Effect of exercise on the human microbiome

Abstract of PhD Thesis

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1. Introduction

The coronavirus epidemic which emerged in 2019 and reached Hungary in 2020 is the first pandemic of the twenty-first century, which can be classified without exaggeration as a global health crisis. The human body is exposed to attacks by various pathogens every day, and epidemics break out on earth year after year, or even month after month. For example in 2009, the H1N1 influenza variant gained special attention due to its major pandemic, and a particularly virulent strain of this subtype was responsible for the infamous Spanish flu of 1918, which is estimated to have claimed over 50 million lives.

The rapid spread of the virus, as well as the scarce knowledge about its pathology, as well as the lack of vaccines resulted in a multitude of unprecedented measures in the 20th and 21st centuries. In addition to social distancing, mask wearing, assembly and confinement restrictions, the introduction of home quarantine was also intended to help reduce the number of new patients. From another perspective, the goal was to ensure that as many people as possible in the population remained healthy (or at least showed mild symptoms) at any given time, as it was essential to reduce the number of those requiring hospital care, thereby avoiding the overload of the healthcare system.

Already at the beginning of the coronavirus pandemic, reports indicated that mortality was higher among older age groups and among infected individuals with other chronic conditions, such as cardiovascular disease or diabetes. Furthermore, it is likely that obese individuals and those with low fitness scores have lower survival chances compared to individuals with average nutrition. A recent study showed that those infected with low physical activity were almost four times more likely to have fatal illness (OR 3.9) compared to those with high physical activity. This information suggests that healthy individuals who move more, are fitter, and have greater physiological reserves are likely to be more resistant to coronavirus and perhaps other infectious diseases as well.

The important question is which variables can be used to define the aforementioned "health" or "physiological reserve". Regular exercise and physical activity appear to trigger much more complex adaptation processes than we might have imagined 15-20 years ago. In addition to the well-known adaptation processes of the cardiovascular system and skeletal muscles, recent studies have also shown that microorganisms living in the gastrointestinal tract respond to exercise. The composition of the microbiome can be linked to immune processes, which play a crucial role in the course of COVID-19 infection, and the severity of the disease outcome is often associated with the modulation of individual immune responses (cytokine storm). It's an intriguing question whether the species composition of the microbiome reacts to COVID-19 infection, and whether trained groups show any beneficial gut microbiological status, such as greater species diversity, which might be correlated with milder disease progression. Can the composition of the microbiome be linked to fitness levels, and what changes in fitness or health indicators correlate with different bacterial taxa?

2. Objectives

Previous studies have uncovered numerous factors influencing the microbiome. Building upon and extending beyond the aforementioned studies, we aim to demonstrate the impact of viral infection and regular exercise on the microbiome. The rapid and global spread of the virus requires studies to develop preventive and therapeutic tools. In this regard, treatments focusing on the immunopathology of SARS-CoV-2 infection have received primary emphasis. Among individuals infected with the virus, factors such as diet, nutrition, age, gender, health status, lifestyle, and environmental conditions greatly influence the course of coronavirus infection, ranging from mild symptoms to death. Careful examination of potential nutritional, lifestyle, and environmental risks and the development of appropriate risk management strategies are important parts of managing the coronavirus pandemic. The aim of the studies outlined in my doctoral dissertation is to find answers to the question of whether the microbiome of athletes and non-athletes plays a role in how trained individuals experience coronavirus infection, and whether indicators of fitness and health are statistically related to the composition of the microbiome. The results of the microbiome study may be crucial for understanding the individual immune response, and later in improving the effectiveness of the immune response or in customizing individual load capacity.

Based on previous research, we hypothesize that the microbiome of trained people will differ from the microbiome of untrained participants, and the composition of the gut microbiome in recovered subjects will differ from the composition of microbiome samples taken at the onset of illness. Another assumption is that the microbiome plays a role in determining whether a trained individual is more likely to overcome COVID-19 infection.

Within another study, which forms part of my dissertation, we aimed to determine whether the microbiological status characterizing the nominal "trained" and "untrained" categories formulated in the first study can be further refined along the lines of some fitness or health markers.

Different athletes often have varying fitness indicators, so it is conceivable that the microbiological composition also shows correlations along these indicators. This study was designed to test the hypothesis that.

1. We assume that in the longitudinal sample, the gut microbiome composition of trained individuals will differ from that of untrained participants (Babszky et al 2021).

2. We assume that in the longitudinal sample, the gut microbiome composition of recovered subjects will differ from the composition of microbiome samples taken at the onset of illness (Babszky et al 2021).

3. We assume that in the longitudinal sample, the microbiome plays a role in determining whether a trained individual is more likely to overcome the COVID-19 virus (Babszky et al 2021).

4. We assume that in the cross-sectional sample, fitness and health indicators are significantly associated with the abundance of different bacterial taxa in the gut microbiome (Torma et al 2024).

3. Material and method

3.1. Subjects of the longitudinal study who have recovered from COVID-19

In the longitudinal study, we examined twenty active, competitive athletes who competed in the first and second divisions of the national championships (16 men and 4 women), with an average age of 24.15 ± 4.7 years, as well as twenty sedentary, non-athlete subjects (15 men and 5 women), with an average age of 27.75 ± 7.5 years. Every member of both groups was diagnosed with COVID-19 through a positive PCR test. At the time of the current study, a significant proportion of the infection in Hungary was caused by the B.1.1.7 variant of the COVID-19 virus, which was first sequenced in south-east England and called α variant.

Classification into the athlete and sedentary categories was based on the number of weekly training sessions and the level of the domestic division of their respective sports. The elite athlete group consisted of those who competed in the first or second division of their sport. The elite athlete group consisted of those who competed in the first or second division of their sport. The non-athlete group included those who did not engage in sports (with weekly training hours not exceeding 2.5 hours, based on the WHO definition of Physical Activity and Adults). Participants from both groups were asked to provide information about their exercise and dietary habits, as well as the severity of the COVID-19 symptoms they experienced.

The four symptom groups were:

- Group 1: COVID-19 infection confirmed by molecular biology methods (PCR), but no symptoms;
- Group 2: Mild disease;
- Group 3: Moderate disease;
- Group 4: Severe disease.

Initially, no one was placed in groups 3 and 4; however, during the study, 2 athletes required hospitalization. They were diagnosed with bilateral pulmonary embolism and were subsequently moved to group 4 during the study.

The completion of the dietary questionnaire occurred during both the first and second sampling periods. During the first sampling, we inquired about the dietary habits of the preceding month, while in the second dietary questionnaire, we were interested in the dietary habits between the two sampling periods. This is because diet, alongside factors such as exercise, age, and numerous others, significantly influences the composition of the microbiome. We utilized Food Frequency Questionnaires (FFQ), tailored to the dietary habits of our country. The questionnaire included discrete-scale frequency variables.

3.1.1. Stool sample collection during the longitudinal study

The composition of the gut microbiome was determined from stool samples using 16S rRNA DNA sequence analysis.

The stool samples were collected two to three days after the diagnosis with the COVID-19 PCR test, and three weeks after a negative COVID-19 PCR test. Participants collected the stool samples at home themselves.

Within one hour of collection, we took the sample and stored it at -80°C until analysis. For DNA extraction, we used 100 mg stool samples. During the collection of stool samples, we ensured proper protective clothing and avoided contact, while adhering to the relevant epidemic situation restrictions.

Following phone or internet consultations, we delivered the stool container, along with the research information sheet and consent form, to the quarantine location while wearing appropriate protective gear. We placed it in front of the entrance, and when we left, the study participant picked it up. The subjects signed the documents wearing appropriate protective gear. We also wore masks and gloves during the process, and disinfected the storage box and our hands after each sampling.

Once the sample was placed in the container, we contacted the participants, and placed a box containing dry ice outside the door of their home quarantine. After our departure, they placed the sample into the box and notified us so we could collect it. Within a maximum of 24 hours after this, we took the samples to the testing laboratory, where the analysis was carried out while adhering to the appropriate epidemic control measures.

The collection of the second stool sample occurred three weeks after the end of the two-week home quarantine, following the same procedure as mentioned above.

3.2. Description of the veteran athletes participating in the cross-sectional study

The subjects of the cross-sectional study were selected from the participants of the 2019 World Rowing Masters Regatta in Venice. The sample collection process was carried out in the same way as the longitudinal study, except for the epidemic situation protocol.

A total of 80 volunteers provided samples. The age of the sample donors ranged from 38 to 84 years old, of which 33 were men and 47 were women.

In this study, we were interested in whether the microbiome composition is related to fitness indicators or markers indicating the health status of the body.

3.2.1. Stool sampling during the cross-sectional examination

During the cross-sectional study, stool sample collection took place in a storage unit similar to the longitudinal study. Upon arrival for the fitness measurements, all participants brought their sample, which was stored anonymously at -80 degrees C until the analysis.

3.2.2. Fitness indicators

The Chester step test was used to estimate the maximum relative aerobic capacity (VO2max).

Hand grip strength was measured with a hand dynamometer.

The explosive strength of the lower extremity was measured with a vertical jump test with maximum knee flexion using a linear encoder.

3.3. Biomarkers of health status

Before the fitness tests, blood was taken from the subjects' elbow veins in tubes containing EDTA anticoagulant. The subjects' blood LDL, HDL, total cholesterol and triglyceride levels were measured using standard laboratory techniques in the analytical laboratory of Semmelweis University.

The irisin level of the subjects was determined using an ELISA kit.

The redox balance was defined as the ratio of BAP and reactive oxygen metabolites to their derivatives.

3.4. Library Preparation and Identification of Prokaryotic Species

The DNA from stool samples was isolated by a QIAmp Fast DNA Stool Mini Kit (Qiagen, Beverly, MA, USA). Fragment libraries were constructed from purified DNA using NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs) according to the manufacturer’s instructions.

3.5. Bioinformatics Analysis

In the first study, the taxonomic classification and sequence data preprocessing was carried out by the HuGe-F laboratory, using the RAPtorU v3.0 pipeline. The quality threshold (Phred score > 30), 52,754 reads/sample on average and 5331 distinct ASVs (amplicon sequence variant covering 659 taxa). The ASVs were assigned to taxonomy using the RDP algorithm with default parameters.

3.6. Statistics

The rare (maximum of 10% were present in all samples) and low-abundance (support of less than 100 reads) taxa were discarded from the subsequent analysis as well as the samples with low coverage (#reads/sample < 30,000). After the filtering process a Bayesian-multiplicative replacement of zeros was carried out using the zCompositions R package, which was followed by a centered log-ratio (CLR) transformation of count and ratio values as implemented in scikit-bio.

The visualization of the microbiome was carried out with biplot PCA (principal component analysis) using the CLR values as an input matrix (scikit-learn v0.24).

The Shannon index was used to measure α diversities, which quantify the entropy of the distributions of taxa proportions.

The compositional similarities between the different groups were investigated with PERMANOVA and the differential abundance testing was done using the Wilcoxon signed-rank test (comparing biome before–after COVID) and the Wilcoxon rank-sum test.

The cross-sectional data were processed using MetaPhlAn3, and the abundance of signaling/biological pathways and other molecular function profiles (gene ontology) were estimated using the HUMANN3 pipeline.

4. Results

4.1. Results of longitudinal research

Two subjects with low levels of reads were excluded from the study. The severity of COVID-19 ranged between grade 2, (mild infection) and grade 4 (severe illness). Interestingly enough, the only two subjects with severe illness symptoms of COVID-19 were professional athletes.

The microbiome analysis at the phylum level revealed that although the bacterial flora of the same subjects during and after COVID-19 infection was quite similar, assessed by correlation (Figure 1), even antibiotic treatment did not show significant alterations.

At the phylum level the relative concentration of *Bacteroidetes* was elevated during COVID-19 infection compared to levels measured three weeks following a negative PCR test ($p< 0.05$) when all subjects were included in the statistical analysis.

The *Bacteroidetes* levels were higher during COVID-19 infection than after recovery. The only significant difference was observed at the phylum and species level: the *Bacteroidetes* had higher abundance at phylum and *B. Vulgatus* at species level during COVID-19 infection than two–three weeks after a negative PCR test ($p <0.05$). $n = 38$. The confidence at *Bacteroidetes* case was 99.19%. CLR: centered log-ratio, $n = 38$.

Significant differences were not found in the bacterial flora of trained and sedentary groups, and we could not identify any bacteria which could be linked to the severity of COVID-19 infection. The Shannon diversity of the microbiome was independent from training status or COVID-19 infection.

Based on the provided data on nutritional habits, significant alterations were not present at the examined periods, during and after COVID-19 infection. The average daily consumption of carbohydrate, protein and fat during the COVID-19 infection of the athletes was 158.6 g, 74.5 g and 75.6 g, and after a negative PCR test was 158.7 g, 65.5 g and 75 g, respectively. The daily carbohydrate, protein and fat consumption of sedentary subjects during COVID-19 infection was 144.2 g, 52.5 g and 56.7 g, and was 146.2 g, 53.2 g and 56.9 g after infection, respectively.

4.2. Results of cross-sectional research

A higher level of physical fitness can be associated with a slower aging process and a longer lifespan. Microbiome changes occur in various diseases and during the aging process, but their relationship with epigenetic clocks has not been investigated. To fill this lack of knowledge, we collected metagenomic, epigenetic, and exercise-related data from physically fit individuals and examined the relationship between gut flora, epigenetic aging, and physical fitness. In this dissertation. I will not go into detail about the data related to epigenetic clocks.

The average relative abundance at the tribe level differed in the case of Firmicutes and Proteobacteria, while in the case of Actinobacteria, Bacteroidetes, Euryarcaeota, and Verrucomicrobia, no significant difference between the sexes was shown.

We examined the correlation separately by gender, since men and women differ significantly in the fitness index due to their biological characteristics.

Based on our study, the racial diversity index in men at different taxonomic levels was negatively correlated with VO2max, maximum jump and redox balance, and a significant positive correlation was detected between the triglyceride level.

In the case of women, a negative correlation was demonstrated between the body mass index (TTI, BMI) and the diversity index.

Taken together, these results suggest that an increase in microbial entropy in the intestinal tract can be negatively associated with fitness and health.

In the case of men, at the tribal level, the presence of Euryarchaeota was positively correlated with the blood triglyceride level, and a similar positive relationship was demonstrated between Bacteroidetes and redox balance. In contrast, there was a negative relationship between Euryarchaeota and redox balance, and between Proteobacteria and VO2max.

In women, the relative abundance of Firmicutes at the tribal level was negatively related to BMI and triglyceride levels, and positively correlated with HDL levels.

We then considered whether, refining our correlation studies to the species level, we would find robust ($p<0.01$) relationships that could assign bacterial species to a fitness or health status marker.

Based on our results, the relative aerobic capacity in men showed an outstanding positive correlation with the abundance of Fusicatenibacter saccharivorans, and a positive correlation was also detected between Coprococcus comes and blood HDL and Blautia obeum and body mass index. Redox balance was positively, while blood LDL and triglycerides were negatively related to the occurrence of Faecalibacterium prausnitzii.

In women, although we did not find a robust correlation with bacterial species, the abundance of Roseburia faecis was associated with a remarkable number of markers. These included the positive VO2max and the negative association with blood irisin, LDL, triglyceride and total cholesterol values.

5. Conclusions

Based on my hypotheses, we can make the following conclusions regarding exercise, gut microbiome and the disease of COVID-19.

1. Our first hypothesis is that the gut microbiome composition of trained people will differ from the gut microbiome composition of untrained participants. **WE DON'T ACCEPT IT**

2. The composition of the gut microbiome of recovered subjects will differ from the composition of the microbiome taken at the onset of the disease. **WE ACCEPT**

3. The microbiome plays a role in whether a trained body is more susceptible to the COVID-19 virus. **WE DON'T ACCEPT IT**

4. We assume that, in a cross-sectional sample, fitness and health indicators have a significant relationship with the abundance of different bacterial taxa of the gut microbiome. **WE ACCEPT**

Furthermore, it can be stated that the bacterial flora, in the case of the same subjects, was quite similar during and after the COVID-19 infection. At the phylum level, the concentration of Bacteroidetes increased when all subjects were included in the statistical analysis. During infection, at the species level, B. vulgatus showed an elevated level.

We found no significant differences in the bacterial flora of the athlete and untrained groups, and we could not identify any bacteria that could be associated with the severity of the COVID-19 infection.

Our important result is that we examined the relationship between cardiovascular fitness (VO2max) and the bacterial composition, in which case significant correlations were demonstrated.

Finally, according to our results, in addition to the physiological variables of training, other health variables also play a role in the development of the appropriate microbiome. It is worth considering these factors and treating the individual as a complex system.

6. List of own publications

Announcements related to the dissertation

- Babszky G, Torma F, Aczel D, Bakonyi P, Gombos Z, Feher J, Szabo D, Ligeti B, Pongor S, Balogh L, Posa A, Radak Zs. (2021) COVID-19 Infection Alters the Microbiome: Elite Athletes and Sedentary Patients Have Similar Bacterial Flora. Genes, 12: 10.
- Torma F, Kerepesi Cs, Jokai M, Babszky G, Koltai E, Ligeti B, Kalcsevszki R, McGreevy K M, Horvath S, Radak Zs. (2024) Alterations of the gut microbiome are associated with epigenetic age acceleration and physical fitness. Aging cell, 23: 4.

Announcements not related to the dissertation

- Jokai M, Torma F, McGreevy K M, Koltai E, Bori Z, Babszky G, Bakonyi P, Gombos Z, Gyorgy B, Aczel D, Toth L, Osvath P, Fridvalszky M, Teglas T, Posa A, Kujach S, Olek R, Kawamura T, Seki Y, Suzuki K, Tanisawa K Goto S, Kerepesi Cs, Boldogh I, Ba Xueqing, Davies K J A, Horvath S, Radak, Zs. (2023) DNA methylation clock DNAmFitAge shows regular exercise is associated with slower aging and systemic adaptation. Geroscience, 45: 2805-2817.
- Torma F, Bakonyi P, Regdon Z, Gombos Z, Jokai M., Babszky G., Fridvalszki M, Virág L, Naito H, Iftikhar B S R, Radak Zs. (2021) Blood flow restriction during the resting periods of high-intensity resistance training does not alter performance but decreases MIR-1 and MIR-133A levels in human skeletal muscle. Sports Med Health Sci, 3: 40-45.
- Škugor K, Gilić B, Karninčić H, Jokai M, Babszky G, Ranisavljev M, Štajer V, Roklicer R, Drid, P. (2023). What Determines the Competitive Success of Young Croatian Wrestlers: Anthropometric Indices. Generic or Specific Fitness Performance?. J Funct Morphol Kinesiol, 8: 3.