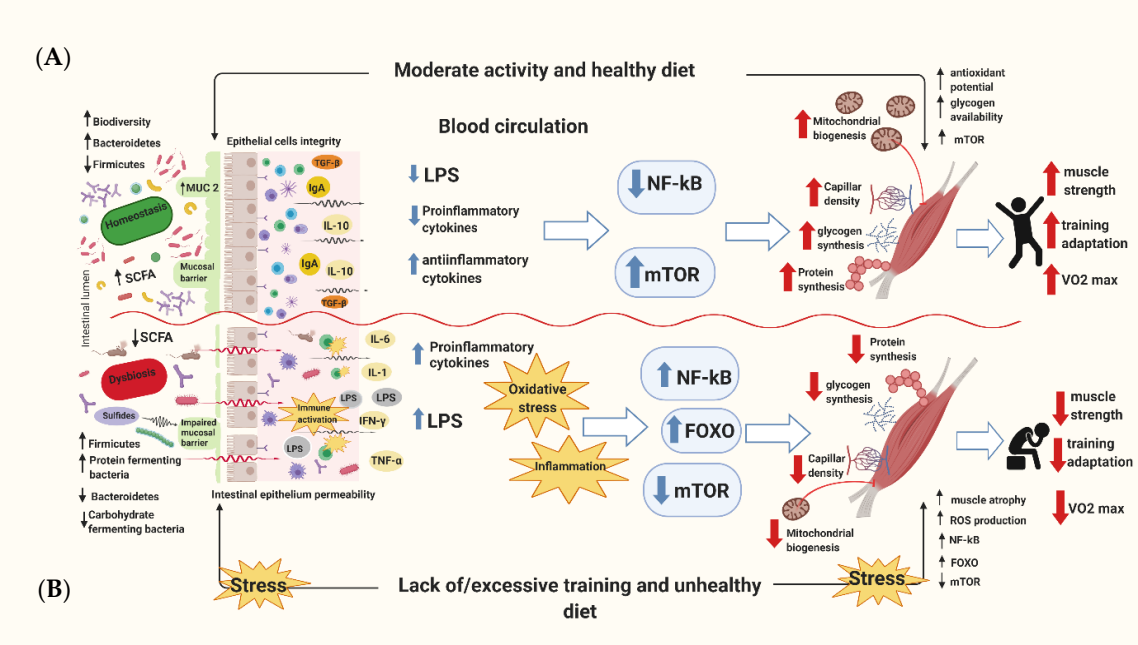

Figure 1. The schematic diagram of combined healthy/unhealthy diet and exercise/lack of exercise action on human skeletal muscle. (**A**) A properly balanced diet and systematic moderate exercise show both direct and indirect effects to benefit skeletal muscle function by reducing oxidative stress and inflammation status. As a result, this shifts to the higher muscle glycogen storage and increased mitochondrial biogenesis and function, as well as the predominance of anabolic signaling pathways, which increasethe aerobic exercise capacity. (**B**) The opposite effects are observed in the case of an inadequately balanced diet and insufficient or excessive physical effort. This leads to an increase in inflammatory and oxidative stress markers, a decrease in the ability to store muscle glycogen and a reduction of mitochondria function, as well as muscle atrophy and the higher accumulation of body fat.

3. The Effect of Microbiota on Anabolic and Catabolic Processes

The intestinal microbiome may affect the metabolism of human skeletal muscles through several pathways. Evidence regarding the relationship between microbiota composition and muscle function have been described in the pathogenesis of age-related sarcopenia. It was noted that muscle atrophy correlates with a decrease in the number of species sending anti-inflammatory and pro-anabolic mediators. Sarcopenia is associated with a reduction of muscle capillaries and a decrease of insulin sensitivity and inflammation severity, leading to declined mitochondrial biogenesis and function as well as protein synthesis disruption [49].

Sarcopenia and systemic weakness among the elderly have been correlated with intestinal dysbiosis, contributing to increased intestinal barrier permeability, elevated blood LPS levels, activation of the immune system, and areduction of insulin sensitivity [50]. Moreover, animal studies clearly emphasize the reduction of muscle atrophy markers (Atrogin-1, MuRF1, LC3 protein, Cathepsin L) in mice supplemented with *Lactobacillus* strains as well as an increase of muscle mass and strength in mice supplemented with *Lactobacillus plantarum* [51,52]. In addition, Buigues et al. demonstrated 13-week multistrain *Lactobacillus* and *Bifidobacterium* probiotic mixture supplementation enhanced endurance and muscular strength in older individuals. The study showed that older patients who received fructooligosaccharides and inulin experienced a significant improvement in hand grip strength andself-reported exhaustion level [53].

The lack of homeostasis was associated with an increased abundance of endotoxic gram-negative bacteria responsible for thesystemic inflammation via LPS. It has also been noticed that *Escherichia/Shigella*, *Klebsiella*, and *Citrobacter* species significantly contribute to the LPS pool [54]. Elevated serum LPS levels have been correlated with the increased *Firmicutes/Bacteroidetes* ratio [55]. Consequently, the presence of LPS in bacterial cell walls causes binding of the lipid A to the surface of immune cells receptors, containing TLR4 and bone marrow differentiation factor 2 (DM2). LPS are recognized by TLR4 in combination with CD14 and DM2, and therefore, may induce the NF-κB activation, which plays a key role in the production of pro-inflammatory cytokines [14,56]. Additionally, elevated LPS levels have been connected with intestinal homeostasis disruption and correlated with an increase in blood intestinal permeability markers such as zonulin and fatty acid-binding protein 2 (FABP2) [57]. This augmented permeability of the intestinal epithelium is associated with bacterial translocation from the intestinal lumen to the lamina propria, activating the immune system and promoting inflammation. However, it should be noted that *Actinobacteria* genus bacteria, such as *Bifidobacterium* or *Collinsella*, have been shown to have anti-inflammatory and immunomodulatory properties that can support intestinal epithelial function. Therefore, probiotics containing *Bifidobacterium* strains may reduce the inflammatory response caused by physical stress [58].

SCFA produced by intestinal bacteria have also been demonstrated to have a positive effect on the integrity of the intestinal barrier, protecting it against inflammation. Specifically, the *Candida Albicans* genus has been shown to be engagedin pro-inflammatory TNF-α induction [59]. Dysbiosis has often been accompanied by an increase in the amount ofgram-negative bacteria that have endotoxic properties and upregulate pro-inflammatory cyotkines like IL-6 [60]. Elevated intestinal permeability level and associated passage of pathogens into the bloodstream induce IL-1, TNF-α, and interferon gamma (IFN-γ) secretion, causing a pro-inflammatory effect [61]. The intestinal microbiota composition can also affect the inflammatory suppression by stimulating secretion of anti-inflammatory cytokines, such as transforming growth factor (TGF-β) and IL-10. It has been proven that *Bacteroides fragilis* bacteria were able to suppress the expansion of Th17 lymphocyte by producing IL-10 via the TLR2 [62]. *Lactobacillus* and *Bifidobacterium* families are associated with inflammation reduction, by affecting the secretion of anti-inflammatory cytokines such as IL-10, TGF-β, and tryptophan-2,3-Dioxygenase (IDO), causing Treg stimulation as well as Th1, Th2, and helper lymphocytes Th17 inhibition [63]. Dysbiosis through the loss of immune tolerance impairs epithelial and intestinal barrier functions. Consequently, this disturbs the balance between pro- Th17 and anti-inflammatory Treg lymphocytes.

Muscle protein synthesis and training adaptation may be limited under chronic inflammation. Notably, satellite cells located between the basal lamina and plasma membrane for muscle fibers play a key role during regeneration and muscle growth [64]. Muscle fiber synthesis and breakdown are under the control of many crossing signaling pathways, which determinate anabolic and catabolic processes. Two E3 ubiquitin ligases, belonging to the ubiquitin-proteasome system, are mainly involved in muscle protein degradation: Atrogin-1 and Muscle RING finger protein (MuRF1). The increase of their transcription activity is regulated by the NF-kB nuclear factor and phosphorylated FOXO proteins. Therefore, the inhibition of these signalling pathways are associated with protection against skeletal muscles atrophy [65,66]. The secretion of pro-inflammatory cytokines also activates NF-kB, contributing to skeletal muscle loss. This is mainly mediated by TNF-α, capable of activating IκB (IKKβ), whose active form may phosphorylate IκB proteins, thereby triggering the NF-kB signaling and changing gene transcription towards catabolism [67].

Myofibrillar protein synthesis is dependent on extracellular signals that activate intracellular molecular pathways. It seems that mTOR plays a crucial role in the process of muscle protein synthesis. Its activation leads to the intensification of anabolic processes, through the integration of signalling pathways that increase translational efficiency and the phosphorylation of initiate substrates [9]. mTOR phosphorylation may be stimulated by either training or nutritional support. Mechanical contractions during resistance training results in the release of IGF-1 from skeletal muscles, capable of mTOR activation. Protein or amino acid intake also contributes to enhanced mTOR signalling,

demonstrating a synergistic effect to the exercise stimulus [68]. IGF-1 secreted into the extracellular matrix is bound by specialized IGF-binding proteins (IGFBP), enabling the activation of specific receptors that process the anabolic signal [69].

Physical training leads to a decrease of adenosine triphosphate ATP level and disturbances in the ATP/AMP (adenosine monophosphate) ratio, causing an energy stress occurrence. The higher concentration of AMP stimulates AMP-activated protein kinase (AMPK) to equalize energy resources by initiating catabolic processes. AMPK promotes aerobic and anaerobic energy production, inhibits glycogen as well as cholesterol synthesis, and induces mitochondrial biogenesis through PGC-1α expression [70]. The biological role of AMPK also controls the circulation of cellular components by reducing mTOR activity and promoting protein breakdown. The elevated AMPK level positively correlates with the increase of FOXO protein activation [71]. The stress response causes FOXO proteins phosphorylation, which intensifies autophagy genes transcription, contributing to the protein breakdown (mainly FOXO3). However, regular exercise does induce autophagy, which isa necessary step prior to muscle fiber rebuilding. It is clear that elevated autophagy is associated with impairments in muscle growth and function [70,72].

Excessive training load and insufficient regeneration periods may cause exhaustion and a temporary weakening of sport performance. Therefore, appropriate regeneration after exercise is an important element in training adaptation [73]. Physical exercise-induced tissue damage is a physiological part of the adaptation process; however, chronic training overload and insufficient regeneration may adversely affect the athlete's well-being and sport capabilities [74]. Specifically, the tissue damage caused by excessive training may result in an acute and local inflammatory response, consisting of cytokines overproduction, mainly interleukin-1b (IL-1b), TNF-a, interleukin-8 (IL-8), and interleukin-6 (IL-6) aimed at rebuilding the damaged structures and promoting muscle adaptations. As a result, there is an activation of circulating monocytes, capable of pro-inflammatory cytokines induction and causing systemic inflammation [75,76], leading to insulin resistance, endoplasmic reticulum stress, and, as a result, muscular atrophy [73]. Moreover, ROS generation may disrupt protein synthesis, promote inflammatory response, and reduce the efficiency of post-workout regeneration processes [77].

Jäger et al. have demonstrated the beneficial effect of using *Streptococcus thermophilus* FP4 and *Bifidobacterium breve* BR03 strains supplementation, to regulate the inflammation state and enhance muscle training adaptation. The study showed that 21 days of probiotic supplementation period decreased blood IL-6 level, 48 h after eccentric exercise in 15 trained men. It also reduced the movement limitations caused by training, contributing to a shortening of the regeneration period [78]. The positive effects on inflammation parameters and muscle functions were also demonstrated by Wen-Ching et al. They have noticed that long-term *Lactobacillus plantarum* PS128 supplementation in triathletes resulted in the reduction of plasma creatine kinse (CK) level. Additionally, other significant improvements were found across various markers of inflammation and oxidative stress during the regeneration phase. These improvments manifested in myeloperoxidase (MPO) and IL-10 elevation as well as a TNF-α, IFN-γ, IL-6, and IL-8 decrease [79]. The effectiveness of probiotic supplementation was also demonstrated by Townsend et al., who showed that 12 weeks of *Bacillus subtilis* DE111 treatment reduced TNF-α levels, without altering other inflammation parameters [80]. Another study, conducted by Roberts et al., clearly displayed the positive effect of using a multi-strain probiotic (*Lactobacillus acidophilus* CUL-60, *Lactobacillus acidophilus* CUL-21, *Bifidobacterium bifidum CUL*-20, and *Bifidobacterium animalis*) for 12weeks on the intestinal permeability of triathlonists. The probiotic combined with fructooligosaccharides and α-lipolic acid supplementation was associated with a reduction in blood endotoxins level compared to the control group [81].

4. Bacterial Products and Their Effect on Muscle Function

Intestinal bacteria can affect the human body by producing a variety of biologically active metabolites. One of the best-known bacterial metabolites are SCFAs. It was considered that SCFAs may

be provide the source of up to 10% of total daily energy demands [82]. Butyrate, acetate and propionate are the most known SCFAs, representing as much as 95% of all SCFAs.

It seems that butyrate plays a key role in regulating cell growth and differentiation [83]. The *Roseburia*, *Clostridia*, and *Eubacteria* genus are main butyrate producers [4]. There are a number of anti-inflammatory properties associated with butyrate, such as enhancing intestinal barrier integrity, promoting antimicrobial peptides secretion, Treg lymphocyte activation, regulation of neutrophil migration, TLR silencing, decrease of pro-inflammatory cytokines production, and lymphocyte or granulocyte activity suppressing. Additoinally, butyrate has been shown to suppress the inflammatory response by altering NF-kB and protein kinase B (AKT) signalling [84] and antagonizing LPS. Additionally, it reduces intestinal permeability, improves tissues' insulin sensitivity, increases lipolysis, and stimulates skeletal muscle glucose uptake [49].

Similar anti-inflammatory properties have been observed for acetate. It affects glucagon-like peptide-1 (GLP-1) and YY peptide secretion, resulting in appetite inhibition, lipolysis, and energy expenditure increase. Moreover, acetate has a beneficial effect on skeletal muscle by stimulating glucose uptake and increasing insulin sensitivity [85]. Propionate and butyrate regulate the secretion of intestinal hormones, improving insulin sensitivity and affecting glucose metabolism [86], becoming a gluconeogenesis precursor and lipogenesis inhibitor [87].

The direct relationship between SCFA and skeletal muscles is mediated by muscular AMP kinase and the deposition of proteins in skeletal muscle tissue. SCFA activate AMPK by increasing the AMP/ATP ratio or via the Ffar2-leptin pathway, but the exact mechanism is not known [88]. Intestinal bacteria may produce secondary bile acids, having antibacterial activity. It has been shown that microbiota may affect the liver and the skeletal muscle receptors, modulating the activity of the farnesoid X receptor (FXR) [89]. This receptor plays an important role in energy metabolic pathways, lipoprotein and glucose turnover. Intestinal microbiota, by alleviating FXR inhibition may contribute to the metabolic balance maintenance and myocyte anabolism. In addition, bile salts may be transformed into immunomodulatory and anti-inflammatory compounds in the intestine [90,91].

5. Microbiome and the Availability of Nutrients

Intestinal microbiota affects the availability and profile of amino acids by participating in their digestion and absorption. Notably, *Fusobacterium*, *Bacteroides*, *Veillonella*, *Megasphaera elsdenii*, and *Selenomonas ruminantium* are all involved in proteolysis, increasing the disposal of amino acids [92]. In addition, some bacterial species such as *Streptococcus bovis*, *Selenomonas ruminantium*, and *Prevotella bryantii*, in the presence of physiological peptide concentrations are involved in *de novo* biosynthesis of amino acids [93]. Intestinal bacteria are crucial for tryptophan metabolism by its direct consumption, thus limiting the availability to the host organism [36]. On the other hand, the intestinal microbiota composition is a key determinant of tryptophan metabolites level in the circulation and serotonin (5-HT) in the brain [92] consequently negatively affects muscle training adaptation.

Another crucial role of microbiota is in the production of vitamins, such as folates, riboflavin (B_2), cobalamin (B_{12}), and vitamin K. Vitamins B are necessary for myocytes anabolic processes through various pathways and a several of metabolic functions, including DNA replication and repair and nucleotide and amino acid synthesis, as well as oxidative stress regulation. *Bifidobacterium longum*, *Bifidobacterium bifidum*, and *Lactobacillus reuteri* are all involved in vitamin synthesis [94]. Intestinal bacteria are also able to metabolize polyphenols, but their efficiency may decrease under unfavourable conditions within the gut. Polyphenols have antioxidant and anti-inflammatory properties and also contribute to mitochondrial biogenesis and function [95].

Lactate utilizing bacteria seem to have an important role for athletic exercise capacity. Lactate is able to penetrate from the serum into the intestinal lumen where it is converted to SCFAs, mainly propionate. Then, the SCFAs enter directly into the circulation where through the Cori cycle transformations, become an additional energy source [96]. Recent studies conducted by Scheiman et al. have shown the important role of *Veillonella atypica* genus, whose only source of carbon is lactate. The number of these

bacterial genera was elevated in the intestines of high-performance athletes. It has also been shown that transplantation of *Veillonella atypica* genus into mice was associated with a significant running time improvement. Therefore, it has been reported that the modulation of enzymes and conversion of lactate to propionate has a role in improving atheletic performance [97]. Lastly, animal models have illustrated the role of SCFA (mainly propionate) in maximizing oxygen uptake and elevating heart rate, while in humans it may cause a resting energy expenditure increase [98,99].

6. Glucose Metabolism

In the light of the current knowledge, the expression of intestinal receptors Gpr41 and Sglt1, involved in glucose transport and energy balance, is associated with an increase in skeletal muscle oxygen metabolism. Bacterial SCFA are able to activate Gpr41 receptors, affecting the endocrine pathway to release the glucagon-like peptide 1 (GLP-1), stimulating insulin secretion [100,101]. A similar mechanism is observed in the case of the sodium glucose co-transporter Sglt1, responsible for glucose homeostasis. Nay et al. have reported that antibiotic-treated mice showed reduced expression of Gpr1 and Sglt1 genes, which is correlated with muscle glycogen content reduction compared to the control group [101].

The intestinal dysbiosis, often caused by antibiotic therapy, contributes to alterations in SCFAs and bile acids (BA) synthesis, which was shown in Zarrinpar et al. The limitation of butyrate, the main energy source for enterocytes, causes glucose compensation. Consequently, this translates into low serum glucose levels as well as insulin sensitivity and increased hepatic gluconeogenesis [102]. It has also been reported that intestinal dysbiosis may reduce skeletal muscle glucose availability, resulting in the reduction of glycogen storage. The glycogen content in muscles is a key factor determining an athletes' aerobic energy metabolism. Glycogen level disturbances may cause muscle strength and function deterioration, leading to bioenergetic metabolism impairment [18]. This concept was supported by another study, which correlated between intestinal microbiota composition and muscle glycogen content. Germ-free mice were shown to have lower muscle glycogen levels compared to individuals with normal microbiome composition [101]. This data demonstrates the important role of microbiota in skeletal muscle function by improving the availability of energy substrates such as glucose.

7. The Interaction between Microbiota and Mitochondrial Function

Intestinal microbiota may affect mitochondrial functions in various ways. LPS, produced mainly by pathogenic bacteria, activates NF-kB signalling and an inflammatory response, through TLRs, resulting in pro-inflammatory cytokine production. TLR activation indirectly increases ETC activation, leading to mitochondrial ROS generation [103]. It has been noted that the growth of pathogenic *Listeria monocytogenes* species contributes to mitochondrial networks fragmentation, disrupting their function [104]. Other intestinal bacteria such as *Mycobacterium tuberculosis* and *Ehrlichia chaffeensis* have been shown to reduce ROS generation, by inhibiting LPS-initiated pathways or by increasing SOD activity [103].

Moreover, it has also been reported that amino acid-reducing bacteria, e.g., *Escherichia coli* and *Salmonella*, are capable of hydrogen sulfide (H_2S) production. In large quantities, H_2S inhibits the mitochondrial ETC, by lowering cytochrome c oxidase activity [103]. Other bacterial metabolites, such as SCFAs, may contribute to the regulation of aerobic energy metabolism in the skeletal muslces. This mainly occurs through butyrate and its ability to enter the Krebs cycle to increase its efficiency [105]. However, recent data has suggested that isovanillic acid 3-O-suflate (IVAS) may also have a positive effect on the glucose absorption and metabolism in human cells. IVAS was shown to increase glucose transport in a dose-dependent manner by activating GLUT-4 and GLUT-1, phosphatidylinositol 3-kinase (PI3K) and AKT phosphorylation [106]. PI3K seems to be crucial for muscle metabolism and mitochondrial homeostasis by modulating insulin sensitivity [107].

8. Microbial Modulation of Neuroactive Molecules

Recently, multiple studies have supported the existence of a gut-brain axis (GBA) that enables bidirectional communication between these two organs. Its signalling pathways consist mainly of afferent and efferent neurons proceeding through the sympathetic and parasympathetic fibers of the autonomic nervous system (ANS). Using that bidirectional cross-talk, intestinal signals are able to affect the brain function, regulating mood or even reflex activity. Similarly, the central nervous system may alter gastrointestinal's (GI) track motility and acid secretion in the stomach and control the defecation process [108,109].

It has been established that the gut microbiota plays a crucial role in gut-brain communication by generating some neuroactive molecules. For example, strains of *Lactobacillus* genus were demonstrated to produce γ-aminobutyricacid (GABA), an important inhibitory transmitter in the brain. Similarly, other bacterial species were shown to be capable of noradrenaline (e.g., *Bacillus mycoides*, *Bacillus subtilis*), dopamine (e.g., *Bacillus cereus*, *Bacillus mycoides*, *Bacillus subtilis*), and serotonin (e.g., *Lactococcus lactis*, *Lactobacillus plantarum*, *Streptococcus thermophilus*) synthesis [108,110,111]. Therefore, it is clear that intestinal bacteria have the potential to alter neurotransmitter activity, thus interacting with the host nervous system to regulate mental health, and consequently, metabolism and exercise capacity.

Supporting these findings, a recent systemic review illustrated how moderate training contributes to the elevation of GABA level in the hypothalamus, which is associated with lowered resting blood pressure, heart rate, and sympathetic tone. In addition, dopamine was shown to be synthesized in the GI tract, during stressful situations. On the other hand, training overload was reported to cause muscle exhaustion and modifications in the CNS leading to mood disturbances, fatigue, insomnia, and depression. The central fatigue was associated with the elevation of 5-HT release and could lead to suboptimal physical performance. Consquently, this reduction in 5-HT levels in the brain could lead to the manifestation of mood disorders, depression, distorted cardiac function, and changes in blood pressure. Overall, gut microbiota was shown to facilitate the production and regulation of neurotransmitters and hormones, which consequently affected athletes well-being, mood, motivation, and subjective sense of regeneration [112].

Interestingly, the study conducted by Bravo et al. presented that chronic supplementation with *Lactobacillus rhamnosus* caused alternations in central GABA receptors expression, reducing stress-induced corticosterone (CORT) as well as anxiety- and depression-related behavior [113]. Furthermore, 5-HT levels were shown to be lower in the blood and colon of GF animals as compared to their typically colonized counterparts. It was suggested that this effect was dependent on bacterial molecules such as SCFA [114]. Additionally, Crumeyrolle-Arias et al. demonstrated the important role of gut bacteria in response to stress. GF mice exhibited higher serum CORT concentrations, elevated corticotropin-releasing factor mRNA expression in the hypothalamus and lower dopaminergic turnover rate in the hippocampus compared with specific-pathogen free mice. These changes suggest that the lack of the gut microbiota exacerbates stress response [115]. Furthermore, the chronic elevation in endogenous glucocorticoids levels may have decreased the rate of protein synthesis and increased proteolysis, to generate amino acids that serve as precursors for hepatic gluconeogenesis. However, in skeletal muscles, this may lead to the development of oxidative stress [116] and skeletal muscle atrophy, as well as muscle weakness [117,118]. Based on these data, we presumed that the gut microbial composition plays a crucial role in the development and function of an appropriate stress response via hypothalamus–pituitary–adrenocortical axis regulation, andas a consequence, exercise abilities in athletes.

9. Impact of the Microbiome on Exercise Capacity

Numerous researches have indicated the validity of intestinal microbiota-targeted strategies to improve training parameters and increase training capabilities, as presented in Table 1. The researches indicate the ability of intestinal microbiota to alleviate oxidative stress and exercise-induced inflammation [78–80,119,120]. A trial conducted by Jager et al. have shown that *Bacillus coagulans*

GBI-30 probiotic supplementation improves the anaerobic capacity measured by the Wingate Test [121]. The positive properties of probiotic supplementation on post-workout regeneration have been presented by Carbuhn et al. as well as Huang et al., using *Bifidobacterium longum* 35624 [122] and *Lactobacillus plantarum* PS128 [79], respectively. In both study groups, athletes reported a feeling of faster recovery time in the probiotic group compared to the placebo groups.

Animal studies have also shown a positive probiotic effect on the aerobic fitness of athletes through extending the exercise to exhaustion time. Hsu et al. have observed muscle mass and endurance augmentation, as well as the antioxidant potential in mice with optimal intestinal microbiota composition [119]. These observations were consistent with the subsequent medical experiment carried out by Chen et al. [52]. Similar reports come from Scheiman et al., who demonstrated the bacterial role in lactate utilization, and thus in increasing exercise capacity [97].

Table 1. The effect of microbiota on exercise.

References	Study Model	Type of Exercise	Intervention	Beneficial Effect of Intervention on Direct and Indirect Parameters of Sports Performance
Hsu et al. 2015 [119]	Mice	Endurance swimming	**Threestudy groups:** germ free (GF) vs. *Bacteroides fragilis* (BF) comparison with no probiotic (specific pathogen-free (SPF)	↑ activity of serum glutathione peroxidase (GPx) and catalase (Cat) ↑ activity of liver GPx ↑ muscle mass ↑ antioxidant properties ↑ free radical damage protection ↑ muscle mass endurance (extended exercise to exhaustion time) No differences in liver superoxide dismutase (SOD) and Cat activity
Unsal et al. 2018 [120]	Rats	Exhaustive swimming trial	**Fourstudy groups**: control, placebo, exercise, exercise+probiotic **Study product**: multi strain probiotic mixture VSL#3 (*Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii subsp. bulgaricus*, *Bifidobacterium longum*, *B. breve*, and *B. infantis*, *Streptococcus salivarius*)	↓ oxidative stress ↑ antioxidative enzymes activity ↑ antioxidative balance
Scheiman et al. 2019 [97]	Mice	Exhaustive treadmill run	**Twostudy groups:** control and supplemented **Studyproduct**: *Veilonella*, propionic	↑ lactate utilization ↑ blood short-chain fatty acid (SCFA) concentration ↑ extended exercise to exhaustion time (treadmill workout) ↑ Cori cycle efficiency
Chen et al. 2016 [52]	Mice	grip strength and endurance swimming	**Threestudy groups:** vehicle, 2.05^108CFU/kg (LP10-1X), and 1.03^109CFU/kg (LP10-5X). **Study product**: *Lactobacillus plantarum* TWK10 (LP10)	↑ relative muscle mass and strength ↑ number of type 1 muscle fibers ↑ extended exercise to exhaustion time (swimming trial) ↓ post-workout lactate blood concentration ↓ post-workout ammonia blood concentration ↓ post-workout CK ↓ post-workout ammonia, albumin, creatinine and triglyceride concentration All above changes were dose-dependent
Hoffman et al. 2019 [123]	Soldiers	vertical jump power, two times 100-m shuttle runs	**Twostudy groups**: *Bacillus coagulans* and placebo **Studyproduct:** *Bacillus coagulans*	↑ interferon gamma (IFN)-γ and interleukin-10 (IL-10) concentration ↑ mean jump power No effects on 60 s pull-ups, 100-m shuttle run, shuttle run fatigue rate No effects on cortisol and testosterone concentration No effects on CK and pro-inflammatory cytokines concentration
Jager et al. 2016 [121]	Recreative training man	Damaging exercise bout	**Twostudy groups**: 20 g of casein consumptionand/or 20 g of casein plus *Bacillus* consumption **Study product**: *Bacillus coagulans* GBI-30	↑ regeneration perception after damaging workout ↑ sport performance in Wingate Test ↓ soreness perception 24 and 72 h after damaging workout ↓ post-exercise blood CK No effects on muscle strength and thickness

Table 1. *Cont.*

References	Study Model	Type of Exercise	Intervention	Beneficial Effect of Intervention on Direct and Indirect Parameters of Sports Performance
Roberts et al. 2016 [81]	untrained men and women	triathlon specific stage times (swim, bike, and run)	**Three study gorups:** probiotics, probiotics +antioxidants and placebo **Study product:** mix of *Bifidobaterium* and *Lactobacillus*	↓ blood lipopolysaccharide (LPS) level up to 6 days after workout ↓ race duration
Toohey et.al. 2018 [124]	volleyballplayers (women)	squat, deadlift, and bench press, vertical jump, pro-agility and isometric midthigh pull test	**Twostudy groups:** probiotic and placebo **Studyproduct:** *Bacillus Subtilis*	↓ fat mass level compared to placebo group No effects on strength or athletic performance.
Jager et al. 2016 [78]	resistance-trained men	eccentric exercise of the elbow	**Twostudy groups:** probiotic and placebo **Study product:** *Streptococcus thermophilus* FP4 *Bifidobacterium breve* BR03	↓ IL-6 concentration up 48 h after damaging training ↑ maximal voluntary isometric peak torque at 24 to 72 h following damaging exercises ↑ flexed arm angle after damaging workout No effect on average maximal voluntary isometric peak No clear effect on plasma CK level after damaging exercises
Carbuhn et al. 2018 [122]	Swimmers (women)	aerobic/anaerobic swim time trials and force plate vertical jump	**Twostudy groups**: probiotic and placebo **Studyproduct:** *Bifidobacterium longum* 35624	↑ post-training regeneration perception No effects on aerobic and anaerobic swim performance testing No effects onconcentric/eccentric force production No differences in serum IL-1, LPS, and LPS Binding Protein (LBP) concentration
Townsend et al. 2018 [80]	baseball players (men)	Ten-yard sprint test, pro-agility test, standing long jump	**Twogroups**: probiotics and placebo **Studyproduct**: *Bacillus subtilis* DE111	↓ post-workout blood TNF-α concentration No significant effecton IL-10, zonulin, testosterone, cortisol concentration and salivary immunoglobulin A (SIgA) secretion No differences in strength, performance and body composition
Huang et al. 2019 [79]	triathletes	triathlon championship	**Twostudy groups**: *Lactobacillus* and placebo **Study product**: *Lactobacillus plantarum* PS128	↓ oxidative stress level ↑ antioxidant potential through thioredoxin (TRX) and MPO modulation ↑ post-workout blood BCAA concentration ↑ post-workout regeneration rate ↑ post-workout blood IL-10 concentration ↓ post-workout blood IL-6, IL-8, TNF-α IFN-γ concentration ↓ CK level during recovery period ↑ anaerobic capacity in Wingate Test No significant differences in body composition No effects on CK, myoglobin, lactate dehydrogenase (LDH), ammonia, lactate and FFA after exercise

Table symbols: ↑—increase; ↓—decrease.

10. Conclusions

In the light of current knowledge, it seems that intestinal microbiota intervention may have beneficial effects on the human body, resulting in better athletic performance. Modulation of the immune response, oxidative stress, metabolic processes, and nutrients bioavailability are considered the main mechanism(s) by which the microbiota affects training adaptation. he microbiome may also have an impact on muscle protein synthesis and mitochondrial biogenesis and function, as well as muscle glycogen storage. Dysbiosis may reduce physiological adaptation, increase inflammatory markers and ROS generation as well as free radical macromolecules devastation, all contributing to skeletal muscle atrophy. On the other hand, numerous studies indicate the beneficial effect of probiotics supplementation on aerobic and anaerobic performance in athletes. Not all of these processes are well understood, and there is a clear need for future studies to explore this intestine-muscle connection. These studies should be focused on athletes and strive to enhance our understanding of their physiological muscle function maintenance.

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Sports Medicine - Open

ORIGINAL RESEARCH ARTICLE

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Effects of Probiotics and Vitamin D_3 Supplementation on Sports Performance Markers in Male Mixed Martial Arts Athletes: A Randomized Trial

Katarzyna Przewłócka[1], Sylwester Kujach[2,3], Piotr Sawicki[4], Paweł Berezka[5], Zofia Kinga Bytowska[1], Marcin Folwarski[6], Kondrat Kowalski[7] and Jan Jacek Kaczor[1,5,8*]

Abstract

Background Strategies targeted at the intestine microbiome seem to be beneficial for professional athletes. The gut–muscle axis is associated with the inflammatory state, glucose metabolism, mitochondrial function, and central nervous system health. All these mechanisms may affect maximal oxygen uptake, muscle strength, and training adaptation. Moreover, the positive effect of certain bacterial strains may be enhanced by vitamin D. Thus, this study aimed to assess and compare the level of selected markers of sports performance of mixed martial arts (MMA) athletes supplemented with vitamin D_3 or probiotics combined with vitamin D_3.

Methods A 4-week randomized double-blind placebo-controlled clinical trial was conducted with 23 MMA male athletes assigned to the vitamin D_3 group (Vit D; $n=12$) or probiotics + vitamin D_3 group (PRO + VitD; $n=11$). Repeated measures of the creatine kinase level, lactate utilization ratio, and anaerobic performance were conducted.

Results After 4 weeks of supplementation, we found lower lactate concentrations 60 min after the acute sprint interval in the PRO + VitD group when compared to the Vit D group (4.73 ± 1.62 and 5.88 ± 1.55 mmol/L; $p<0.05$). In addition, the intervention improved the total work (232.00 ± 14.06 and 240.72 ± 13.38 J kg^{-1}; $p<0.05$), and mean power following the anaerobic exercise protocol (7.73 ± 0.47 and 8.02 ± 0.45 W kg^{-1}; $p<0.05$) only in the PRO + VitD group. Moreover, there was an improvement in the lactate utilization ratio in the PRO + VitD group compared with the Vit D group as shown by the percentage of T60/T3 ratio (73.6 ± 6.9 and 65.1 ± 9.9%, respectively; $p<0.05$). We also observed elevated serum 25(OH)D_3 concentrations after acute sprint interval exercise in both groups, however, there were no significant differences between the groups.

Conclusion Four weeks of combined probiotic and vitamin D_3 supplementation enhanced lactate utilization and beneficially affected anaerobic performance in MMA athletes.

Key Points

1. A single bout of high-intensity exercise induced an elevation in the serum 25(OH)D_3 concentration in both groups, both before and after supplementation.

*Correspondence:
Jan Jacek Kaczor
jan.kaczor@ug.edu.pl
Full list of author information is available at the end of the article

2. Combined supplementation of vitamin D_3 with a multistrain probiotic mixture improved LA utilization after exercise.
3. MMA athletes who were supplemented with a combination of vitamin D_3 and probiotics achieved better results, specifically in average power and total work during the first 30 s of exercise.

Keywords MMA, Supramaximal sprints, Vitamin D_3, Probiotics, Anaerobic capacity, LA utilization

Background

Mixed martial arts (MMA) is a sport that dates back to 649 BC as a part of an ancient competition. The first MMA tournaments were limited to applying as few rules as possible to resemble real fights [1]. With the development and growth in popularity, more rules have been introduced to ensure safety and equal opportunities for athletes. Moreover, certain weight classes have been introduced. Currently, a typical professional MMA fight lasts three 5-min rounds (or five rounds in the case of a title fight), separated by a 1-min break. Physical performance in MMA is characterized as interval exercise, and athletes perform high-intensity actions during the fight, interrupted by short breaks or low-intensity activity.

This sport requires energy supplied to the most extent by anaerobic metabolic pathways [2]. During high-intensity bouts of exercise, the capacities for phosphocreatine (PCr) hydrolysis and glycolysis processes are crucial to meet the maximal demand for adenosine triphosphate (ATP) resynthesis [3]. Moreover, it has been proven that the blood lactate (LA) concentration increases significantly immediately after a fight, and the values might reach up to 20.1 mmol/L, indicating high-intensity exertion with a significant proportion of anaerobic LA metabolism [4]. However, the contribution of aerobic pathways to energy yield is also elevated. It has been established that even during submaximal exercise, a certain pool of ATP is resynthesized via oxidative phosphorylation [3]. Therefore, pyruvate dehydrogenase (PDH) activity positively correlates with increased oxygen uptake, enhancing LA utilization and pyruvate oxidation [5]. The pyruvate dehydrogenase complex is composed of three subunits that require the cofactors thiamine pyrophosphate, lipoic acid, and reduced form of flavin adenine dinucleotide (FADH2), and reduced nicotinamide adenine dinucleotide (NADH) is also needed for the reaction to shift forward. This complex is a key transition point between cytosolic and mitochondrial metabolism, converting pyruvate produced by glycolysis into acetyl coenzyme-A (acetyl-CoA), which is further oxidized by the tricarboxylic acid cycle in mitochondria. Thus, training programs for MMA athletes should focus on enhancing both anaerobic and aerobic energy metabolism processes to increase the function of mitochondria, improving the proteins concentrations of substrate transport, the activity of certain enzymes of LA utilization, and glycolytic pathways.

Strategies targeted at obtaining maximal performance and skeletal muscle physiological adaptation together with the maintenance of a low risk of injuries and enhancement of regeneration processes thus represent the most important objectives among MMA athletes. In addition, an increasing number of studies indicate that the intestinal microbiome may be an indirect factor in enhancing the effect of training on physiological adaptation. This phenomenon has been called the gut–muscle axis [6, 7]. The human intestinal microbiota has been established as one of the most complex sites of the human body, with the estimated number of microorganisms exceeding 10^{14} cells. The most abundant population represents bacteria. The biodiversity and overall composition of the gut microbiota play a crucial role in maintaining homeostasis within the human body [8, 9]. It has been shown that gut microorganisms may impact the host's nutritional status, energy metabolism pathways, and immune system function, and contribute to maintaining the integrity of epithelial cells of the gut [10]. The main mechanism through which the gut microbiome impacts muscle function is its ability to modulate oxidative stress and the inflammatory process through certain metabolic pathways, such as mammalian target of rapamycin (mTOR) kinase [11], nuclear factor kappa B (NF-kB) and Forkhead box O (FOXO) protein [12]. Moreover, some bacterial species may utilize LA, which seems to be especially important in MMA. Scheiman and coworkers have reported that animals supplemented with *Veillonella atypical* show higher LA utilization levels enhancing the Cori cycle. Improvement in the extended exercise to exhaustion time has also been observed [13]. In addition, it has been shown that serum LA crosses the epithelial barrier and is metabolized by some bacterial species to propionate, improving athletic performance [13]. It has been described that the gut–muscle axis could also be associated with glucose metabolism, mitochondrial function, and central nervous system health. All of these mechanisms may affect maximal oxygen uptake, muscle strength, and training adaptation [7]. Additionally, some studies suggest that certain strains of bacteria

may decrease the blood creatine kinase (CK) level after exercise, which is a specific marker of muscle damage [14]. Moreover, in previous studies, it has been shown that certain bacterial strains such as *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W22, *Levilactobacillus brevis* W63, and *Lactococcus lactis* W58, reduced the exercise-induced tryptophan degradation rate, limited exercise-induced drops in the tryptophan concentration and reduced incidents of upper respiratory tract infections [15]. In another study, the same probiotic strains decreased the levels of Zonulin, a marker of intestinal permeability, in feces. The authors have also observed an improvement in the exercise-induced inflammatory state in trained men [16]. These data demonstrate the benefits of some bacterial strains on sports performance via the improvement in gut homeostasis and intestinal permeability. Therefore, strategies targeted at the intestinal microbiome seem to be substantial for professional athletes.

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) define probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [17]. In light of current knowledge, it seems that the positive effect of certain bacterial strains may be enhanced by the synergistic effect of vitamin D. The discovery of vitamin D receptor (VDR) in skeletal muscle has provided evidence showing the beneficial results of cholecalciferol for proper muscle metabolism. Moreover, its role in signaling pathways appears to largely overlap with the potential intestine–muscle axis signaling pathway. The synergistic intestinal microbiota and vitamin D 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. Further research on the impact of probiotics and vitamin D_3 on brain functions, chronic stress [18], and neuroprotection seems to be crucial, as these factors indirectly affect skeletal muscles and exercise capacities [19]. Thus, we assume that combining probiotics with vitamin D_3 supply may have a beneficial effect on muscle function in MMA athletes. Moreover, it has been shown that reaching optimal serum concentrations of vitamin D_3 in patients with low back pain reduced markers of oxidative stress and inflammation [20] as well as elevated the biogenesis and function of mitochondria and decreased skeletal muscle atrophy [21]. Vitamin D_3 deficiency commonly occurs in the Polish population and is found in 85% of Poles. Currently, it is clear that an optimal serum 25-hydroxy cholecalciferol ($25(OH)D_3$) concentration (≥ 30 ng/mL) is crucial for human health and sport performance. Thus, the aim of the study was to assess and compare the level of selected markers of sports performance of MMA athletes supplemented with vitamin D_3 or probiotics combined with vitamin D_3.

Materials and Methods

Study Design

The parallel study was designed as a randomized double-blind placebo-controlled clinical trial. Participants were randomly divided into two research conditions, with the probiotic group (PRO + VitD) receiving a multistrain probiotic together with vitamin D_3 daily and the placebo group (Vit D) receiving a placebo preparation and vitamin D_3 for four weeks. All athletes were Caucasian. The randomization and product allocation were conducted using an Excel random generator by an independent researcher who was not engaged in any study procedures. Athletes and investigators remained blinded until the end of the data analysis. All study procedures were performed twice: before intervention (PRE) and immediately after four weeks of intervention (POST). The protocol was approved by the Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (No. NKNNB/643/2019-2020) in accordance with the Declaration of Helsinki. To ensure that all sufficient details were provided, Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) was adhered to [22, 23]. The study was conducted between the end of September 2020 and December 2020 in Gdansk, Poland. The research project was carried out during the autumn–winter season when sun exposure was minimal. This assumption was a priority, as we wanted to avoid any interference with the project goals by exposing MMA athletes to the sun's rays. Furthermore, according to the website Meteoblue, there were only eleven days with significant sun exposure during these three months and a couple of days in 2020. The project has been registered with Clinical Trials under the identifier NCT04759729. The study flow diagram is presented in Fig. 1.

Participants

Twenty-five well-trained MMA male athletes were enrolled in the study after being screened based on the inclusion and exclusion criteria. However, twenty-three of them completed the protocol. Athletes who were adults who had more than 3 years of MMA training experience, fought at least three fights and well trained a minimum of five times a week were included in the study, while participants who had a history of inflammatory bowel diseases, heart failure, antibiotic therapy within the last 2 months or a chronic injury within the previous 6 months were excluded. Some studies reported sex- and age-related differences in the gut microbiome

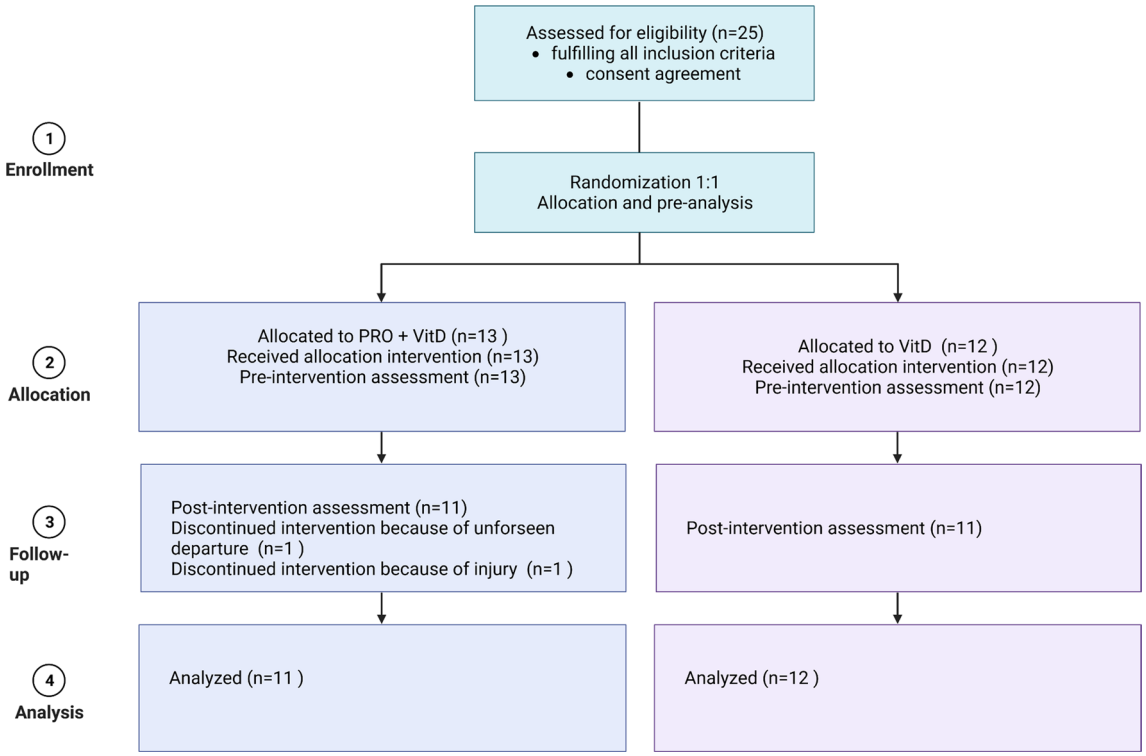

Fig. 1 Participants flow diagram

composition [24, 25], so to reduce data variability, female sex and age < 18 years old were selected as exclusion criteria. All participants enrolled in the study were fully informed about the study's protocol and were obligated to provide a signed informed consent form before any assessment or intervention. The sample was recruited from Gdansk and surrounding areas. All athletes who participated in the study were in the same training period throughout the clinical trial and were engaged in typical MMA workouts based on kickboxing, Brazilian jiu-jitsu, and wrestling practice, including endurance and strength sessions. Moreover, athletes were asked not to change any training or dietary habits to minimize the risk of external factors influencing obtained data.

Intervention

The study product was a combination of lyophilized strains of bacteria with positive effects on host health (*Bifidobacterium lactis* W51, *Levilactobacillus brevis* W63, *Lactobacillus acidophilus* W22, *Bifidobacterium bifidum* W23 and *Lactococcus lactis* W58) [15, 16], maize starch, maltodextrin, plant proteins, and hydroxypropyl ethylcellulose tablet coating. The trade name of this probiotic mixture is Sanprobi® Active & Sport (Szczecin, Poland). The total cell count was adjusted to 2.5×10^9 colony forming units (CFUs) per gram (≥ 500 million CFUs in a capsule). Identical-looking capsules containing only 40 mg of maltodextrin and plant proteins were used as a placebo. The product has been tested by means of pharmacopea methods, that is culture techniques. The number of CFUs was guaranteed by the end of the shelf life, as evidenced by the use of a climatic chamber. Each pack of probiotics or placebo contained 40 capsules. At the baseline visit, all athletes received three packs of probiotics or a placebo, depending on the random allocation. Participants were also given specific instructions on how to take the product and were informed to take 4 capsules daily with meals (total daily dose of 2×10^9 CFUs; 2 capsules in the morning and two capsules in the evening). The capsules were stored at room temperature in a dry place before distribution, and subjects were instructed to maintain these conditions. Athletes reported no adverse events and adhered to taking the supplements during the intervention. In addition to probiotics, athletes from both the Vit D and PRO + VitD groups received 5 ml of vitamin D_3 supplement (in oil) containing 0.5 mg of cholecalciferol per 1 ml, which encompasses 20,000 IU, and Miglyol 812 as an excipient. Athletes were instructed to supplement 3–4 drops (3000–4000 IU) of vitamin D_3 daily during the 4 weeks of the intervention period, together with the morning dose of probiotics.

Study Protocol

Participants reported twice to the laboratory: before intervention (PRE) at the baseline visit and after four weeks of the intervention period (POST) at the follow-up visit. All PRE evaluations were performed during the baseline visit, and POST evaluation were performed one day after intervention. During both visits, athletes completed an assessment of body composition, were interviewed about their dietary habits, and completed an anaerobic interval exercise—a triple Wingate test-based protocol. Furthermore, blood samples were drawn before and after the triple Wingate test to assess selected parameters of the muscle damage state and the 25(OH) D_3 and LA concentrations. All assessments and analyses are specified in a separate section. Participants were instructed to continue their nutrition habits and the training program. Athletes were obligated to perform at least 5 specific MMA workouts per week to maintain the competitive character of the study. To assess the quality of workouts, participants were asked to note the duration of all training sessions and to evaluate the subjective feeling of fatigue in a training diaries. The study products and training diary were distributed among athletes at the baseline visit.

Background Information

Background information was collected during the baseline visit. Athletes were asked about their health status, especially past and present injuries, diseases, medical procedures, and any mental or physical problems as well as the usage of concomitant medications.

Diet and Training Assessment

To assess whether any habitual changes, such as diet or training programs, might influence the gut microbiome and sports performance among athletes, a specific interview was evaluated by a qualified sports nutritionist. All athletes were obligated to perform at least 5 typical MMA trainings per week, with an average duration of 60–90 min. Typical MMA training is a mix of standing combat, grappling, ground fighting, and striking and contains strength and endurance elements. We also assessed current supplement intake with a specially prepared survey. All interviews were performed PRE and POST intervention.

Body Composition

Body composition was evaluated at the same time of day under the same nutritional conditions before and after the intervention. We used multifrequency bioelectrical impedance analysis (BIA) using a Tanita MC 720 analyzer to assess lean body mass (LBM), total fat mass (TFM), and body mass (BM). BIA is obtained from the measures of two parameters: resistance and reactance based on the flow of electricity through the body [21]. Before each assessment age, height, and sex were manually entered, and the participant's feet and hands were cleaned with Tanita provided tissues. During the assessment, it was necessary for the participants to stand fully erect on the measurement electrodes, hold the hand electrodes, and refrain from moving, talking, or touching the sides of the body.

Assessments and Data Collection

Anaerobic Performance—Interval Exercise Session

Anaerobic performance assessments were evaluated at two-time points: the baseline and follow-up visits, in the morning, after a balanced breakfast (80 g of wheat roll, 60 g of strawberry jam, and one banana). Testing sessions were performed on a cycle ergometer (884E Sprint Bike, Monark, Sweden). Before the examination, the saddle was individually adjusted. The exercise protocol started with a 5-min warm-up at 100 watts, including two all-out sprints lasting 3–5 s in the last minute of the warm-up. Next, subjects were allowed a 3-min rest for the final preparation and then immediately started the interval exercise including three 30 s "all-out" supramaximal sprints—SIE (Wingate anaerobic test based—WAnT). The flywheel resistance equaled 7.5% of each individual's body mass and was applied at the onset of the sprints. The interval rest periods between cycling bouts were set to 2 min. The athletes were instructed to accelerate to their maximal pedaling rate and were verbally encouraged to maintain this pedaling.

Venous Blood Collection and Analysis

Blood samples were collected during sports tests at two points in time, that is, PRE and POST intervention, under the same conditions. A nurse drew blood samples from the arm vein into appropriate standardized tubes containing K_2EDTA and a coagulation activator. Each time, up to 4 ml of blood was collected. Blood samples were taken indirectly before the start of exercise tests and 30 min and 24 h after the end of the test. The blood was centrifuged at 3000×g to separate serum and plasma. The material was placed into separately labeled microcentrifuge tubes and stored at − 80 °C for further analysis.

The collected venous blood samples were subjected to biochemical analysis. To evaluate the level of muscle damage, we determined the activity of creatine kinase (CK) in plasma through the kinetic method at 37 °C, using a RANDOX CK-NAC assay (No. CK522, Randox Laboratories LtD., Crumlin, UK) according to the "manufacturer's instructions" with a spectrophotometer (CE9200, Cecil instruments Ltd., Cambridge, UK). The CK activity is expressed as U/l. The serum 25(OH)

D_3 concentration was assessed using the isotope dilution method by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). All samples were prepared and analyzed using the Eksigent ExionLC analytical HPLC system with a CTC PAL autosampler (Zwinger, Switzerland) coupled with a QTRAP®4500 MS/MS system (Sciex, Framingham, MA, USA).

Capillary Blood Collection and Analysis

Capillary blood samples (100 µl) were taken by a qualified researcher each time sports tests were performed (PRE and POST) and collected into a sterile graduated microcapillary. The measurements were performed on fingertip capillary blood. The blood was then transferred to a microcentrifuge tube containing 500 µl 0.6 mol perchloric acid and stored at − 20 °C until the determination was carried out. Capillary blood was collected before exercise testing and at 5-time points within an hour before and after the end of the anaerobic test (TB, before; T3, 2–3 min after exercise; T15, 15 min after exercise; T30, 30 min after exercise, and T60, 60 min after the test). To determine the concentration of LA, collected capillary blood samples underwent biochemical analysis using the colorimetric kinetic method based on reaction speed. We used a lactate analyzer (Biosen C line, 173 5214 09 0045, EKF Diagnostic, Germany).

Statistical Analysis

All raw data were analyzed using the statistical program Statistica 13.3 (StatSoft Inc., Tulsa, OK, USA). We included only full data from subjects who completed all intervention periods and all study procedures ($n = 23$). Data were previously tested for normality using the Shapiro–Wilk *W*-test. The descriptive statistics for background information and to examine the trends in the analyzed parameters with mean values and 95% confidence intervals were used. Statistical analysis was performed using two-way ANOVA test. To determine statistical significance, post hoc testing for specific differences was performed using the least significant difference (LSD) test. The statistical significance was set at $p < 0.05$.

Results

A total of 25 MMA athletes were enrolled in the study, of whom 23 completed the protocol. Two participants in the PRO + VitD group of the study had to drop out due to unforeseen circumstances—one due to departure and the other due to injury. There were no statistically significant anthropometric differences between the groups at the baseline visit. The average age was 26.18 ± 4.05 years old in the Vit D group and 24.67 ± 6.46 years old in the PRO + VitD group. Mean body weight was 80.23 ± 9.83 kg and 81.08 ± 12 kg; fat-free mass was 71.94 ± 8.19 kg and 73.61 ± 9.33 kg; and fat mass was 8.29 ± 2.78 kg and 7.48 ± 3.85 kg in the Vit D and the PRO + VitD group, respectively. Athletes in both groups meet the criteria related to the training. The average weekly training time was 11.4 ± 3.1 h in the Vit D group and 11.8 ± 3.4 h in the PRO + VitD group. The characteristics of the MMA athletes are shown in Table 1.

Effects of Supplementation on Anaerobic Performance

Anaerobic performance was assessed by applying supramaximal sprints (SIE; a triple WAnT). The obtained data indicated that post-supplementation values of total work (W_{tot}) as well as the mean power (MP) during the first 30 s bout (WanT) were significantly different. We found an increase in W_{tot} ($p < 0.05$) and MP ($p < 0.05$) in the PRO + VitD group after supplementation. There were no statistically significant differences in the Vit D group ($p > 0.05$). Individuals in the Vit D group maintained their performance between pre- and post-supplementation. No positive or negative correlations were observed in this group. We found no differences between groups in the fatigue index (FI), maximal power (P_{max}), and time to

Table 1 Participant characteristics

Participants' information	Vit D group range	Vit D group Mean ± SD	PRO + VitD group range	PRO + VitD group Mean ± SD
Age	19–34	26.2 ± 4.0	18–40	24.7 ± 6.5
Height (cm)	168–194	179.3 ± 7.7	173–200	182.2 ± 9.3
Weight (kg)	69–98	80.2 ± 9.8	66.2–107.5	81.1 ± 12.0
FFM (kg)	59.3–87.7	71.94 ± 8.2	62.0–90.8	73.61 ± 9.3
FM (kg)	4.2–11.9	8.29 ± 2.8	2.6–16.7	7.48 ± 3.9
Years of training	5–17	10.1 ± 4.4	5–17	9.9 ± 4.0
Years of competition	3–14	7.9 ± 4.1	2–13	7.4 ± 4.1
Quantity of training (hours/week)	8.5–16	11.4 ± 3.1	9–16	11.8 ± 3.4

FFM fat-free mass, *FM* fat mass, *SD* standard deviation

Table 2 Results obtained during the first bout of supramaximal sprints

Variable	Vit D			PRO + VitD		
	PRE Mean ± SD	POST Mean ± SD	*p* value	PRE Mean ± SD	POST Mean ± SD	*p* value
W_{tot} [J kg^{-1}]	239.34 ± 19.46	238.37 ± 14.35	0.99	232.00 ± 14.06	240.72 ± 13.38	**0.04***
P_{max} [W kg^{-1}]	10.47 ± 0.95	10.39 ± 1.22	0.96	9.80 ± 0.83	10.00 ± 0.65	0.22
P_{max} time [s]	6.93 ± 2.02	6.38 ± 1.56	0.53	6.67 ± 1.13	6.93 ± 1.88	0.84
MP [W kg^{-1}]	7.98 ± 0.65	7.95 ± 0.48	0.99	7.73 ± 0.47	8.02 ± 0.45	**0.04***
FI [w/kg/s]	0.20 ± 0.04	0.18 ± 0.06	0.06	0.17 ± 0.05	0.17 ± 0.04	0.97

The bold text indicates a significant difference (*$p < 0.05$) between PRE and POST supplementation in the PRO + VitD group

PRE before supplementation, *POST* after supplementation, W_{tot} total work, P_{max} maximal power, P_{max} *time* time to obtain max power, *MP* mean power, *FI* fatigue index (decrease rate of P_{max}), *SIE* supramaximal sprints

P_{max}. There were no significant differences between the groups (Table 2).

Effects of Supplementation on LA

The blood LA concentration in both groups before supplementation, and exercise as well as after SIE was not significantly different between the groups. The LA concentration before exercise (TB) was 1.82 ± 0.87 mmol/L (Vit D) and 1.77 ± 0.82 mmol/L (PRO + VitD) and increased at 3 min (T3; Vit D 16.74 ± 3.50 mmol/L and PRO + VitD 15.71 ± 3.49 mmol/L after SIE and then decreased to values of 5.54 ± 1.36 mmol/L and 4.73 ± 1.63 mmol/L within 60 min (T60) in the Vit D and PRO + VitD groups, respectively (Fig. 2A). After four weeks of supplementation we found a significantly higher concentration of LA in the Vit D group (5.88 ± 1.55 mmol/L) than in the PRO + VitD group (4.73 ± 1.63 mmol/L) 60 min (T60) after SIE (*$p < 0.05$; Fig. 2B). In addition, we also observed that LA was more effectively utilized in the PRO + VitD group (73.6 ± 6.9%) compared with the Vit D group (65.1 ± 9.9%) from the highest concentration at T3 to T60 after SIE. The rate of LA oxidation is shown as the percentage of the T60/T3 ratio (*$p < 0.05$; Fig. 2C).

Effects of Supplementation on the Serum 25(OH)D_3 Concentration

The mean value of all MMA athletes regarding the serum 25(OH)D_3 concentration before the supplementation period was 27.22 ± 11.23 ng/mL, and after four weeks of supplementation with an average amount of 3500 IU/day vitamin D_3, the serum 25(OH)D_3 concentration was 28.35 ± 11.03 ng/mL (*$p < 0.05$; Fig. 3A). To our surprise, despite vitamin D_3 supplementation at a dose of 3 500 IU/day, the serum concentration of 25(OH)D_3 was still below the minimum (30 ng/mL). On the other hand, in both pre-supplementation groups, the serum concentration of 25(OH)D_3 was significantly elevated 30 min after SIE (*$p < 0.05$ BT0 vs. BT30; Fig. 3B). Moreover, after 4 weeks of intervention and 30 min after the SIE, there was an even higher increase in 25(OH)D_3 in serum (**$p < 0.001$ AT0 vs. AT30; Fig. 3B).

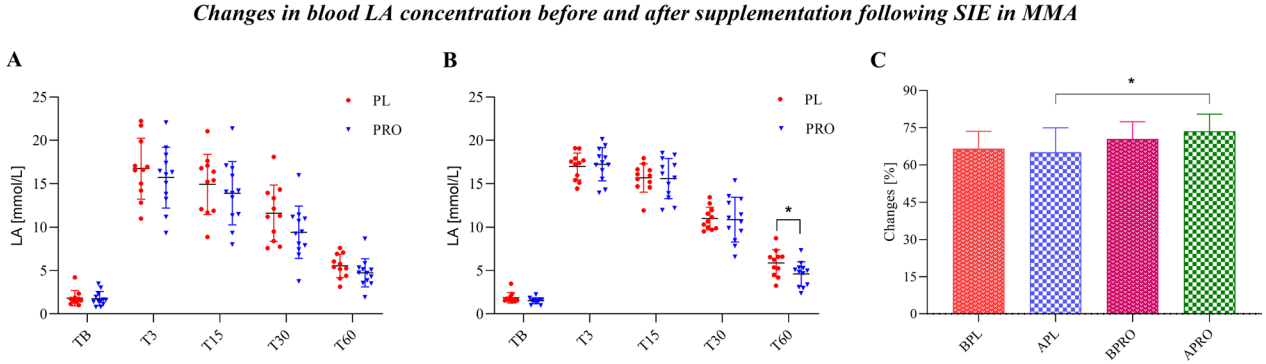

Fig. 2 **A** The concentration of LA before and in 4-time points within an hour after the end of SIE in both groups before supplementation (PRE). **B** LA concentration after 4 weeks of supplementation (POST) was lower in the Vit D vs. PRO + VitD group 60 min after SIE (*$p < 0.05$; LSD). **C** The ratio of LA utilization (T60/T3) expressed as a percentage (*$p < 0.05$; LSD)

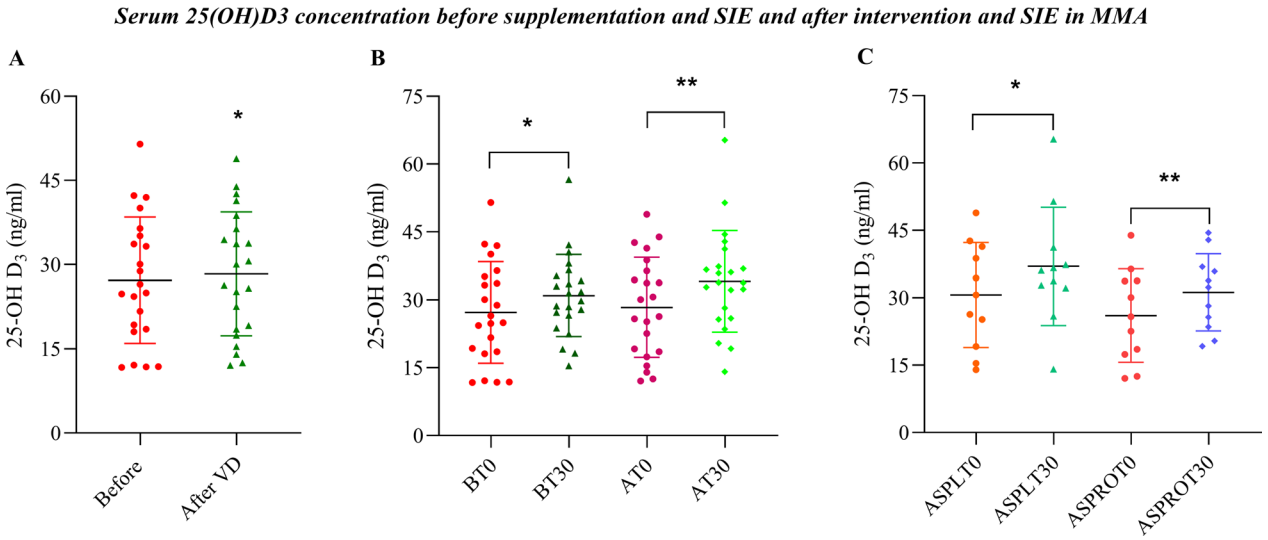

Fig. 3 **A** The concentration of 25(OH)D_3 before (PRE) and after 4 weeks (POST) of vitamin D_3 supplementation (*$p < 0.05$; LSD). **B** Serum 25(OH)D_3 concentration in MMA at BT0 (PRE; before supplementation and exercise) compared to BT30 (before supplementation after 30 min of SIE) and AT0 (POST; after intervention before exercise) versus AT30 (after supplementation 30 min after SIE). Data are expressed as ng/mL (*$p < 0.05$; LSD). **C** The concentration of 25(OH)D_3 after 4 weeks of combined vitamin D_3 and probiotics supplementation (*$p < 0.05$; LSD) before and 30 min after SIE; ASPLT0-after supplementation of vitamin D_3 before SIE; ASPLT30-after supplementation of vitamin D_3 after 30 min SIE; ASPROT0-after combined supplementation vitamin D_3 with probiotics before SIE, ASPROT30-after combined supplementation vitamin D_3 with probiotics after 30 min SIE

Furthermore, the release of 25(OH)D_3 into the bloodstream, 30 min following SIE after 4 weeks of supplementation was higher, in the Vit D group (*$p < 0.001$; ASPLT0 vs. ASPLT30; 6.4 ng/mL) as well as in the PRO + VitD group (**$p < 0.01$; ASPROT0 vs. ASPROT30; 5.1 ng/mL). We noticed a trend toward a higher release of 25(OH)D_3 after 30 min of SIE following supplementation in the Vit D group than in the PRO + VitD group; however, the differences did not reach statistical significance (Fig. 3C).

Effects of Supplementation on CK Activity

We observed that plasma CK levels were slightly higher 24 h after SIE before the supplementation period (PRE) as well as after supplementation (POST) compared with levels before exercising in both groups. However, we noticed no statistically significant differences between the Vit D and the PRO + VitD groups after the supplementation period. In addition, the changes in CK activity after supplementation were not significant before SIE in the Vit D and PRO + VitD groups or 24 h after SIE in either group. The plasma CK activities are presented in Table 3.

Discussion

To the author's knowledge, this is the first study to investigate a multistrain probiotic mixture combined with vitamin D_3 in the mixed martial arts (MMA) athlete population. The current study examined the influence of 4 weeks of probiotic and vitamin D_3 supplementation on anaerobic performance, LA utilization, and muscle damage. Athletes tolerated both supplements well and did not experience any side effects. We found that a single bout of high-intensity exercise elevated the serum 25(OH)D_3 concentration in both groups, both before and after supplementation, which is in agreement with recently published data [26, 27]. Moreover, combined vitamin D_3 with multistrain probiotic mixture supplementation

Table 3 Effects of supplementation on plasma creatine kinase level

Variable	Vit D			PRO + VitD		
	PRE Mean ± SD	POST Mean ± SD	*p* value	-PRE Mean ± SD	POST Mean ± SD	*p* value
Before SIE	154.6 ± 103.7	195.0 ± 183.4	0.969	152.2 ± 143.1	269.4 ± 214.7	0.726
24 h After	316.0 ± 142.5	317.3 ± 255.9	0.999	447.8 ± 347.9	339.2 ± 270.6	0.911

PRE before supplementation, *POST* after supplementation, *SIE* supramaximal sprints, *SD* standard deviation Statistical significant ($p < 0.05$)

also showed improvement in LA utilization after SIE (between 15 and 30 min) compared to the VIT D group. This may support the assumption that changes in the gut microbiome caused the enrichment of species able to metabolize LA and thus enhanced its faster utilization [28]. MMA athletes supplemented with vitamin D_3 and probiotics achieved better results in anaerobic performance tests. We found a beneficial effect on average power and total work during the first 30 s of SIE.

Recent studies have established the crucial role of the gut microbiota composition in immune function [29–31] and brain health [32, 33], which may be indirect factors influencing physiological adaptation to training. However, the potential beneficial connection between intestinal microbiota composition, muscle function, and sports performance is not clearly understood. Increasing evidence has confirmed the importance of the interplay between gut homeostasis, inflammatory processes, and skeletal muscle adaptation to training, which we demonstrated in our previous review [7].

Short-term, high-intensity interval exercises are essential to an MMA athlete's training program. It is well known that the metabolic response to this type of physical effort leads to the accumulation of LA and hydrogen in skeletal muscles and blood circulation, which as a consequence, may impair physical performance [34]. It was suggested that intramuscular LA accumulation and the associated pH decrease among muscle cells due to the development of fatigue during exercise exert a detrimental effect on glycolytic energy provision and potassium release [35]. Therefore, athletes' ability to remove or metabolize LA seems to be a crucial factor influencing sports performance. Moreover, the blood LA response to exercise can be a useful factor in assessing exercise capacity. In our study, we observed that 4 weeks of probiotic supplementation contributed to a higher rate of LA utilization after SIE than at baseline. In contrast, no changes in the Vit D group occurred. In the PRO + VitD group, the blood LA concentration decreased significantly within 1 h after SIE to the value obtained 3 min after exercise. The utilization rate at baseline was 66.5% in the Vit D group and 70% in the PRO + VitD group. After the supplementation period, the utilization rate was approximately 66.5% in the Vit D group and 74% in the PRO + VitD group. Significant changes were observed 15 min after the SIE. The LA produced during high-intensity exercise mostly comes from fast-twitch muscle fiber, which consume large amounts of glucose to generate energy, and it is mainly removed by slow twitch muscle fiber. According to Brooks' theory, improvement in LA utilization may be caused by the enhancement of clearance complex processes engaging lactate-specific enzymes and transporters. In addition, LA may be used as an additive energy source as well as a gluconeogenesis substrate that enhances glycolytic processes [36].

In light of current knowledge, the composition of the intestinal microbiome of athletes differs from that of inactive people, mainly due to the greater abundance of bacterial species, biodiversity, and a higher proportion of some bacterial species such as *Veillonella*, *Bacteroides*, *Akkermansia*, *Methano brevi bacteria* and *Prevotella* [37, 38]. Sheiman et al. indicated, that the relative abundance of *Veillonella* strains increases in long-distance runners after a marathon. In addition, another group showed that each gene in the major metabolic pathway that metabolizes LA to propionate was more abundant after exercise than before exercise and that LA can overcome the epithelial barrier to the intestinal lumen [28]. The same researchers isolated *Veillonella atypica* from marathon runners' stool samples and transplanted them into mouse intestines. As a result, there was a significant increase in running time on a treadmill to exhaustion [28]. According to these data, it is very likely that exercise-induced LA is released from muscle cells into the bloodstream and then crosses the epithelial barrier, where it is metabolized to propionate by *Veillonella atypical* and other bacterial species using it as the sole carbon source. LA is converted to propionate via the methylmalonyl-CoA pathway [39], and lactate dehydrogenase (LDH), a crucial enzyme engaged in LA metabolism, is present in a phylogenetically diverse group of bacteria [28]. Taken together, some bacteria may improve physical performance via a microbial-encoded enzymatic process, which enhances LA utilization and delivers extra energy and gluconeogenesis substrates.

In our study, we observed improved LA utilization after 4 weeks of multistrain probiotic mixture supplementation, including some species that metabolized it. Therefore, it is very likely that changes in the gut microbiome caused the enrichment of species able to metabolize LA and thus enhanced its utilization. A similar effect was observed in the study by Huang and coworkers, where 6 weeks of *Lactiplantibacillus plantarum* TWK 10 supplementation significantly improved LA accumulation both during exercise and after 90 min in the recovery period in healthy adults. Researchers found that the beneficial effect of Lactiplantibacillus*plantarum* TWK 10 supplementation on LA utilization was stronger during the recovery phase [40]. Interestingly, a previous study indicated that mice supplemented with the same bacterial strain showed positive changes in the gut microbiome composition, significantly affecting the relative abundances of *Bacteroidetes* and *Firmicutes* [40]. Moreover, *Lactiplantibacillus plantarum* TWK 10 intake resulted in a decrease in LA blood accumulation after a 15-min swimming trial [41]. Another animal model study

showed that a multistrain probiotic mixture significantly decreased the postexercise LA concentration in horses subjected to athletic activity. These findings also suggest that probiotics may promote physical performance by enhancing short-chain fatty acid (SCFA) production, which is used by muscles as an energy source instead of carbohydrates [42]. Scientific data confirm that the *Lactobacillus* genus may alter the hindgut pH and induce the proliferation of LA-utilizing bacteria such as *Veillonell asp.* Thus, the source of energy usage is modified during exercise [42]. Our data are in line with Lighi and coworkers. However, in contrast to a study in triathlon athletes, 8 weeks of supplementation with *Lactiplantibacillus plantarum* PS 128 did not affect the LA concentration, although it did beneficially affect physical performance and inflammatory markers after exercise [43].

According to current knowledge, it seems that intestinal microbiota-targeted strategies may improve training parameters as well as increase training capabilities. In our study, 4 weeks of multistrain probiotic supplementation improved anaerobic performance. We found an improvement in average power and total work during the first bout of SIE in the PRO + VitD group, while no significant changes were found in the Vit D group. Similar results were shown by the study of Jager et al., who supplemented men exercising recreationally with *Weizmannia coagulants* GBI-30, 6086 (BC30). Probiotic ingestion prevented the decline in peak power in the probiotic group, but no changes occurred in strength or vertical jump. The authors showed that probiotic supplementation significantly decreased postexercise CK levels [14]. Interestingly, no muscle damage as measured by CK was observed in our study. Although we observed an increase in the plasma CK concentration 24 h after SIE, it was not high enough to indicate muscle damage. Moreover, we did not detect any differences in plasma CK levels between the groups. The lack of significant changes in CK levels may be due to the large standard deviations in both groups. It is well known that CK levels are elevated after excessive training and may reach thousands of units per liter. However, substantial individual variability was reported. The magnitude of muscle damage marker differences may be influenced by the intensity and duration of exercise, training state of athletes, sex, genetic predisposition, distribution of muscle fiber type, kind of muscle contraction, and type of exercise [44]. A single bout of the 30-s Wingate test triggered muscle damage manifested by elevated CK concentration in non-athlete individuals [45]. In our study, SIE did not increase muscle damage measured by CK levels in MMA athletes. It is possible that this kind of exercise, which resulted in high LA concentrations, as well as high self-reported fatigue, was not sufficient to damage the skeletal muscle tissue among combat sports athletes. Perhaps this may be due to the high physiological adaptation to this exercise type and intensity. It is worth noting that scientific reports investigating the effect of probiotics on CK concentration are ambiguous. Some animal and human model studies have supported the hypothesis that some bacterial strains may enhance muscle regeneration by attenuating CK levels and thus muscle damage [14, 41, 43]. Our results are in line with the study by Jager et al., where *Streptococcus thermophilus* FP4 and *Bifidobacterium breve* BR03 supplementation improved the maximal voluntary isometric peak torque and flexed arm angle after a damaging workout without plasma CK induction among resistance-trained male participants [46]. Similarly, supplementation of Weizmannia coagulants GBI-30, 6086 (BC30) in soldiers positively affected muscle strength and attenuated the inflammatory response during intense training (mean jumping power) but did not influence the level of CK [47].

Concerning anaerobic performance, a similar effect was reported by Huang et al. in triathletes after 8 weeks *Lactiplantibacillus plantarum* PS128 supplementation. According to our results, the probiotic intervention improved the mean peak power, mean power, and fatigue index. Moreover, the authors showed a positive effect on oxidative stress markers, CK, and proinflammatory cytokine plasma concentrations [43]. Interestingly, the test was performed on the same day as the official triathlon competition, after 6 h of rest. It is well known that excessive physical exercise due to neuromuscular deficits is responsible for forcing transmission impairment. In this scenario, a lower than average positive force and a slower rate of force development were observed [48]. Thus, some bacterial strains may be a potential alternative ergogenic aid, contributing to the maintenance of mean power and the fatigue index (FI) via the positive effect on blood metabolites and thus on muscle physiological adaptation. There is a high probability that this effect may be enhanced through proper vitamin D_3 concentration (> 30 ng/mL), which we found in both PRE and POST supplementation after SIE. Therefore, we assume that regular physical exercise may have a beneficial impact on nervous system and muscle health. Thus, the synergistic effect of probiotics and vitamin D_3 could have particular importance in this area.

Recently, data from our laboratory reported that a serum vitamin D_3 deficit is associated with oxidative stress, negatively affecting mitochondrial function and skeletal muscle metabolism [19]. Moreover, the discovery of vitamin D receptor (VDR) in skeletal muscle provided evidence showing the beneficial effects of cholecalciferol on proper muscle metabolism, and its role in signaling pathways appears to largely overlap

with the potential intestine–muscle axis signaling pathway. Thus, we aimed to detect whether cosupplementation of vitamin D_3 and probiotics is favorable to sports performance and if this strategy is more effective than supplementation of vitamin D_3 alone. In the current study, we found a significant increase in the serum 25(OH)D_3 concentration 30 min after the entire SIE in both groups compared to the initial value. Our data are consistent with a study by Dzik et al., showing a significant increase in serum 25(OH)D_3 concentrations after an acute 30 s WAnT in young, trained boys. Moreover, this elevation positively correlated with fat-free mass (FFM), suggesting the release of 25(OH)D_3 from the muscle tissue [26]. Interestingly, a recent study indicated that a 20-week resistance training program increased the plasma 25(OH)D_3 concentration from 42.4 to 51.2 nmol/L and induced *CYP27B1* gene expression, having a positive impact on VDR regulation. In this investigation, no vitamin D_3 supplementation was implemented [26]. Thus, it is highly probable that skeletal muscles may store and release 25(OH)D_3. Our data suggest that physical exercise may trigger the release of some metabolites of vitamin D_3 into the blood circulation. It seems that the training process may enhance this increase. In our study, we observed a slight increase in 25(OH)D_3 in blood serum after 4 weeks of supplementation. MMA athletes reached the minimum recommended value, but their levels were still not optimum. We suggest that the doses of vitamin D_3 were too low to induce physiological effects. Thus, no spectacular changes in MMA athletes were observed. It was also shown that seven months of vitamin D_3 supplementation (1200 IU daily) had no effect on calcium, parathormone, cortisol, and testosterone blood concentrations, as well as hand grip strength among Estonian soldiers [49].

Similarly, no effect on peak power output was reported by Hew-Bulter et al., who performed a 12-week supplementation of 4000 IU vitamin D_3 in basketball players [50]. To summarize, vitamin D_3 recommended doses for healthy, active adults are probably too low for a population of elite athletes. Moreover, because of the potential high muscle usage of vitamin D_3, athletes seem to be at risk of vitamin D_3 deficiency. It appears that supplementation of vitamin D_3 combined with probiotics may potentially result in enhanced serum vitamin D_3 concentrations. The potential mechanism is associated with the similarity of lipid and vitamin D_3 absorption in the gut. It seems that some microorganisms, via improved intestinal emulsification of lipids can enhance vitamin D_3 gut absorption. This hypothesis was confirmed by Castagliuolo et al., who supplemented mice with *Lacticaseibacillus paracasei* DG and oil-based cholecalciferol. The authors observed that supplementation resulted in maintaining adequate vitamin D_3 levels in the blood [51]. This preclinical study suggests that the coadministration of probiotics and vitamin D_3 may be more effective in preventing and treating vitamin D_3 deficits.

Limitations of the Study

A possible study limitation was the lack of diet standardization; even though participants were asked not to change their previous eating habits (to avoid the influence of diet on the gut microbiome), participants also declared that they were not taking any medications, not drinking alcohol, and not smoking during the study and 1 month before the study. There was no true control group in this study either. It is also possible that the proposed exercise protocol was insufficient to damage the skeletal muscle tissue in combat sports athletes. However, the SIE modality resulted in high LA concentrations and a high self-reported fatigue score reflecting sport fighting conditions. Finally, on average, 3500 IU/day of vitamin D_3 supplementation seemed to be too low for professional MMA athletes.

Conclusion

We found that exercise increased serum concentrations of 25(OH)D_3 in both groups before and after supplementation. In addition, the combined intake of vitamin D_3 and a probiotic mixture containing several strains showed an improvement in LA utilization after SIE in the supplemented group. This finding may support the notion that changes in the gut microbiome led to an enrichment of species that can metabolize LA, allowing for more rapid utilization of lactate. MMA athletes supplemented with combined vitamin D_3 and probiotics showed total work and mean power improvement in the anaerobic test. However, further studies should be conducted with higher vitamin D_3 doses, gut microbiome composition analysis, serum vitamin D binding protein determination, and different exercise types and intensities to better understand the mechanisms involved in the physiological adaptation.

Abbreviations

MMA	Mixed martial arts
PCr	Phosphocreatine
LA	Lactate
PDH	Pyruvate dehydrogenase
FADH2	Reduced form of Flavin Adenine Dinucleotide
NADH	Reduced form of Nicotinamide Adenine Dinucleotide
mTOR	Mammalian target of rapamycin
NF-kB	Nuclear factor kappa B
FOXO	Forkhead box O protein
FAO	The Food and Agriculture Organization of the United Nations

WHO	The World Health Organization
VDR	Vitamin D receptor
$25(OH)D_3$	25-Hydroksy cholecalciferol
PRO + VitD	Probiotic plus vitamin D_3 group
Vit D	Vitamin D_3 group
PRE	Before intervention
POST	4 Weeks after intervention
SPIRIT	Standard Protocol Items: Recommendations for Interventional Trials
CFUs	Colony forming units
LBM	Lean body mass
TFM	Total fat mass
BM	Body mass
WAnT	Wingate anaerobic test
SIE	Supramaximal sprints based on WAnT
TB	Time point before exercise
T3	2–3 Minutes after exercise
T15	15 Minutes after exercise
T30	30 Minutes after exercise
T60	60 Minutes after exercise
FFM	Fat-free mass
W_{tot}	Total work
MP	Mean power
P_{max}	Maximal power
P_{max} time	Time to obtain maximal power
FI	Fatigue index
BT0	Before supplementation, before exercise
BT30	Before supplementation, 30 min after exercise
AT0	After supplementation, before exercise
AT30	After supplementation, 30 min after exercise
CK	Creatine kinase
LDH	Lactate dehydrogenase
SCFA	Short-chain fatty acid

Author Contributions

KP, JJK, and MF contributed to conceptualization and methodology; KP, PB, and SK performed investigation; KP, PB, SK, ZKB, PS, and KK carried out laboratory analysis; JJK and KP carried out statistical analysis; KP and JJK performed writing—original draft preparation; KP, SK, MF, and JJK performed writing—review and editing; JJK and MF performed supervision; all authors have read and agreed to the published version of the manuscript.

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Availability of Data and Materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability

Not applicable.

Declarations

Ethics Approval and Consent to Participate

The study was approved by Independent Bioethics Committee for Scientific Research at the Medical University of Gdańsk (No. NKNNB/643/2019-2020). All participants enrolled to the study were fully informed about the study's product and protocol and provided a signed informed consent form before any assessment or intervention.

Consent for Publication

Not applicable.

Competing interests

Marcin Folwarski received remuneration for lectures from probiotic company. The rest of the authors declare that they have no competing interest.

Author details

[1]Division of Bioenergetics and Exercise Physiology, Medical University of Gdańsk, Gdańsk, Poland. [2]Department of Physiology, Gdansk University of Physical Education and Sport, Gorskiego 1, 80-336 Gdańsk, Poland. [3]Department of Human Physiology, Medical University of Gdańsk, Tuwima 15, 80-210 Gdańsk, Poland. [4]Department of Gymnastics and Dance, Faculty of Physical Education, Gdansk University of Physical Education and Sport, Gorskiego 1, 80-336 Gdańsk, Poland. [5]Department of Animal and Human Physiology, Faculty of Biology, University of Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland. [6]Department of Clinical Nutrition and Dietetics, Medical University of Gdańsk, 80-210 Gdańsk, Poland. [7]Masdiag Laboratory, 01-882 Warsaw, Poland. [8]Department of Animal and Human Physiology, Faculty of Biology, University of Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland.

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Publisher's Note

Combined probiotics with vitamin D_3 supplementation improved aerobic performance and gut microbiome composition in Mixed Martial Arts athletes

Katarzyna Przewłócka[1], Marcin Folwarski[2], Mariusz Kaczmarczyk[3], Karolina Skonieczna-Żydecka[4], Joanna Palma[4], Zofia Kinga Bytowska[1], Sylwester Kujach[5,6] and Jan Jacek Kaczor[1,7*]

[1]Department of Bioenergetics and Exercise Physiology, Medical University of Gdansk, 80-211 Gdansk, Poland
[2]Department of Clinical Nutrition and Dietetics, Medical University of Gdansk, 80-210 Gdansk, Poland
[3]Department of Clinical and Molecular Biochemistry, Pomeranian Medical University in Szczecin, Szczecin, Poland
[4]Department of Biochemical Research Pomeranian Medical University in Szczecin, Szczecin Poland
[5]Department of Physiology, Gdansk University of Physical Education and Sport, Gdansk, Poland
[6]Department of Neurophysiology, Neuropsychology and Neuroinformatics, Medical University of Gdansk, Gdansk, Poland
[7]Department of Animal and Human Physiology, University of Gdansk, Gdansk, Poland

*** Correspondence:**
Jan Jacek Kaczor; jan.kaczor@ug.edu.pl; ORCID: 0000-0002-6370-2160
Department of Animal and Human Physiology, Faculty of Biology,
University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland

Keywords: probiotics, gut microbiome, intestinal permeability, MMA athletes, aerobic capacity, inflammation

Abstract

Introduction: Mixed Martial Arts (MMA) is characterized as an interval sport in which the training program focuses on enhancing both aerobic and anaerobic capacities. Therefore, strategies targeting the intestinal microbiome may be beneficial for MMA athletes. Moreover, vitamin D supplementation may amplify the positive effects of certain bacterial strains. We previously demonstrated that the combined of probiotics and vitamin D_3 supplementation improved the lactate utilization ratio, total work, and average power achieved during anaerobic tests in MMA. Therefore, this study aimed to investigate whether combined probiotic and vitamin D_3 ingestion can modify the composition of the gut microbiome and epithelial cell permeability, influence the inflammatory response, and ultimately enhance aerobic capacity.

Methods: A 4-week clinical trial was conducted with 23 male MMA athletes randomly assigned to either the probiotic + vitamin D_3 (PRO+VIT D) group or the vitamin D_3 group (VIT D). The trial employed a double-blind, placebo-controlled design and involved measurements of serum inflammatory markers, gut microbiome composition, epithelial cell permeability, and aerobic performance.

Results: After 4-week of supplementation, we found a significantly lower concentration of calprotectin in the PRO+VIT D group (34.79 ± 24.38 mmol/L) compared to the value before (69.50 ± 46.91) supplementation ($p = 0.030$), augmentation of beta diversity after the intervention in the PRO+VIT D group ($p = 0.0005$) and an extended time to exhaustion to 559.00 ± 68.99; compared to the value before (496.30 ± 89.98; $p = 0.023$) after combined probiotic and vitamin D_3 supplementation in MMA athletes. No effect was observed in the VIT D group.

Conclusion: Our results indicate that combined treatment of probiotics and vitamin D_3 may cause alterations in alpha and beta diversity and the composition of the gut microbiota in MMA athletes. We observed an improvement in epithelial cell permeability and an extended time to exhaustion during exercise in MMA athletes following a 4-week combined probiotic and vitamin D_3 treatment.

1 Introduction

The human intestinal microbiome is inhabited by approximately 10^{14} microorganisms and has been recognized as one of the most complex sites in the human body (1). The most abundant population is bacteria. It seems that some bacterial species may affect the nutritional status of the host, metabolic pathways, and the immune system function and predominantly contribute to maintaining the integrity of epithelial cells (2). Moreover, the intestinal microbiome may indirectly affect physiological adaptations during the training process. This phenomenon is called the gut-muscle axis and is based on the assumption that certain microbes can impact muscle function (3).

Moderate physical activity has a positive influence on human gut microbiome composition, especially in the field of the diversity of bacteria species as well as bacteria genes involved in carbohydrate and protein metabolism and production of short-chain fatty acids (SCFA) (4,5). However, training overload may disturb the homeostasis among intestine microbes, which is particularly evident in professional athletes (6). Specifically, deterioration of intestinal blood perfusion caused by high-volume exercises leads to temporary gastrointestinal tract ischemia as well as gut mucous barrier dysfunction (7). As a consequence, increased intestinal permeability occurs, referred to in the literature as „leaky gut" (8). In this scenario, negative changes in gut microbes profile are observed, promoting the growth of potentially harmful bacteria such as *Peptostreptococcus*, *Staphylococcus*, *Peptoniphilus*, *Acidaminococcus*, and *Fusobacterium* instead of bacteria species producing anti-inflammatory mediators including *Bacteroides*, *Faecalibacterium*, *Collinsella*, and *Roseburia* (9). Moreover, bacteria and their associated toxins translocate into the bloodstream, leading to the exacerbation of both local and systemic inflammatory responses (10). It is clear that chronic inflammation and oxidative stress increase catabolism, negatively impact regeneration processes, and lead to a decline of muscle function (3). Therefore, strategies aimed at enhancing regeneration and decreasing inflammatory response are important for professional athletes.

Mixed Martial Arts (MMA) is characterized as an interval sport, where high-intensity actions during the fight are interspersed with low-intensity actions or short breaks. Therefore, during MMA training, anaerobic pathways exceed aerobics and constitute the main source of energy (11). Thus, the MMA training program focuses on both aerobic and anaerobic capacity enhancements.

In a previous review, we described that the gut-muscle axis is associated with the modulation of inflammatory pathways, oxidative stress, anabolic and catabolic processes, glucose metabolism, mitochondrial function, and central nervous system health. All of these affect maximal oxygen uptake, muscle function, and training adaptation (3). Furthermore, numerous studies have provided evidence that probiotic intake may reduce inflammatory response as well as improve antioxidant potential (12,13). In some cases, it is related to improved athletic performance (14–16). Moreover, certain data have demonstrated the benefits of some bacteria strains on sports performance *via* the improvement of gut homeostasis and intestinal permeability. It has been shown that a formula containing

Bifidobacterium bifidum W23, *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W22, *Levilactobacillus brevis* W63, *Lactococcus Lactis* W58 decreased zonulin in feces and improved the exercise-induced inflammatory state in trained men (17). According to the stance of the International Society of Sports Nutrition on probiotics, probiotic intake is linked to a multitude of health benefits. Probiotic supplementation has been described as contributing to promoting a healthy immune response, improving recovery and responses to physical or mental stressors, reducing lactate levels, and increasing neurotransmitter synthesis(18). Additionally, the positive effect of certain bacterial strains may be enhanced by vitamin D_3, the deficiency of which is observed widely in the Polish population and affects 85% of Poles (19). The detection of the vitamin D receptor (VDR) in skeletal muscle has provided evidence that highlights the beneficial effects of cholecalciferol on muscle metabolism. Therefore, supplementation of protective doses of vitamin D_3 is necessary for athletes' health and performance. In our previous study, it has been shown that combined probiotics with vitamin D_3 supplementation improved the lactate utilization ratio, total work, and average power obtained during the anaerobic in the MMA athletes (20). Therefore, the present study is intended to cover the remaining objectives of the project and answer the question of whether combined probiotics with vitamin D_3 ingestion can modify the composition of the gut microbiome and epithelial cell permeability, influence the inflammatory response, and ultimately improve aerobic capacity. The purpose of the study is to provide the evidence and answer the question if combined probiotics and vitamin D_3 may enhance sport performance and muscle health in professional athletes. Moreover, we try to explain potential mechanisms of action through, which intestinal homeostasis can support the exercise capacity of athletes.

2 Materials and methods

2.1. Study design

As described in our previously published study, the parallel study was a double-blind, placebo-controlled clinical trial (20). Athletes were randomly divided into the groups receiving a multistrain probiotic mixture and vitamin D_3 (PRO+VIT D) or vitamin D_3 (VIT D) receiving a placebo instead of probiotics and vitamin D_3. Vitamin D deficiency commonly occurs in the Polish population and is found in 85% of Poles (19); therefore, we decided to supplement both groups with vitamin D_3. All study procedures were performed twice: before and immediately after four weeks of supplementation. In accordance with the Declaration of Helsinki, the project has been approved by the Independent Bioethics Committee (No. NKNNB/643/2019-2020) and was registered in Clinical Trials under the identifier NCT04759729. We adhered to the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) (21,22).

2.1.1. Participants

A total of 25 male athletes who were well trained in MMA were initially enrolled in the study. The participants were recruited from Gdansk, Poland, and the surrounding areas. They were actively involved in typical mixed martial arts workouts that encompassed various disciplines such as kickboxing, Brazilian jiu-jitsu, and wrestling practice and included endurance and strength training sessions. Regarding the inclusion criteria, participants were required to have a minimum of 3 years of

MMA training experience, participation in at least 3 fights, and a minimum of five training sessions per week. On the other hand, individuals with a history of inflammatory bowel diseases, heart failure, recent antibiotic therapy within the past 2 months, or chronic injuries within the last 6 months were excluded from the study. Previous research has highlighted that there are differences in gut microbiome composition related to gender and age (23,24). To minimize data variability, we exclude females and subjects under the age of 18.

2.1.2. Intervention

We used a probiotic mixture composed of lyophilized strains of bacteria: *Bifidobacterium lactis* W51, *Levilactobacillus brevis* W63, *Lactobacillus acidophilus* W22, *Bifidobacterium bifidum* W23, and *Lactococcus lactis* W58. This probiotic mixture was combined with maize starch, maltodextrin, and plant proteins and coated with hydroxypropyl ethylcellulose tablets. The probiotic mixture is commercially known as Sanprobi® Active & Sport and is produced in Szczecin, Poland. Detailed product characteristics, which were consistent with a previously published study (20), have been shown in supplementary data. Besides probiotics, athletes from both VIT D and PRO+VIT D groups received 5 ml of Vitamin D_3 supplement (in oil) containing 0.5 mg of Cholecalciferol per 1 ml, which encompasses 20 000 IU, and Miglyol 812 as an excipient (Juvit D3). Participants were instructed to supplement 3500 IU (3 – 4 drops) daily during the intervention period. Athletes reported no adverse events and adhered to taking the supplements during the intervention.

2.1.3. Study protocol

The participants underwent two examinations: the first was conducted BS during the baseline visit, and the second was conducted AS at the follow-up visit. During both evaluations, the athletes completed assessments of body composition, a 3-day nutritional interview, and a cardio-respiratory fitness evaluation. Additionally, fecal samples and blood samples were collected before and after a sport test at both the BS and AS visits to assess specific parameters of inflammation. Participants were instructed to maintain their regular nutrition habits and training program throughout the study. Moreover, the athletes were advised not to make any changes to their training or dietary habits during the study period to minimize the risk of external factors influencing the collected data. All participants were required to engage in a minimum of 5 typical MMA training sessions per week, which included a combination of standing combat, grappling, ground fighting, and striking, as well as strength and endurance exercises. The average duration of these training sessions ranged from 60 to 90 minutes. A visit program is shown in Figure 1.

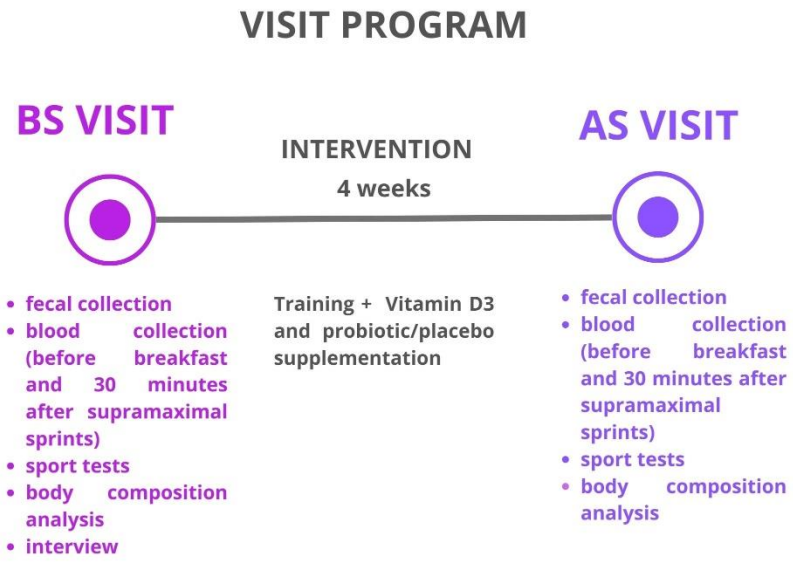


Figure1.Visit program.

2.1.4. Background information, diet, and training assessment

During the baseline visit, background information was gathered from the athletes. They were asked about their past and present injuries, diseases, medical procedures, as well as any mental or physical problems they may have experienced. Additionally, the athletes were questioned about their dietary habits and current use of supplements. Body composition analysis was also conducted during these interviews. The same interviews and assessments were performed both before and after the intervention (BS and AS). The specific procedures for collecting this information and conducting the assessments are shown in supplementary data.

2.2. Assessments and data collection

2.2.1. Aerobic fitness assessment

The cardiopulmonary exercise test (VO_2 max test) was applied in the two-time points: baseline and follow-up visit, in the morning, after a balanced breakfast (60 g of wheat bread, 60 g of strawberry jam, and 60 g of banana). Athletes were instructed not to make any physical effort one day before testing. To determine aerobic capacity, participants performed a graded cycle ergometry (ViaSprint 150P, Ergoline, Germany) test. Before the examination, the bicycle saddle was individually adjusted to obtain the athletes position with the slightly bent knee in the lowest pedal location to prevent hyperextension of the limbs during the test. Participants were allowed a 5-min warm-up at an intensity of 50 W with a pedaling cadence of 60 rpm. Immediately after the warm-up, the participants began cycling, in which resistance was increased every 3 minutes by 50 W until the subjects reached the point of volitional exhaustion. Breath by breath pulmonary gas exchange was measured by an Oxycon-Pro analyzer (Jaeger Oxycon Champion, Viasys Healthcare GmbH, Germany). Heart rates were monitored continuously by telemetry (Polar Monitors, Electro, Kempele, Finland). Maximal heart rate (HRmax),

maximal respiratory exchange ratio (RER), and maximal aerobic power (MAP) were calculated at the VO_2 max level.

2.2.2. Venous blood collection

The blood samples were collected at two points time: before breakfast and 30 minutes after supramaximal sprints (Wingate anaerobic test based), as described in the supplementary data (20). Blood samples from the antecubital vein (v. mediana cubiti) were collected by a professional nurse into appropriate standardized tubes containing clot activator. Each time, up to 4 ml of blood was collected. The blood samples were centrifuged at 3000 x g to separate the serum. The material was placed into separately labeled microcentrifuge tubes and stored at -80 °C for later determination. We assessed the level of pro- (IL-6 and TNF-α) as well as anti-inflammatory cytokines (L-2 and IL-15) using a commercially available enzyme-linked immunosorbent assay (ELISA) kits Diaclone SAS, Company of Medix Biochemica Group, France (No. 950.035.192, 950.090.192, 850.870.192 and 873.000.192, respectively) according to the "manufacturer's instructions".

2.2.3. Fecal collection

The subjects were provided a fecal sample indirectly before and after the intervention period. The material was collected by participants into a special standardized container. Subjects received adequate instructions for collecting and handling the material. Stool samples were immediately frozen and stored at – 80 °C. Collected fecal samples were subjected to quantitative and qualitative content of the intestinal microbiota using the new generation sequencing method (NGS).

2.3.4. New generation sequencing

Metagenomic DNA was sequenced using Illumina 2x151bp shallow shotgun sequencing approach (25). Initial quality control, adapter removal, and merging of paired-end reads were performed using a self-learning pipeline SHI7 (26). Taxonomic profiling was performed using a shallow-shotgun computational pipeline SHOGUN (10.1093/bioinformatics/btaa277) using pipeline command with a bowtie2 aligner. The composition of the microbiome was characterized primarily by alpha and beta diversity. The diversity of microbial communities in one sample (alpha) was measured using several indicators, such as Chao1, ACE, Shannon, and inverted Simpson. All indicators were calculated based on species level data (without any pre-processing, e.g., removal of rare species) after rarefying to an even sequencing depth of 23,424. The diversity of microbial communities between samples (beta) was measured using the Bray-Curtis distance calculated on genus level after rarefying to an even sequencing depth of 23,137. Both rarefactions were done using the rtk R package (version 0.2.6.1). Permutation multivariate analysis of variance (PERMANOVA) with strata (to block by individual) was used to assess the significance of the change in microbiota composition during intervention. Collected fecal samples were analyzed in the context of the presence of intestinal permeability parameters such as calprotectin and zonulin, as well as bacterial metabolism products such as short chain fatty acids (SCFA).

2.3.5. SCFA analyses

The synthesis of SCFA was done using gas chromatography with the Agilent Technologies 1260 A GC system with a Flame Ionization Detector (FID). A silica capillary column with a free fatty acid phase (DB-FFAP, 30m x 0.53mm x 0.5mm) was used. Hydrogen was supplied as a carrier gas at a flow rate of 14.4 ml/min. The initial temperature was 100 °C. It was held for 0.5 minutes, then raised to 180 °C at a rate of 80 °C/min and held for 1 minute. The temperature was then increased to 200 °C (20 °C/min) and finally held at 200 °C for 5 minutes. The injection volume was 1 µl and the duration of each analysis was approximately 17.5 minutes. SCFAs were identified qualitatively by comparing the retention times to a standard, namely 2-ethyl butanoic acid. For quantitative analysis, ChemStation Software (Agilent Technologies, UK) is used. The concentrations of individual acids were converted according to the internal standard.

2.3.6. Gut barrier integrity parameters

Gut barrier integrity markers: zonulin and calprotectin were evaluated using commercially available ELISA kits (Immunodiagnostic AG, Bensheim, Germany; No K5601 and K6927, respectively). The procedures followed the manufacturers' instructions.

2.4. Statistical analysis

The statistical analysis of all raw data was conducted using the software program Statistica 13.3 (StatSoft Inc., Tulsa, OK, USA) and R (R Core Team (2023). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.). Only complete data from participants who completed all intervention periods and study procedures (n=23) were included in the analysis. Before analysis, the data were assessed for normality using the Shapiro-Wilk W-test. Descriptive statistics, including mean values with 95% confidence intervals, were utilized to examine trends in the analyzed parameters and provide background information. Statistical analysis was performed using a two-way ANOVA test and general linear mixed-effect models (lme4 R package). Following ANOVA, to determine statistical significance, post-hoc testing for specific differences was conducted using Tuckey's Honestly Significant Difference (Tukey's HSD) method. To compare predictions made by linear mixed-effects models for different regressor values (average marginal effects), the function *comparison* from the R marginal effects (0.12.0) package was used. The threshold for statistical significance was set at $p < 0.05$.

3 Results

The study initially enrolled a total of 25 MMA athletes, but two athletes from the PRO+VIT D did not complete the protocol, more details related to MMA athletes were partially presented by Przewłócka et al. (20). There were no statistically significant anthropometric differences between groups at the baseline visit. The characteristics of MMA athletes are shown in Table 1.

Table 1. Participants characteristics.

Participants' Information	VIT D	PRO+VIT D
	Mean ± SD	Mean ± SD

Age	26.02 ± 4.00	24.70 ± 6.50
Height (cm)	179.30 ± 7.70	182.20 ± 9.30
Weight (kg)	80.20 ± 9.80	81.10 ± 12.00
FFM kg)	71.94 ± 8.20	73.61 ± 9.30
Vit D BS [ng/mL]	29.56 ± 12.62	24.89 ± 9.68
Vit D AS [ng/mL]	30.63 ± 11.66	26.07 ± 10.39
HR max	185.00 ± 10.73	187.00 ± 11.34
Years of training	10.10 ± 4.40	9.90 ± 4.00
Quantity of training (hours/week)	11.40 ± 3.10	11.80 ± 3.40

FFM – fat-free mass; BS – before supplementation; AS – after supplementation; SD, standard deviation, HR max – maximal heart rate obtained during VO_2 max test.

3.1.Effects of supplementation on aerobic performance

Analyzing the effect of PRO+VIT D supplementation on aerobic capacity parameters, significant differences observed in exercise time to exhaustion were found before supplementation (BS) compared to after supplementation (AS); *p = 0.023). There were no statistically significant differences in the VIT D group (p = 0.685). We found no differences between groups in maximal oxygen uptake value (VO_2 max), maximal aerobic power (MAP) as well as maximal respiratory exchange ratio (RER) during the VO_2 max test (Table 2).

Table 2. Results obtained during aerobic fitness assessment.

Variable	VIT D			PRO+VIT D		
	BS	AS	p-value	BS	AS	p-value
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
RER [vCO_2/vO_2]	1.19 ± 0.10	1.19 ± 0.06	0.999	1.21 ± 0.08	1.19 ± 0.05	0.869
VO_2 max [$ml/min/kg^{-1}$]	52.33 ± 5.06	52.48 ± 3.76	0.999	56.92 ± 0.83	56.37 ± 7.09	0.969
MAP [W]	306.82 ± 33.70	311.36 ± 40.87	0.940	317.50 ± 45.72	330.00 ± 42.16	0.096
Time [s]	489.91 ± 72.02	468.55 ± 102.03	0.668	496.30 ± 89.98	559.00 ± 68.99	0.023*

RER – respiratory exchange ratio; VO_2 max – maximal oxygen uptake*; MAP* - maximal aerobic power; Time – exercise to exhaustion time; *BS – before supplementation; AS – after supplementation; SD, standard deviation; Statistical significant (*p < 0.05).*

3.2.*Effects of supplementation on inflammatory state*

No statistically significant differences were observed in the serum concentrations of IL-2, IL-6, IL-15, and TNF-α between the VIT D and PRO+VIT D groups after the supplementation period. We found a statistically significant increase in serum IL-6 concentration after exercise in both groups, both before and after supplementation (BS – $p < 0.001$ in both groups, AS – $p = 0.033$ in the PRO+VIT D; $p = 0.029$ in the VIT D group). Similarly, it was observed a significant increase in serum IL-15 concentration after intervention in the VIT D group ($p = 0.038$). However, the pairwise analysis did not show statistically significant changes between groups. Predicted outcomes based on fitted linear mixed-effects models are presented in Table 3.

Table 3. Average marginal effects as a difference in predicted outcomes (Before workout *versus* After workout) for the combination of levels of supplementation and intervention.

Outcome	Time	Intervention	Est	SE	P	$Est_{pairwise}$	$SE_{pairwise}$	$P_{pairwise}$
IL2	BS	VIT D	-0.008	0.056	0.888	0.024	0.077	0.757
	BS	PRO+VIT D	0.016	0.053	0.763			
	AS	VIT D	0.022	0.056	0.693	0.004	0.077	0.962
	AS	PRO+VIT D	0.026	0.053	0.631			
IL6	BS	VIT D	1.26	0.35	<0.001**	-0.02	0.48	0.975
	BS	PRO+VIT D	1.24	0.33	<0.001**			
	AS	VIT D	0.76	0.35	0.029*	-0.05	0.48	0.915
	AS	PRO+VIT D	0.71	0.34	0.033*			
IL15	BS	VIT D	-0.64	1.51	0.675	2.04	2.09	0.330
	BS	PRO+VIT D	1.40	1.45	0.332			
	AS	VIT D	3.13	1.51	0.038*	-3.86	2.09	0.065
	AS	PRO+VIT D	-0.73	1.45	0.616			
TNF-α	BS	VIT D	168.2	108.6	0.122	-201.8	147	0.170
	BS	PRO+VIT D	-33.6	99.2	0.734			
	AS	VIT D	68.0	108.6	0.531	-83.1	147	0.572
	AS	PRO+VIT D	-15.2	99.2	0.878			

*Est – estimates; SE – standard error; statistically significant (**p < 0.001); statistically significant (*p < 0.05). BS-before supplementation; AS-after supplementation.*

3.3.*Effects of supplementation on microbiome profile*

Analysis of the gut microbiome was performed using a general linear mixed-effects model, where the time (BS and AS) of the intervention (PRO+VIT D or VIT D) interactions were examined. The predictor effect plot indicated that the interaction was significant because the alpha-diversity increase in the probiotic group was greater, but the baseline (PRE) values were lower, so the follow-up (POST) values between interventions were very similar. In addition, following FDR adjustment, none of the p-values remained statistically significant. Beta diversity measured by Bray-Curtis showed statistically significant shifts in microbiota composition during the intervention in the PRO+VIT D group (p = 0.0005), but there were no significant changes in the VIT D group (p = 0.145, Figure 2).

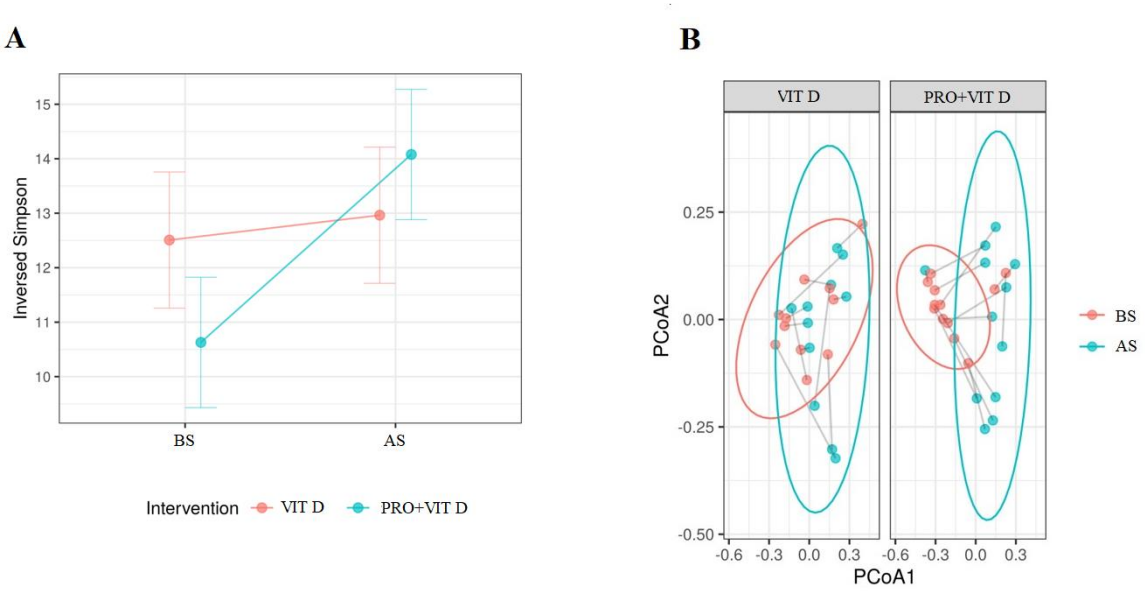
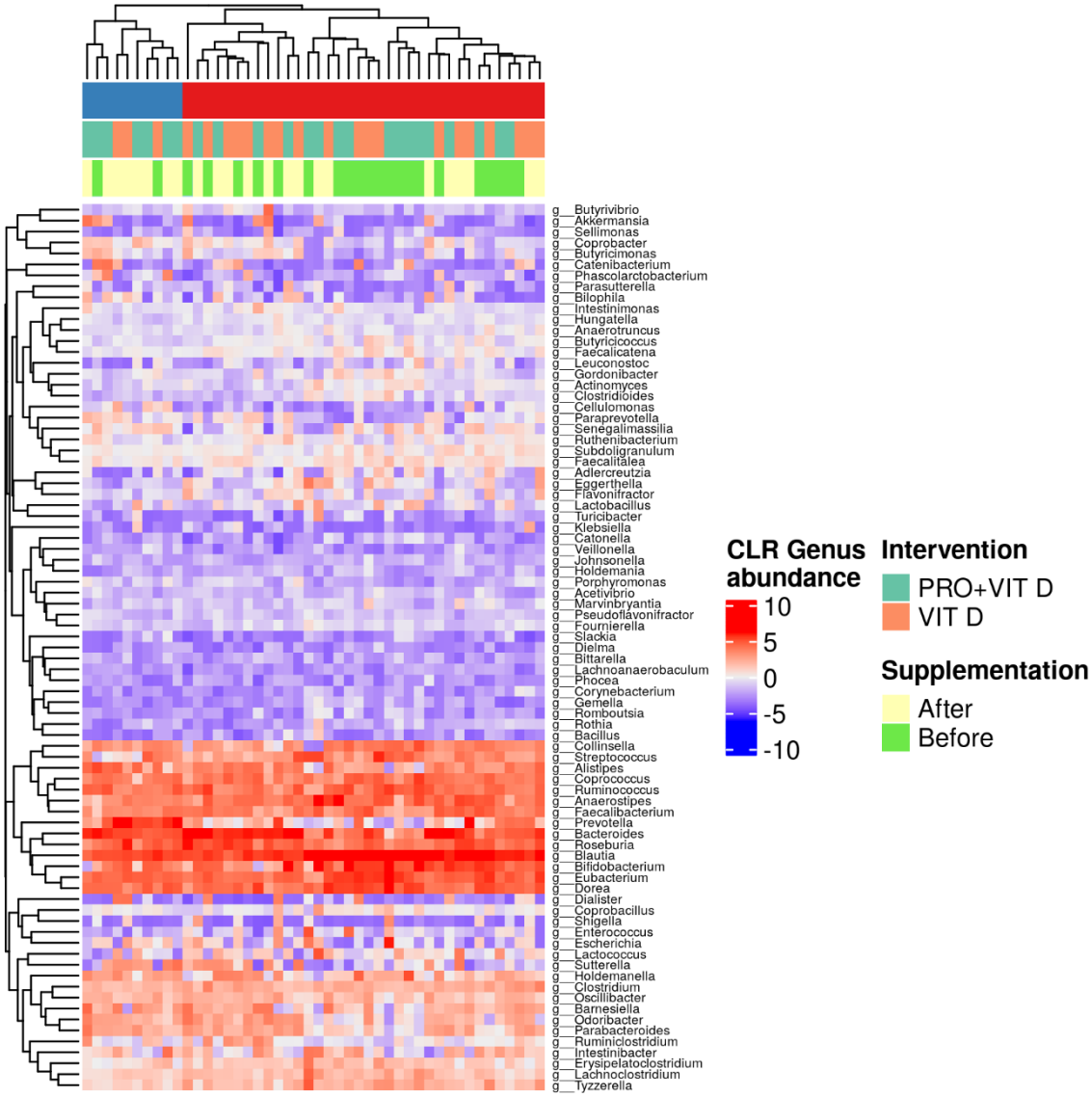


Figure 2. The comparison of changes in alpha diversity between groups as a result of the intervention (p = 0.086), FDR adjusted p value (Q) = 0.166). B The comparison of changes in beta diversity measured via Bray-Curtis between groups as a result of the intervention (***p = 0.0005). *BS – before supplementation; AS – after supplementation.*

Moreover, we observed significant changes in the gut microbiome composition after probiotics supplementation, when individual taxa were considered. A total change in bacteria profile and the abundance of the gut microbiota at the genus level among each group is presented in Figure 3. Our results indicate that supplementation significantly affected intestinal microbes' profile and contributed to the growth of bacteria having a potentially beneficial effect on the host health (e.g. Bacteroides genus, Roseburia inulinivorans, Prevotella genus, Lactobacillaceae family). We found a significant growth of Negativicutes class in the PRO+VIT D group (Est = 1.98, p = 0.006), but not in the VIT D group (Est = -0.25, p = 0.738). The changes in the selected bacteria are presented in Table 4. The total observed changes are shown as supplementary data.



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343 Figure 3. Heatmap of the abundance of the gut microbiota at the genus level center-log transformed
344 (CLR) among each group.

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346 Table 4. Total abundance of selected bacteria before and after 4 weeks of probiotics supplementation.

Outcome	Intervention	Est	SE	P	$Est_{pairwise}$	$SE_{pairwise}$	$P_{pairwise}$
Bacteroides fluxus	VIT D	0.08	0.48	0.859	2.02	0.66	0.002**
	PRO+VIT D	2.11	0.46	<0.001			
Lachnospiraceae bacterium	VIT D	-0.28	0.27	0.309	-1.27	0.38	<0.001**
	PRO+VIT D	-1.55	0.26	<0.001			
Roseburia inulinivorans	VIT D	-0.66	0.36	0.065	1.40	0.49	0.005**
	PRO+VIT D	0.74	0.34	0.030			

Peptostreptococcaceae bacterium	VIT D	0.84	0.68	0.222	-2.69	0.95	0.004**
	PRO+VIT D	-1.86	0.65	0.005			
Bacteroides genus	VIT D	0.30	0.67	0.657	2.29	0.92	0.013*
	VIT D	0.30	0.67	0.657			
Collinsella genus	VIT D	0.37	0.46	0.413	-1.65	0.63	0.009**
	PRO+VIT D	-1.28	0.44	0.003			
Faecalibacterium genus	VIT D	-0.19	0.35	0.601	1.21	0.49	0.013*
	PRO+VIT D	1.03	0.34	0.002			
Prevotella genus	VIT D	0.62	0.96	0.516	-1.01	0.33	0.002**
	PRO+VIT D	3.62	0.92	<0.001			
Lactobacillaceae family	VIT D	0.88	0.62	0.158	-2.04	0.86	0.018*
	PRO+VIT D	-1.17	0.60	0.050			
Negativicutes class	VIT D	-0.25	0.75	0.738	2.23	1.04	0.032*
	PRO+VIT D	1.98	0.72	0.006			
Firmicutues class	VIT D	-0.75	0.32	0.021	-0.93	0.45	0.038*
	PRO+VIT D	-1.68	0.31	<0.001			

*Est – estimates; SE – standard error; Statistically significant (**p < 0.01). statistically significant (*p < 0.05).*

3.4.*Effects of supplementation on SCFA*

The analysis of the percentage changes in selected SCFA concentrations showed no significant differences between PRO+VIT D and VIT D groups. We noted that propionate decreased after supplementation in both groups; however, this decrease was greater in the VIT D group. This trend was close, but not statistically significant (p = 0.061). The total changes in the SCFA profiles are presented in Table 5.

Table 5. Short-chain fatty acids - average marginal effects as a difference in predicted outcomes (BS *versus* AS) for PRO+VIT D and VIT D groups.

Outcome	Intervention	Est	SE	z	P	$Est_{pairwise}$	$SE_{pairwise}$	$P_{pairwise}$
C2:0 (%)	VIT D	-0.39	2.57	-0.15	0.879	-3.19	3.51	0.364
	PRO+VIT D	-3.58	2.39	-1.50	0.134			
C3:0 (%)	VIT D	-2.30	0.81	-2.86	0.004	2.07	1.10	0.061
	PRO+VIT D	-0.23	0.75	-0.31	0.756			
C4n (%)	VIT D	2.26	2.04	1.11	0.266	1.20	2.79	0.668
	PRO+VIT D	3.46	1.90	1.82	0.069			
C5n (%)	VIT D	-0.21	0.21	-1.01	0.311	0.13	0.29	0.650
	PRO+VIT D	-0.08	0.20	-0.43	0.670			

C2:0-acetic acid; C3:0 – propionic acid; C4:0- butyric acid; C5:0 – Valeric acid; Est – estimates; SE – standard error; z – Est/SE

3.5.*Effects of supplementation on intestinal permeability parameters*

The fecal zonulin and calprotectin concentrations in both groups before supplementation were not significantly different. After 4-week of supplementation, we found a significantly lower concentration of calprotectin in the PRO+VIT D group (34.79 ± 24.38 mmol/L) compared to the value before (69.50 ± 46.91) supplementation ($p = 0.030$; Fig. 4A). No significant effects were observed in the VIT D group. We did not observe significant differences in the fecal concentrations of zonulin in both groups (Figure 4B).

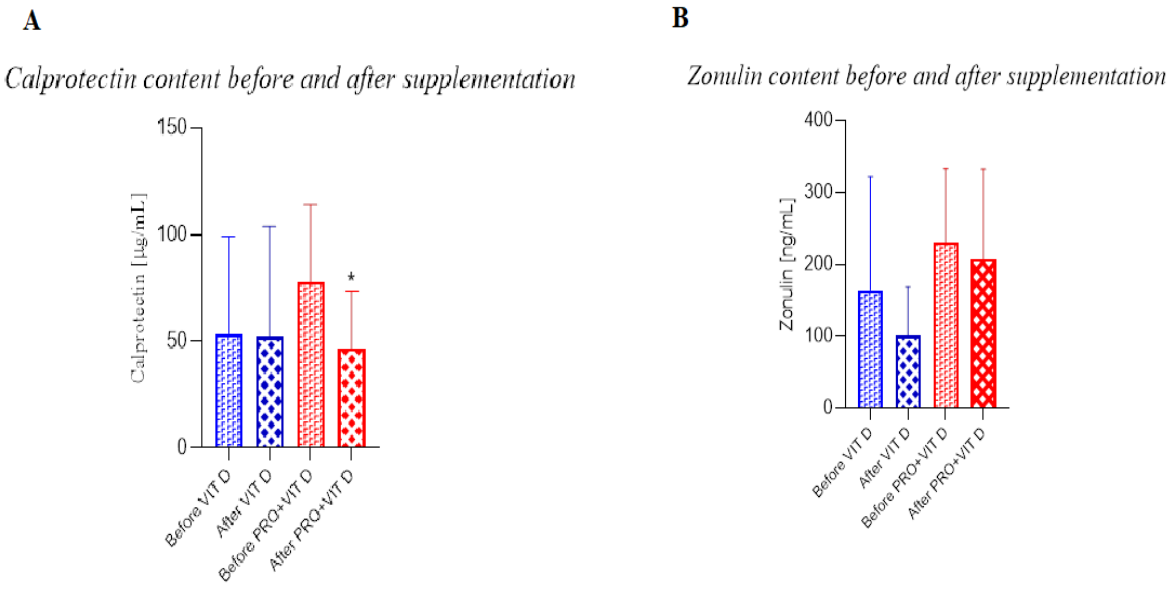

Figure 4. Calprotectin and zonulin content before and after supplementation A) Calprotectin Statistical significant ($^{*}p < 0.05$) BS vs AS in the group PRO+VIT D; B) Zonulin the lack of changes in both groups.

4. Discussion

To the best of our knowledge, this is the first study to comprehensively assess the influence of a combined multistrain probiotic mixture and vitamin D_3 supplementation in a Mixed Martial Arts (MMA) athlete population. The current study examined the influence of a 4-week supplementation on aerobic performance, inflammatory state, gut microbiome composition, and intestinal permeability. We found that the 4-week treatment positively affected the gut microbiome profile measured via inverted Simpson distance and Bray-Curtis distance, as well as the abundance of certain bacteria. The intervention positively correlated with the total time to exhaustion obtained in the aerobic test; however, it did not affect the maximal oxygen uptake and lactate threshold. Probiotic supplementation also improved intestinal permeability. Our results suggest that the combined supplementation of a multistrain probiotic mixture with vitamin D_3 improved physical performance and was associated with

significant changes in the gut microbial profile. Therefore, combined probiotic and vitamin D_3 supplementation may be considered a strategy for promoting exercise performance among competitive MMA athletes.

Recently, it was reported that the gut microbiota profile plays a crucial role in immune function (27–29) and brain health (30,31), which may be indirect factors influencing physiological adaptation to training. However, the potential beneficial connection between intestinal microbiota composition, muscle function, and exercise performance is not clearly understood. Increasing evidence has confirmed the importance of the interplay between gut homeostasis, inflammatory processes, and skeletal muscle adaptation to training, as demonstrated in a previous review (3).

The MMA training program focused on both aerobic and anaerobic capacity enhancements. Increased ATP resynthesis via oxidative phosphorylation during submaximal exercise enhances sports performance by improving LA utilization as well as pyruvate oxidation (11). In the present study, we observed a significant improvement in time to exhaustion during the VO_2 max test, reflecting endurance capacity but not the VO_2 max value. Recent studies indicated that VO_2 max may increase mainly as a result of an effective training program that includes interval sessions (32,33). Moreover, training programs more effectively influence VO_2 max than any nutritional strategy. According to the literature, certain nutritional strategies do not significantly elevate oxygen uptake in the athletic population (34,35). In our study, athletes were instructed to maintain their MMA training program. It is important to note that typical MMA training does not specifically target to improvement VO_2 max. Consequently, the increase in total time to exhaustion observed in our study was not associated with changes in VO_2 max value, instead, it was likely attributed to alterations in the gut microbiome profile. Some bacteria may enhance physical performance through a microbial-encoded enzymatic process, facilitating LA utilization while also providing substrates for gluconeogenesis and additional energy.

We found that athletes who were supplemented with a multistrain probiotics mixture combined with vitamin D_3 extended their exercise to exhaustion time. Our results are in line with those of a study conducted by Huang et al., who supplemented triathletes with *Lactobacillus plantarum* PS128. The authors showed that the intervention improved endurance running performance through intestinal microbiome alteration but did not affect maximal oxygen uptake (14). A similar effect was observed by Lin et al. after 5 weeks of *Bifidobacterium longum subsp. longum* OLP-01 supplementation period in the well-trained middle- and long-distance runners. Researchers detected that the change in the 12-min Cooper's test running distance significantly increased, as well as the total abundance of the gut microbiota (36). Roberts et al. obtained similar results after 12 weeks of supplementation of the probiotic mixture containing ed *Lactobacillus* and *Bifidobacterium* species. In the group of recreative training adults, improved certain stage time of triathlete race and endotoxemia were a result of probiotic intake (37). Scheiman et al. reported that mice supplemented with *Veilonella atypical* showed improvement in extended exercise to exhaustion time as well as higher LA utilization levels (38). Similarly, animals supplemented with Bacteroides fragilis showed improved extended exercise-to-exhaustion time (39). These data support the hypothesis that probiotic intake may have a positive effect on endurance capacity through the alteration of gut microbes; however, the influence on VO_2 max is limited. In our previous study, we demonstrated that a 4-week combined supplementation of a multistrain probiotic and vitamin D_3 resulted in a significant increase in the rate of lactate (LA) utilization among MMA athletes after supramaximal sprints (20). The efficient metabolism of LA is

of utmost importance for athletes' sports performance. It is widely recognized that the accumulation of LA within muscle tissues and the subsequent decrease in pH levels among muscle cells contribute to the onset of fatigue during training. These effects are primarily attributed to the detrimental impact on glycolytic energy production and the release of potassium ions (40). Consequently, we posit that probiotics, although limited in their impact on maximal oxygen uptake, may enhance endurance capacity through favorable modulation of the gut microbiome profile, potentially leading to improve LA utilization.

In contrast to our results, there are some studies indicating no effect on aerobic capacities after certain probiotics supplementation. In the study conducted by Carbuhn et al., 12 weeks of *Bifidobacterium longum* did not affect aerobic swim performance in female swimmers. However, in this study, the gut microbiome composition was not assessed, thus, it is not known whether the intervention affected the composition of the intestinal bacteria profile. The number of researchers evaluating the influence of probiotic supplementation on the gut microbiome composition in athletes is limited. Therefore, more research is needed in this area to explore the potential mechanism(s) via certain bacteria species that improve endurance capacity in athletes.

One of the most known mechanisms by which the gut microbiome may affect sports performance is their ability to alter the inflammatory response. It is well established that probiotics may suppress intestinal inflammation by down-regulation of Toll Like Receptors (TLR) expression (41) as well as enhanced innate immunity via different mechanisms, like upregulation of immunoglobulins, antimicrobial proteins, phagocytic activity, natural killer cells activity, and T and B lymphocytes function improvements (27). Microbial ability to SCFA production and thus enhance the integrity of intestinal mucus is described as a potential mechanism protecting against excessive activation of the immune system and hence maintaining appropriate pro- and anti-inflammatory cytokines ratio (42). It was shown that chronic inflammation, manifested in pro-inflammatory cytokines overproduction, may disrupt regeneration processes and inhibit muscle protein synthesis and metabolic adaptations to training (43,44).

In our study, we did not observe any significant changes of pro- as well as anti-inflammatory markers in serum. Our results are not consistent with data obtained by Vaisberg et al. who demonstrated that 30 days of ingestion of fermented milk containing 40 billions of *Lactobacillus casei* Shirtota of marathon runners was able to modulate both immunological and inflammatory response. The authors found a higher level of TNF-α in serum in the placebo group and lower levels of pro-inflammatory cytokines (IL-1, IL-5, IL-6, IL-13, and TNF-α) as compared to the supplemented group after a marathon race. There were no significant changes in serum IL-6 and IL-10 between groups (13). It might have been caused by the fact that the increase of this interleukin is not solely in response to inflammation. It may also physiologically appear as a result of muscle contraction and glycogen regulation. Similarly, there was a substantial reduction of TNF-α in basketball players supplemented 12 weeks with *Bacillus subtilis* DE111, without changes in other parameters such as IL-10, zonuline, testosterone and cortisol concentration as well as sport performance parameters (12). The influence of probiotics supplementation on inflammatory response was also observed in the clinical trial conducted by Jager et al. In their study, men on resistance training supplemented with *Streptococcus thermophilus* FP4 and *Bifidobacterium breve* BR03 showed a lower IL-6 concentration up to 48 h after damaging

training. Improvement of maximal voluntary isometric peak torque at 24 to 72 h following damaging exercises as well as flexed arm angle after the damaging workout was also shown (16). In our study, any significant changes in the level of TNF- α, IL-6, Il-2, and IL-15 were not observed. It is important to note that both groups received vitamin D3 supplementation. Therefore, it can be assumed that the treatment with vitamin D3 had a beneficial impact on reducing pro-inflammatory markers in MMA athletes. Furthermore, vitamin D3 supplementation may serve as an attenuating factor in regulating inflammation and inhibiting the immune system, making it a modifiable risk factor for reducing inflammation. This may explain why we did not observe any significant differences of pro- as well as anti-inflammatory markers in serum between the PRO+VIT D and VIT D groups. Similarly, Hoffman et al. also detected no difference in pro-inflammatory cytokines levels after *Bacillus coagulant* supplementation period among soldiers (45).

Numerous data report that an increase of microbiome diversity and a higher abundance of health-promoting bacteria species are associated with enhanced fitness (27). In our study, we observed that 4 weeks of supplementation was enough to significantly increase beta but not alpha diversity. The effect was not detected in the VIT D group. We found positive changes in the gut bacteria profile, as increase of *Bacteroides, Peptostreptococcaceae bacterium, Roseburia inulinivorans* species, and *Prevotella* genus, and decrease of potentially harmful *Lachnospiraceae bacterium*. Moreover, we detected that the *Lactobacillaceae* class augmented despite the decline of *Firmicutes* species.

The vast majority of intestinal bacterial species are *Firmicutes* and *Bacteroidetes*, thus the relative ratio between Firmicutes to Bacteroidetes (F:B) is used to describe the gut microbiota homeostasis (46). It is established that in obese individuals F:B ratio is elevated (46). However, it seems that physical training may increase bacterial species within the Firmicutes phyla. This finding was confirmed by Durk et al., who indicated that VO_2 max was associated with an elevation in the F:B ratio among young healthy individuals (47). In the current study, we observed no differences in VO_2 max in both groups, but we found a decrease of the F:B ratio. The combined supplementation significantly increased the total abundance of the *Bacteroides* genus and caused a reduction of the total abundance of *Firmicutes* phyla. However, some beneficial bacteria within Firmicutes phyla, e.g., *Roseburia inunilivorans, Lactobacillaceae,* and *Negativicutes* increased.

The analysis of the gut microbiome indicated a higher level of *Feacilibacterium* genus after combined intake. This bacteria was correlated with the improvement of intestinal health via an increase of butyrate production as well as by lowering the oxygen tension (48). The effect was confirmed by Yoonmi et al., who supplemented mice with *Feacilibacterium prausnitzi* and *Akkermansia muciniphila* (49). The authors observed recovery of the gut barrier function and increased zonulin production. In contrast, our results showed no difference in zonulin level. However, we found a reduction of fecal calprotectin – a marker of intestinal inflammation. A similar effect was found by MinAh et al., who supplemented patients with functional diarrhea with *Lactobacillus plantarum* CJLP243. The authors showed that two months of intervention resulted in a reduction of fecal calprotectin concentration (50).

Interestingly, our results displayed a significant increase of the *Collinsella* genus after combined probiotic and vitamin D3 supplementation. It was reported that this bacteria grows during a high-carbohydrate diet and was associated with improved time-trail performance by +6.5% (51). Similarly,

an increase of the relative abundance of the Collinsella genus was observed by van Zanten et al., who supplemented healthy humans with synbiotics (*Lactobacillus acidophilus* NCFM and cellobiose) (52). However, the authors did not investigate any parameters of sport performance. The *Collinsella* genus is described as having favorable anti-inflammatory and immunomodulatory effects (9). Moreover, the study conducted by Kassinen et al. presented that a lower abundance of *Collinsella* genera occurs in people with irritable bowel syndrome (IBS) (53). Therefore, these bacteria may play a protective role against intestinal barrier dysfunction during stress. Furthermore, it was shown that *Collinsella* genera are associated with high blood insulin levels and have broad dietary carbohydrate metabolizing potential (51). Similarly, recent metagenomic analysis linked the growth of *Prevotella* with the increased ability of intestinal microbes to carbohydrate metabolize (54). The link between a high-fiber diet and *Prevotella* abundance was present by Kovatcheva-Datchary et al. who observed that this kind of diet resulted in the growth of the *Prevotella* genus. Moreover, the authors indicated that changes in the gut microbiome composition positively correlated with improved glucose metabolism, partially by promoting increased glycogen storage (55). Thus, we suggest that improvement in exercise performance may be related to enhancement of the efficiency of energy processes involved in carbohydrate metabolism via shifts in microbes engaged in glucose metabolism. However, there is a lack of studies investigating the effect of probiotic supplementation on the gut microbiome composition in MMA athletes.

Our previous published data showed that athletes who took a combined probiotic and vitamin D_3 mixture improved lactate metabolism rate after SIE (20). Interestingly, the analysis of the gut microbiome indicated a higher abundance of *Negativicutes* class after 4 weeks supplementation period, whereas the VIT D group showed a slight decrease of this class. It is known that some human bacteria belonging to the *Negativicutes,* e.g., *Phascolarctobacterium succinatutens,* can convert succinate to propionate, and the other one, like *Veilonella spp.* convert lactate to propionate (56). The accumulation of LA and hydrogen ions in skeletal muscle and blood circulation impair physical performance due to the limitation of glycolysis and the development of fatigue during exercise (57). Thus, we suppose that *Negativicutes* class increasement might enhance endurance capacity, partially via improvement of lactate metabolism and thus provide additional energy. By our results, a link between members of *Negativicutes* class (*Veilonella* genus) and exercise performance was identified by Scheiman et al. Researchers observed that the relative abundance of *Veillonella* is higher in marathon runners after marathons and that inoculation of *Veilonella atypica* into mice improved exhaustive treadmill runtime (38).

One of the strengths of our study was that we not only evaluated the direct effects but also examined changes in the composition of the intestinal microbiome. We demonstrated that the intervention resulted in significant changes in the gut microbiome, which had a beneficial effect on exercise capacity. One limitation of the study was the lack of diet standardization. However, in order to minimize the potential influence of diet on the gut microbiome, athletes were instructed not to make any changes to their existing eating habits, and they were also advised to refrain from taking any medications, consuming alcohol, or smoking.

Our results indicate that combined probiotic and vitamin D_3 treatment is beneficial for MMA athletes and may lead to shifts in both alpha and beta diversity as well as in the composition of the gut microbiota. We found a decrease in calprotectin concentration after probiotic supplementation, indicating an improvement in epithelial cell permeability. It shows that probiotics supplementation may protect athletes against intestinal inflammation. Furthermore, this supplementation extended the time to exhaustion during exercise in MMA athletes. This is a result directly indicating the benefits of supplementation with probiotics in sports, which shows that in fact, the optimization of the intestinal microbiota has a positive effect on exercise capacity. However, an effect on blood inflammatory markers and gut SCFA profiles in both groups was not observed. Our data suggest a bidirectional communication pathway between muscle cells and gut microbiota, confirming the beneficial effects of combined probiotics and vitamin D_3 in competitive athletes.

5. Figures and tables

5.1.Figures

Figure Legends

Figure 1. Visit programme

Figure 2. The comparison of changes in alpha diversity between groups as a result of the intervention ($p = 0.086$), FDR adjusted p-value ($Q = 0.166$). B The comparison of changes in beta diversity measured via Bray-Curtis between groups as a result of the intervention (***$p = 0.0005$). *BS – before supplementation; AS – after supplementation.*

Figure 3. Heatmap of the abundance of the gut microbiota at the genus level center-log transformed (CLR) among each group.

Figure 4. Calprotectin and zonulin content before and after supplementation A) Calprotectin Statistical significant ($^{*}p < 0.05$) BS vs AS in the group PRO+VIT D; B) Zonulin the lack of changes in both groups.

5.2.Tables

Table 1. Participants characteristics.

Participants' Information	VIT D Mean ± SD	PRO+VIT D Mean ± SD
Age	26.02 ± 4.00	24.70 ± 6.50
Height (cm)	179.30 ± 7.70	182.20 ± 9.30
Weight (kg)	80.20 ± 9.80	81.10 ± 12.00
FFM kg)	71.94 ± 8.20	73.61 ± 9.30

Vit D BS [ng/mL]	29.56 ± 12.62	24.89 ± 9.68
Vit D AS [ng/mL]	30.63 ± 11.66	26.07 ± 10.39
HR max	185.00 ± 10.73	187.00 ± 11.34
Years of training	10.10 ± 4.40	9.90 ± 4.00
Quantity of training (hours/week)	11.40 ± 3.10	11.80 ± 3.40

FFM – fat-free mass; BS – before supplementation; AS – after supplementation; SD, standard deviation, HR max – maximal heart rate obtained during VO_2 max test.

Table 2. Results obtained during aerobic fitness assessment.

Variable	VIT D			PRO+VIT D		
	BS	AS	p-value	BS	AS	p-value
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
RER [vCO_2/vO_2]	1.19 ± 0.10	1.19 ± 0.06	0.999	1.21 ± 0.08	1.19 ± 0.05	0.869
VO_2 max [$ml/min/kg^{-1}$]	52.33 ± 5.06	52.48 ± 3.76	0.999	56.92 ± 0.83	56.37 ± 7.09	0.969
MAP [W]	306.82 ± 33.70	311.36 ± 40.87	0.940	317.50 ± 45.72	330.00 ± 42.16	0.096
Time [s]	489.91 ± 72.02	468.55 ± 102.03	0.668	496.30 ± 89.98	559.00 ± 68.99	0.023*

RER – respiratory exchange ratio; VO_2 max – maximal oxygen uptake*; MAP* - maximal aerobic power; Time – exercise to exhaustion time; *BS – before supplementation; AS – after supplementation; SD, standard deviation; Statistical significant (*p < 0.05).*

Table 3. Average marginal effects as a difference in predicted outcomes (Before workout *versus* After workout) for the combination of levels of supplementation and intervention.

Outcome	Time	Intervention	Est	SE	P	$Est_{pairwise}$	$SE_{pairwise}$	$P_{pairwise}$
IL2	BS	VIT D	-0.008	0.056	0.888	0.024	0.077	0.757
	BS	PRO+VIT D	0.016	0.053	0.763			
	AS	VIT D	0.022	0.056	0.693	0.004	0.077	0.962
	AS	PRO+VIT D	0.026	0.053	0.631			
IL6	BS	VIT D	1.26	0.35	<0.001**	-0.02	0.48	0.975
	BS	PRO+VIT D	1.24	0.33	<0.001**			
	AS	VIT D	0.76	0.35	0.029*	-0.05	0.48	0.915
	AS	PRO+VIT D	0.71	0.34	0.033*			

IL15	BS	VIT D	-0.64	1.51	0.675	2.04	2.09	0.330
	BS	PRO+VIT D	1.40	1.45	0.332			
	AS	VIT D	3.13	1.51	0.038*	-3.86	2.09	0.065
	AS	PRO+VIT D	-0.73	1.45	0.616			
TNF-α	BS	VIT D	168.2	108.6	0.122	-201.8	147	0.170
	BS	PRO+VIT D	-33.6	99.2	0.734			
	AS	VIT D	68.0	108.6	0.531	-83.1	147	0.572
	AS	PRO+VIT D	-15.2	99.2	0.878			

*Est – estimates; SE – standard error; statistically significant (**p < 0.001); statistically significant (*p < 0.05). BS-before supplementation; AS-after supplementation.*

Table 4. Total abundance of selected bacteria before and after 4 weeks of probiotics supplementation.

Outcome	**Intervention**	**Est**	**SE**	**P**	**$Est_{pairwise}$**	**$SE_{pairwise}$**	**$P_{pairwise}$**
Bacteroides fluxus	VIT D	0.08	0.48	0.859	2.02	0.66	0.002**
	PRO+VIT D	2.11	0.46	<0.001			
Lachnospiraceae bacterium	VIT D	-0.28	0.27	0.309	-1.27	0.38	<0.001**
	PRO+VIT D	-1.55	0.26	<0.001			
Roseburia inulinivorans	VIT D	-0.66	0.36	0.065	1.40	0.49	0.005**
	PRO+VIT D	0.74	0.34	0.030			
Peptostreptococcaceae bacterium	VIT D	0.84	0.68	0.222	-2.69	0.95	0.004**
	PRO+VIT D	-1.86	0.65	0.005			
Bacteroides genus	VIT D	0.30	0.67	0.657	2.29	0.92	0.013*
	VIT D	0.30	0.67	0.657			
Collinsella genus	VIT D	0.37	0.46	0.413	-1.65	0.63	0.009**
	PRO+VIT D	-1.28	0.44	0.003			
Faecalibacterium genus	VIT D	-0.19	0.35	0.601	1.21	0.49	0.013*
	PRO+VIT D	1.03	0.34	0.002			
Prevotella genus	VIT D	0.62	0.96	0.516	-1.01	0.33	0.002**
	PRO+VIT D	3.62	0.92	<0.001			
Lactobacillaceae family	VIT D	0.88	0.62	0.158	-2.04	0.86	0.018*
	PRO+VIT D	-1.17	0.60	0.050			
Negativicutes class	VIT D	-0.25	0.75	0.738	2.23	1.04	0.032*
	PRO+VIT D	1.98	0.72	0.006			
Firmicutues class	VIT D	-0.75	0.32	0.021	-0.93	0.45	0.038*
	PRO+VIT D	-1.68	0.31	<0.001			

*Est – estimates; SE – standard error; Statistically significant (**p < 0.01). statistically significant (*p < 0.05).*

Table 5. Short-chain fatty acids - average marginal effects as a difference in predicted outcomes (BS *versus* AS) for PRO+VIT D and VIT D groups.

Outcome	Intervention	Est	SE	z	P	$Est_{pairwise}$	$SE_{pairwise}$	$P_{pairwise}$
C2:0 (%)	VIT D	-0.39	2.57	-0.15	0.879	-3.19	3.51	0.364
	PRO+VIT D	-3.58	2.39	-1.50	0.134			
C3:0 (%)	VIT D	-2.30	0.81	-2.86	0.004	2.07	1.10	0.061
	PRO+VIT D	-0.23	0.75	-0.31	0.756			
C4n (%)	VIT D	2.26	2.04	1.11	0.266	1.20	2.79	0.668
	PRO+VIT D	3.46	1.90	1.82	0.069			
C5n (%)	VIT D	-0.21	0.21	-1.01	0.311	0.13	0.29	0.650
	PRO+VIT D	-0.08	0.20	-0.43	0.670			

C2:0-acetic acid; C3:0 – propionic acid; C4:0- butyric acid; C5:0 – Valeric acid; Est – estimates; SE – standard error; z – Est/SE

6. Conflict of Interest

Marcin Folwarski received remuneration for lectures from probiotic company. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization and methodology: KP, MF and JJK; investigation: KP, SK; Laboratory analysis: KP, KS-Ż, JP, ZKB, statistical analysis: MK, KS-Ż, KP and JJK; writing—original draft preparation: KP, JJK; writing—review and editing: KP, SK, MF, KS-Ż, ZKB, MK and JJK; supervision: JJK All authors have read and agreed to the published version of the manuscript.

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8. Supplementary Material

Supplementary Material should be uploaded separately on submission, if there are Supplementary Figures, please include the caption in the same file as the figure. Supplementary Material templates can be found in the Frontiers Word Templates file.

12 Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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