Heliyon 10 (2024) e40663

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Effect of elite sport activity on salivary microbiota: The case of water polo

Iolanda Veneruso [a,b,1], Cristina Mennitti [a,1], Alessandro Gentile [a], Gennaro Di Bonito [a,b], Jacopo Ulisse [a], Carmela Scarano [a,b], Barbara Lombardo [a,b], Daniela Terracciano [c], Raffaela Pero [a,d], Giovanni D'Alicandro [e], Giulia Frisso [a,b], Valeria D'Argenio [b,d,f,*], Olga Scudiero [a,b,d]

[a] Department of Molecular Medicine and Medical Biotechnologies, Federico II University, Via Sergio Pansini 5, 80131, Napoli, Italy
[b] CEINGE-Biotecnologie Avanzate Franco Salvatore, via G. Salvatore 486, 80145, Naples, Italy
[c] Department of Translational Medical Sciences, University of Naples Federico II, 80131, Naples, Italy
[d] Task Force on Microbiome Studies, University of Naples Federico II, 80100, Naples, Italy
[e] Department of Neuroscience and Rehabilitation, Center of Sports Medicine and Disability, AORN, Santobono-Pausillipon, 80122, Naples, Italy
[f] Department of Human Sciences and Quality of Life Promotion, San Raffaele Open University, via di Val Cannuta 247, 00166, Roma, Italy

ARTICLE INFO

Keywords:
water polo
Microbiome
Salivary microbiota
Elite athletes

ABSTRACT

It has been well established that the human gut microbiota plays a pivotal role in humans' health, since it is involved in nutrients' uptake, vitamins' synthesis, energy harvest, inflammatory modulation, and host immune responses. Moreover, gut microbiota alterations have been associated to an increasing number of diseases and its composition can be affected by several factors, including physical exercise. In particular, it has been reported that intense physical activity can induce metabolic changes which translate in alterations of specific biomarkers that can lead to the onset of infections, inflammation and hepatic or kidney disorders. Recently, the oral microbiota has shown its relevance not only for the health of oral cavity but also for human host's health, emerging as an ecological niche with a great potential for the study of gut microbiome alterations due also to its accessibility respect to other tracts that can be inferred through fecal samples analysis. Thus, the purpose of this study has been to assess the effect of intense physical activity, i.e., elite water polo, on the human salivary microbiota. Thirteen professional water polo players and nineteen sedentary controls were recruited for this study. The salivary microbiota analysis was performed in oral rinse collected from both controls and athletes three months after the beginning of the agonist season. Our results showed significant differences in the salivary microbiota between athletes and controls. In particular, three species, namely *Oribacterium sinus*, *Oribacterium parvum* and *Oribacterium asaccharolyticum*, were found to be significantly increased in the water polo players compared to controls. Even if these data have to be further validated, also to assess the role of these identified species, they strengthen the hypothesis that elite sports can influence and alter the status of the gut microbiota. Moreover, the saliva is confirmed as a

* Corresponding author. CEINGE-Biotecnologie Avanzate Franco Salvatore, via G. Salvatore 486, 80145, Naples, Italy.
E-mail addresses: venerusoi@ceinge.unina.it (I. Veneruso), cristinamennitti@libero.it (C. Mennitti), alexgenti98@libero.it (A. Gentile), dibonito.g@gmail.com (G. Di Bonito), jacopo.ulisse@gmail.com (J. Ulisse), scaranoc@ceinge.unina.it (C. Scarano), barbara.lombardo@unina.it (B. Lombardo), daniela.terracciano@unina.it (D. Terracciano), pero@unina.it (R. Pero), ninodalicandro@libero.it (G. D'Alicandro), gfrisso@unina.it (G. Frisso), dargenio@ceinge.unina.it (V. D'Argenio), olga.scudiero@unina.it (O. Scudiero).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.heliyon.2024.e40663
Received 6 April 2024; Received in revised form 30 October 2024; Accepted 22 November 2024
Available online 23 November 2024

suitable sample for microbiome evaluations that may improve athletes' status evaluation and monitoring.

1. Introduction

The human microbiota consists of the 10–100 trillion symbiotic microbial cells harbored by each person, primarily bacteria and mainly in the gut; the genes carried by these cells build up the human microbiome [1,2]. Human body has 150 times more microbial genes than are present in the total human genome and contains at least 1000 different types of known bacteria [2]. During host growth, this colony of microorganisms progressively transforms into a highly varied ecosystem. Host-bacterial partnerships have evolved into advantageous connections throughout life [2]. In recent decades, several studies have demonstrated that the human microbiota plays a significant role in human physiology through various mechanisms. Firstly, the microbiota might boost the amount of energy that is extracted from meals, enhance the amount of nutrients harvested, and modulate appetite signaling [3]. Humans have access to specialized enzymes and biochemical pathways thanks to the microbiota, which has significantly more metabolic genes with a wide range of functions than the human genome. Furthermore, a significant fraction of the host-beneficial metabolic microbial activities, such as the metabolism of undigested carbohydrates, as well as vitamin biosynthesis, are involved in either nutrients absorption or xenobiotic processing [4]. Secondly, the human microbiota acts as a physical barrier to shield the host against outside pathogens by producing antimicrobial substances and engaging in competitive inhibition. Subsequently, the microbiota plays a key role in the host's immune system and intestinal mucosa development [5].

It has been largely assessed that microbial colonization involves the whole body, including body niches previously considered as sterile, and that this process begins already before birth [2,6]. Nevertheless, the majority of the human microbiota is localized in the gut where, as mentioned above, it plays a crucial role for human's host health establishment and maintenance, so much so that its alteration, or dysbiosis, has been associated to a large number of diseases. In this context, the oral microbiota has recently emerged as a powerful model to study microbial communities: it contributes to humans' health, is involved in the pathogenesis of an increasing number of diseases, and can be directly assessed offering an advantage respect to other less-accessible gut tracts that are usually inferred through the analysis of fecal samples [7]. In particular, the saliva has recently emerged as suitable sample to study the gut microbiota: indeed, alterations in salivary microbial communities have been identified in an increasing number of intestinal and systemic disorders, supporting the idea that factors able to modify the gut microbiota act on the whole digestive tract [8]. Moreover,

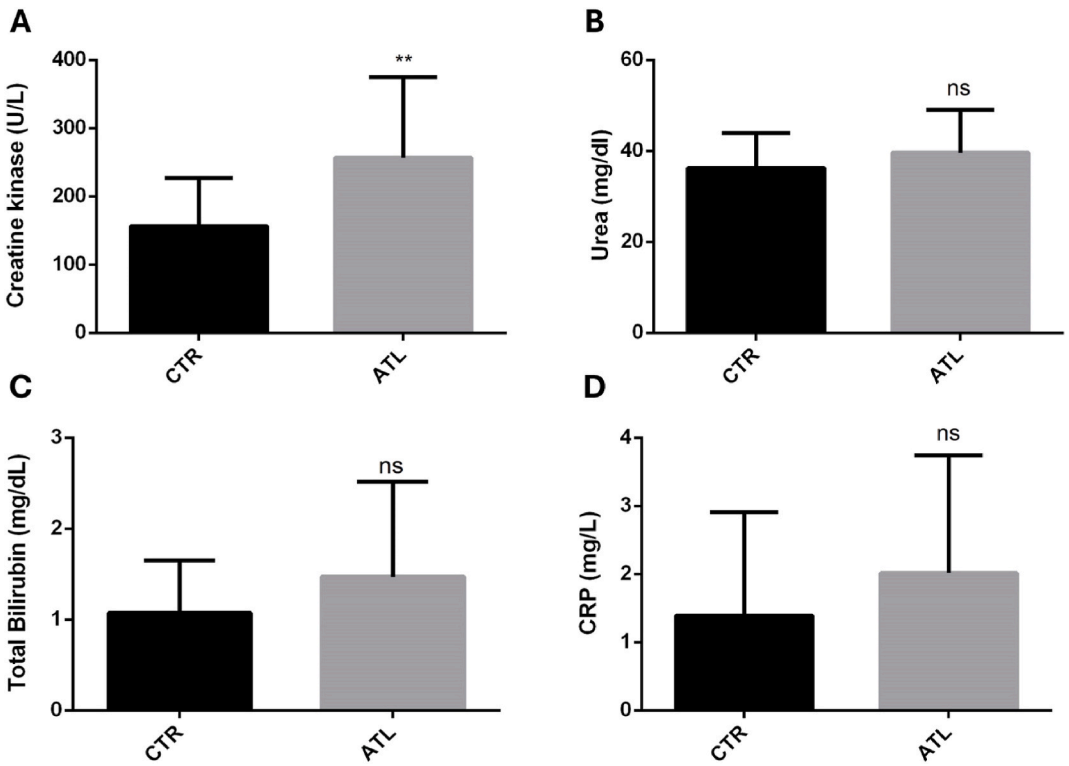

Fig. 1. Serum concentration of Creatine Kinase (A), Urea (B), Total Bilirubin (C) and CRP (D) in athletes compared to controls. The data are expressed as the means ± SDs. The significance was determined by the Student's t-test: ** ($p < 0.01$) represents significance compared to sedentary controls.

saliva is a non-invasive and easy-to-collect sample, so that the interest in its potential use is progressively increasing.

Physical activity may influence the composition and functional activity of the human microbiome independently of other external factors, according to cross-sectional and longitudinal research studies [9]. It is known that intense and prolonged exercise can cause metabolic adaptations. The variations in these parameters represent the changes that occur in the body in response to the intensity and duration of physical exercise [10]. These adaptations translate into alterations in specific parameters, in terms of concentration and activity, and their identification could represent a new method for monitoring the health of an athlete [11].

Here, we investigated the composition of the saliva microbiota in professional water polo athletes to verify any possible qualitative and/or quantitative microbiome alterations significantly associated with this physical activity practiced at elite level, and to assess also any correlation with other biochemical parameters. The results of this study highlight microbial adaptation to physical exercise that may influence athletes' health, thus suggesting that microbiome analysis may be a useful additional parameter to be included in the so-called athlete's passport [10].

2. Results

Thirty-two subjects, including 19 sedentary individuals ("Control" group) and 13 water polo professional players ("Water polo" group) were totally enrolled and analyzed as described under "Materials and Methods" section. All the study subjects were male and aged between 24 and 32 years.

2.1. Biochemical determinations

Biochemical serum evaluations were performed to verify any possible alterations related to sport activity. Our results have showed that creatine kinase levels are significantly increased in athletes if compared to controls; on the other hand, we observed an upward trend in urea, total bilirubin, and c-reactive protein (CRP) levels in the water polo respect to the control group (Fig. 1). No differences were identified in the other tested analytes (Table 1).

2.2. Microbiome analysis

Saliva microbiome profile was then carried out to identify qualitative and/or quantitative microbial features specifically associated to the Water polo group. Totally, we obtained an average of 131,946 reads/sample allowing for the identification of 1072 operational taxonomic units (OTUs). The negative controls, processed together with the collected saliva samples as internal control of the whole analytic procedure, were used for reads filtering in order to exclude possible environmental contaminations.

First, diversity analyses were performed. Richness (number of microbial taxa rep-resented in each study group) and evenness (representation of the identified taxa within each group) were both evaluated using respectively Observed species (Fig. 2A) and Chao1 (Fig. 2B) measures, and Shannon index (Fig. 2C). Interestingly, even if no significant differences were highlighted, both richness and evenness were reduced in the Water polo group suggesting that the saliva microbiome of the athletes may have a reduced biodiversity with a non-uniform representation of its taxa.

Additionally, beta diversity, measured using unweighted (Fig. 2D) and weighted UniFrac (Fig. 2E) distance measures, resulted significantly different ($p = 0.001$ and $p = 0.012$, respectively), thus suggesting that the differences between the 2 study groups are due more to the presence of different taxa rather than to their abundances.

Taxonomic assignment allowed to identify the taxa present in the analyzed communities. In particular, 4 most abundant phyla

Table 1
Serum biochemical parameters evaluated in athletes and controls. The data are ex-pressed as the means ± SDs. The significance was determined by the Student's t-test.

Parameters	Athletes (n = 13)	Controls (n = 19)	P-value
Urea (mg/dL)	39,62 ± 9,38	36,29 ± 7,65	0.2786
Total Bilirubin (mg/dL)	1,47 ± 1,05	1,07 ± 0,58	0.1759
Fructosamine (μmol/L)	292,77 ± 20,79	290,65 ± 22,90	0.7915
CK (U/L)	256,77 ± 118,15	156,24 ± 70,70	0.0052
Glucose (mg/dL)	86,92 ± 7,72	84,35 ± 7,74	0.3631
Uric Acid (mg/dL)	5,45 ± 0,72	5,97 ± 1,72	0.3131
Total Cholesterol (mg/dL)	192,46 ± 31,56	191,71 ± 41,68	0.9566
LDL (mg/dL)	133,00 ± 28,37	128,00 ± 42,55	0.7139
HDL (mg/dL)	54,92 ± 10,74	50,35 ± 15,62	0.3674
Triglycerides (mg/dL)	75,85 ± 38,30	117,65 ± 70,61	0.0617
Aldolase (U/L)	5,58 ± 1,36	5,98 ± 1,26	0.3977
AST (U/L)	27,00 ± 5,40	24,76 ± 5,63	0.2701
ALT (U/L)	29,38 ± 10,49	31,76 ± 16,65	0.6504
GGT (U/L)	18,31 ± 8,93	28,24 ± 18,99	0.0874
ALP (U/L)	83,69 ± 37,90	68,71 ± 9,77	0.1082
LDH (U/L)	183,85 ± 33,61	194,94 ± 29,38	0.3304
CRP (mg/dL)	2,02 ± 1,72	1,39 ± 1,52	0.2836

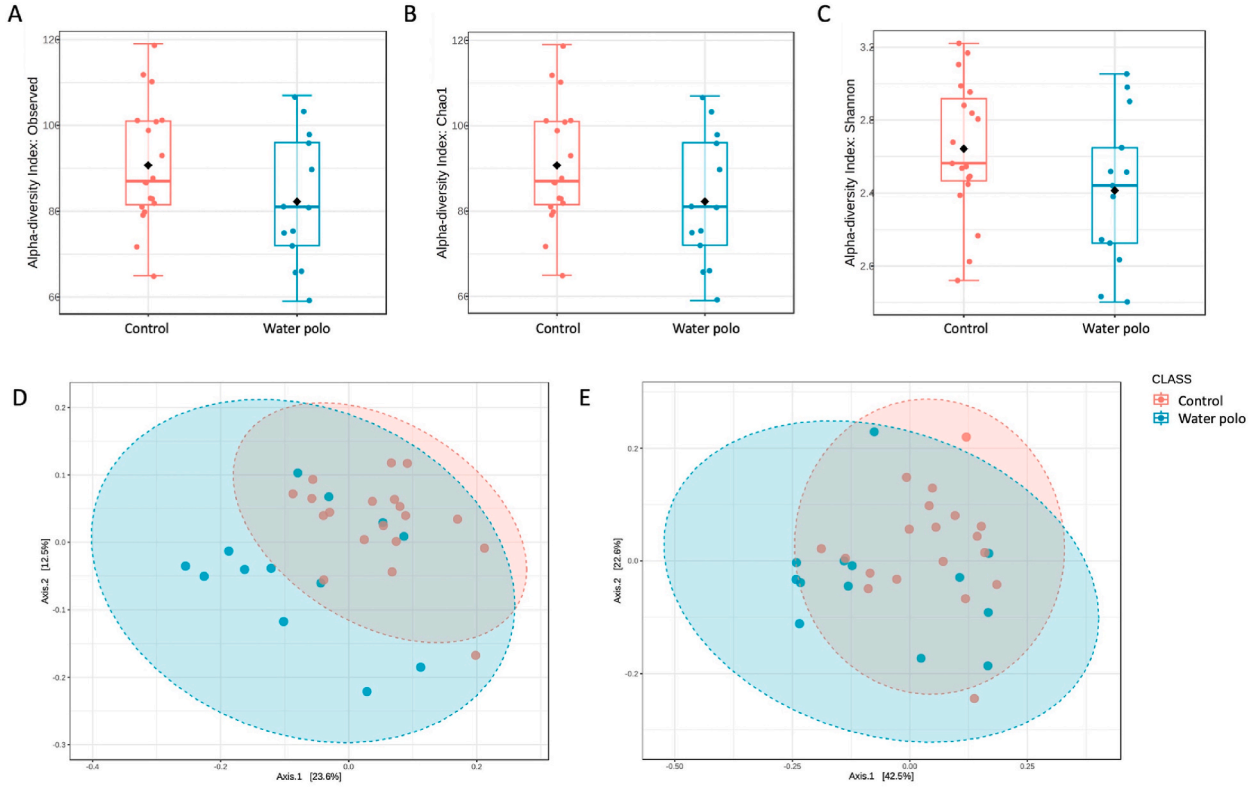

Fig. 2. Alpha and beta diversity analyses highlighted differences between Control and Water polo groups. In particular, alpha diversity was evaluated using Observed species (A) and Chao1 (B) measures, and Shannon index (C). No significant differences were found ($p = 0.13$ for all indexes), even if richness (A and B) and evenness (C) were all reduced in the water polo players. Moreover, beta diversity was assessed by using the unweighted (D) and weighted (E) UniFrac distance measures. Statistical significance was measured by the PERMANOVA test resulting significant in both cases ($p = 0.001$ and $p = 0.012$, respectively).

(percentage abundance >1 % in at least one of the tested conditions) were identified, Proteobacteria being the most represented in both groups (56 % and 52 % of relative abundance, respectively in Control and Water polo groups), followed by Firmicutes (23 % and 24 %) and Fusobacteria (3.9 % and 4 %). The Actinobacteria phyla showed a slight increase in the Water polo group (from 3.8 % to 6 %) (Fig. 3A). At genus level, 9 genera were the most represented (Fig. 3B).

To verify the presence of statistically significant differences in the microbial taxa identified in the 2 analyzed groups, differential abundance analysis was carried out. Interestingly, several significant features were identified, including 1 phylum, 2 classes, 3 orders, 9 families, 19 genera and 13 species. The full list of these 45 significant taxa is reported in Table 2 according to their taxonomic level and corresponding *p*-values. In particular, among the 13 differentially expressed species, 10 were more abundant and 3 less abundant in the Water polo group respect to the controls. To assess the potential significance of these differences, both Linear Discriminant Analysis Effect Size (LEfSe) and Random Forest analyses were performed (Fig. 4).

LEfSe analysis couples Kruskal-Wallis test, able to identify significant differentially abundant taxa between the compared groups, to Linear Discriminant analysis (LDA) to assess the effect size (i.e., the relevance) of these differential abundant features. In our study population, 15 significant features were identified both at genus and species level (Fig. 4A and D). Random Forest analysis was then performed, including the whole set of identified taxa, to highlight the presence of predictive features able to discriminate the 2 tested conditions. The obtained decision trees both at genus (Fig. 4B) and at species levels (Fig. 4E) differentiated the 2 groups providing also a list of predictive taxa mainly contributing to this difference (Fig. 4C and F for genus and species, respectively). Finally, to verify a possible correlation between the biochemical parameters altered in the athletes and their saliva microbiome, multivariate analysis was also performed. Interestingly, we found significant associations by using creatine kinase as covariate factor with 1 genus and 2 species significantly differentially abundant between Water polo and Control groups. In particular, the *Oribacterium* genus ($p = 6.58e\text{-}4$), the *Oribacterium sinus* ($p = 1.56e\text{-}06$) and the *Oribacterium parvum* ($p = 1.56e\text{-}06$) were significantly more abundant in the Water polo group respect to the Controls.

3. Discussion

Physical exercise impacts several body functions and is known to exert a beneficial effect on health [12]. Indeed, it has been established that regular sport activity improves immune system functions, ameliorates muscular and bone resistance and fitness, and

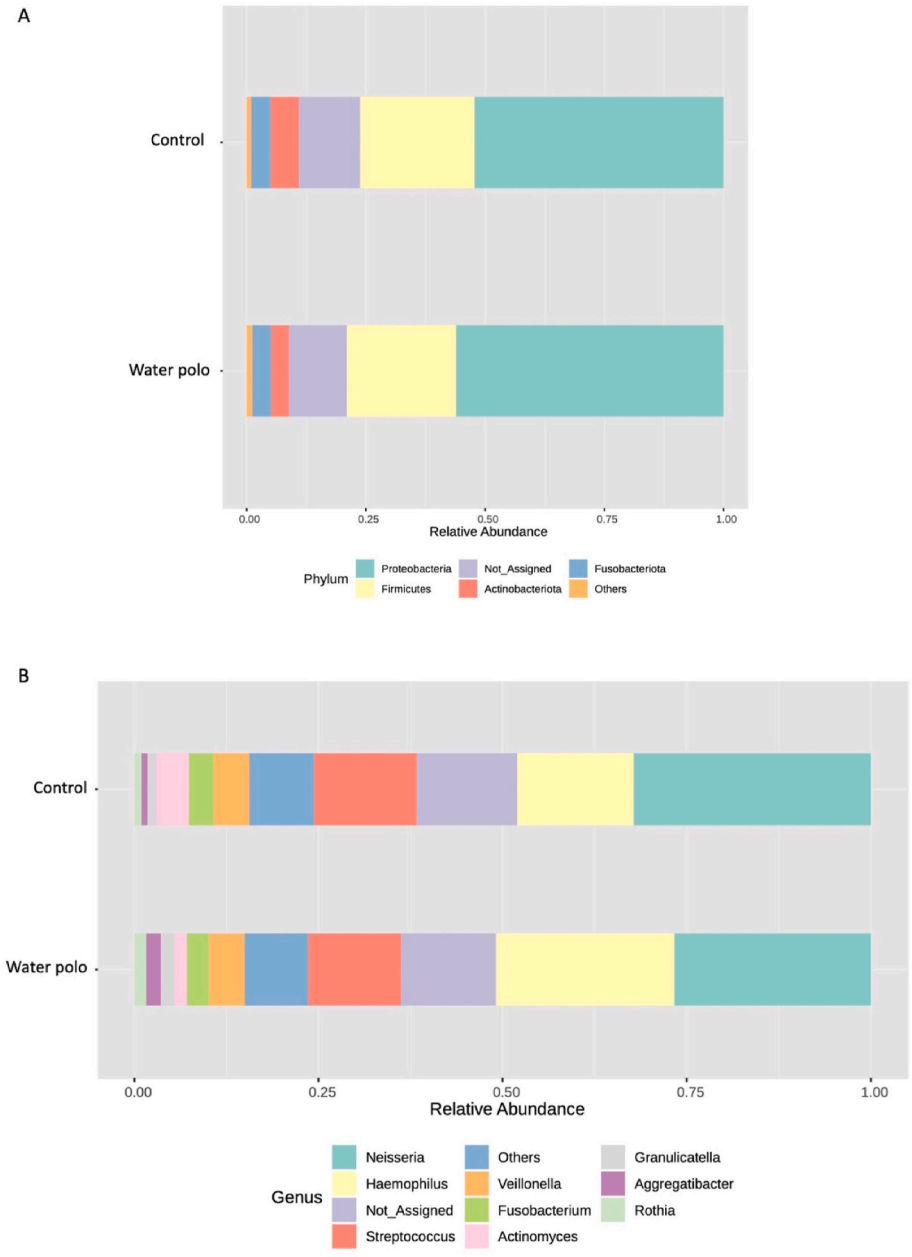

Fig. 3. Taxonomic assignment shows a different microbial composition in Control and Water polo groups. Relative abundance (%) highlighted a different bacterial composition in the 2 tested conditions at phylum (A) and down to genus level (B).

reduces stress and the risk of several diseases, including diabetes and obesity [13]. Despite these well-known advantages, it has to be mentioned that, while mild to moderate physical exercise has proven to be beneficial, intense training may be dangerous leading to the onset of diseases [14]. This occurs especially in elite athletes that, due to their engagement, are exposed to prolonged physical stressors [15]. As a consequence, monitoring athletes health to avoid diseases onset and reduce the risk of physical injuries is becoming an even more important point [16].

Human microbiota has emerged as a novel potential biomarker for monitoring human's health since its alterations have been reported in an increasing number of diseases. Indeed, microbiome analysis has the potential to identify specific microbial alterations as hallmark of a specific condition, can be easily monitored over time, and offer the opportunity to develop therapeutic approaches aimed at its manipulation. Among others, sport activity is a factor able to modify microbiome composition. Thus, there is an increasing interest in understanding the relationship between microbiota and sport, also to shed light on the molecular mechanisms linking physical exercise to diseases onset. Moreover, microbiome evaluation may be an additional biomarker for athlete's health monitoring, and/or a target for the development of specific interventions [17].

Table 2
Significant taxa identified by differential abundance analysis as assessed by EdgeR (adjusted p-value<0.05) between Control and Water polo groups. Microbial taxa are reported for each taxonomic rank, from phylum to species and ordered based on p-values (from the most significant value). The kind of variation (increased or reduced abundance in the water polo group) is also shown.

Rank	Taxon	P-value	FDR	Fold change (Water polo vs Control)
Phylum	Spirochaetota	0.00049086	0.0044177	down
Class	*Clostridia*	2.8594e-05	0.00034312	up
Class	*Spirochaetia*	0.00017958	0.0010775	down
Order	*Lachnospirales*	9.9143e-10,	2.3794e-08	up
Order	*Actinomycetales*	8.5648e-06	0.00010278	up
Order	*Spirochaetales*	0.0001328	0.0010624	down
Family	*Lachnospiraceae,*	1.3238e-11	4.2363e-10	up
Family	*Actinomycetaceae,*	4.7018e-07,	7.5229e-06	up
Family	*Eggerthellaceae*	4.081e-05	0.00043531	down
Family	*Erysipelotrichaceae*	0.00038588	0.003087	up
Family	*Spirochaetaceae,*	0.00061866	0.0039594	down
Family	*Parvimonas,*	0.00093831	0.0050043	down
Family	*Fusobacteriaceae*	0.0084756	0.038746	up
Family	*Atopobiaceae*	0.010377	0.041508	up
Family	*Burkholderiaceae*	0.011768	0.041842	down
Genus	*Oribacterium*	1.834e-15	9.9037e-14	up
Genus	*Actinomyces*	5.2473e-07	1.4168e-05	up
Genus	*Butyrivibrio*	3.414e-06	6.1452e-05	up
Genus	*Lachnoanaerobaculum*	4.6054e-05	0.00059961	up
Genus	*Stomatobaculum*	5.552e-05	0.00059961	up
Genus	*Slackia*	7.3152e-05	0.00065837	down
Genus	*Johnsonella*	0.00028502	0.0021988	up
Genus	*Solobacterium*	0.00043556	0.00294	up
Genus	*Necropsobacter,*	0.00057673	0.0033852	down
Genus	*Treponema*	0.00062689	0.0033852	down
Genus	*Actinobacillus*	0.001072	0.0052624	up
Genus	*Catonella*	0.0033121	0.014904	up
Genus	*Shuttleworthia*	0.0039727	0.016502	down
Genus	*Atopobium*	0.0057528	0.022189	up
Genus	*Alysiella,*	0.0083698	0.030131	down
Genus	*Fusobacterium*	0.011179	0.037728	up
Genus	*Megasphaera*	0.01189	0.03777	up
Genus	*Mogibacterium*	0.01509	0.044405	up
Genus	*Lautropia*	0.015624	0.044405	down
Species	*Oribacterium_sinus*	1.1811e-12	5.5513e-11	up
Species	*Oribacterium_parvum*	9.7891e-11	2.3004e-09	up
Species	*Actinomyces_graevenitzii*	6.3183e-10	9.8987e-09	up
Species	*Actinomyces_odontolyticus*	6.5943e-08	7.7483e-07	up
Species	*Oribacterium_asaccharolyticum*	2.2135e-06	2.0807e-05	up
Species	*Fusobacterium_periodonticum*	7.1483e-05	0.00055995	up
Species	*Campylobacter_concisus*	0.00019504	0.0013095	up
Species	*Slackia_exigua*	0.00054873	0.002918	down
Species	*Solobacterium_moorei*	0.00055877	0.002918	up
Species	*Atopobium_parvulum*	0.0011123	0.0052279	up
Species	*Megasphaera_micronuciformis*	0.0020518	0.0087669	up
Species	*Veillonella_massiliensis*	0.0094344	0.036951	down
Species	*Leptotrichia_massiliensis*	0.013421	0.048523	down

In this context, salivary microbiota exploitation, being an easily accessible sample, is showing a great potential [18]. Indeed, the oral cavity hosts about 500–700 different bacterial species and this microbial community appears to be stable over time, even if it is featured by high inter-individual variability, being affected by several factors including host genetics, diet and lifestyle, [18]. In particular, it has been reported that the Mediterranean diet can positively regulate the abundance of salivary microbial species that have been associated with a better macronutrient metabolism [19]. Moreover, it can also modify the composition of gut microbiota enhancing the growth of Bifidobacteria, *Faecalibacterium prausnitzii* and species producing short-chain fatty acids (i.e., butyrate), such as *Clostridium leptum* and *Eubacterium rectale*, and reducing the growth of Firmicutes and *Blautia* species [20].

Interestingly, Li et al. reported that saliva and fecal samples share the same phyla, even if with different abundances, thus suggesting that the study of gut microbiota may be applied not only to fecal samples, but to saliva as well [21]. Accordingly, alterations of salivary microbiota have been described in an increasing number of oral and systemic diseases [18].

Based on this evidence, the evaluation of the salivary microbiota may be particularly useful for monitoring athletes' health, since this kind of samples collection is not invasive, easy to organize, allows to obtain stable samples and is well-accepted by the study subjects respect to stool collection. In this context, the effects of the interaction of diet and exercise on the salivary microbiota have to be taken also into account. Murtaza et al., investigating the effects of three diets (High Carbohydrate diet, Periodised Carbohydrate diet and ketogenic Low Carbohydrate High Fat diet) on the oral microbiota of elite athletes, highlighted that the ketogenic Low

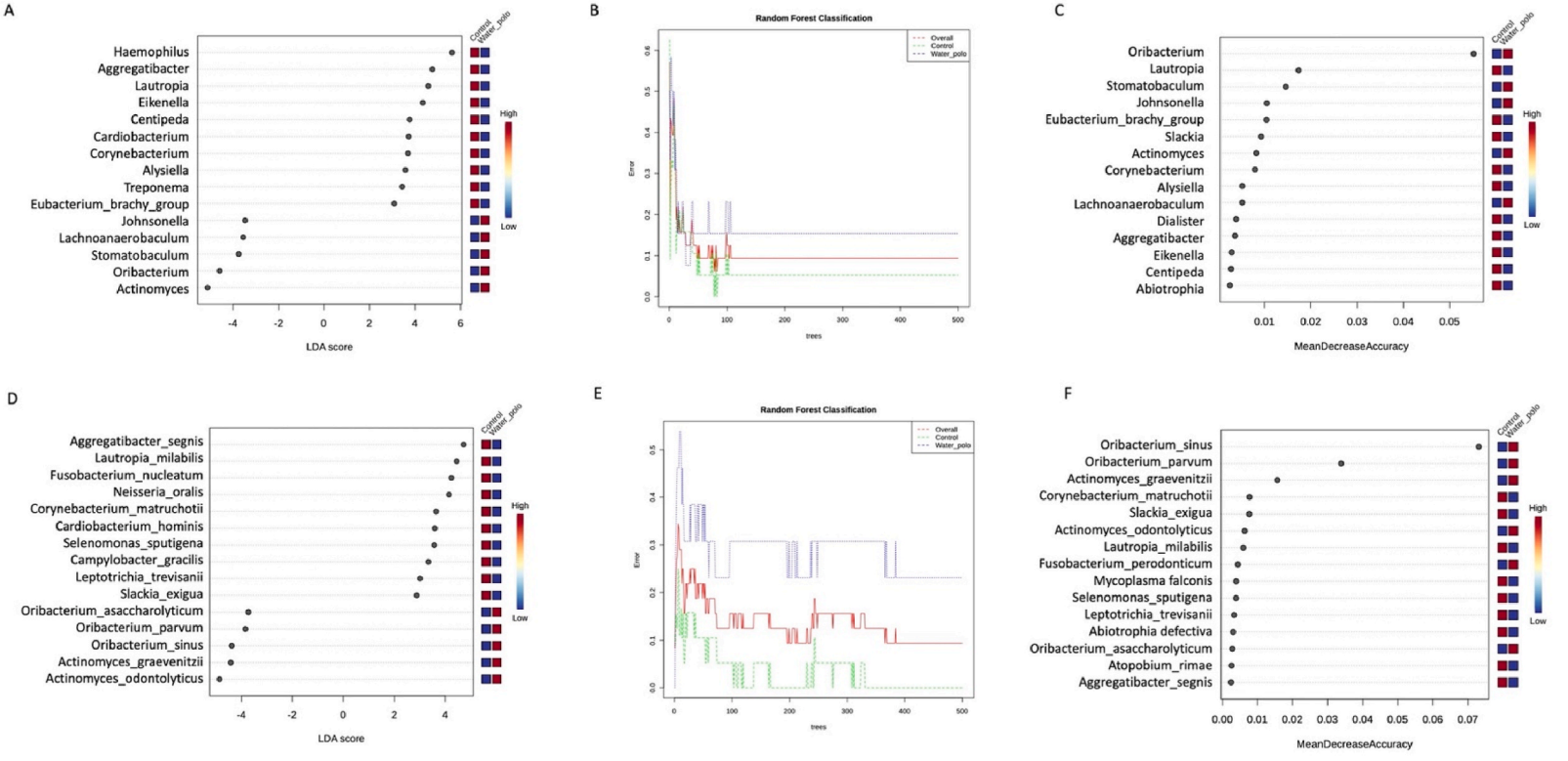

Fig. 4. Identification of the taxa most likely to explain the differences between the Water polo and Control groups. Linear discriminant analysis (LDA) effect size (LEfSe) was performed identifying 15 genera (A) and 15 species (D) significantly differentially abundant between the 2 tested conditions. Random Forest highlighted different decision trees for the Water polo and Control groups at both at genus and species level (B and E, respectively). The lists of the taxa contributing to these differences were generated ranking them based on their contribution to classification accuracy (C and F at genus and species level, respectively).

Carbohydrate High Fat diet resulted in the most dramatic effects on the oral microbiota, with reductions in the relative abundance of *Haemophilus*, *Neisseria* and *Prevotella*, and with a coincident increase in the relative abundance of *Streptococcus* spp. [22]. Uchida et al., showed that physical exercise modifies the saliva microbiota of non-alcoholic fatty liver disease (NAFLD) patients by increasing microbial diversity and reducing LPS-producing taxa [23]. Lamb et al., by analyzing "in-season" versus "off-season" time an undergraduate athletic team, identified alterations in the saliva microbial communities potentially related to a positive effect on student-athlete health [24]. Moreover, Tripodi et al. recently reported that sport practice, irrespective of the kind of activity, should be considered as a risk factor for the development of oral diseases [25]. Water polo players appear to be a particularly at-risk category. This kind of physical activity expose to traumatic and overuse injuries; in addition, the prolonged exposure to both water and chlorine can increase the risk for several diseases, including eye irritation, asthma, otitis and allergies [26]. Nevertheless, the effects of such sport on the microbiota have been poorly investigated so far. A very recent study by Kalabiska and colleagues, by analyzing the saliva microbiome of 29 water polo players and 16 non-athletes, found significant differences in bacterial composition that may unfavorably modify the composition of the oral microbial community by increasing the abundance of pro-inflammatory taxa [27]. However, in this study, a population of young athletes was enrolled (16–20 years as range of age) showing microbiome features specific of this phase of life; in addition, both male and female athletes were analyzed, highlighting microbial differences specifically related to gender. To avoid these potential confounding factors, here, we carried out the saliva microbiome characterization of adult, male water polo players (24–32 years as range of age). In our study, we identified significantly different bacterial communities between water polo athletes and controls, as highlighted by significantly different beta diversity. Subsequent univariate analysis allowed to identify several significant differentially abundant taxa explaining for this diversity. In particular, 19 significant genera and 13 species were identified; among these, the *Oribacterium* genus was the most significantly different between the two compared groups and resulted more abundant in the athletes' respect to the controls. Accordingly, three species, namely *Oribacterium sinus*, *Oribacterium parvum* and *Oribacterium asaccharolyticum*, were significantly more abundant in the water polo players. It has to be noticed that the same genus and its related species were identified also as features able to discriminate the two groups by using 2 independent algorithms (Fig. 4) and were correlated to the biochemical alterations found in the athletes. Our results showed also a significant increase of serum creatine kinase in athletes compared to controls (Fig. 1A) and a similar increasing trend of urea, total bilirubin and reactive protein c in athletes compared to controls (Fig. 1). Creatine kinase (CK) is considered a marker of extreme exercise and was used to compare exercise levels in athletes and controls. In literature, a positive correlation has been found between increased serum CK and urea levels and microbial diversity. For this reason, physical exercise could be identified as a driver of diversity in athlete's microbiome [28,29].

The *Oribacterium* genus and its functions within the human microbiota and in relationship with human host' health have been poorly investigated so far.

Carlier and colleagues firstly isolated in a young child an unknown anaerobic bacillus from the sinus pus and characterized it as a novel species belonging to the *Lachnospiraceae* family (phylum Firmicutes) within the *Oribacterium* genus and named it "*Oribacterium sinus*" [30]. Next, *Oribacterium* spp. were isolated from human subgingival dental plaque and were found to be able to produce, as major metabolic fermentation end products, short-chain fatty acids (SCFAs) including acetate and lactate [31]. A longitudinal study, aimed to analyze the development of oral human microbiota during infancy, identified the *Oribacterium sinus* as a component of the core oral microbiome, whose abundance was not significantly associated to caries status [32]. Tian et al. recently suggested that the *Oribacterium* genus may regulate both skin symptoms and renal function in chronic kidney disease patients playing a probiotic role [33]. In a study aiming to evaluate the effect of cigarette smoking on the salivary microbiota, the *Oribacterium* genus increase was found to be positively correlated to the lifetime exposure to smoking; even if this association needs to be further studied, the authors suggested that the salivary microbiota modifications they found (including the *Oribacterium* genus increase) may be responsible for an increased risk of developing oral malodor and future periodontitis in the smokers [34]. Finally, Medeiros and colleagues, studying the modifications of the salivary microbiome following chemoradiotherapy in patients affected by oral cancer, found an increase of the *Oribacterium* genus that was positively correlated with an increased expression of the salivary DMBT1, an anti-microbial protein present in human saliva [35]. Interestingly, DMBT1 is a highly abundant protein in human healthy saliva that is poorly expressed in patients with oral cancer; after treatment, the DMBT1 levels increased and positively correlated with an increased abundance of *Oribacterium,* suggesting that in may be used as novel biomarker of oral cancer to monitor patients' response to treatment [35].

To the best of our knowledge this is the first report describing *Oribacterium* genus in elite sports athletes. A limitation of the present study is the small sample size. However, we analyzed a well-defined group of athletes since they belong to the same water polo team allowing for an adequate characterization of possible source of variability, such as nutrition, lifestyle and metabolic adaptions. Moreover, considering that athletes and controls followed both Mediterranean diet roles, we assume that the differences found in the salivary microbiota between the two investigated groups are most likely due to the intense physical activity than diet. Future studies, including larger groups of athletes even from different types of sports, are required to confirm our finding. Finally, this was an observational study highlighting an intriguing association between water polo and the *Oribacterium* genus and also a correlation with the creatine kinase levels. Further mechanistic studies are required to better understand *Oribacterium* genus functions and the possible role in athletes' physiology and responses to stressors.

4. Materials and Methods

Ethical Approval

The study was conducted in accordance with the ethical guidelines of Helsinki Declaration of the World Medical Association and

was approved by ethics committee (protocol 200/17) of the School of Medicine, University of Naples Federico II.

4.1. Participants

For this study, we recruited thirteen professional water polo players and nineteen sedentary controls. All participants were informed about the procedures and purpose of the study, and informed consent was obtained from everyone. The physical characteristics of the players as mean (±SD) were: age 24 ± 6 years, weight 89 kg ± 9 kg, height 187 cm ± 4 cm. On the other hand, controls characteristics were age 28 ± 5 years, weight 86 kg ± 8 kg, height 185 cm ± 15 cm. None of the subjects smoked, drank alcohol or consumed drugs known to alter chemical parameters. The athletes included in the study are all men, with a competitive activity of 7 years.

Their calorie intake is around 3000–3500 kcal every day, in particular: carbohydrates about 55–70 % of the daily calorie intake, protein about 15–20 % of the daily calorie intake, and total lipids 20–30 % of the daily calorie intake [36], minerals and vitamins according to the recommendations valid for the general population (LARN, recommended levels of energy and nutrient intake for the Italian population - Italian Society of Human Nutrition '96), water at least 1.5–2 L per day; on the other hand, the controls population follows a Mediterranean diet and consumes a daily quantity of approximately 2000–2500 kcal, divided as follows: 45–65 % of the daily caloric intake composed of carbohydrates, approximately 10–15 % of proteins and 20–35 % of fats, water at least 1.5–2 L per day [37]. It is important to underline that, in both the analyzed groups, the diet followed the rules of Mediterranean diet based on daily intake of cereals (1–2/meal), vegetables (>2/meal), fruits (1–2/meal) and olive oil (every meal), and weekly intake of legumes (>2/week), potatoes (<3/week), red meat (<2/week), white meat (2/week), eggs (2–4/week) and fish (>2/week).

The players followed the same training program: on Mondays they practice strength training in the gym with low repetitions and high load, then in the water part of resistance swimming with aerobic work (never less than 2,5 km) and ball shooting technique; on Tuesdays they train only in the water, focusing on high intensity over short distances (es. 50m) and part with ball dribbling and shooting technique; on Wednesdays they practice resistance work in the gym with low load and high repetitions and explosiveness training in the water with part of swimming on very short distances (es. 12,5/25m), finally, work with the ball and shooting and refinement training to prepare the weekend's match; On Thursdays and Fridays they practice a swimming warm-up of about 700–800 m and a technical refinement training in the water for the weekend's match. The sedentary controls, instead, are people who do not practice any type of physical activity, even as a hobby and carry out very static jobs such as working in the office or in smart working.

4.2. Biochemical analyses

Serum samples were collected in the morning (8.00 a.m.) before training period. The athletes and controls respected a period of 72 h of rest and 8 h of fasting. All the determinations were performed immediately, and all the samples were stored at −80 °C to permit appropriate repetitions. The dosage of creatine kinase (CK), lactic dehydrogenase (LDH), glutamyl transferase (GGT), aspartate transferase (AST), alanine-transferase (ALT), total bilirubin, fructosamine, was performed on the serum of the entire study population by Architect c16000 through a spectrophotometric method (Creatine Kinase, Lactate Dehydrogenase, Glutamyl Transferase, Aspartate Aminotransferase, Alanine Aminotransferase, total bilirubin and fructosamine assays; ABBOTT Diagnostics, USA). Statistical analysis was performed using GraphPad Prism 8.4.0 (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as the means ± standard deviations. Stu-dent's *t*-test was used to compare the two groups, with values of $p < 0.05$ considered significant.

4.3. Saliva samples collection

We selected oral rinse samples for our study. The collection of oral rinse from athletes has been performed in December, three months after the beginning of the agonistic season. Study participants rinsed their mouth (swish/gargle) with 15 mL sterile phosphate buffered saline (PBS) for 1 min and expectorated the contents of the mouth into a 50 mL centrifuge tube. The collected samples were centrifuged at 4000 rpm for 20 min at 4 °C to separate the cells (pellet) from extracellular soluble components (supernatant). The obtained pellet was immediately used for the DNA extraction or stored at −80 °C until the analysis. The same oral rinse was taken for the controls. The analysis of the oral microbiota was applied to the oral rinse of the entire study population.

4.4. Oral microbiome analysis

Genomic DNA was extracted from all the collected and pelleted saliva samples by using the Maxwell® RSC Buccal Swab DNA Kit and the Maxwell RSC instrument (both from Promega, Madison, WI, USA). In particular, 200 μl of pelleted saliva samples were transferred into a fresh tube containing 200 μl of saline solution and mixed by pipetting. This diluted sample was added to a freshly prepared solution containing 300 μl of Lysis Buffer and 30 μl of Proteinase K (PK) Solution and incubated for 20 min at 56 °C. The pretreated samples were loaded into a RSC cartridge to complete the extraction. The DNA samples were eluted in 80 μl of Elution Buffer.

During DNA extraction two blank/negative samples were processed together with the collected samples; the same was carried out also for the subsequent PCR amplifications steps thus obtaining a total of 6 controls used to check for any potential environmental contamination during the different analytical procedures.

To analyze the oral microbiome, 16S rRNA libraries for next generation sequencing (NGS) analysis were prepared. In detail, the V4-V6 hypervariable regions of the 16S rRNA gene were amplified by using custom primers, as previously reported [38,39]. For the amplification mix, AmpliTaq Gold polymerase, GC enhancer (both from Thermo Fisher Scientific, Waltham, MA, USA) and 20 μM of

forward and reverse custom primers were used. After amplicons' quality assessment through 2 % agarose gel electrophoretic analysis, all the amplicons were purified by using the AMPure XP beads (Beckman Coulter, Brea, CA, USA) and analyzed by using D1000 ScreenTapes and the Tape Station System (both from Agilent Technologies, Santa Clara, CA, USA). These purified amplicons were quantified by using the Qubit HS (Qubit, dsDNA HS Assay, Life Technologies, Carlsbad, CA, USA) and diluted to 2 ng/μL. A second-round PCR was per-formed to add the Nextera DNA CD Indexes (Illumina, San Diego, CA, USA) in order to add the universal adapters required for the subsequent NGS reactions and also univocally tag each sample, thus allowing for their multiplexing. Finally, each sample, together with the six negative controls, was quantified with the Qubit fluorometer (Life Technologies), diluted at 4 nM and pooled with the others in equimolar concentrations for NGS. The sequencing reactions were performed loading 9 pM of the libraries' pool with 30 % PhiX on the MiSeq instrument (Illumina, San Diego, CA, USA) with a MiSeq reagent Kit V2.5, 500 cycles (250X2).

The obtained FASTQ files were analyzed by the CEINGE Biotecnologie Avanzate Bioinformatic facility. Quality check analysis was carried out by using FastQC software. Next, SILVA NR99 v.138 database was used for reads alignment and correct OTUs assignment. The generated OTU and taxonomy tables were used as input files for the web-based tool Microbiome Analyst (version 2.0, last accession January 2024), that integrates several tools for the deeper analysis of the bacterial community composition [40]. In particular, α diversity analysis was carried out by using different metrics (namely, Observed Species, Chao1 and Shannon index) able to assess both richness and evenness and applying the ANOVA test to verify statistically significant differences. Beta diversity was evaluated by analyzing Unweighted and weighted UniFrac distance measures using the PERMANOVA test to highlight significant differences. Differential abundance analysis was performed using the EdgeR algorithm as univariate statistical method and the FDR measure for p-values adjustment. Kruskal-Wallis rank sum test followed by Linear Discriminant analysis were performed as part of the LEfSe algorithm. Random Forests machine learning algorithm was also used for biomarkers discovery. The MaAsLin2 package was used for the multivariable association between biochemical and microbiome features (adjusted *p*-value cutoff: 0.05).

5. Conclusions

The results presented here support the hypothesis that physical activity direct influences the oral microbiota. Research in the newly growing field of microbiota analysis is relevant to many aspects of the human physiology. It is well recognized that the microbiota may affect the development and treatment of a wide range of human disorders; nevertheless, the precise functional contribution of bacteria to athletes' physiology is poorly described. It is unreasonable to hypothesize that a specific "healthy microbiota configuration" could be defined for all the various sports and athletes, due to the complexity and variability of the sports' tasks (and associated nutrition - related approaches), and the significantly larger inter-variability of microbiota characteristics and related response to diet. Here, we focused on water polo athletes and were able to identify specific microbial signatures related to elite sport activity. The enrichment of the *Oribacterium* genus deserves further investigation to assess its role and verify if it may be specifically manipulated to improve athletes' health and performance, and/or may be used as a biomarker of water polo player status during the sport season. First, the *Oribacterium* genus increase should be confirmed in a larger cohort of water polo players, if possible including different age groups and also female athletes, so as to highlight any gender or age-related changes. It would also be interesting to check for variations in the abundance of the genus *Oribacterium* during the league so that any identified modifications may be linked to changes in the type of training and/or levels of stress related to competitions. Finally, the use of culture-based strategies to isolate the *Oribacterium* strain directly from the water polo players saliva samples could allow the study of the characteristics of this bacterium in order to better understand its functions and, consequently, highlight any pro-inflammatory factors which may be modulated.

In conclusion, the results reported herein shown the suitability of the oral microbiota analysis related to sport. Indeed, the salivary sample has been proven to be a reliable and non-invasive biological sample for the analysis of the oral microbiota, which means that could become a useful tool in future studies for athletes' health monitoring in different stage of the sport season.

CRediT authorship contribution statement

Iolanda Veneruso: Writing – original draft, Methodology, Data curation. **Cristina Mennitti:** Writing – original draft, Methodology, Data curation. **Alessandro Gentile:** Methodology, Data curation. **Gennaro Di Bonito:** Methodology. **Jacopo Ulisse:** Methodology. **Carmela Scarano:** Methodology. **Barbara Lombardo:** Validation. **Daniela Terracciano:** Validation. **Raffaela Pero:** Validation. **Giovanni D'Alicandro:** Methodology. **Giulia Frisso:** Writing – review & editing, Validation. **Valeria D'Argenio:** Writing – review & editing, Conceptualization. **Olga Scudiero:** Writing – review & editing, Conceptualization.

Informed consent statement:

Informed consent was obtained from all subjects involved in the study.

Institutional review Board Statement:

The study was conducted in accordance with the ethical guidelines of Helsinki Declaration of the World Medical Association and was approved by ethics committee (protocol 200/17) of the School of Medicine, University of Naples Federico II.

Data availability statement

Data are contained within the text and were not deposited into a publicly available repository. Raw data will be available on request.

Funding

This research received no external funding

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] P.J. Turnbaugh, R.E. Ley, M. Hamady, C.M. Fraser-Liggett, R. Knight, J.I. Gordon, The human microbiome project, Nature 449 (2007) 804–810.
[2] V. D'Argenio, Human microbiome acquisition and bioinformatic challenges in metagenomic studies, Int. J. Mol. Sci. 19 (2018) 383.
[3] P.J. Turnbaugh, R.E. Ley, M.A. Mahowald, V. Magrini, E.R. Mardis, J.I. Gordon, An obesity-associated gut microbiome with increased capacity for energy harvest, Nature 444 (2006) 1027–1031.
[4] S.R. Gill, M. Pop, R.T. Deboy, P.B. Eckburg, P.J. Turnbaugh, B.S. Samuel, et al., Metagenomic analysis of the human distal gut microbiome, Science 312 (2006) 1355–1359.
[5] A.J. Macpherson, N.L. Harris, Interactions between commensal intestinal bacteria and the immune system, Nat. Rev. Immunol. 4 (2004) 478–485.
[6] V. D'Argenio, The prenatal microbiome: a new player for human health, High Throughput 7 (2018) 38.
[7] J.L. Baker, J.L. Mark Welch, K.M. Kauffman, J.S. McLean, X. He, The oral microbiome: diversity, biogeography and human health, Nat. Rev. Microbiol. 22 (2024) 89–104.
[8] A. Acharya, Y. Chan, S. Kheur, L.J. Jin, R.M. Watt, N. Mattheos, Salivary microbiome in non-oral disease: a summary of evidence and commentary, Arch. Oral Biol. 83 (2017) 169–173.
[9] A.E. Mohr, R. Jäger, K.C. Carpenter, C.M. Kerksick, M. Purpura, J.R. Townsend, N.P. West, K. Black, M. Gleeson, D.B. Pyne, S.D. Wells, S.M. Arent, R.B. Kreider, B.I. Campbell, L. Bannock, J. Scheiman, C.J. Wissent, M. Pane, D.S. Kalman, J.N. Pugh, C.P. Ortega-Santos, J.A. Ter Haar, P.J. Arciero, J. Antonio, The athletic gut microbiota, J. Int. Soc. Sports Nutr. 17 (2020) 24.
[10] C. Mennitti, M. Brancaccio, L. Gentile, A. Ranieri, D. Terracciano, M. Cennamo, E. La Civita, A. Liotti, G. D'Alicandro, C. Mazzaccara, G. Frisso, R. Pero, B. Lombardo, O. Scudiero, Athlete's passport: prevention of infections, inflammations, injuries and cardiovascular diseases, J. Clin. Med. 9 (2020) 2540.
[11] B. Lombardo, V. Izzo, D. Terracciano, A. Ranieri, C. Mazzaccara, F. Fimiani, A. Cesaro, L. Gentile, E. Leggiero, R. Pero, B. Izzo, A.C. D'Alicandro, D. Ercolini, G. D'Alicandro, G. Frisso, L. Pastore, P. Calabro, O. Scudiero, Laboratory medicine: health evaluation in elite athletes, Clin. Chem. Lab. Med. 57 (2019) 1450–1473.
[12] C. Malm, J. Jakobsson, A. Isaksson, Physical activity and sports—real health benefits: a review with insight into the pub-lic health of Sweden, Sports 7 (2019) 127.
[13] J.P. Thyfault, A. Bergouignan, Exercise and metabolic health: beyond skeletal muscle, Diabetologia 63 (2020) 1464–1474.
[14] D.C. Nieman, L.M. Wentz, The compelling link between physical activity and the body's defense system, J. Sport Health. Sci. 8 (2019) 201–217.
[15] A. Gentile, C. Punziano, M. Calvanese, R. De Falco, L. Gentile, G. D'Alicandro, C. Miele, F. Capasso, R. Pero, C. Mazzaccara, B. Lombardo, G. Frisso, P. Borrelli, C. Mennitti, O. Scudiero, R. Faraonio, Evaluation of antioxidant defence systems and in-flammatory status in basketball elite athletes, Genes 14 (2023) 1891.
[16] R. Pero, M. Brancaccio, C. Mennitti, L. Gentile, S. Arpino, R. De Falco, E. Leggiero, A. Ranieri, C. Pagliuca, R. Colicchio, et al., Urinary biomarkers: diagnostic tools for monitoring athletes' health status, Int. J. Environ. Res. Public Health 17 (2020) 6065.
[17] M. Clauss, P. Gérard, A. Mosca, M. Leclerc, Interplay between exercise and gut microbiome in the context of human health and performance, Front. Nutr. 8 (2021) 637010.
[18] A. Acharya, Y. Chan, S. Kheur, L.J. Jin, R.M. Watt, N. Mattheos, Salivary microbiome in non-oral disease: a summary of evidence and commentary, Arch. Oral Biol. 83 (2017) 169–173.
[19] S. Daniele, G. Scarfò, L. Ceccarelli, J. Fusi, E. Zappelli, D. Biagini, T. Lomonaco, F. Di Francesco, F. Franzoni, C. Martini, The mediterranean diet positively affects resting metabolic rate and salivary microbiota in human subjects: a comparison with the vegan regimen, Biology 10 (2021) 1292.
[20] T.M. Barber, S. Kabisch, A.F.H. Pfeiffer, M.O. Weickert, The effects of the mediterranean diet on health and gut microbiota, Nutrients 15 (2023) 2150.
[21] J. Li, I. Nasidze, D. Quinque, M. Li, H.P. Horz, C. André, R.M. Garriga, M. Halbwax, A. Fischer, M. Stoneking, The saliva microbiome of Pan and Homo, BMC Microbiol. 13 (2013) 204.
[22] N. Murtaza, L.M. Burke, N. Vlahovich, B. Charlesson, H.M. O'Neill, M.L. Ross, K.L. Campbell, L. Krause, M. Morrison, Analysis of the effects of dietary pattern on the oral microbiome of elite endurance athletes, Nutrients 11 (2019) 614.
[23] F. Uchida, S. Oh, T. Shida, H. Suzuki, K. Yamagata, Y. Mizokami, H. Bukawa, K. Tanaka, J. Shoda, Effects of exercise on the oral microbiota and saliva of patients with non-alcoholic fatty liver disease, Int. J. Environ. Res. Public Health 18 (2021) 3470.
[24] A.L. Lamb, D.E. Hess, S. Edenborn, E. Ubinger, A.E. Carrillo, P.M. Appasamy, Elevated salivary IgA, decreased anxiety, and an altered oral microbiota are associated with active participation on an undergraduate athletic team, Physiol. Behav. 169 (2017) 169–177.
[25] D. Tripodi, A. Cosi, D. Fulco, S. D'Ercole, The impact of sport training on oral health in athletes, Dent. J. 9 (2021) 51–54.
[26] E.P. De Oliveira, R.C. Burini, The impact of physical exercise on the gastrointestinal tract, Curr. Opin. Clin. Nutr. Metab. Care 12 (2009) 533–538.
[27] I. Kalabiska, D. Annar, Z. Keki, Z. Borbas, H.P. Bhattoa, A. Zsakai, The oral microbiome profile of water polo players aged 16-20, Sports (Basel) 11 (2023) 216.
[28] S.F. Clarke, E.F. Murphy, O. O'Sullivan, A.J. Lucey, M. Humphreys, A. Hogan, P. Hayes, M. O'Reilly, I.B. Jeffery, R. Wood-Martin, D.M. Kerins, E. Quigley, R. P. Ross, P.W. O'Toole, M.G. Molloy, E. Falvey, F. Shanahan, P.D. Cotter, Exercise and associated dietary extremes impact on gut microbial diversity, Gut 63 (2014) 1913–1920.
[29] W. Barton, N.C. Penney, O. Cronin, I. Garcia-Perez, M.G. Molloy, E. Holmes, F. Shanahan, P.D. Cotter, O. O'Sullivan, The microbiome of professional athletes differs from that of more sedentary subjects in composition and particularly at the functional metabolic level, Gut 67 (2018) 625–633.
[30] J.P. Carlier, G. K'ouas, I. Bonne, A. Lozniewski, F. Mory, Oribacterium sinus gen. nov., sp. nov., within the family 'Lachno-spiraceae' (phylum Firmicutes), Int. J. Syst. Evol. Microbiol. 54 (2004) 1611–1615.
[31] M.V. Sizova, P.A. Muller, D. Stancyk, N.S. Panikov, M. Mandalakis, A. Hazen, T. Hohmann, S.N. Doerfert, W. Fowle, A.M. Earl, K.E. Nelson, S.S. Epstein, Oribacterium parvum sp. nov. and Oribacterium asaccharolyticum sp. nov., obligately anaerobic bacteria from the human oral cavity, and emended description of the genus Oribacterium, Int. J. Syst. Evol. Microbiol. 64 (2014) 2642–2649.
[32] S.G. Dashper, H.L. Mitchell, K.A. Lê Cao, L. Carpenter, M.G. Gussy, H. Calache, S.L. Gladman, D.M. Bulach, B. Hoffmann, D.V. Catmull, S. Pruilh, S. Johnson, L. Gibbs, E. Amezdroz, U. Bhatnagar, T. Seemann, G. Mnatzaganian, D.J. Manton, E.C. Reynolds, Temporal development of the oral microbiome and prediction of early childhood caries, Sci. Rep. 9 (2019) 19732.

[33] Y. Tian, C. Gu, F. Yan, Y. Gu, Y. Feng, J. Chen, J. Sheng, L. Hu, P. Jiang, W. Guo, N. Feng, Alteration of skin microbiome in CKD patients is associated with pruritus and renal function, Front. Cell. Infect. Microbiol. 12 (2022) 923581.
[34] N. Suzuki, Y. Nakano, M. Yoneda, T. Hirofuji, T. Hanioka, The effects of cigarette smoking on the salivary and tongue microbiome, Clin. Exp. Dent. Res. 8 (2022) 449–456.
[35] M.C. Medeiros, S. The, E. Bellile, N. Russo, L. Schmitd, E. Danella, P. Singh, R. Banerjee, C. Bassis, G.R. rd Murphy, M.A. Sartor, I. Lombaert, T.M. Schmidt, A. Eisbruch, C.A. Murdoch-Kinch, L. Rozek, G.T. Wolf, G. Li, G.Y. Chen, N.J. D'Silva, Salivary microbiome changes distinguish response to chemoradiotherapy in patients with oral cancer, Microbiome 11 (2023) 268.
[36] R. Jäger, C.M. Kerksick, B.I. Campbell, P.J. Cribb, S.D. Wells, T.M. Skwiat, M. Purpura, T.N. Ziegenfuss, A.A. Ferrando, S.M. Arent, A.E. Smith-Ryan, J.R. Stout, P.J. Arciero, M.J. Ormsbee, L.W. Taylor, C.D. Wilborn, D.S. Kalman, R.B. Kreider, D.S. Willoughby, J.R. Hoff-man, J.L. Krzykowski, J. Antonio, International society of sports nutrition position stand: protein and exercise, J. Int. Soc. Sports Nutr. 14 (2017) 20.
[37] M. Ryan-Harshman, W. Aldoori, New dietary reference intakes for macronutrients and fibre, Can. Fam. Physician 52 (2006) 177–179.
[38] V. D'Argenio, I. Veneruso, C. Gong, V. Cecarini, L. Bonfili, A.M. Eleuteri, Gut microbiome and mycobiome alterations in an in vivo model of alzheimer's disease, Genes 13 (2022) 1564.
[39] I. Veneruso, F. Cariati, C. Alviggi, L. Pastore, R. Tomaiuolo, V. D'Argenio, Metagenomics reveals specific microbial fea-tures in males with semen alterations, Genes 14 (2023) 1228.
[40] A. Dhariwal, J. Chong, S. Habib, I.L. King, L.B. Agellon, J. Xia, MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data, Nucleic Acids Res. 45 (2017) W180–W188.