International Doctoral Thesis / Tesis Doctoral Internacional

Microbiome in women: a focus on physical activity and reproductive health

El microbioma en la mujer:
un enfoque centrado en la actividad física y la salud reproductiva

UNIVERSIDAD DE GRANADA

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2023

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LIST OF ABBREVIATIONS

ABC: ATP-binding cassette

ANCOM-BC: analysis of compositions of microbiomes with bias correction

ART: assisted reproductive technologies

BMI: body mass index

CI: confidence interval

CoDA: compositional data analysis

CST: community state type

DNA: deoxyribonucleic acid

FASN: fatty acids synthase

FC: fold change

FDR: false discovery rate

FT: Fallopian tubes

HMP: human microbiome project

IQR: interquartile range

ITS: internal transcribed spacer

KEGG: Kyoto encyclopedia of genes and genomes

KO: KEGG orthology groups

LPA: light physical activity

MET: metabolic equivalent of task

MVPA: moderate-to-vigorous physical activity

NGS: next generation sequencing

NMDS: nonmetric multidimensional scaling

Non-RCT: non-randomized controlled trial

PA: physical activity

PCoA: principal coordinate analysis

PCOS: polycystic ovary syndrome

PERMANOVA: permutational analysis of variance

PRISMA: preferred reporting items for systematic reviews and meta-analyses

PROSPERO: international prospective register of systematic reviews

RCT: randomized controlled trial

RNA: ribonucleic acid

rRNA: ribosomal RNA

SB: sedentary behavior

SCFA: short-chain fatty acid

SD: standard deviation

SMD: standardized mean difference

ABSTRACT

The human body harbors at least as many microorganisms as own cells that synergistically coexist and regulate several physiological functions supporting host life. Much is known on how the gut microbiome influences human physiology and has a deep influence on human metabolism. These gut microbial communities are shaped by host genetic and lifestyle factors, such as diet, physical activity or medications, among others. Because an imbalance in the gut microbial communities (that is, dysbiosis) has been linked to numerous pathological states, microbiome modulation by lifestyle factors emerges as a promising therapeutic strategy. Conversely, little is known about the presence and composition of the microbiome along the female reproductive tract and its role in the development of gynecological diseases. Thus, despite of the strong correlation between the microbiome and human health and disease, there is still lack in characterizing and understanding the compositional and functional profiles of the host-associated microbes.

The present Doctoral Thesis examines the relationships of the microbiome with female health in association with physical activity and reproductive health performing meta-omics analyses. Thus, the specific aims of this Doctoral Thesis were: 1) to summarize and meta-analyze the state-of-the-art of the association of physical activity and sedentary behavior with the microbiome across different body sites in different human populations; 2) to study the associations between accelerometer-measured sedentary behavior and physical activity on different intensities (light and moderate-to-vigorous) with the gut microbiome using a compositional data analysis in middle-aged women; 3) to characterize the endometrial and Fallopian tubes' microbial composition in fertile women in order to identify the female upper reproductive tract microbiome; 4) to profile and compare the gut microbiome in women with and without endometriosis in a

large cohort in order to identify microbes and microbial pathways associated with the development of the disease. To address the objectives of the Thesis, four studies were performed, which were organized into two sections. **Section I**, Physical activity and microbial composition (**Studies I** and **II**); and **section II**: The microbiome in female reproductive health (**Studies III** and **IV**).

The main findings of this Doctoral Thesis were: 1) a systematic review of 91 studies and meta-analyses uniting more than 2600 participants, where we gathered the current knowledge of the association of physical activity and sedentary behavior with the microbiome. Most studies reported higher abundances of short-chain fatty acids-producing microbes in more active individuals or after a physical activity intervention. While influence of physical activity on the microbial diversity is unclear, athletes seem to have a richer microbiome compared to non-athletes (**Study I**); 2) physical activity and sedentary behavior did not associate with the gut microbial diversity in the middle-aged women, however, several butyrate-producing microbes were significantly more abundant when increasing time of moderate-to-vigorous physical activity from light physical activity/sedentary behaviors (**Study II**); 3) the endometrium and Fallopian tubes harbor an endogenous microbiome with high inter-individual variability, which share around 70% of microbes and have specific bacteria of each anatomical site (**Study III**); 4) the gut microbiome profiles were not significantly different between the women with and without endometriosis, suggesting that the gut microbiome imbalance does not seem to directly impact the pathogenesis of the disease, nevertheless further research considering endometriosis severity stage and treatment strategies is warranted (**Study IV**).

RESUMEN

El cuerpo humano alberga al menos tantos microorganismos como células propias que coexisten sinérgicamente y regulan varias funciones fisiológicas sustentando la vida del hospedador. Se conoce bien cómo el microbioma intestinal influye en la fisiología humana y tiene una profunda influencia en el metabolismo humano. Estas comunidades microbianas intestinales están influenciadas por factores genéticos y de estilo de vida del hospedador, como la dieta, la actividad física o los medicamentos, entre otros. Dado que el desequilibrio de las comunidades microbianas intestinales (es decir, la disbiosis) se ha relacionado con numerosos estados patológicos, la modulación del microbioma mediante factores del estilo de vida se postula como una prometedora estrategia terapéutica. Por el contrario, poco se sabe sobre la presencia y composición del microbioma a lo largo del tracto reproductivo femenino y su papel en el desarrollo de enfermedades ginecológicas. Por lo tanto, pesar de la fuerte correlación entre el microbioma y la salud y la enfermedad humanas, sigue habiendo carencias en la caracterización y comprensión de los perfiles composicionales y funcionales de los microbios asociados al huésped.

La presente Tesis Doctoral examina las relaciones del microbioma con la salud femenina en asociación con la actividad física y la salud reproductiva realizando análisis meta-ómicos. De esta forma, los objetivos de esta Tesis Doctoral fueron: 1) resumir y meta-analizar la evidencia existente respecto a la asociación de la actividad física y el sedentarismo con el microbioma de diferentes sitios corporales en diferentes poblaciones humanas; 2) estudiar las asociaciones entre el sedentarismo y la actividad física de diferente intensidad (ligera y de moderada a vigorosa) medidos por acelerometría con el microbioma intestinal, utilizando un análisis de datos composicional en mujeres de mediana edad; 3) caracterizar la composición microbiana del endometrio y las trompas de Falopio en mujeres fértiles para identificar el microbioma del tracto reproductor

superior femenino; 4) perfilar y comparar el microbioma intestinal en mujeres con y sin endometriosis en una gran cohorte para identificar microbios y rutas microbianas asociadas al desarrollo de la enfermedad. Para abordar los objetivos de esta Tesis, se realizaron cuatro estudios, organizados en dos secciones. **Sección I**, Actividad física y composición microbiana (**Estudios I** y **II**); y **sección II**: El microbioma en la salud reproductiva femenina (**Estudios III** y **IV**).

Los principales hallazgos de esta Tesis Doctoral fueron: 1) una revisión sistemática de 91 estudios y meta-análisis que reunieron más de 2600 participantes, donde recogimos el conocimiento actual de la asociación de la actividad física y el sedentarismo con el microbioma. La mayoría de los estudios reportaron una mayor abundancia de microbios productores de ácidos grasos de cadena corta en individuos más activos o tras una intervención de actividad física. Aunque la influencia de la actividad física en la diversidad microbiana no está clara, los deportistas tienden a mostrar un microbioma más diverso en comparación con no deportistas (**Estudio I**); 2) la actividad física y el sedentarismo no se asociaron con la diversidad microbiana intestinal en las mujeres de mediana edad, sin embargo, varios microbios productores de butirato fueron significativamente más abundantes al aumentar el tiempo de actividad física de intensidad moderada a vigorosa respecto a la actividad física ligera/sedentarismo (**Estudio II**); 3) el endometrio y las trompas de Falopio albergan un microbioma endógeno con una gran variabilidad interindividual, compartiendo alrededor del 70% de los microbios y presentando bacterias específicas de cada lugar anatómico (**Estudio III**); 4) los perfiles del microbioma intestinal no fueron significativamente diferentes entre las mujeres con y sin endometriosis, lo que sugiere que el desequilibrio del microbioma intestinal no parece influir directamente en la patogénesis de la enfermedad; no obstante, se justifica la

realización de nuevas investigaciones que tengan en cuenta el estadio de gravedad de la endometriosis y las estrategias de tratamiento (**Estudio IV**).

1. GENERAL INTRODUCTION

1.1. The microbiome in human

Early predictions of the human genome estimated that ~100,000 genes would be discovered. However, after finalizing the Human Genome Project, many researchers were surprised by the finding that it contains only ~25,000 protein-coding genes. If the view of what constitutes a human is extended, this estimate is probably underestimated, which brought the microscopic world that humans harbor into a research topic of increasing interest. After the Human Microbiome Project (HMP) was launched in 2007 [1], a new sight of the human being as a combination of microbial and mammalian cells has emerged leading to the picture of a human as a "holobiont" [2] (**Figure 1**).

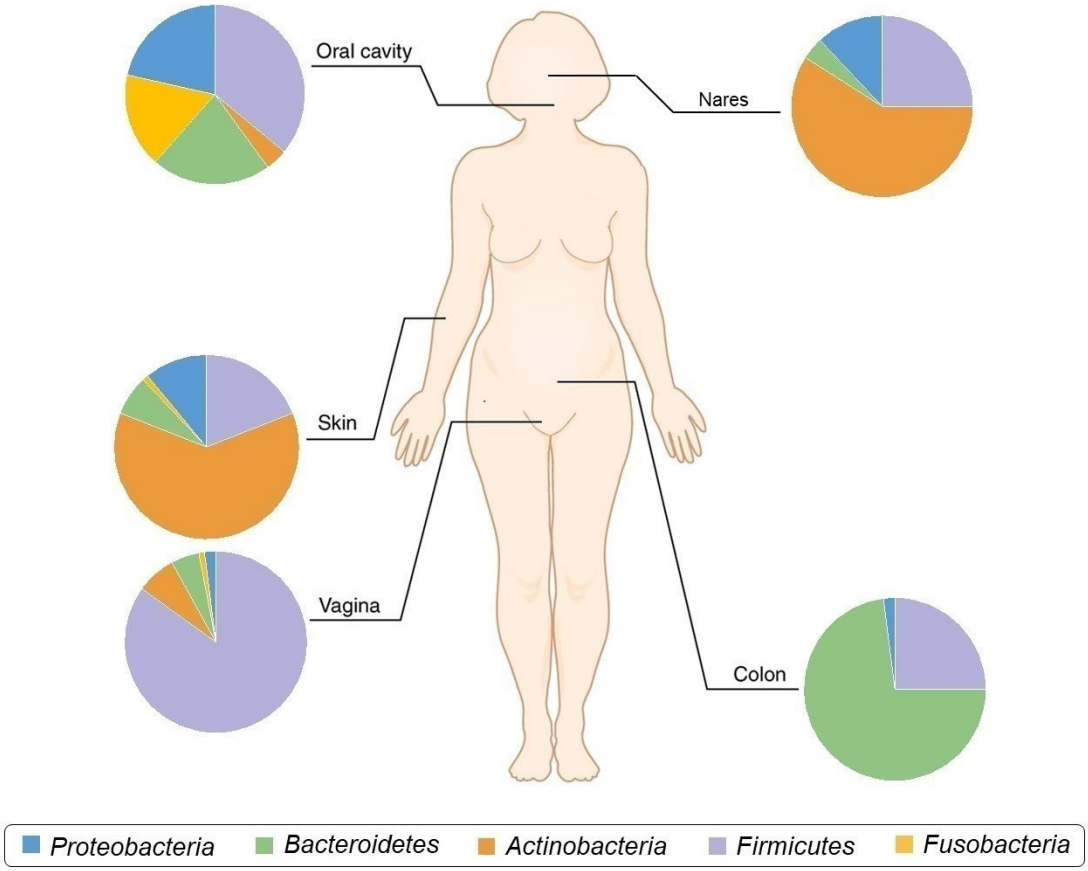

Figure 1. Microbial composition at phylum-level across different body sites. Data resulting from the NIH Human Microbiome Project (HMP). Distinct sites of the body were collected and analyzed separately: oral samples; skin specimens; nares specimens; stool samples; and in females, vaginal specimens. *Figure adapted from Gilbert et al. 2018 [3] under a Copyright Clearance Center's RightsLink® service (License number 5575971072526).*

The collection of microorganisms encompasses bacteria, archaea, viruses, protozoa and fungi that inhabits the human surfaces and all body cavities is defined as the microbiota. According to last estimates, the microbial cells equal to that of the human [4], however, these estimates only take into consideration bacteria, while viruses and phages could equal or outnumber bacterial estimates [5]. The collection of all microbial genes, termed as microbiome, represents a genetic repertoire that is more than one order of magnitude higher in genes than the human genome [6,7]. Therefore, it has been considered "our second genome" and "our last organ" due to its important role in human physiology [8,9]. As a result of billions of years of coevolution, the vast majority of these microbial communities are balanced through symbiotic relationships with the host and support human life. Some relevant microbial activities include promoting different physiological functions such as gut barrier protection, energy production, neurologic signaling, endocrine and immune functions [10,11], that ultimately shape our health and resistance to a disease [3,12].

Over the past two decades, fast advances in the next-generation sequencing (NGS) technologies have driven microbiology into a new microbiome era based on "meta-omics" approaches, including 16S ribosomal RNA (rRNA) gene sequencing, metagenomics, meta-transcriptomics, meta-proteomics, and meta-metabolomics [13]. Collectively, these approaches have vastly improved our understanding about the impact of microbes on human health and disease.

1.1.1. The gut microbiome

In humans, the gastrointestinal tract is the most diverse microbial environment, harboring several trillions of microbes that are influenced by the nutrition, lifestyle, medication and host genetics, among others factors [14,15]. A large number of these microbes reside in the distal gut and have a profound influence on the human metabolism

and nutrition, contributing to essential amino acids and vitamins biosynthesis, dietary components digestion, immune and endocrine function, or drug and exogenous toxins metabolism, among others [10,11,16]. Consequently, the gut microbiome has been proposed as a new biomarker of overall health and emerges as the most thoroughly studied ecosystem within the human body. Indeed, the Metagenomics of the Human Intestinal Tract (MetaHIT) Consortium, created by the European Commission in 2008, united 13 academic and industry partners from 8 countries with the aim to establish a catalogue of the gut microbial genes and to discover the associations between the bacterial genes and different health conditions [17].

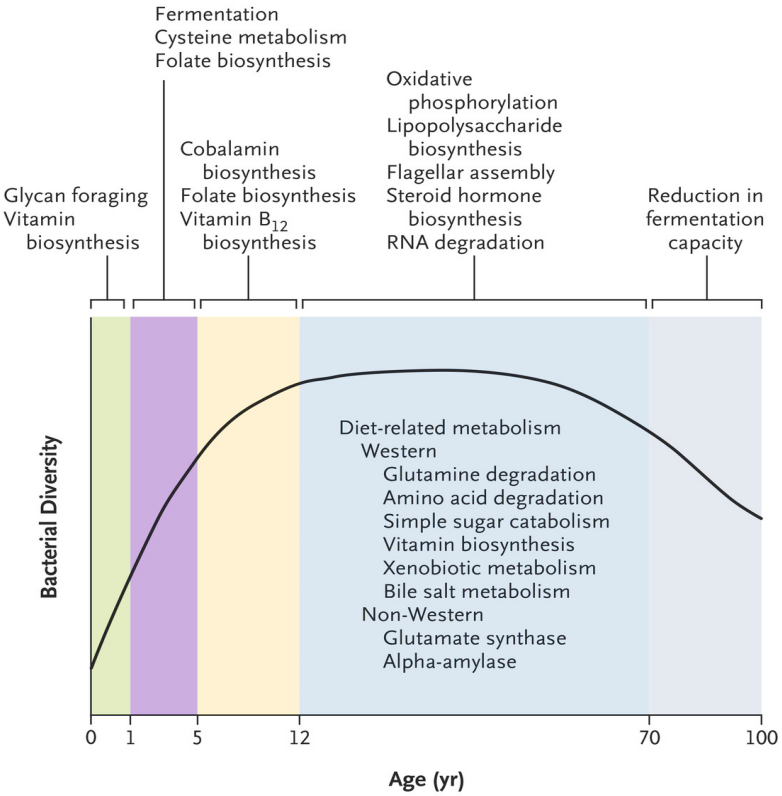

Figure 2. Temporal dynamics of the human gut microbiome. During childhood, the rapid rate of expansion in bacterial diversity observed in infancy slows and becomes more stable, with a high abundance of butyrate-producing microbes from *Bacteroidetes* phylum. In preadolescents, *Anaerovorax*, *Bifidobacterium*, *Faecalibacterium*, and *Lachnospiraceae* as well as pathways involved in vitamin B_{12} and folate biosynthesis increase. The healthy adult microbiome is dominated by *Bacteroidetes* and *Firmicutes* phyla, showing functional pathways involved in amino acids, vitamins and hormones biosynthesis, among others. *This figure is reproduced with permission from Lynch & Pedersen, 2016 [12], Copyright Massachusetts Medical Society.*

Shaping of the gut microbiome starts at birth, when it is noted for lacking diversity and stability, with 500–1000 species colonizing the gastrointestinal tract. After weaning, the gut microbiome becomes consolidated in a stable way leading to a lifelong adult gut microbiome where fluctuations are infrequent and normally related to the pathological processes [18]. In the elderly, it becomes compositionally unstable and less diverse [19] (**Figure 2**). Despite these fluctuations in the microbial dynamics, diversity and composition during the lifespan, it is generally considered that the higher the microbial diversity the better for a healthy state [20].

Regardless of the active research of the gut microbiome and its role in health and disease, there is a lack of integrating gut microbiome profiles with host, clinical and environmental factors associated with inter-individual taxonomic variation [21]. Hence, while it is widely demonstrated that the gut microbiome consists of app. 90% of bacteria belonged to *Bacteroidetes* and *Firmicutes* phyla, with other less predominant bacteria from *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia*, a universal healthy microbiome profile has not been established at more profound taxonomic levels [14].

1.1.2. The microbiome in the female reproductive tract

Unlike the high microbial diversity of the gut, the lower reproductive tract is characterized by a low diversity of bacterial species and it fluctuates dynamically during the menstrual cycle [22]. In the vagina, *Lactobacillus* spp. represent 90-95% of the total bacteria in reproductive-aged women (**Figure 3**) and create a low pH (3.4-4.5) environment through lactic acid production, which provides hostile conditions for opportunistic pathogens. Scientific knowledge identifies a healthy vaginal microbiome through the dominance by four lactobacilli species: *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus jensenii*, and *Lactobacillus gasseri* [23]. In 2011, Ravel

et al. identified 5 vaginal microbial community state types (CST); four of them (I, II, III and V) dominated by *L. iners*, *L. crispatus*, *L. gasseri*, or *L. jensenii*, whereas CST-IV was characterized by increased abundance of strictly anaerobic bacteria and reduced *Lactobacillus* spp. [24]. Indeed, *Lactobacillus* species depletion and increased microbial diversity are biomarkers of the pathological processes including bacterial vaginosis, increased risk of sexually transmitted infections, infertility and adverse pregnancy outcomes [25]. Hence, the Vaginal Human Microbiome Project was launched in order to reveal the diversity of microbial communities in human vagina and investigate their associations with physiological and infectious states (http://vmc.vcu.edu/vahmp).

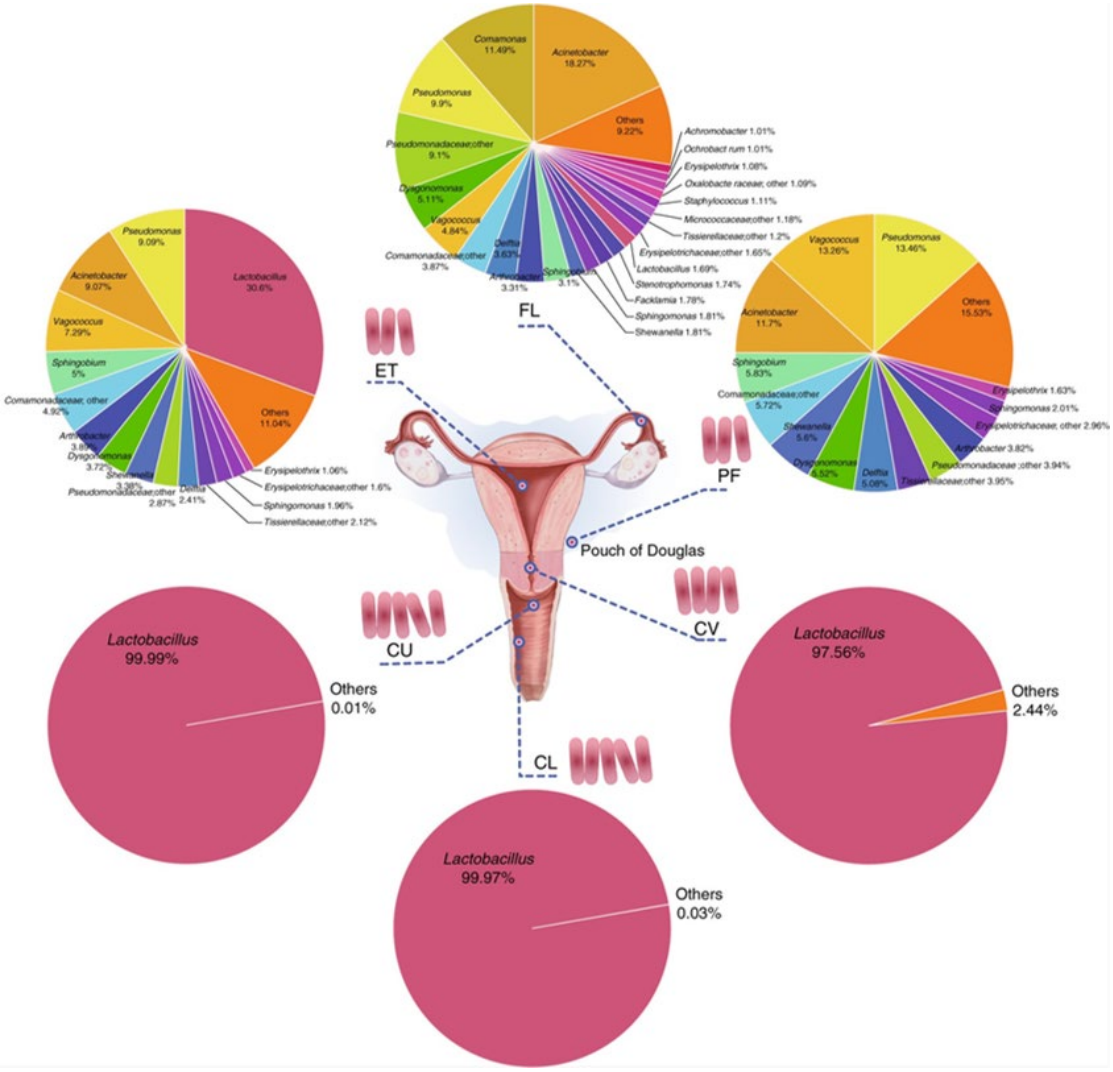

Figure 3. Microbiome of the female reproductive tract. CL – lower third of the vagina; CU – posterior fornix; CV – cervical mucus drawn from cervix; ET – endometrium; FL – Fallopian tubes; PF – peritoneal fluid from the pouch of Douglas [26]. *This figure is reproduced under a*

In contrast to the lower reproductive tract (vagina), the upper reproductive tract (uterus, Fallopian tubes -FT- and ovaries) remains scarcely explored. Traditionally, the uterus was considered sterile with the presence of microorganisms only in the context of infection or pathological states [27], which is now confirmed with the culture-independent NGS techniques to present its own microbial communities that differ from that of the vagina [26]. Growing evidence supports that the endometrium harbors its own functionally active microbiome, hosting 100-10,000 times fewer bacteria than the vagina [28]. Despite its potential role in maintaining female reproductive health, the composition of the endometrial microbiome is still inconclusive due to differences in study design and absence of proper negative and positive controls [29,30]. Furthermore, FT and ovaries are less studied due to the difficulty in samples collection, especially in fertile women in disease-free conditions.

1.2. The microbiome in health and disease

Epidemiological studies have reported a global burden of human multifactorial diseases through the past two centuries [31]. This increasing rate of incidence makes it unlikely that these disorders can be exclusively explained by genetic factors, and has driven attention to investigate the influence of other factors, including the coexisting microbes that inhabit our body and, more extensively, the gut microbiome [11]. Contrary to a healthy balanced microbiota (a state also called eubiosis), dysbiosis refers to compositional and functional alterations of the microbial communities that lead to a disruption in the host homeostasis. A dysbiotic environment has been associated with the development of several clinical conditions such as obesity, type-2 diabetes, cancer, bowel diseases, cardiovascular, and neurological disorders, among others [12,32]. This avenue of

research raises the prospects for developing new diagnostic and therapeutic strategies through determining the composition of a healthy core microbiome, identifying specifically those factors that induce alterations, and manipulating the microbial composition for improved health [13]. A summarized overview of the microbial effects along the human systems in health and disease is shown in **Figure 4**.

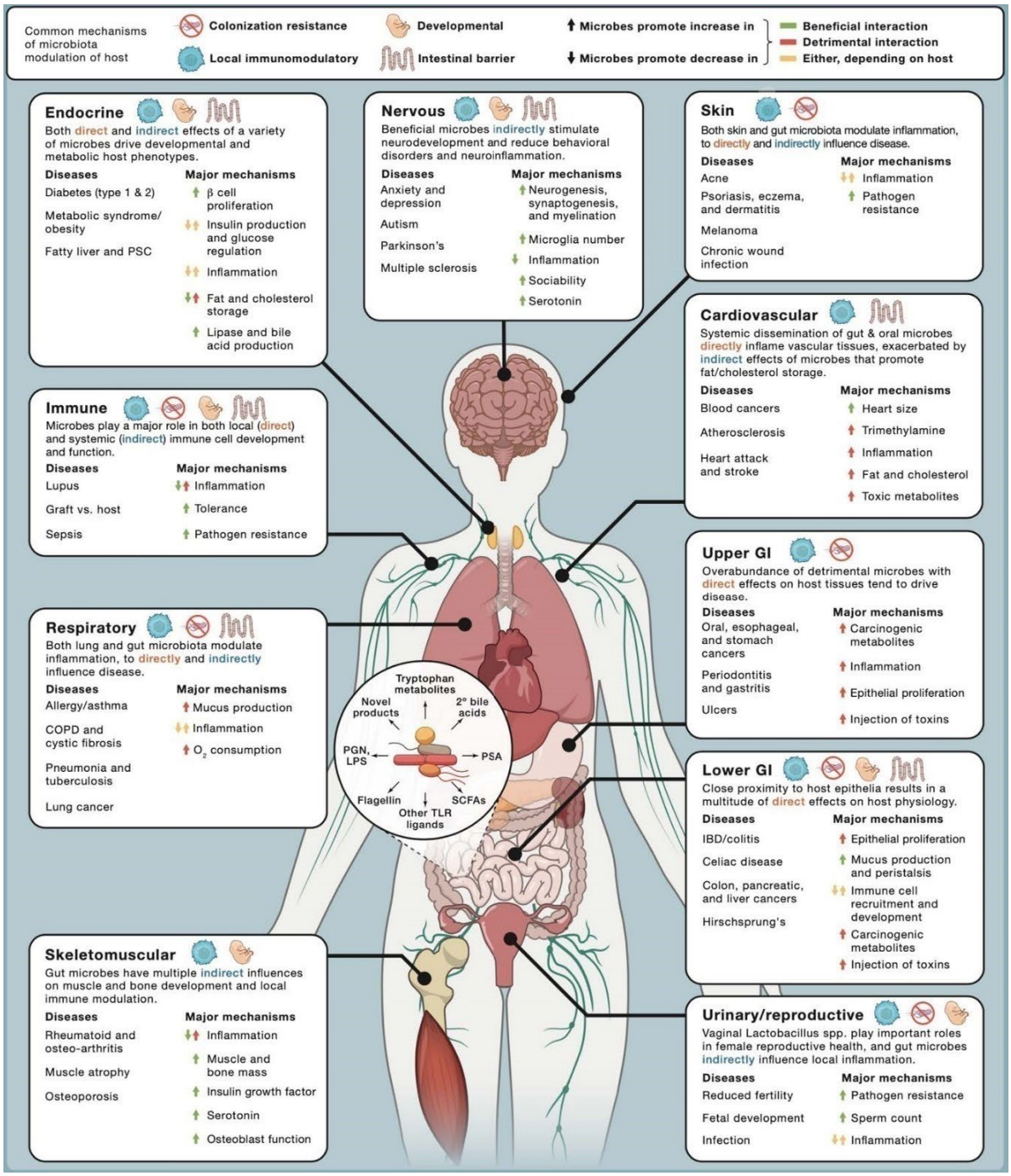

Figure 4. Microbiome physiological effects on human health and disease. The contribution of host-associated microbes to the maintenance of homeostasis in the major organ systems is

represented according to germ-free animal models [33]. *This figure is reproduced under a Copyright Clearance Center's RightsLink® service (License number 5573020733330).*

Nevertheless, large inter- and intra-individual microbial variability even in the absence of a disease, hampers the establishment of a core microbial profile and, therefore, the identification of specific microbial imbalances that either cause or reflect a pathological process [34]. Indeed, only a third of the microbial genes are shared between healthy individuals [35], which means that the integration of evidence from microbiome studies in different populations remains a challenge. Host genetics and lifestyle factors are known to influence microbial composition and play a role in this variability. For example, diet, physical activity (PA), lack of sleep, exposure to drugs, and stress conditions may induce changes in the gut microbial ecosystem and impact host health [3]. Thus, it is necessary to recognize first which microbial signatures are relevant to health and as a next step, which ones contribute to the disease development.

1.2.1. The microbiome association with physical activity

There is robust evidence that habitual moderate PA (i.e., any body movement produced by skeletal muscles which demands a higher energy expenditure than in resting conditions) has multiple benefits on human health, and it has been described as a polypill that offers protection to almost every chronic disease [36,37]. Contrary, sedentary lifestyle, including physical inactivity and more time spent in sedentary behavior (SB) (i.e., a behavior characterized by an energy expenditure of 1.5 or fewer metabolic equivalents - METs), is one of the leading causes of numerous diseases and is associated with all-cause mortality [38,39].

More recently, growing evidence supports that PA also influences the gut microbiome by increasing microbial diversity and functional activity in both humans and

animal models [40–42]. However, much less is known about the molecular mechanisms underlying this interaction and whether SB has an impact on microbial composition. As diet is one of the most important modulators of the gut microbiome, the isolated effect of PA on the gut microbiome independently from dietary habits is difficult to discern [43]. Although numerous observational studies have investigated the association between PA and the gut microbiome in the last years, highly heterogeneous study designs, lack of controlling for relevant covariates and wide use of self-reported PA data complicate obtaining conclusive findings. Self-reported methods such as questionnaires relies on recall, have poor reliability and validity, and tend to homogenize PA of different intensities (i.e., light PA -LPA, moderate-to-vigorous PA -MVPA) [44,45]. Otherwise, the reference or "gold-standard" methods (e.g., direct and indirect calorimetry, doubly label water) have limited use to research studies, being expensive, complex and not very viable in most circumstances [46]. As an alternative, accelerometry is an objective and continuous measuring method of PA and SB, that allows to collect the entire range of PA intensities during the day [47] (**Figure 5**).

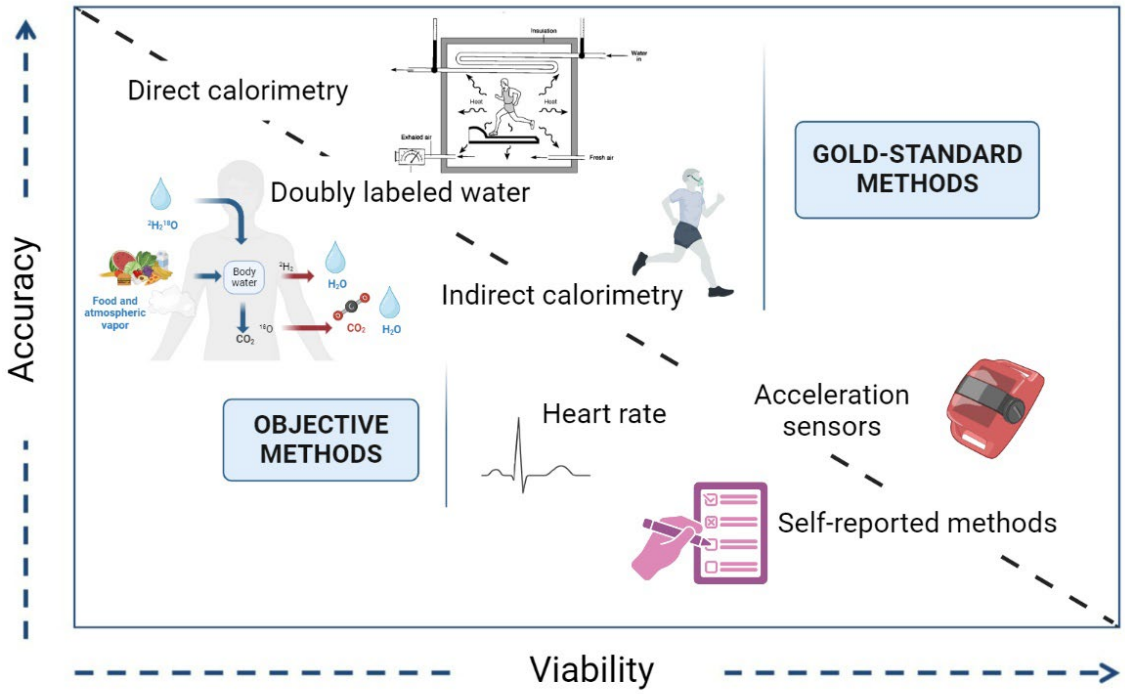

Figure 5. Methods to evaluate physical activity. Representation of reference methods such as indirect and direct calorimetry, and measurement of energy expenditure using doubly labeled water; objective methods including techniques such as measuring heart rate or acceleration by sensors; and subjective methods consisting of self-reported questionnaires. *Figure adapted from Gil Hernández et al. 2017* [46].

First evidence for the role of PA in shaping the human gut microbiome emerged from cross-sectional studies in athletes [48,49], where greater microbial diversity and several differentially abundant gut microbes were found in athletes compared to lean sedentary controls [48]. In non-athletes, cross-sectional studies in different human populations show inconclusive results [50–54], although findings reporting a positive association of butyrate-producing bacteria with PA have been described in several studies [50,51,54]. However, all these studies were limited by their inability to control for dietary effects and variability of PA modality and intensity. These limitations highlight the need for future well-designed studies to determine whether PA independently alters the gut microbiome composition.

To date, previous studies have analyzed PA or SB as isolated components, without taking into consideration the interplay between these behaviors [55]. However, the newer methods to assess PA with accelerometers, allow to record continuously the 24 hours of the day, and classify the time into sleep time and movement at different intensities. In fact, in a 24-hour day, the time is spent across movement behaviors of PA of different intensities, SB and sleep, which means that spending more time in one behavior directly reduces the time in the others. Hence, there has been a shift of paradigm in the PA research field towards the investigation of the composition of the entire day spent on the different behaviors and its association with health related outcomes [56,57].

Compositional data analysis (CoDA) emerges as a novel analytic approach to study the codependency between different movement behaviors and how their interaction

may synergistically impact health. In this context, a scientific workshop was held in Granada in 2019 to discuss advances in accelerometer-based methods and provide recommendations about the most appropriate analytical approach for analyzing accelerometry data [58]. This Granada consensus importantly highlighted the need of extending future investigations to understand the interplay of physical behaviors (PA, SB and sleep) and their relationship with health [58].

The CoDA approach has not been applied in PA-microbiome studies and the effect of 24-hour activity/sedentary behavior in totality is utterly unexplored in association with the microbial composition. Future research on CoDA would provide a holistic picture of the PA/inactivity effects on the microbiome in human.

1.2.2. The microbiome association with reproductive health

The "microbiome revolution" has arrived at the human reproduction field. Strong evidence links the vaginal and endometrial microbiome with female reproductive health as well as pregnancy outcomes [29,30,59,60]. Indeed, microbial dysbiosis have been widely associated with different gynecological diseases, including endometriosis, chronic endometritis, polycystic ovary syndrome (PCOS), dysfunctional menstrual bleeding, endometrial cancer, and infertility [61–63]. Importantly, many of these conditions have no cure so far, and require invasive procedures for diagnosis confirmation. Particularly, the estrobolome term emerges to define the gene repertoire encoding estrogen-metabolizing enzymes, specifically in the gut microbiome. Since hormonal fluctuations, especially estrogens, drive the proliferative state of many of these pathologies, an involvement of the gut microbial communities in the development of female reproductive diseases has been proposed [64,65]. However, identifying the composition of a healthy female

reproductive tract microbiome is the first step before applying therapeutic strategies based on microbiome modulation to diagnose and treat gynecological disorders.

1.2.2.1. Endometriosis

Endometriosis is a chronic inflammatory disease characterized by the growth of endometrial-like tissue (glands and endometrial stroma) outside the uterus, causing a wide range of associated symptoms, including dysmenorrhea, pelvic pain, dyspareunia and infertility, among others [66]. Although it is one of the most prevalent female reproductive disorders, affecting 5-10% of reproductive-aged women, it has been historically understudied and is challenging to diagnose and treat [67]. The most well-accepted hypothesis for endometriosis is based on the retrograde menstruation where adhesion and growth of endometrial tissue occur after migration from the uterus toward the peritoneal cavity [68]. Other proposed models include coelomic metaplasia (transformation of peritoneal mesothelium into endometrial-like cells) and Müllerian remnants, among others [69]. However, none of these theories explain the etiology of all the different phenotypes of endometriosis (i.e., superficial, ovarian and deep infiltrating endometriosis). Consequently, to date, the etiology of endometriosis remains unknown, although it seems to have a multi-factorial origin due to the interplay of genetic predisposition, environmental factors, inflammation, immune dysregulation, hormonal imbalance and possibly the microbiome [70].

The influence of the microbes on immunomodulation and the development of several inflammatory diseases is well established [71]. Much is known how the gut microbial composition maintains the integrity of the gastrointestinal epithelial lining as well as immune homeostasis, preventing bacterial translocation, which can cause low-grade systemic inflammation [72]. Endometriosis is considered a chronic inflammatory disease, and the role of the microbiota in possibly driving local and systemic

inflammation and the relationship to the pathophysiology of endometriosis is proposed [65]. Women with endometriosis have higher incidence of chronic endometritis, more severe pelvic inflammatory disease, a higher risk of surgical site infection after hysterectomy, and a higher incidence of lower genital tract infection [73]. In fact, a new concept of "bacterial contamination hypothesis" in endometriosis has been proposed [74], where the lipopolysaccharide inflammatory mediator could be the initial trigger and bacterial contamination its source in the intrauterine environment that could lead to the growth regulation of endometriosis [74]. Nevertheless, little is known about the presence and composition of the microbes along the female reproductive tract and in the gut in endometriosis [63,65,75].

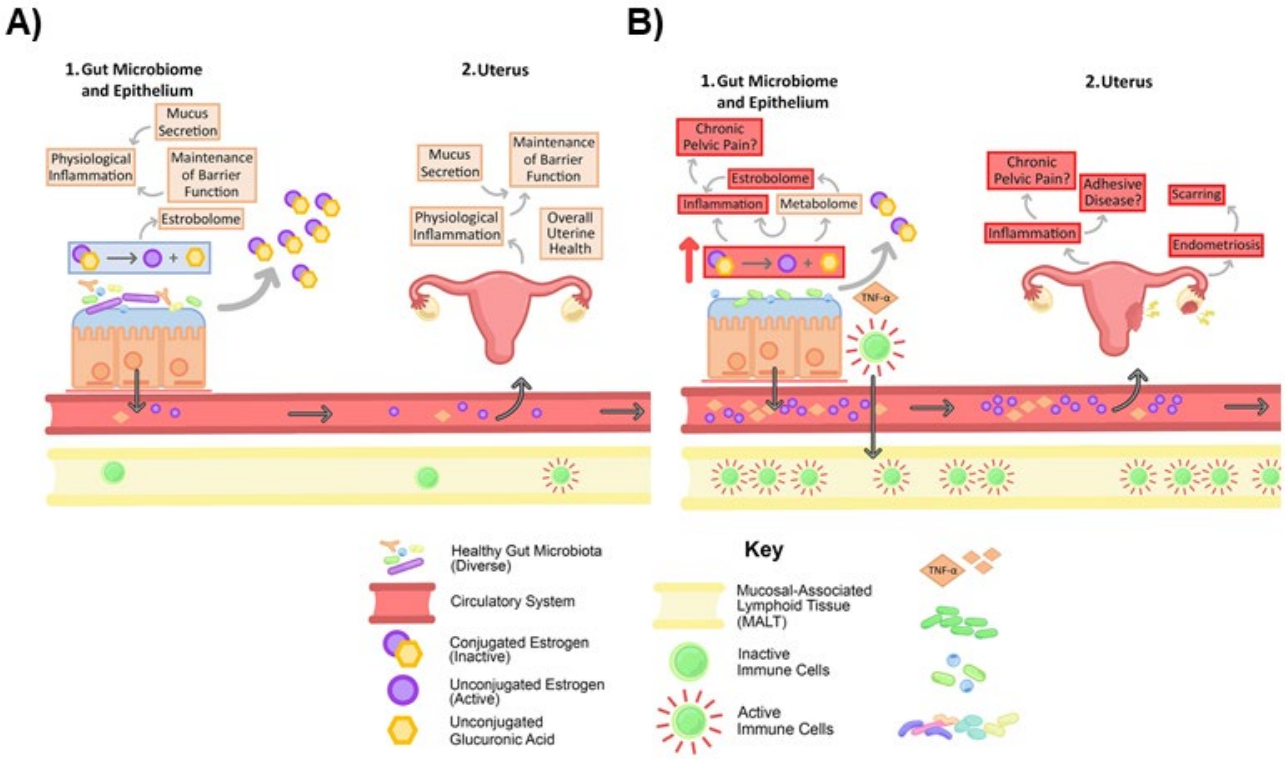

Figure 6. Role of the gut microbiome and estrobolome in endometriosis. A) A healthy diverse gut microbiome sustains homeostatic levels of β-glucuronidase activity that maintains circulating estrogen, resulting in menstrual cycle regulation. B) Gut dysbiosis negatively impacts the estrobolome and the female reproductive tract through altering circulating estrogen levels, increasing systemic inflammation and immune dysregulation. *Figure adapted from Sallis et al. 2022[65] under a Copyright Clearance Center's RightsLink® service (License number 5573050626405).*

Furthermore, estrogen metabolism has been described as a keystone factor to the development of proliferative disorders, including endometriosis [76–78] (**Figure 6**). Particularly, estrobolome alters the estrogen circulating levels through deconjugation from its conjugate (glucuronic acid) by the β-glucuronidase action [79]. Considering the estrogen-driven and inflammatory state of endometriosis, a dysbiotic microbiome has been postulated as a contributing factor in the development of endometriosis [80]. Indeed, recent evidence suggests that the gut dysbiosis may lead to hyperestrogenic conditions mediated by the estrobolome through the increased abundance of β-glucuronidase-producing bacteria [65,80]. Although in the last years intense research is reporting associations of several microbes from the vagina, cervix, endometrium, follicular fluid and gut with endometriosis [81,82], small-sample sizes hampers to reach definite conclusions. Future investigations on bigger sample size and well-controlled studies are warranted to explore the complex relationship between the microbiome and endometriosis.

1.3. Methods of microbiome analysis

Rapid advances in the NGS technologies have enhanced our knowledge in the field of the microbiome in human and its association with host health [13]. These developments have brought the microbiomics to a golden era as a research field, via the combination of molecular biology methods, analytical platforms and *in silico* approaches [83].

Microbial analysis methods have been drastically evolved in the past few years [7,84], being primarily classified into different levels of meta-omics analysis: DNA-, RNA-, protein- and metabolite-level analyses (**Figure 7**). DNA-based approaches are currently well characterized for studying the compositional and functional capacity of the human

microbiomes, since DNA is easy to extract, preserve, and sequence. The commonly used DNA-based methods are amplicon sequencing (also called marker gene, e.g., 16S rRNA gene) and shotgun metagenomics (sequencing the microbial whole genomes) [85]. At RNA-level, meta-transcriptomics surpasses the limitation of amplicon and metagenomics approaches (since the mere presence of DNA sequences is not a sufficient indication of microbial activity), and detects living and functional microorganisms using RNA sequencing to snapshot a transcriptionally active microbiome profile [86]. Complementary to these microbiome analysis methods, meta-proteomics and meta-metabolomics identify and quantify the microbial proteins and metabolites in a sample, which provides new insights into the functional microbiota and to the understanding of host-microbes interactions [87,88].

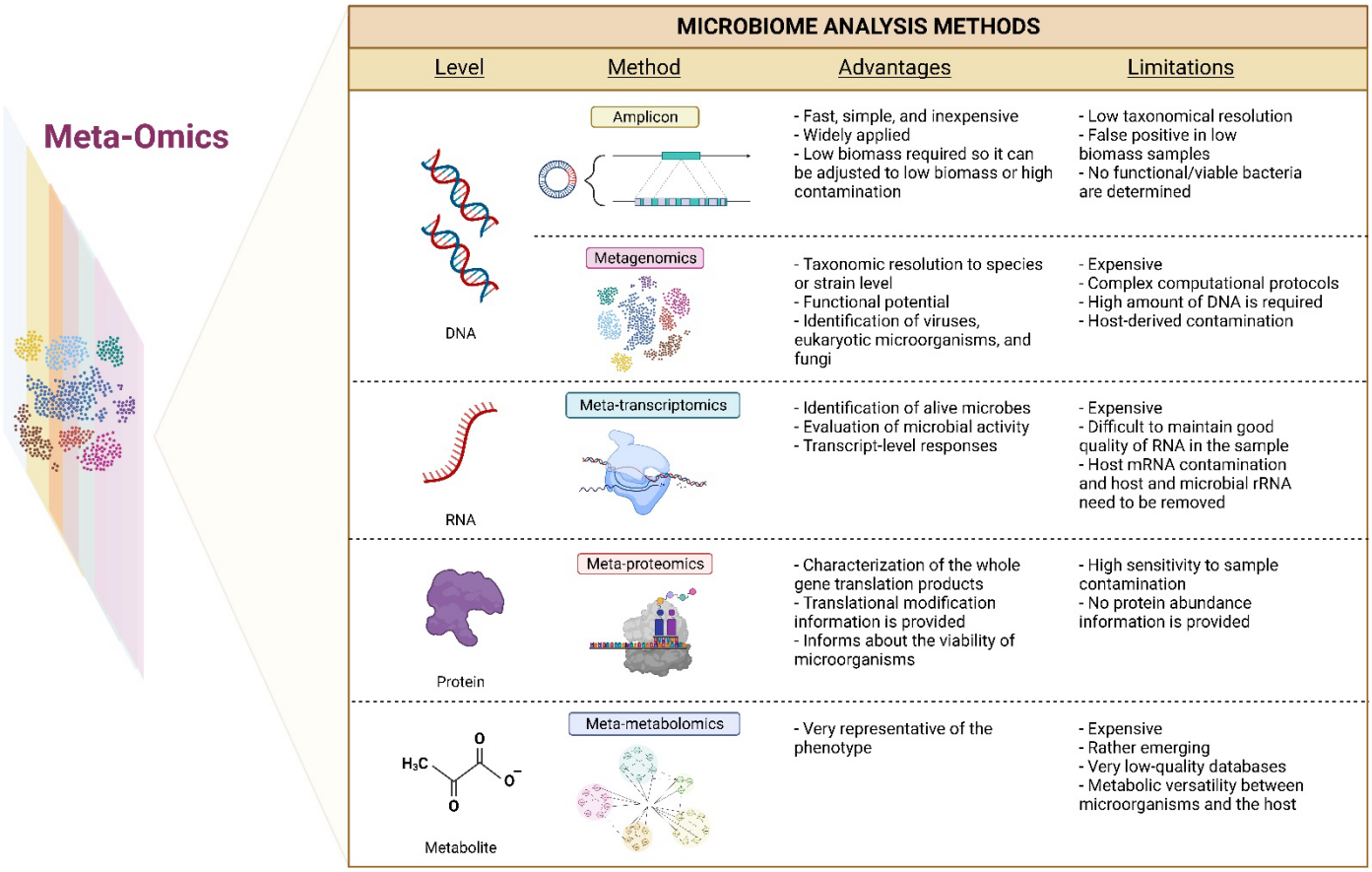

Figure 7. Methodologies used in human microbiome studies: amplicon, metagenomics, meta-transcriptomics, meta-proteomics, and meta-metabolomics analyses. Created with BioRender.

Despite the rapid growth of microbiome analysis, challenges arise from the statistical and experimental design issues. Since microbiome data is high dimensional and heterogeneous, a major concern for the field is the integration of different databases and multiple meta-omics approaches. Therefore, recent efforts are being directed to standardize computational frameworks to combine different cohorts and to integrate and interpret different molecule-level data in light of each other, paving the way for future multi-omics studies in microbiome research [89].

1.3.1. Amplicon analysis

Amplicon or marker gene sequencing, the most common method for microbiome analysis, uses primers that target a specific sequence of a gene to identify the taxonomic composition in a sample. The major marker genes used in amplicon sequencing include 16S rRNA gene for prokaryotes (bacteria and archaea) and 18S rDNA and internal transcribed spacers (ITS) for eukaryotes [90]. The 16S rRNA gene comprises both conserved and hypervariable regions (V1-9). Analysis of the hypervariable regions provides a deep classification of the microorganisms into kingdoms, phyla, classes, orders, families and genera, although lacking accuracy at the species level [91] (see **Figure 7** for further details).

1.3.2. Metagenomics analysis

Metagenomics, also called shotgun metagenomics, consists of the sequencing of all microbial genomes within a sample. This method provides a deeper detection of taxonomic composition than marker gene sequencing, detecting viral and eukaryotic DNA. Contrary to marker gene, metagenomics sequencing allows to reach species level resolution, can be used to profile the functional activity of the microbial communities and makes it possible the assembly of microbial genomes from short reads [92]. However,

shotgun metagenomics has limitations due to the relatively high sequencing cost and the introduction of potential bias during experimental protocols [93]. Furthermore, it does not perform well for samples with high risk of host genome contamination such as low-biomass samples [85] (**Figure 7**).

1.3.3. Controlling for contamination

A crucial step in microbiome analysis is to deal with cross-contamination during sample collection or processing, especially regards to low microbial biomass sites. As a result, it is still under debate whether several body sites such as the placenta [94], upper reproductive tract [95] and blood [96], in fact, possess their own microbiomes. Indeed, it has been demonstrated that bacterial contaminant DNA of extraction and sequencing reagents can dominate the composition of low microbial biomass samples [97]. This highlights the importance of including negative and positive controls in the study design. Additionally, applying *in silico* analyses are becoming mandatory to remove contaminating taxa. For example, Decontam and microDecom are recommended packages to generate cleaner data in low-biomass tissues [98].

1.3.4. Statistical analysis and visualization

Microbiome composition analysis mainly comprises of three steps: 1) data preprocessing, which consists of filtering and quality control of the raw sequences, 2) quantification and taxonomy annotation, and 3) statistical analysis and visualization [99]. In the final step, multiple statistical methods are used to explore the diversity and composition of the human microbiomes. Compared with many software developed for raw microbiome data analysis, the downstream analysis relies basically on the R language environment.

The vast majority of statistics and visualization methods use the most important output files from amplicon and metagenomics analysis pipelines: the taxonomic and functional tables [85]. Downstream analysis of these outputs usually includes alpha- and beta-diversity, taxonomic composition, differential abundance analysis, and additionally deeper correlation, network and machine learning analyses (**Figure 8**).

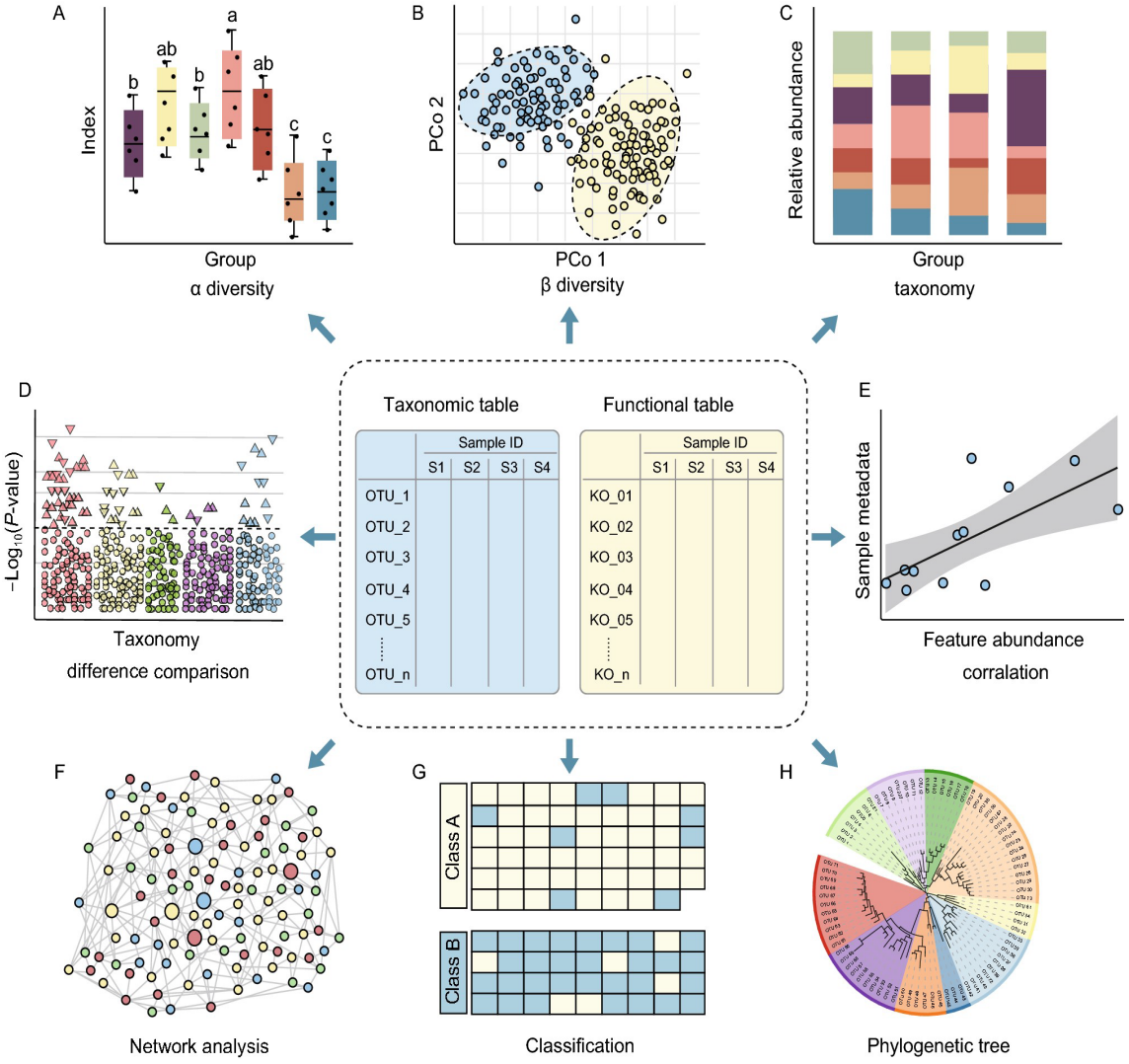

Figure 8. Overview of microbial diversity and compositional analyses commonly used in microbiome studies. Most commonly statistics and visualization methods resulted from the taxonomic and functional tables are represented [85]. *This figure is reproduced under a Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/.*

Alpha-diversity evaluates the microbial diversity (number [richness] and distribution [evenness]) within a sample. Another different concept is beta-diversity, that

measures differences in the microbial communities among samples and is normally represented by dimensional reduction methods such as principal coordinate analysis (PCoA) or non-metric multidimensional scaling (NMDS). Both analyses can be performed in different sequences analysis software, such as Qiime2 or USEARCH, and they are also implemented in the R vegan package. Differential abundance analysis is based on statistical comparison methods (e.g., Mann Whitney *U* test, Kruskal-Wallis test) or tools (ALDEx2, LEfSe or ANCOMBC) to compare the abundance of different features (phyla, genera, species, pathways) and identify those that significantly differ between the groups of interest [85,99].

Due to the rapid emergence of this great variety of computational pipelines and analysis tools, there is a need to perform detailed studies based on well-informed choices. To ensure reproducibility and comparability of the results, standardized practices for performing a microbiome study, including experimental design, choice of molecular analysis technology and pipeline for analysis, and integration of multiple meta-omics data sets are warranted for future studies [84].

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2. AIMS

The overall aim of this Doctoral Thesis was to increase our understanding of the microbial composition in women, with a focus on its association with PA and female reproductive health. This general aim is divided into specific aims and addressed in four studies organized into two sections:

SECTION I. Physical activity and microbial composition

Aim 1. To study the associations of PA and SB with the microbial communities across human tissues.

Specific aim 1.1. To summarize and meta-analyze the current knowledge about PA, SB and the microbiome across different body sites and in different human populations through marker gene and metagenomics approaches (**Study I**).

Specific aim 1.2. To study the associations between accelerometer-assessed behaviors (i.e. SB, LPA and MVPA) with the gut microbiome using CoDA in middle-aged women (**Study II**).

SECTION II. The microbiome in female reproductive health

Aim 2. To characterize the microbial composition in different body sites and investigate its association with female reproductive health.

Specific aim 2.1. To analyze the microbial composition of endometrium and FT in fertile women in order to identify the female upper reproductive tract microbiome in the absence of a disease (**Study III**).

Specific aim 2.2. To analyze and compare the gut microbiome profiles in women with and without endometriosis in a large cohort with the objective to identify microbial species and pathways potentially associated with the pathogenesis of the disease (**Study IV**).

3. MATERIAL AND METHODS

This section summarizes the methodology applied along this Doctoral Thesis. A methodological summary of the four studies is presented in **Table 1**. A detailed methodological explanation can be found in each respective study.

Table 1. Methodological overview of the studies included in this Doctoral Thesis.

Study	Design	Participants characteristics	Exposure	Sample origin	Main outcome	Methodological approach and statistical analysis
Study I	Systematic review and meta-analysis	Healthy (e.g. non-athletes and professional athletes), unhealthy populations (e.g. obesity, diabetes, cancer) at different stages of life (i.e., children, young and older adults)	SB and PA levels or PA intervention	Different body sites (e.g. gut, saliva, vagina)	Microbial diversity and composition (16S rRNA gene or shotgun metagenomics)	Search strategy performed following PRISMA 2020 guidelines Review protocol registered in PROSPERO (CRD42022298526) Systematic search performed in PubMed, Web of Science, Scopus, and Cochrane databases independently conducted by two researchers Random-effects model meta-analyses of cross-sectional studies and trials reporting the chronic effect of PA interventions on gut microbial alpha-diversity in healthy individuals
Study II	Cross-sectional study	289 women from the Northern	SB, LPA and MVPA assessed	Fecal samples	Microbial diversity and composition	Microbiome analysis based on 16S rRNA gene analysis, V3-V4 hypervariable regions

	Finland Birth Cohort 1966 (NFBC1966) (age=46±0 years, BMI=27.9±5.4)	by accelerometry	Raw sequences demultiplexed with Illumina bcl2fastq2 and imported to Qiime2 DADA2 used for denoising step Annotation of ASVs performed with the SILVA 16S v132_99 database Alpha- and beta-diversity and differential abundance analyses performed using R packages (e.g., vegan, ANCOMBC packages) Group comparisons analyzed by LME (alpha-diversity), PERMANOVA (beta-diversity) and ANCOM-BC (differential abundance) Associations of movement behaviors with the gut microbiome explored using CoDA

Study III	Cross-sectional study	19 women from a total laparoscopic hysterectomy (age=45.0±3.0 years, BMI=28.5±4.7) and 5 women from tubal ligation (age=37.0±4.0 years, BMI=28.3±4.0)	NA	Endometrial and FT samples	Microbial diversity and composition	Microbiome analysis based on 16S rRNA gene analysis, V2, V3, V4, V6, V7, V8, and V9 hypervariable regions Raw sequences analyzed by Ion Reporter™ Software Annotation of OTUs performed with the Greengenes v13.5 database Decontam decontamination *in silico* approach applied Alpha- and beta-diversity and differential abundance analyses performed using R packages (e.g., vegan, ANCOMBC packages) Group comparisons analyzed by Mann-Whitney *U* or Wilcoxon signed-rank test for paired data (alpha-diversity and differential abundance), PERMANOVA (beta-diversity) and ANCOM-BC (differential abundance)

Study IV	Case-control study	1000 women from the Estonian Microbiome (EstMB) cohort, including 136 women with endometriosis (age=48.9±10.4; BMI=26.1±5.4) and 864 control women (age=45.6±10.3; BMI=25.7±5.6)	NA	Fecal samples	Microbial diversity and composition, and microbial pathways	Microbiome analysis performed with shotgun metagenomics approach Host reads removal conducted by SOAP2.21 Metagenomic assembly performed by SOAPdenovo Gene prediction performed by MetaGeneMark Taxonomic annotation performed with the NCBI nonredundant database using DIAMOND Microbial functional pathways annotated using KEGG Microbial clustering performed by PAM algorithm Alpha- and beta-diversity and differential abundance analyses performed using R packages (e.g., vegan, ANCOMBC packages)

	Case-control comparisons analyzed by LME (alpha-diversity), PERMANOVA (beta-diversity) and ANCOM-BC (differential abundance)

Note: Participants characteristics were presented as mean±standard deviation. Abbreviations: ANCOM-BC: analysis of compositions of microbiomes with bias correction; ASV: amplicon variant sequence; BMI: body mass index; CoDA: compositional data analysis; DADA2: divisive amplicon denoising algorithm 2; FT: Fallopian tubes; KEGG: Kyoto encyclopedia of genes and genomes; LME: linear-mixed effect model; NA: not applicable; OTU: operational taxonomic unit; PA: physical activity; PAM: partitioning around medoids; PERMANOVA: permutational analysis of variance; rRNA: ribosomal RNA; SB: sedentary behavior.

4. RESULTS AND DISCUSSION

SECTION I. Physical activity and microbial composition

Study I: Physical Activity, Sedentary Behavior and Microbiome: A Systematic Review and Meta-analysis

ABSTRACT

Background. The effects of physical activity and sedentary behavior on human health are well known, however, the molecular mechanisms are poorly understood. Growing evidence points to physical activity as an important modulator of the microbial composition, while evidence of sedentary behavior is scarce. We aimed to synthesize and meta-analyze the current evidence about the effects of physical activity and sedentary behavior on microbiome across different body sites and in different populations.

Methods. A systematic search in PubMed, Web of Science, Scopus and Cochrane databases was conducted until September 2022. Random-effects meta-analyses including cross-sectional studies (active *vs*. inactive / athletes *vs*. non-athletes) or trials reporting the chronic effect of physical activity interventions on gut microbiome alpha-diversity in healthy individuals were performed.

Results. Ninety-one studies were included in this systematic review. Our meta-analyses of 2632 participants indicated no consistent effect of physical activity on microbial alpha-diversity, although there seems to be a trend toward a higher microbial richness in athletes compared to non-athletes. We observed an increase in short-chain fatty acids-producing bacteria such as *Akkermansia*, *Faecalibacterium*, *Veillonella* or *Roseburia* in active individuals and after physical activity interventions.

Conclusions. Physical activity levels were positively associated with the relative abundance of short-chain fatty acids-producing bacteria. Athletes seem to have a richer microbiome compared to non-athletes. However, high heterogeneity between studies avoids to obtain conclusive information on the role of physical activity in microbial composition. Future multi-omics studies would enhance our understanding of the molecular effects of physical activity and sedentary behavior on the microbiome.

1. INTRODUCTION

It is well-known that physical activity (PA) (i.e., any movement produced by skeletal muscles which demands a higher energy expenditure than in resting conditions) can improve different health-related outcomes such as insulin resistance, adiposity, and fitness, among others [1,2]. A related yet different construct is sedentary behavior (SB) (i.e., a behavior characterized by an energy expenditure of 1.5 or fewer metabolic equivalents [METs]), and is associated with a higher risk of different diseases [3,4]. Thus, increasing PA and reducing SB have been considered to prevent and treat multiple chronic diseases [5]. However, the molecular mechanisms underlying the health benefits of PA (acute or chronic effects) and the adverse effects of SB on health are poorly understood [6].

In the last decades, a new sight of the human being as a set of microbial and human cells has emerged [7]. The collection of microorganisms encompasses bacteria, viruses, fungi and archaea that inhabits our body is defined as the microbiota and is at least as abundant as the number of human cells [8]. The genomes of the abovementioned microorganisms (i.e., microbiota) are called the microbiome, which is considered "our second genome" and "our last organ" due to its important role in human physiology [9,10]. Metagenomics studies (e.g., marker gene sequencing and whole metagenome sequencing) led characterization of microbiome composition using three common analyses: (1) alpha-diversity, that characterizes the microbial diversity within a sample considering richness and evenness (i.e., the number and the relative abundance of microbes); (2) beta-diversity, which measures the diversity between samples assigning a numerical value for every pair of samples to determine microbial community-level dissimilarities; (3) differential abundance analysis, that identifies those microorganisms that differ in abundance when compared different samples.

There is evidence indicating that environmental and lifestyle factors such as pollutants, antibiotics, diet, lack of PA and increased SB, among others, may have a negative impact on microbiome composition and function leading to the disruption of the microbial homeostasis (i.e., dysbiosis) [11–13]. In fact, microbial dysbiosis has been associated with the development of multiple diseases such as obesity [14,15], type-2 diabetes [16], and cancer [17,18], among others. Thus, there is a growing interest to determine the composition of the "healthy core" microbiome and the factors that could shape the microbial communities, such as PA and SB, in order to design new therapeutic interventions [19,20].

Particularly, PA has been described as one of the most important modulators of the microbiome, while little is known about the effect of SB on microbial communities due to the limited number of studies [21,22]. Recent advances in meta-omics-based studies (i.e., marker gene sequencing, metagenomics, meta-transcriptomics, meta-proteomics, and meta-metabolomics) allow the identification of the molecular pathways regulated by PA [23]. Thus, the effect of PA on the microbiome, especially on the gut microbial communities, is a research topic of increasing interest [24,25]. In the last years, several systematic reviews reported the effects of PA on the gut microbiome of healthy adults [26–29], older adults [30] and adults with obesity and type-2 diabetes [31,32]. In addition, a systematic review on the effect of aerobic athletic performance has been recently published [33]. However, the aforementioned systematic reviews showed inconsistent findings from observational and intervention studies [26–32]. Therefore, there is a need to synthesize the whole body of knowledge about the effect of PA and SB on the microbiome including healthy (e.g. non-athletes and professional athletes), unhealthy populations (e.g. obesity, diabetes, cancer), different stages of life (i.e., children, young and older adults), and different body niches (e.g. gut, saliva, vaginal, etc.) through metagenomics approaches.

The current study aimed: 1) to summarize all the studies available about the relationship of PA and SB (observational and intervention studies) with microbiome performing metagenomics in humans and 2) meta-analyse the available data.

2. MATERIAL AND METHODS

This systematic review and meta-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (PRISMA) [34]. The review protocol was registered in the International Prospective Register of Systematic Reviews (PROSPERO; http://www.crd.york.ac.uk/PROSPERO) with the reference number: CRD42022298526.

2.1. Search strategy

A systematic search was conducted in PubMed, Web of Science, SCOPUS, and Cochrane electronic databases from inception to September 29, 2022. Search terms were included based on the sports science and microbiome terms of interest. **Table 1** includes a list with the main terms and their definitions related to microbiome field used in this systematic search. **Supplementary Table S1** illustrates the search terms and strategy for each database.

Table 1. Definition of main microbiome-related terms used in this systematic review.

Term	Definition
Microbiota	Collection of microorganisms, including bacteria, arquea, viruses and fungal communities, that collectively inhabit a particular environment (eg., gut, blood, vagina, etc.)
Microbiome	Collection of genomes of the microorganisms inhabiting a particular environment
Healthy core microbiota	Set of microbial taxa universally present in healthy individuals who lack overt disease phenotypes

Alpha-diversity	Diversity within a sample taking into account both the number of microorganisms in a sample (richness) and their distribution (evenness)
Shannon Diversity Index	Alpha-diversity estimator of microbial richness and evenness within a sample or niche
Chao1 Index	Alpha-diversity abundance-based estimator of microbial richness within a sample or niche
Beta-diversity	Diversity between samples taking into account a distance matrix that reflects how compositionally different the samples are from one another (i.e., dissimilarity between samples)
Meta-omics	Refers to those techniques, including marker gene sequencing, metagenomics, meta-transcriptomics, meta-proteomics, and meta-metabolomics, which directly examine the phylogenetic markers, genes, transcripts, proteins, or metabolites from a microbial community
Marker gene sequencing (e.g. 16S rRNA gene sequencing)	DNA sequencing method to identify the microbes present in a given microbial community through the analysis of a sequence variation (i.e., hypervariable region) of a single ubiquitous gene (e.g. 16S ribosomal RNA gene)
Metagenomics (or shotgun metagenomics)	DNA sequencing method to assess the entire functional gene content of a given microbial community. It provides a much greater specific identification of the microbes compared to marker gene analysis (e.g. 16S rRNA gene), in which classification is normally limited to the genus level as multiple species may have the same sequence within the studied hypervariable region
Meta-transcriptomics	RNA sequencing method to assess the transcriptionally active microbes of a given microbial community, providing knowledge of the functional activity of these microorganisms
Meta-proteomics	Shotgun method to characterize the entire microbial protein complement of a sample
Meta-metabolomics	Shotgun method to identify the microbial metabolites present in a sample

2.2. Study selection criteria

The inclusion criteria were as follow: (1) all observational studies (longitudinal or cross-sectional) that report the association of PA and/or SB with microbiome; (2) all original studies that included the effect of PA (acute and/or chronic effects) on microbiome. The exclusion criteria were: (1) studies addressing the effect of PA (acute or chronic effects) on microbiome containing diet modifications, probiotics and prebiotics supplements or caloric restriction, in which it was not possible to isolate the independent effect of PA, (2) non-eligible publication types, such as editorials, study protocols, letters to the editors, meeting abstracts, or review articles, (3) studies written in any language other than English or Spanish.

The selection process of the studies resulting from the literature search was performed using the software "Covidence" (https://www.covidence.org/), which detected duplicates. After removing the duplicates, the articles were first independently filtered by title/abstract screening by two researchers (I.P.P and A.P.F). Those articles that met the inclusion criteria were selected for the full-text review. Conflictive articles were solved through common consensus by the same researchers (I.P.P and A.P.F). Any article that did not meet the eligibility criteria was excluded. The quality assessment of the included studies was independently conducted by I.P.P and A.P.F (see **Supplementary Material Appendix S1**).

2.3. Data extraction

For each study, one researcher (I.P.P) conducted the data extraction including the following information: (1) author's name and date of publication, (2) study design, (3) characteristics of the population (number of participants, sex, age and ethnicity), (4) characteristics of the exposure (i.e., PA or SB), (5) sample origin, (6) dependent outcome

(i.e., DNA extraction method, detection method of the microbiome and sequencing platform), (7) dietary record, (8) main findings and (9) raw data availability. A second researcher (A.P.F) performed a double-check for data correction.

2.4. Data synthesis and meta-analysis

We conducted three meta-analyses including cross-sectional studies (active *vs*. inactive / athletes *vs*. non-athletes) or trials reporting the chronic effect of PA interventions on gut microbiome diversity (specifically alpha-diversity, expressed by the Shannon diversity and Chao1 indexes) in healthy individuals (see **Supplementary Material Appendix S1** for detailed explanation).

Statistical analyses were performed using the Comprehensive Meta-Analysis software (version 3; Biostat Inc.,1385, NJ, USA). The effect size was calculated using Cohen's d and 95% confidence intervals (CIs) for standardized mean difference (SMD). Pooled SMD was estimated using a random-effects model. Heterogeneity between studies was assessed using the I^2 statistics, which represents the percentage of total variation across studies, considering I^2 values of 25%, 50%, and 75% as low, moderate and high heterogeneity, respectively [35]. A p value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. General overview

PRISMA checklist 2020 reflects the appropriateness of the methods performed in this systematic review and meta-analysis (**Supplementary Tables S2 and S3**). **Figure 1** illustrates the PRISMA flow diagram of the search process.

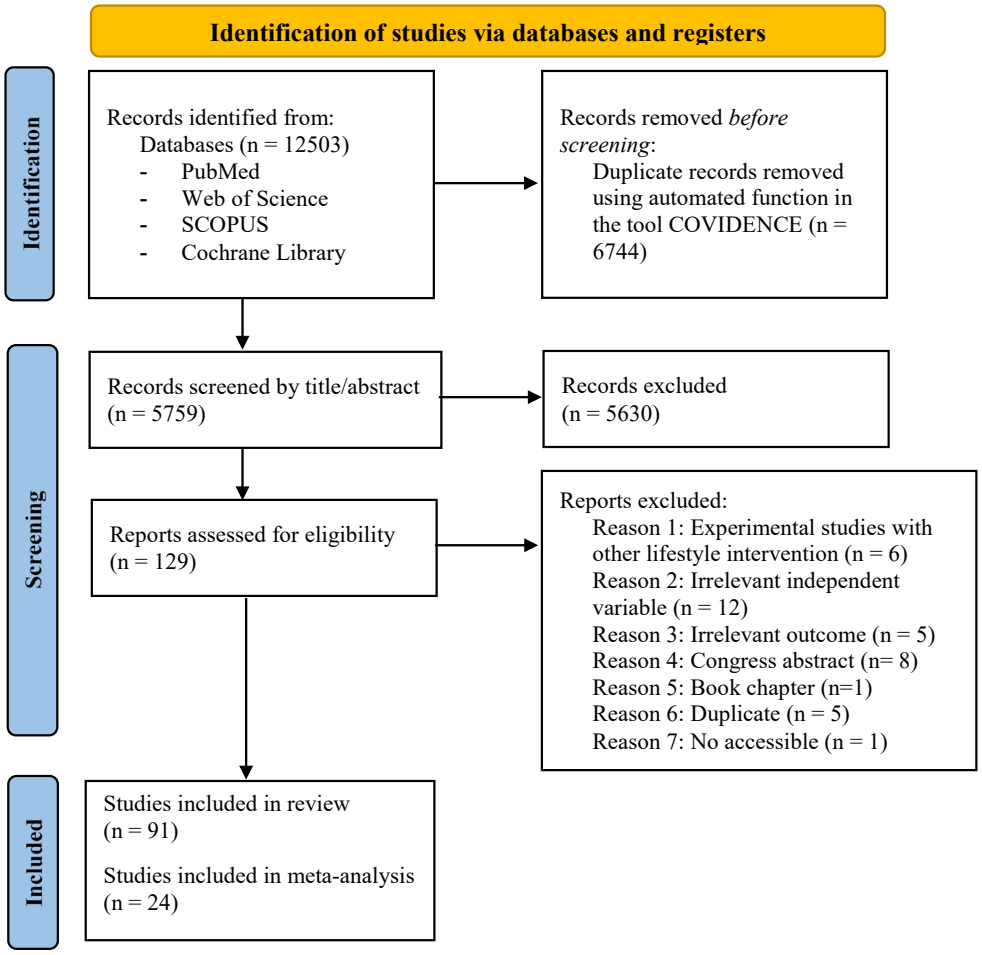


Figure. 1 Search process according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 flow diagram

A total of 12503 articles were detected across the four databases, and after removing the duplicates and non-eligible articles, 91 studies were included in this systematic review: 50 observational studies (all cross-sectional) [36–85], 9 studies reported the acute effects of PA (e.g., following a marathon, rowing, etc.) on microbiome [23,86–93], and 32 studies reported the chronic effects of PA on microbiome (17 non-RCT, 13 RCT and 2 randomized controlled cross-over trials) [94–125]. Of the 50 cross-sectional studies, 8 were eligible (based on availability of microbiome diversity data and healthy participants) for the first meta-analysis comparing groups of high and low PA levels in non-athletes [44,66,69,70,78,81,83,85], and 11 were included in the second meta-analysis comparing athletes *vs*. non-athletes [47,49,51,56,60,74,76,78,79,81,104]. Of the 32 intervention studies, 7 were selected

for the third meta-analysis, to evaluate the chronic effects of PA on microbiome alpha-diversity [96,97,99,109,112,119,124].

Seventeen studies reported significant associations between PA [38,41,43,44,59,63,65–68,72,83–85] or SB [48,64,70] and microbial diversity (i.e., alpha- and/or beta-diversity), and 19 studies found significant differences in the relative abundance of specific bacteria in active *vs*. inactive participants [36,38,39,41,42,44–46,48,59,63,66,68–71,78,84,85] (**Supplementary Table S4**). Sixteen studies found significant differences in microbial diversity [47,49,51,52,54–58,61,62,74–76,79,80] and 13 in the abundance of specific microbial taxa [47,52,53,55–58,74–76,78,79,104] between athletes *vs*. non-athletes, professional *vs*. amateur or athletes from different sports. Three studies detected significant differences in alpha-diversity [89,90,92], while 8 studies described significant changes in the relative abundance of certain bacteria after acute PA interventions [23,86,87,89–93]. Seventeen studies detected significant differences in alpha- and/or beta-diversity [94,95,98,101,102,104,108,111,112,114,115,117–120,123,125], and 24 studies described significant changes in the relative abundance of certain bacteria after chronic PA interventions [94,95,97–99,101,102,105,107–109,111–117,119,121–125].

The sample sizes ranged from 1 [90] to 2183 [65] (**Supplementary Table S4**). Fifty-three studies involved both male and female participants [23,36,38,39,41,42,45,46,50,54–57,59,63–73,77–81,83,85–87,92,94,96,98,100,103–108,114,115,117,119–121,123,125], while 12 were exclusively conducted on women [37,43,48,51,58,74,82,88,95,99,110,118] and 25 on men [40,44,47,49,52,53,60–62,75,76,84,89–91,93,97,101,102,109,112,113,116,122,124]. One study did not report the gender of the participants [111]. Regarding age, 5 studies recruited children (i.e., 7-12 years) and/or adolescents (i.e., 13-17 years) [59,64,68,73,123], 67 included young and middle-aged adults (i.e., 18-64 years) [23,36,40,42–55,60–62,65,66,70,71,74,76–80,82,85–96,98,100–113,115–118,120–122,124,125], 12 older adults (i.e., ≥65 years) [37–39,41,57,63,69,72,84,97,99,114], 3 studies combined adolescents and adults [56,58,119] and 4 adults of different age [67,75,81,83]. Fifty-nine studies were performed on healthy individuals

[23,36,37,40,42–45,47–50,52–62,64–66,68,70,71,74–76,78–81,83,85,87,89–93,96–100,103–105,109–113,116,119], while 32 studies included participants with different diseases such as obesity or breast cancer, among others [38,39,41,46,51,63,67,69,72,73,77,82,84,86,88,94,95,101,102,106–108,114,115,117,118,120–125].

Regarding the exposure, 26 cross-sectional studies recorded PA using self-reported questionnaires [36–46,48,59,65–68,70–73,78,81,83–85], whereas 8 studies included PA data registered by accelerometry [42,48,63,66,69,82,84,85] (**Supplementary Table S4**). Additionally, four studies reported SB data expressed as time per sedentary breaks/bouts or screen time [48,64,70,73]. Twenty-two cross-sectional studies recruited athletes from different sports such as rugby, athletics or football, among others [47,49–58,60–62,74–80,104]. Six studies analyzed the effects of a marathon, footrace or rowing race on microbiome [23,87,89,90,92,93], three reported the effect of a single bout of PA (i.e., no sport competition) on microbiome [86,88,91] and 32 conducted a long-term PA intervention ranged from two weeks [121] to thirty-four weeks [115], mostly consisting of aerobic training [94,95,97,98,103,105,107,109,111–113,115,119–121] or a combination of aerobic and resistance training [96,99–102,104,106,108,110,114,116–118,122–125].

Most of the studies analyzed the gut microbiome, with the exception of ten which collected saliva, oral, oropharyngeal, muscle, blood or vaginal samples [36,43,52,64,80,86,88,102,113,116] (**Supplementary Table S4**). Concerning the detection method, 78 studies conducted the 16S rRNA gene sequencing approach to characterize the microbiome [23,36–48,51–53,55,56,58–64,66–87,90–95,97–100,102–105,107–113,115,116,118–121,123–125], 16 performed metagenomics analyses [23,49,50,54,57,60,65,89,95,96,101,106,114,115,117,122] and two studies focused on meta-transcriptomics (i.e., microbial RNA-sequencing) [50,88]. Twenty-one studies did not report dietary data for all the participants [36,39,74,76,79,80,86,88,98,99,102,104,106,111,113,115–117,119,121,123]. One study performed a control of diet (each participants received the same kind of food) during the PA intervention [87].

Figure 2 shows a graphical summary of the main findings**.** Specific outcomes of microbial composition identified in the articles are discussed and interpreted in the context of the current knowledge in the Discussion section. For further details, see **Supplementary Appendix S2**.

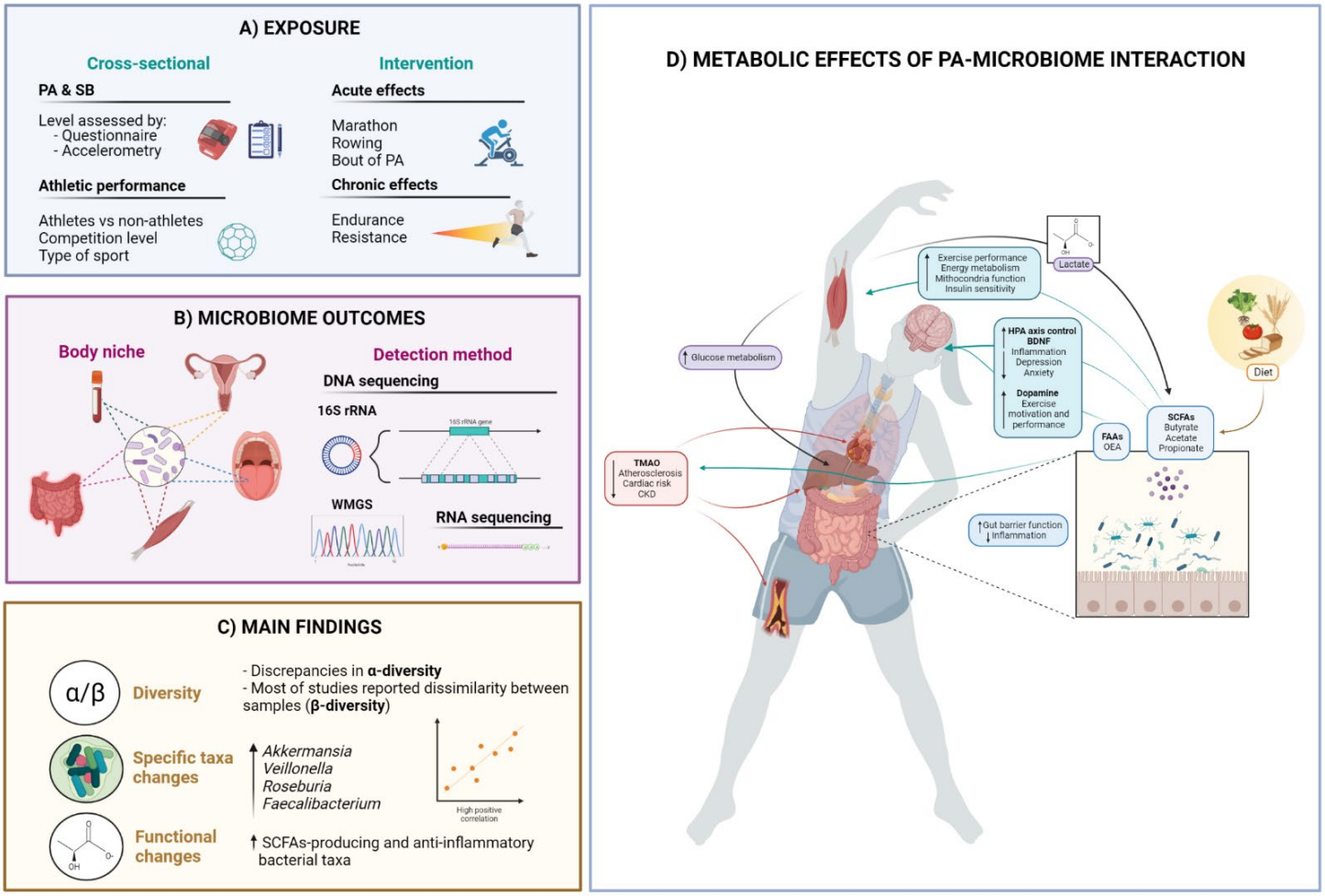


Figure. 2 Summary of the main characteristics and findings of the studies included in this systematic review. A) Exposure: in cross-sectional studies, the effect of physical activity (PA), sedentary behavior (SB) or athletic performance on microbiome was analyzed. In intervention studies, the acute or chronic effects of PA on microbiome were evaluated. B) Microbiome outcomes: samples from different body sites (gut, saliva, blood, muscle and vagina, among others) were analyzed by distinct detection methods (16S rRNA sequencing and whole metagenome sequencing [WMGS] for DNA-based microbiome analysis; meta-transcriptomic [RNA sequencing] for RNA-based microbiome analysis). C: Main findings: relevant results concerning alpha- and beta-diversity and differential abundance analysis are shown. D) Metabolic effects of PA-microbiome interaction. Growing evidence indicates that PA increases the abundance of members of the *Firmicutes* phylum, bacteria able to produce short-chain fatty acids (SCFAs). SCFAs produced by the gut microbiome by processing nutrients from diet may have positive effects in the intestine, improving barrier function and inflammation state. A crosstalk between the gut microbiome and skeletal muscle through lactate (generated during PA) and its conversion

to SCFAs may improve athletic performance. SCFAs have been also linked to promoting neurogenesis (through brain-derived neurotrophic factor [BDNF]), improving hypothalamic–pituitary–adrenal (HPA) axis control, reducing inflammation and the risk of psychological diseases (e.g. depression, anxiety). A microbiome-dependent gut-brain connection mediated by microbial metabolites (i.e., fatty acid amides [FAAs], such as N-oleoylethanolamide [OEA]) has been discovered in mice, which enhances exercise performing and motivation by increasing dopamine signaling during PA. Recent studies suggest that dysbiosis may lead to the growth of proteolytic microbes able to produce trimethylamine-N-oxide (TMAO), an important metabolite that in elevated concentration has been linked to adverse cardiac events and chronic kidney diseases (CKD). This figure was created with BioRender.com

3.2. Quality assessment

Among the 50 cross-sectional studies, 26 were categorized as high quality (quality score≥75%), whereas 24 as low quality (quality score<75) (**Supplementary Table S5**). Regarding the 9 studies about the acute effects of PA, 8 studies were considered to have a high quality and 1 showed a low quality (**Supplementary Table S6**). Concerning the 32 studies (15 RCTs and 17 non-RCTs) that reported the chronic effects of PA interventions, one RCT presented a high quality and 14 a low quality (**Supplementary Table S7**), while 12 non-RCTs were categorized as high quality and 5 as low quality studies (**Supplementary Table S8**).

3.3. Meta-analysis

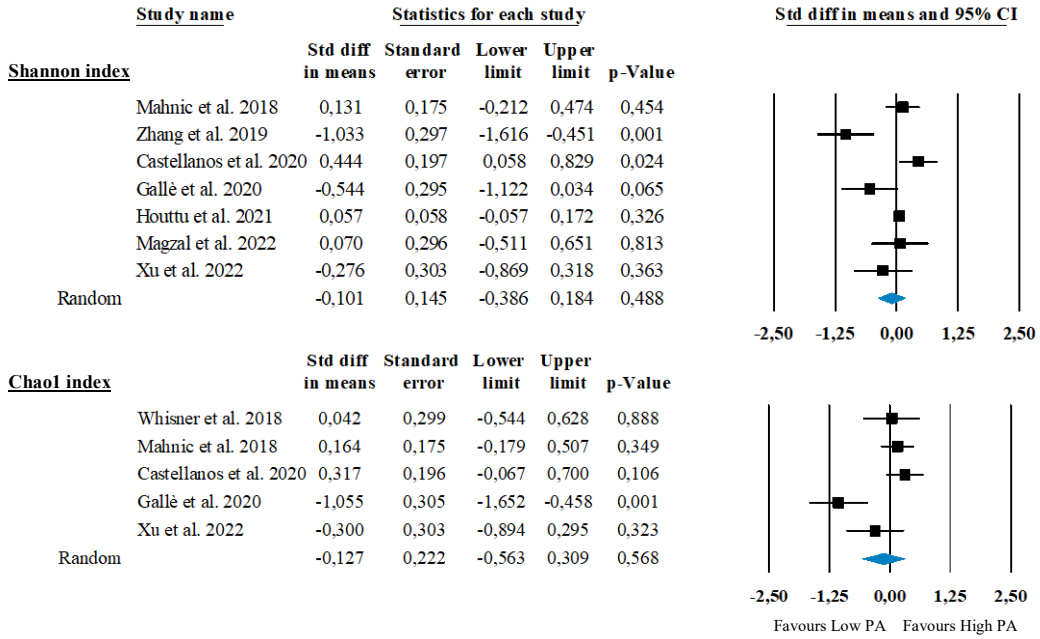
3.3.1. First meta-analysis (cross-sectional studies): high *vs*. low PA levels

This meta-analysis united 1814 participants from 8 studies, where 1157 belonged to the high PA and 657 participants to the low PA groups. No significant differences were reported between the groups of high and low PA levels on alpha-diversity represented by the Shannon diversity index (SMD=-0.101, 95% CI -0.386-0.184, p=0.488, I^2=33.581) and Chao1 index (SMD=-0.127, 95% CI -0.563-0.309, p=0.568, I^2=13.774) (**Figure 3A**).

3.3.2. Second meta-analysis (cross-sectional studies): athletes *vs*. non-athletes

This meta-analysis comprised 651 participants from 11 studies, including 329 athletes and 322 non-athletes. No significant differences were reported between the groups of athletes and non-athletes on alpha-diversity using the Shannon diversity index (SMD=-0.113, 95% CI -0.441-0.215, p=0.501, I^2=0.000). However, athletes tended to present a higher alpha-diversity compared to non-athletes' when Chao1 index was used as an indicator of microbial alpha-diversity (SMD=0.482, 95% CI -0.026-0.991, p=0.063, I^2=0.000) (**Figure 3B**).

A)



B)

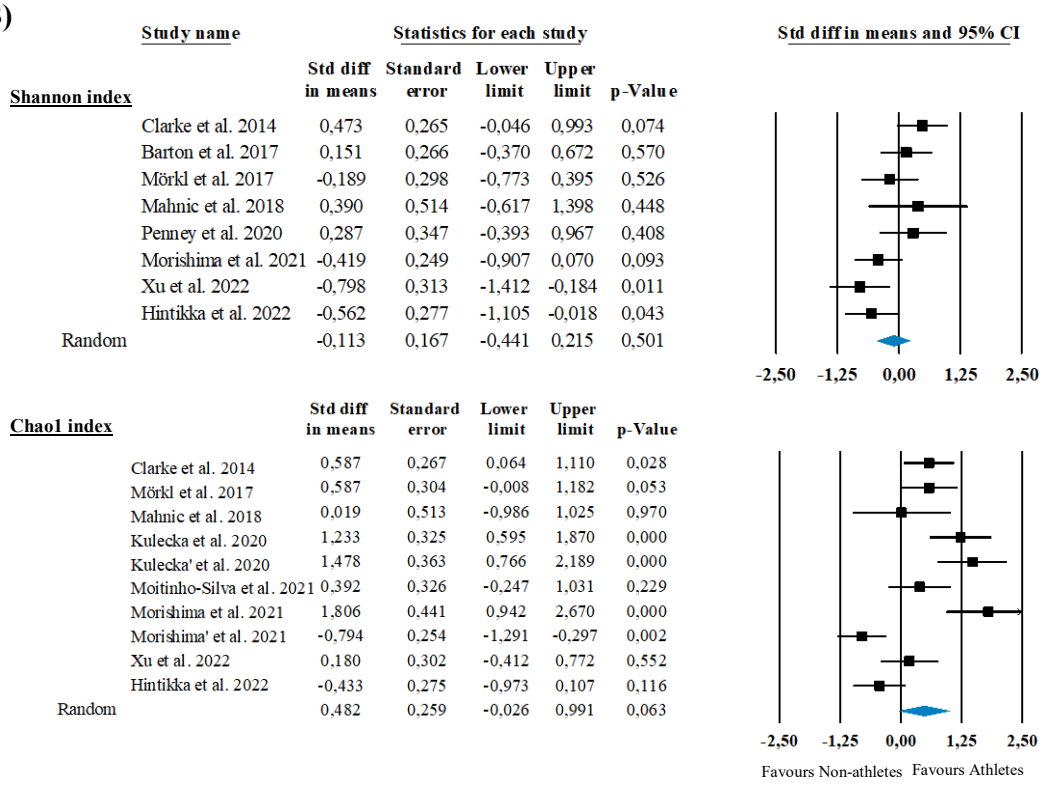


Figure 3. Panel A) shows the meta-analysis of the high-PA *vs*. low-PA level's effects on Shannon diversity and Chao1 indexes (i.e., alpha-diversity metrics). Eight studies were finally included (Shannon diversity index [44,66,69,78,81,83,85]; Chao1 index [44,70,78,81,85]). Panel B) indicates the meta-analysis of the athletes *vs*. non-athletes' effects on both alpha-diversity metrics, i.e., Shannon diversity (8 studies [47,49,51,60,76,78,79,81]) and Chao1 indexes (9 studies [47,51,56,74,76,78,79,81,104]).

3.3.3. Third meta-analysis (intervention studies): chronic effects of PA

The third meta-analysis united 167 participants from 7 studies, where 118 were allocated to a PA group and 49 to a control group. No significant differences were found between the PA and control groups on alpha-diversity using the Shannon diversity index (PA group: SMD=0.132, 95% CI -0.124-0.388, p=0.312, I^2=0.000; control group: SMD=0.110, 95% CI -0.288-0.508, p=0.587; I^2=0.000) or Chao1 index (PA group: SMD=-0.080, 95% CI -0.454-0.295, p=0.677, I^2=0.000; control group: SMD=0.001, 95% CI -0.454-0.457, p=0.995; I^2=0.000) (**Figure 4**).

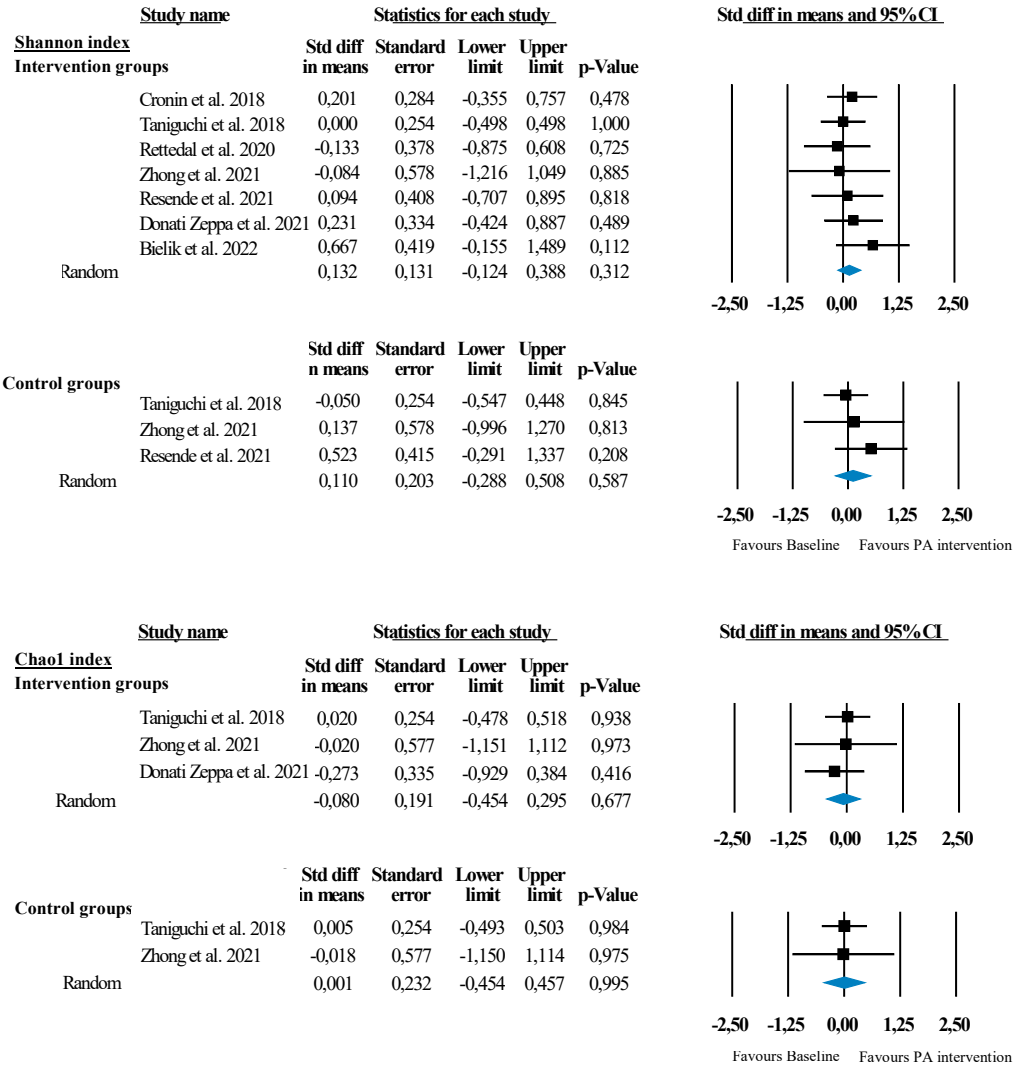


Figure 4. Meta-analysis of the PA intervention (up) *vs*. control's effects (down) on the Shannon diversity (7 studies [96,97,99,109,112,119,124]) and Chao1 index (3 studies [97,99,112]). Due to the lack of studies, we included both RCTs [96,97,99,109,119] and non-RCTs [112,124] in the same meta-analysis. The bottom meta-analyses reflect the effect of time in the absence of PA intervention since only

includes the control groups that were available from the RCTs. We did not use the control groups of Cronin *et al*. 2018 and Bielik *et al*. 2022, as they consumed a protein or probiotic supplement [96,119]

4. DISCUSSION

The main findings of this systematic review and meta-analysis were: 1) there was no consistent effect of PA on modifying microbial alpha-diversity, although most of studies support that PA (observational and intervention studies) induces changes in microbiome composition with the increase of short-chain fatty acids (SCFAs)-producing bacteria such as *Akkermansia*, *Roseburia* or *Veillonella*, among others; 2) there is very limited evidence of the effect of SB on microbiome; 3) few studies assessed PA data by objective methods (i.e., accelerometry); 4) there are few studies about the acute effect of PA on microbiome; 5) available studies are hardly comparable due to heterogeneity of the participants (i.e., age, sex, health status), wide use of different self-reported questionnaires to record PA, lack of standardized criteria to stratify participants in active/sedentary groups in cross-sectional studies and different characteristics of PA interventions (e.g., type, intensity, duration); 6) most of studies did not include diet as a confounder in their statistical analyses; 7) well-designed multi-omics studies (i.e., metagenomics, meta-transcriptomics, meta-proteomics and meta-metabolomics) are warranted to clarify the effect of PA and SB on microbiome.

4.1. Cross-sectional studies: physical activity and sedentary behavior (non-athletes)

Microbiome diversity is considered a direct measure of gut health, and a loss of diversity has been linked to a higher risk of obesity, type-2 diabetes, and cancer, among others [126]. In this systematic review, four studies found that the gut microbiome of children and adults with higher PA levels showed higher alpha-diversity, compared to

those who rarely or never exercised [59,65,66,85]. Similarly, two studies reported a positive association between PA level and gut alpha-diversity in participants with different diseases [38,67]. A positive correlation between average PA intensity and vaginal microbiome alpha-diversity was also found in healthy college-aged women [43]. However, other studies in individuals with different age and health conditions reported negative or no associations [36,39,41,44,48,63,68–71,78,81–84], as is also detected in our meta-analysis of 1814 participants (**Figure 3A**). Heterogeneity in study population (i.e., health status, sex, age), methodological aspects (i.e., use of diverse self-reported questionnaires, different pipelines to analyze the microbiome, etc.), varying criteria to stratify participants based on PA level and lack of control of relevant covariates (e.g. diet) in statistical analyses may contribute to the discrepant findings across studies. In fact, Langsetmo *et al*. demonstrated different results depending on the method for measuring PA, where self-reported PA was positively associated with beta-diversity [84], while objectively measured PA recorded by accelerometry (expressed as step counts) showed no associations [84].

Regarding SB, Bressa *et al*. reported that less time in sedentary bouts was positively associated with alpha-diversity (Shannon and Chao1 indexes) in premenopausal women [48]. In contrast, there were no significant differences in alpha-diversity when compared the gut microbiome of physically active women (those who perform at least 3 hours of PA per week) and sedentary women (i.e., those who perform <3 hours) [48]. Whisner *et al*. did not find any significant differences in alpha-diversity parameters across quartiles of SB in a cohort of college students [70]. However, a later study detected an increased alpha-diversity in the saliva of children who reported less screen time [64]. Interestingly, recent evidence indicates a positive association between SB and *Streptococcus,* detected in feces and saliva [63,64]. *Streptococcus* has been described as a key bacteria in disease such as old-onset colorectal cancer [127]. The existence of an oral-

gut microbiome crosstalk has been proposed, highlighting a possible association between oral dysbiosis, oral–gut microbiome axis and the pathogenesis of different diseases such as gastrointestinal disease or colorectal cancer [128]. Thus, more future research is needed to unravel the role of SB as a potential modulator of microbial communities.

There are more consistent findings about the associations between PA and the gut microbiome, mostly at lower taxonomic categories. At phylum level, *Firmicutes* seems to be more abundant in the gut of those individuals with higher PA levels [41,59], although several studies found the inverse association [44–46]. Since *Firmicutes* has been associated with fiber [129], different dietary habits may be partially explaining variability between the studies. Interestingly, growing evidence supports that PA increases the abundance of a *Firmicutes*-belonging group of commensal bacteria able to produce SCFAs from non-digestible carbohydrates ingested through diet, such as butyrate, propionate and acetate [130]. Most of the included studies reported higher abundances of SCFAs-producing bacteria from *Lachnospiraceae* and *Erysipelotrichaceae* families [42,59,63,66,70], and *Roseburia*, *Coprococcus*, *Lachnospira*, *Blautia* and *Faecalibacterium* genera, among others, in more active individuals compared to those with lower PA levels [42,59,70,71,84,85]. Particularly, Bressa *et al*. quantified the relative abundance of *Akkermansia muciniphila*, *Faecalibacterium prausnitzii* and *Roseburia hominis* by real-time PCR (qPCR) and detected higher abundances in physically active compared to inactive women [48]. SCFAs have been linked to good health due to their role on metabolic function, being substrates for energy metabolism as well as important signaling molecules implicated in the gut-microbiota axis and in the regulation of the immune response [131,132]. Since the availability of SCFAs are influenced by both, the ingestion of nutritional components and their digestion directed by the gut microbes [130], the previous results could indicate SCFAs as the key molecular link between PA, diet and microbiome.

4.2. Cross-sectional studies: athletes *vs.* non-athletes

Available evidence generally agrees on a trend towards the increase of the gut microbial diversity in athletes of different sports disciplines compared to non-athletes (see our meta-analysis of 651 participants; **Figure 3B**). Further, a recent meta-analysis evaluated microbial alpha-diversity of shotgun metagenomics data of the gut microbiomes of 207 athletes of different sports and 107 non-athletes and found a significantly higher species richness in athletes compared to non-athletes [133]. However, it is also well known that specific dietary requirements are usually implemented based on the duration and intensity of PA training [134], which makes difficult to determine the isolated effect of athletic performance on the microbial communities. In 2014, Clarke *et al*. reported, for the first time, a positive association between athletic performance and alpha-diversity parameters, when compared the gut microbiome by 16S rRNA sequencing of a group of professional rugby players and sedentary participants with low and high BMI (i.e., BMI≤25 or >28, respectively) [47]. However, the athletes' enhanced diversity was also associated with high protein consumption in this group. Barton *et al*. [49] re-analyzed the participants from Clarke *et al*. to evaluate the microbiome diversity with the whole metagenome shotgun sequencing, confirming the previous results [47]. More recently, Penney *et al*. analyzed the combined effects of diet and athletic performance in the gut microbiome of those participants, and found a significant association with alpha-diversity when combined the effect of both athletic performance and dietary habits [60]. Later studies described an enriched microbial diversity in athletes with special diets, compared to sedentary participants [61,62], and others did not find any significant differences between athletes and non-athletes with similar dietary patterns [52,53,57]. In contrast, 2 studies reported a higher alpha-diversity in athletes compared to sedentary participants with similar dietary habits [51,56]. Large variety of sports disciplines included in the

abovementioned studies (marathon runners, bodybuilders, cross-country skiers, rugby players, etc.) can be also contributing to inconsistency of the results. So far, the isolated effect of athletic performance, independently of diet, is still unclear.

Since diet is one of the most important modulators of the microbiome, differences in nutritional habits may also affect the relative abundance of specific microorganisms [135]. In fact, high-digestible carbohydrate diets have been related to the growth of SCFAs-producing bacteria. Clarke *et al*. reported a higher abundance of *Firmicutes* phylum and a decreased abundance of *Bacteroidetes* in rugby players compared to sedentary individuals with high BMI [47]. Both groups presented a distinct nutritional profile, with an increased consumption of protein, fiber, carbohydrate and monounsaturated and polyunsaturated fat in the athletes group. A later study also described a higher abundance of *Firmicutes* and lower levels of *Bacteroidetes* in rugby players compared to non-athletes [76]. Accordingly to these findings, animal and human studies have positively associated *Firmicutes* to fiber intake but negatively to fat consumption, while *Bacteroidetes* showed the opposite association [129]. Additionally, later metabolic pathway analyses revealed that rugby players had an enriched profile of SCFAs [49]. Other SCFAs-producer, *F. prausnatzii,* was also found to be more abundant in senior athletes compared to older sedentary participants after adjusting for different covariates, including diet [57]. Morishima *et al*. found an increase of *Faecalibacterium* in female runners compared to non-athletes, and a higher abundance of succinate, a SCFA that can be produced by *Faecalibacterium* [74].

Liang *et al*. reported that professional martial arts athletes had an enriched microbiome compared to amateurs, and identified changes in the abundance of several bacteria after adjusting for different confounders including diet [55]. Furthermore, one study found higher diversity and *Firmicutes/Bacteroidetes* ratio in female elite compared to

non-elite athletes [58]. However, metagenomics and meta-transcriptomics analyses conducted by Petersen *et al*. only detected differences at transcriptomic (RNA) level, highlighting the need for more microbiome studies at functional level [50].

Few studies have identified significant microbial shifts in relation with the type of sport [53,54,56]. Interestingly, O'Donovan *et al*. compared athletes from 16 different sports and found specific bacterial taxa such as *Anaerostipes hadrus*, *F. prausnitzii* and *Bacteroides caccae*, differently abundant between sports with a moderate-dynamic component (e.g. fencing), high-dynamic and low-static components (e.g. field hockey), and high-dynamic and static components (e.g. rowing) [54].

4.3. Acute effects of PA

Most of the studies aimed to analyze potential changes in the gut microbial composition following a marathon [23,87,90]. In this sense, two studies detected an increase in *Firmicutes/Bacteroidetes* ratio of the gut microbiome in long-distance runners post-race [90,92]. Significantly, Grosicki *et al*. also detected a higher abundance of *Veillonella*, accordingly to the results obtained by Scheiman *et al*. [23,90]. The last study proposed a microbiome-encoded enzymatic mechanism that could partially explain how microbiome and its metabolites (i.e., SCFAs) may contribute to enhance athletic performance [23]. After detecting a higher abundance of *Veillonella* in runners after the race, they observed that administration of *Veillonella atypica* in a mouse model improved run time and demonstrated its capability of metabolically converting the exercise-induced lactate into propionate in the colon to subsequently re-enter the systemic circulation. In search of confirming these findings, Moitinho-Silva *et al*. quantified the relative abundance of *V. atypica* by qPCR and sequencing in a subset of elite athletes (mainly cyclists and triathletes) and sedentary participants, but failed to find any significant differences between the groups [104]. These contrasting results could be partially explained by several

limitations of the last study such as the lack of dietary data for the athletes group. Other studies have detected an increase in several SCFA-producing bacteria, including *Coprococcus*_2, *Dorea* or *Roseburia* after a marathon or a transoceanic rowing race [87,89]. Although the number of human studies is still limited, these findings support emerging evidence of the existence of a crosstalk between the gut microbiota and skeletal muscle through lactate (generated during exercise) and its conversion to SCFAs by the gut microbes which, consequently, could improve athletic performance [25]. In fact, SCFAs have been recently defined as "biotics" (substances able to modulate the microbiome by increasing the abundance of beneficial microbes) that could be used as an exogenous microbiome modulation approach for improving health and athletic performance [136]. Interestingly, a recent study discovered a gut–brain connection in mice that enhances athletic performance by increasing dopamine signaling during PA [137]. These results indicate that motivation for PA is influenced by the gut microbes derived-metabolites, suggesting a microbiome-dependent mechanism for explaining inter-individual variability in PA motivation and performance.

On the other hand, the acute effect of a bout of PA on the microbiome continues to be a scarcely investigated topic. Tabone *et al*. followed this approach analyzing fecal samples from athletes who underwent a moderate-intensity treadmill session until volitional exhaustion and detected changes in six bacteria (*Romboutsia*, *Escherichia coli* TOP498, *Ruminococcaceae* UCG-005, *Blautia*, *Ruminiclostridium* 9 and *Clostridium phoceensis*) [91]. Overall, acute interventions collect serum samples where potential changes can be detected earlier compared to fecal ones. In this context, one study collected blood and fecal samples of myalgic encephalomyelitis / chronic fatigue syndrome participants and detected changes at major bacterial phyla such as *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, in both samples after a

cycle ergometer maximal exercise test [86]. A later study exclusively analyzed viral reads (i.e., virome) from blood samples by RNA-seq and did not detect any differences after acute PA [88]. Thus, more research directed to analyze blood microbiome is needed to accurately assess the short-term effect of PA, specially, meta-transcriptomics and meta-metabolomics could be a novel and useful approach to study the active microbiome in the context of acute effects of PA.

4.4. Chronic effects of PA

To further deepen the overall knowledge of the chronic effects of PA on human microbiome and, generally, the host health, several clinical trials have been published in the last years [94–125]. Our meta-analysis of 167 participants is the first analysis that quantifies those trials in healthy participants, indicating that controversial results for alpha-diversity are consistently found (**Figure 4**). Two studies performed in healthy adults that underwent a 12-week aerobic PA intervention (3 sessions of 30 min per week) or 7-week high-intensity interval training (consisting of swimming lengths) reported an increase in microbial alpha-diversity [111,119]. Conversely, Moitinho-Silva *et al*. detected a slight decrease in alpha-diversity after an aerobic PA intervention (6 weeks; 3 sessions of 30 min per week) in healthy adults, although no differences were observed in another group subjected to a strength training [104]. Another study recruited healthy adults to undergo a PA intervention (aerobic and resistance training; 8 weeks; 3 sessions of 90 min per week), but no significant changes in alpha-diversity were detected after the intervention [96]. Most of the studies in unhealthy individuals did not report any significant changes in alpha-diversity after a PA intervention [94,95,102,106–108,114,115,118,121,122,124,125]. However, the chronic effect of PA on microbiome composition becomes clearer in the beta-diversity analysis, where more studies agree on a significant dissimilarity in the microbial communities of the individuals after long-term PA

[94,95,98,101,102,108,111,112,114,115,118–120,123,125]. Interestingly, Allen *et al*. observed how differences in the beta-diversity detected at baseline between the participants with normal-weight and obesity disappeared after an aerobic PA intervention (6 weeks; 3 of 30-60 min sessions per week) [94]. Different study designs (17 non-RCT, 13 RCT and 2 randomized controlled cross-over trials), health status of participants (15 studies with healthy and 17 with unhealthy populations), characteristics of PA interventions (type, duration, and intensity), and methodological differences in microbiome analysis, diet, among other factors, might partially influence the varying results obtained.

In accordance with observational studies [38,48,59,63,66,69,71,74,85], an increase in SCFAs-producing bacteria such as *Lachnospiraceae*, *Verrucomicrobiaceae, Lachnospira*, *Akkermansia*, *Veillonella*, *Faecalibacterium, Bifidobacterium* and *Roseburia* was also reported in participants with different age and health conditions (including obesity, prediabetes and insulin resistance, among others) after PA interventions ranging from 2 to 34 weeks [94,95,101,102,107,108,112,114,115,121–124]. More specifically, Liu *et al*. described an increase in *A. muciniphila* and an improvement in insulin sensitivity after a 12 weeks-concurrent PA intervention in men with prediabetes that were classified as responders compared to non-responders [122]. Later studies have also reported an increase in *A. muciniphila* in participants with overweight/obesity or type-2 diabetes after long-term PA [101,114]. *A. muciniphila* has been related to prevention of multiple metabolic diseases such as obesity, metabolic syndrome and type-2 diabetes [138]. In a recent publication, a multi-omics approach (transcriptomics, proteomics, metabolomics and lipidomics) investigated the underlying molecular mechanism of *A. muciniphila* in obesity. It concluded that *A. muciniphila* reduced lipid accumulation and downregulated the expression of genes related to adipogenesis and lipogenesis in adypocites [139]. These studies point to *A. muciniphila* as a promising microbial target

(potentially modulated by PA) with therapeutics effects in obesity and other metabolic diseases. At genus level, *Akkermansia* has also been widely found to be positively associated with PA in cross-sectional studies [47,48] and increased after PA interventions [95,122].

4.5. Future Directions

Our understanding about the effect of PA (little research is conducted on SB) on microbial communities is still in its infancy, vastly limited to the amplicon sequencing approach (i.e., 16S rRNA sequencing) and is highly variable between the studies. This heterogeneity highlights the need to perform well-designed studies focusing on specific detailed populations and establishing reference pipelines to ensure the accuracy and comparability of the results. To ensure the reproducibility and comparability of the future studies in the field, we recommend the researchers to follow the recent good practice guidelines [140,141] when microbiome analyses are performed.

Most of cross-sectional studies in this systematic review recorded PA measures by self-reported questionnaires. Accelerometry has been widely demonstrated to be a more valid and comparable method for objectively collecting participants' PA and SB levels [142]. Therefore, more accelerometry-based studies will allow researchers to apply standardized criteria to classify participants based on the use of cut-points for PA and SB which will reduce the inconsistency between study findings and reveal the accurate association of PA and SB with microbiome. In intervention studies that assess the chronic effects of PA on microbiome, we detected a low quality in the RCTs. These results could be partially explained by the use of a checklist [143] with a stricter scale for the quality assessment.

Since most of the studies analyzed DNA sequences regardless of microbial variability or functionality (only two studies performed a meta-transcriptomic analysis), we are not close to determine the functional microbes susceptible to PA. Moreover, future multi-omics analyses (i.e., combining metagenomics, meta-transcriptomics, meta-proteomics and meta-metabolomics) would further unravel the complex host-microbial molecular pathways implicated in the molecular response to PA. In this regard, the Molecular Transducers of Physical Activity Consortium (MoTrPAC) [6] will provide a powerful source of information to advance our understanding of PA's effects on the microbiome in humans and animal models performing multi-omics analyses.

4.6. Limitations and strengths

Due to the lack of available information, an important limitation of our meta-analysis was the use and transformation of directly reported data from the articles instead of re-analyzing raw data to reduce potential bias introduced by applying different methodologies and pipelines across studies. Besides, limited information prevented us from additionally analyzing other microbiome outcomes of interest, such as the differential abundance of key bacteria. Future studies should make publicly available raw sequences generated from sequencing platforms to allow future meta-analyses to cover these gaps in the literature. Nevertheless, our meta-analysis has been performed including specifically those studies in healthy population and conducting sub-groups analysis according to study design to gain homogeneity. Moreover, we followed a rigorous and reliable methodology previously validated [144,145] to obtain numerical data when they were unavailable. Additional strengths of our systematic review are the elaboration according to PRISMA guidelines, use of four different search databases (PubMed, Web of Science, SCOPUS and Cochrane), and performance of quality assessment with validated tools specific for each study design, which ensure the scientific rigor.

5. CONCLUSIONS

Our systematic review summarizes the available knowledge about the relationship between PA and SB and the microbiome from multiple body sites and across different human populations. So far, growing evidence points to higher abundances of SCFAs-producing bacteria in more active individuals or after a PA intervention. Our meta-analysis uniting 2632 participants indicated no consistent effect of PA on microbial alpha-diversity, although there seems to be a trend toward a higher richness in athletes compared to non-athletes. Thus, accelerometry-based observational studies and RCTs are needed to face this inconsistency. Additionally, there are scarce information about the effect of SB on microbiome. In conclusion, precisely-designed, well-controlled and multi-omics studies are needed to reduce heterogeneity, obtain comparable results and, therefore, gain reliable knowledge about the effect of PA and SB on the human microbiome.

SUPPLEMENTARY MATERIAL

Supplementary material can be downloaded in this link:

https://osf.io/bepqc/

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Study II: Association of accelerometer-determined physical activity and sedentary behavior with the gut microbiome in middle-aged women: A compositional data approach

ABSTRACT

Introduction. The beneficial effects of physical activity (PA) on gut microbiome have been previously reported, nevertheless the findings are inconsistent, with main limitation of using subjective methods for assessing PA. It is well-accepted that using an objective assessment of PA will reduce the measurement error of this complex behavior and will allow also objective assessment of sedentary behavior (SB). We aim to study the associations between the accelerometer-assessed behaviors (i.e. SB, light-intensity physical activity -LPA- and moderate-to-vigorous physical activity -MVPA) with the gut microbiome using compositional data analysis, a novel approach in the field that enables to study these behaviors accounting for their inter-dependency.

Methods. This cross-sectional study included 289 women from the Northern Finland Birth Cohort 1966. Microbiome analysis was performed on fecal samples collected at age 46. Movement behaviors were measured during 14 days by wrist-worn accelerometers. Univariate analyses on a single behavior and compositional data analyses on MVPA, LPA and SB in association with the gut microbiome data were performed.

Results. The microbial alpha- and beta-diversity in the gut were not significantly different between the higher *vs*. lower MVPA or SB groups, and no differentially abundant microorganisms were detected. Accordingly, compositional data analysis did not show any statistically significant associations between increasing one behavior while proportionally reducing the others on microbial alpha-diversity (all p-values>0.05). Butyrate-producing microbes such as *Agathobacter* and *Lachnospiraceae* CAG56 were significantly more abundant when reallocating time from LPA or SB to MVPA (γ=0.609 and 0.113, both p-values=0.007). A sensitivity analysis on a subset of samples corroborated our results of the whole cohort.

Conclusions. While PA of different intensities and SB did not seem to have a significant effect on microbial diversity, we found associations of these behaviors with specific gut bacteria, suggesting that PA of at least moderate intensity (i.e., MVPA) could exert synergistic effects on short-chain fatty acids-producing microbes.

1. INTRODUCTION

Strong scientific evidence supports that regular physical activity (PA) exerts beneficial effects on different health conditions, such as cardiovascular, insulin resistance and physical fitness, among others [1,2]. In 2020, the World Health Organization (WHO) approved the new guidelines on PA and sedentary behavior (SB) to enable people of all ages and conditions to maintain healthy levels of PA and mitigate diseases [3]. In the last decades, epidemiological studies detected a decrease in the levels of PA in adolescence, with one-third of adults not meeting the minimum WHO recommendations [4,5]. This emergence of physical inactivity has been defined as a "pandemic" and prevails as the fourth leading cause of death worldwide with 3.9–5.3 million annual premature deaths [6,7]. Therefore, implementation of PA promotion strategies is a global health priority to reverse this trend and reduce the health risks and economic burden associated with physical inactivity [8].

PA consists of a wide range of different intensity behaviors, such as moderate-to-vigorous PA (MVPA) and light intensity PA (LPA), both domains associated with lower risks of chronic diseases and all-cause mortality [9]. Meanwhile, the detrimental consequences of SB on health are well-reported [10,11]. To date, most studies independently investigate the associations of SB, LPA, or MVPA without considering the interplay between these behaviors [12]. Since the hours of the day are limited to 24h or to the wake

up time, increase in the time spent in one behavior necessarily comes with a reduction in the time spent in other behaviors that day, and this closure effect and inter-dependency of the variables should be mathematically modelled, which has not been done in the past. In this context, compositional data analysis (CoDA) emerges as a shift from a univariate to a 24-hour time-use paradigm that analyzes how the daily time spent in different codependent movement behaviors (i.e., MVPA, LA, SB) synergistically impacts our health (since dedicating more time in one of these behaviors means a reduction of time in the others) [13,14]. This new analytic tool provides a more advanced approach of the time spent on each activity during the day and whether a single ideal combination of these movements actually exists in the context of a study outcome [9,15].

Humans are host to trillions microbes, including bacteria, viruses, fungi and archaea, where the gut presents the most diverse microbiome (i.e., the collection of genomes from the microorganisms) within the human body [16]. The human gut microbiome consists of app. 90% of bacteria belonging to *Bacteroidetes* and *Firmicutes* phyla, with other less predominant bacteria from *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* [17]. These microbes are balanced through symbiotic relationships and support human life by promoting gut barrier protection, energy production, endocrine and immune function [18,19]. Thus, gut microbiome imbalance (i.e., dysbiosis) can lead to a disruption in the gastrointestinal homeostasis and drive the development of several diseases such as obesity, type-2 diabetes, cancer, bowel diseases, cardiovascular, and neurological disorders, among others [20–23].

Given the importance of the gut when it comes to maintain human health status and prevention of disease, the gut microbiome has been proposed as a new biomarker of overall health [24]. More research is currently focusing on studying the factors that influence its composition in order to alter the microbiome deliberately for preventive or therapeutic

purposes [25]. Particularly, there is evidence indicating that PA (little is known about SB) seems to be a cornerstone in promoting an optimal ecosystem for a healthy diverse gut microbiome, which in turn may benefit overall host health [26,27]. Although numerous studies have investigated the association between PA and the gut microbiome, heterogeneous study populations and the prevalence of using self-reported PA data provide contrasting and inconclusive results [28]. Thus, there is a need for studies on bigger sample size and homogenous study groups with rigorous PA data (objectively measured PA using accelerometry). Furthermore, the association of a movement behavior (relative to the others) with microbiome diversity and composition and whether there is an ideal combination of PA of different intensities associated with a healthy gut microbiome remains unexplored.

In the present study, we aimed to determine the joint associations between the accelerometer-measured MVPA, LPA and SB with microbiome diversity and composition in a subset of women from a wide population-based birth cohort study from Northern Finland, using CoDA. To the best of our knowledge, this is the first study investigating the associations of combinations of daily time spent in PA of different intensities and SB using CoDA in relation to the gut microbiome.

2. MATERIAL AND METHODS

2.1. Study population

This cross-sectional study analyzed a subset of women of the Northern Finland Birth Cohort 1966 (NFBC1966), a longitudinal population-based cohort study which includes all expected births in the year 1966 in the two northernmost provinces of Finland

[29]. During the follow-up, anthropometric measures, clinical examinations, health and personal information and blood and fecal samples were collected. Our study population consisted of a total of 304 women, where 102 women had been diagnosed with polycystic ovary syndrome (PCOS) and 202 were age- and BMI-matched controls with no PCOS at 46 years of age [30]. Women with hormonal contraceptive or antibiotic, antimycotic, letrozole or tamoxifen treatment within the last 3 months preceding sample collection were excluded. Since a previous study in this subset did not find any statistically significant differences in the gut microbiome profile of women with PCOS and controls [30], we included the whole population in a single group, considering diagnosis as a covariate for statistical analyses. The study has been approved by the ethical committee of Northern Ostrobothnia hospital district. All participants of the NFBC1966 provided informed consent for the data and samples to be used for scientific purposes.

2.2. Accelerometer assessed physical activity

Accelerometry has been widely demonstrated to be a more valid and comparable method to obtain a more precise estimation of each PA and SB components, compared to self-reported questionnaires [31,32]. Accelerometers are wearable monitors that allow to objectively and continuously measure the composition of the accelerations (movements) of the body segment in which the devices are attached during the entire day. Participants were asked to wear a wrist-worn accelerometer (Polar Electro Oy, Kempele, Finland) during 24 h/day for at least 14 days on the non-dominant hand. Polar Active is a waterproof activity monitor that records MET values every 30 s based on daily PA, and using clinical data (height, weight, age) as predefined inputs [33]. Accelerometers were blinded during the data collection period, and participants were asked to mail it back after clinical examinations ended. Briefly, these devices categorize PA according to five levels: very light: 1–2 metabolic equivalents of task (METs), light: 2–3.5 METs, moderate: 3.5–

5 METs, vigorous: 5–8 METs, and vigorous ≥8 METs) [34,35]. For the present study, very LPA was considered the SB component and PA level of ≥3.5 was classified as MVPA. Daily duration spent in each PA category was calculated in min/day for all participants. The first accelerometer monitored day was not included in the analysis. Women with less of 4 valid days (those with a wear time of at least 600 min/day) were excluded from the analyses.

2.3. Sample collection and DNA extraction

The fecal samples were collected at home by the study participants. It was recommended that the fecal sample should be delivered in a cooler on the day of collection. When it was not possible, the sample was stored for 1-2 days in a freezer at -20ºC until delivery. After delivery, the fecal samples were initially stored at -20ºC and then at -70ºC until analyses were performed.

For microbial DNA isolation, the fecal samples were first homogenized in a Stomacher-400 blender. DNA was extracted using QIAamp Stool Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions, except for the incubation step where samples were mixed with lysis buffer and incubated at 95ºC instead of 70ºC in order to ensure the lysis of both Gram-negative and Gram-positive bacteria. The extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, DE, USA). DNA yield was determined by measuring the absorbance ratios spectrophotometrically, adjusting A260/280 nm for protein and A260/230 nm for salt and phenol contamination.

2.4. 16S rRNA gene sequencing

Gut microbiome analysis was profiled by amplifying the hypervariable V3-V4 regions of the 16s rRNA gene using the forward 5'-CCTACGGGNGGCWGCA-3' and

reverse 5'-GACTACHVGGGTATCTAATCC-3' primers pair and sequencing on a MiSeq Illumina instrument. All PCR reactions were carried out in a 25 µl final reaction volume containing 12.5 µl 2X KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, MA, USA), 5 µL of each primer (1 µM), and 2.5 µL of extracted DNA (10 ng) under the following cycling conditions: initial denaturation at 95ºC for 3 min, 35 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 30 s, and elongation at 72ºC for 30 s, with a final extension step at 72ºC for 5 min. PCR clean-up was done with AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). Next, a PCR to index the amplicons was performed using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) with conditions: 95ºC for 3 min; 8 cycles of 95ºC for 30 s, 55ºC for 30 s, 72ºC for 30 s, with a final extension of 5 min at 72ºC, and hold at 4ºC. The pooled PCR products were purified with AMPure XP beads (Beckman Coulter) before quantification. The final library was paired-end sequenced (2 x 300 bp) using a MiSeq Reagent Kit v.3 on the Illumina MiSeq sequencing platform (Illumina).

2.5. Microbiome analysis

Raw sequences were demultiplexed with Illumina bcl2fastq2 Conversion Software v2.20 and imported to Qiime2 software v.2022.11 with a PairedEndFastqManifestPhred33 input format. DADA2 was used for the denoising step. Low-quality regions were trimmed considering a quality score below 25 to create high quality forward and reverse reads, using the "q2-dada2" function with the following parameters: trunc_len_f=288, trunc_len_r v=241, trim_left_f=16, and trim_left_r=0. Taxonomy assignment of amplicon sequence variants (ASVs) was performed using the "classify-sklearn" function against the SILVA 16S v132_99 database, along with a similarity threshold of 99%.

2.6. Statistics

Descriptive characteristics of the study participants were reported as mean and standard deviation (SD) or geometric mean and covariance, as appropriate. Since fiber intake is well-known to influence the gut microbiome [36,37], a fiber score based on the weekly frequency consumption of fresh/boiled vegetables, fruits and grain-contained products was calculated. Six women did not provide any information about the fiber consumption. As such, we estimated these values using multiple imputation method in SPSS v.28.0.1.0. Body mass index, accelerometer wear time, PCOS diagnosis and fiber score were considered confounders in our statistical analyses.

All statistical analyses were performed in R (v.4.2.1) under RStudio (v.2022.07). Statistically significance was set to 0.05 (i.e., p-value or q-value<0.05 for analyses using Benjamini-Hochberg false discovery rate [FDR] for multiple correction). To compare the microbial profile between groups, we categorized participants into groups (higher *vs*. lower) according to time spent in MVPA and SB (min/day). CoDA captures a more real approach of the movement composition of the day, quantifying the effect of increasing a specific behavior while reducing the others on a continuous scale. Hence, we investigated the cross-sectional associations of increasing time spent in one single behavior (while proportionally reducing the others) with microbiome outcomes (i.e., Shannon diversity index, richness and relative abundances of bacteria >0.1%). For this purpose, one time-use composition was defined and included SB, LPA, and MVPA. Isometric log ratios were calculated in sequential binary partition and included as explanatory variables as previously reported [38]. Compositional models were adjusted by BMI, PCOS diagnosis, fiber score and accelerometer wear time. The strength and direction of each association were indicated by gamma (γ) coefficients. Prediction analyses for specific time reallocations were additionally performed for each behavior.

Microbiome diversity analyses were conducted and visualized using phyloseq, vegan, microviz and ggplot2 packages. Microbial taxa were aggregated to phylum and genus level in further analysis. Within-sample microbiome diversity (i.e., alpha-diversity) was estimated by Shannon diversity index and richness (i.e., number of microbial taxa), using the "diversity" and "specnumber" functions from the vegan package. Between-sample microbiome dissimilarity (beta-diversity) was visualized using nonmetric multidimensional scaling (NMDS) ordination, based on the Bray Curtis distance. For alpha-diversity comparisons, linear-mixed effect models (LME) were used for significance testing among MVPA and SB groups with the function "aov" from the stats package, to include BMI, PCOS diagnosis and fiber score as potential covariates. For beta diversity significance testing, PERMANOVA was permuted using the "adonis2" function from vegan package. Differential abundance analysis was performed in those bacterial taxa with a relative abundance >0.1% using an Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) from the ancombc2 package. ANCOM-BC models the microbial absolute abundances using a linear regression framework [39], providing statistically significant differentially abundant taxa between MVPA and SB groups. Furthermore, sensitivity analyses were conducted by excluding women with PCOS to corroborate the previous analyses.

3. RESULTS

Of the initial study population of 303 women, a total of 289 presented valid objectively measured PA data. All these participants wore the devices for at least 4 days and provided valid accelerometer data whose mean daily wear time (SD) was 971.5 min (57.0). Descriptive characteristics and accelerometer data of study participants are summarized in **Table 1**.

Table 1. Descriptive characteristics of the study participants.

Variables	Study participants N=289 women
Age, mean±SD	46±0.0
BMI, mean±SD	27.9±5.4
Accelerometer valid days, mean±SD	13.5±1.8
Accelerometer wear time, mean±SD (min/day)	971.5±57.0
Moderate-to-vigorous PA, mean±SD (min/day)	61.1±29.7
Light PA, mean±SD (min/day)	297.5±72.0
Sedentary behavior, mean±SD (min/day)	612.9±85.7

Note: Data presented as mean±SD. Abbreviations: BMI: body mass-index, min: minutes; PA: physical activity

3. 1. Microbiome composition across the study population

After 16S rRNA gene sequencing, 72742 ASVs were detected, with a total of 20 phyla and 523 genera. The most dominant phyla were *Firmicutes* (53.8%) and *Bacteroidetes* (32.6%), followed by other sub-dominant phyla such as Proteobacteria (6.7%), *Actinobacteria* (3.2%), and *Verrucomicrobia* (2.0%), representing >98% of the gut microbiome. At genus taxonomic level, 103 taxa were present in a relative abundance over 0.1%, where *Bacteroides* (18.4%), *Alistipes* (7.0%), *Faecalibacterium* (4.5%), *Blautia* (2.4%) and *Ruminococcaceae* UCG-002 (2.4%) and *Roseburia* (2.2%) were the most abundant bacteria in the gut.

3.2. Microbiome analysis in groups of MVPA and SB

As the first descriptive step, we compared the gut microbiome profile of women with different MVPA and SB levels (as independent components). Therefore, we stablished two study groups based on the median of each behavior (median [q1; q3] of MVPA: 57.6 [39.7; 73.5]; SB: 616.8 [561.0; 673.3]). Women in the higher MVPA group

presented a lower BMI compared to the other group (25.6 [5.6] *vs*. 27.2 [7], p=0.018). The frequency of PCOS diagnosis and fiber score was similar in the higher *vs*. lower groups of MVPA and SB (all p-values>0.05).

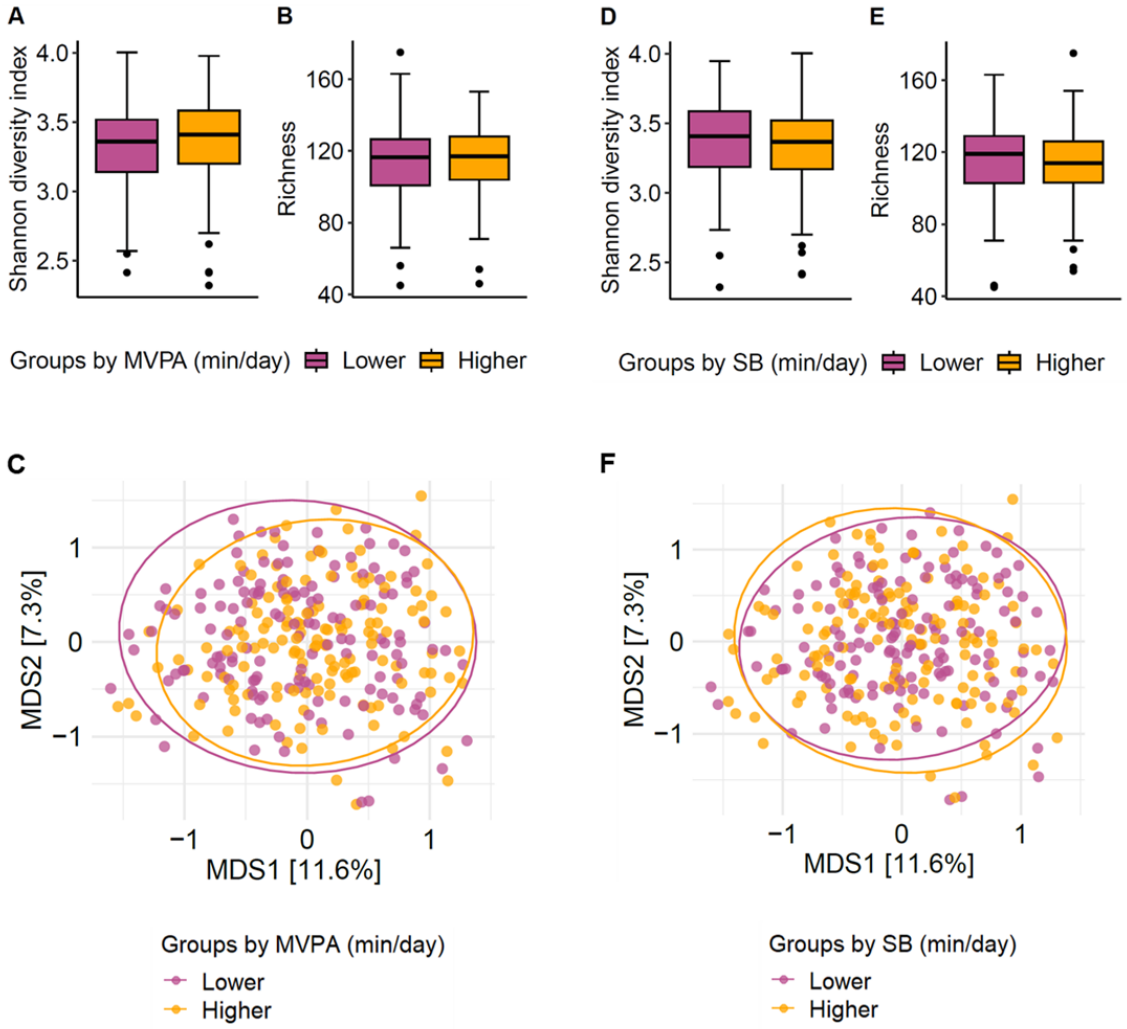

Figure 1. Microbial diversity measures in MVPA (A, B, C) and SB groups (D, E, F). Panels A, B, D and E represent alpha-diversity analysis (i.e. Shannon diversity index and richness). Higher (N=145) and lower (N=144) study groups were stablished based on the median of each behavior (MVPA: 57.6 min/day; SB: 616.8 min/day). Groups comparisons indicate no significant differences between higher and lower groups (Linear-mixed effects: all p-values>0.05). Panels C and F represent beta-diversity analysis: Principal coordinate analysis of Bray-Curtis distances. There is no significant dissimilarity between higher and lower groups (Adonis PERMANOVA: MVPA: R^2=0.003, p=0.765; SB: R^2=0.004, p=0.330). Abbreviations: MVPA: moderate-to-vigorous physical activity; SB: sedentary behavior

Alpha-diversity evaluated by Shannon diversity index and richness indicated no significant differences between the MVPA or SB groups (**Figure 1A, B, D** and **E**,

Shannon: MVPA groups: p=0.245; SB groups: p=0.316. Richness: MVPA groups: p=0.845; SB groups: p=0.498. All models included BMI, PCOS diagnosis and fiber score as potential confounders). Beta-diversity analysis based on Bray Curtis distances did not detect any significant dissimilarity between the microbial populations of each group (**Figure 1C** and **F**, PERMANOVA test accounting for covariates, MVPA: R^2=0.003, p=0.765; SB: R^2=0.004, p=0.330).

Next, we performed a differential abundance analysis using ANCOM-BC to detect specific taxa that could be differentially abundant in the gut microbiome of higher relative to lower MVPA and SB groups. *Intestinimonas*, *Rikenellaceae* RC9 gut group and a member of *Ruminococcaceae* family were slightly more abundant in the higher MVPA group compared to the lower group. *Dorea* was increased in the higher SB group, while *Bifidobacterium*, *Sutterella* and *Collinsella* were less abundant compared to the lower SB group. However, these bacteria did not remain statistically significantly different after FDR correction (all p-values>0.05, **Supplementary Tables S2 and S3**).

3.3. Compositional data analysis

Movement composition behavior of the women included in this study was visualized in a ternary plot illustrated in **Figure 2**. The geometric mean for each behavior was 54 min/day of MVPA, 289 min/day of LPA and 608 min/day of SB. Multiple regression models over compositional data were conducted to investigate the effect of increasing MVPA, LPA or SB while decreasing the rest of behaviors on multiple microbiome outcomes, adjusting for BMI, PCOS diagnosis, fiber score and the total amount of accelerometer wear time.

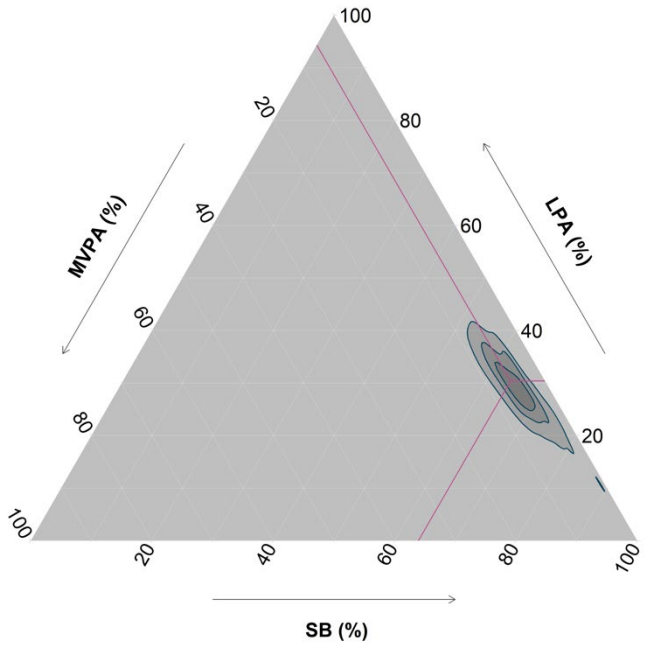

Figure 2. Ternary plot for the daily time-use in the movement behaviors in the study participants. The crosshair marks represent the geometric mean of the behaviors (i.e., MVPA: 54 min/day, LPA: 289 min/day, SB: 608 min/day). Concentric rings represent the 25, 50 and 75% confidence regions. MVPA: moderate-to-vigorous physical activity, LPA: light physical activity, SB: sedentary behavior

Figure 3 represents the curves relative to increasing one behavior while proportionally reducing others on microbial diversity. MVPA, LPA or SB (relative to the remaining behaviors) were not associated with any alpha-diversity metric (all p-values>0.05). Next, we investigated the associations between compositional data and the relative abundance of bacteria present in a relative abundance >0.1% (**Figure 4**). At phylum level, increasing time spent in LPA while reducing proportionally the other behaviors was negatively associated with Tenericutes (γ=-0.844, p=0.035). For example, increasing 30 min per day of LPA was associated with a 0.09% decrease in its relative abundance (expected change [CI]: -0.089 [-0.195; 0.017]).

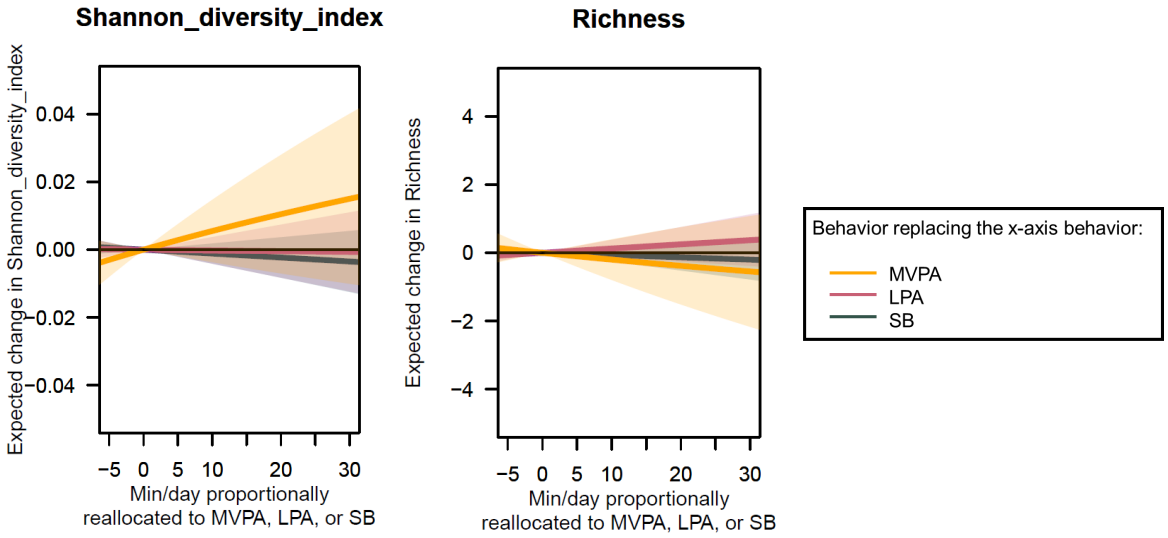

Figure 3. Joint associations of the movement behavior (i.e., MVPA, LPA and SB) composition with alpha-diversity metrics, i.e., Shannon diversity index and richness, both p>0.05. The models are adjusted by BMI, PCOS diagnosis, accelerometer wear time and fiber score consumption. Each line represents time in a behavior while proportionally reducing the others. Shaded areas represent the 95% confidence intervals.

At genus level, 10 bacteria showed significant associations with accelerometer-assessed behaviors. Reallocating time from LPA or SB to MVPA was positively associated with the relative abundance of *Agathobacter* (γ=0.609, p=0.007), *Lachnospiraceae* CAG56 (γ=0.113, p=0.007) and an unidentified bacterium from *Muribaculaceae* family (γ=0.473, p=0.015). For example, increasing 30 min per day of MVPA was associated with a 0.2% (expected change [CI]: 0.164 [0.048; 0.280]), 0.03% (expected change [CI]: 0.030 [0.009; 0.052]), and 0.1% (expected change [CI]: 0.127 [0.027; 0.227]) higher relative abundance of *Agathobacter*, *Lachnospiraceae CAG56* and *Muribaculaceae*'s bacterium, respectively. However, increasing time in MVPA was negatively associated with *Asteroleplasma* (γ=-1.329, p=0.041). Reallocating 30 min per day to MVPA at expenses of the other behaviors was associated with a 0.4% decrease in the relative abundance of this genus (expected change [CI]: -0.379 [-0.713; -0.045]).

Next, increasing time in LPA while proportionally reducing the other behaviors was positively associated with *Asteroleplasma* (γ=3.387, p=0.001), while negatively

associated with *Agathobacter* (γ=-0.892, p=0.014), *Lachnospiraceae* CAG56 (γ=-0.157, p=0.020), the bacterium from *Muribaculaceae* family (γ=-0.662, p=0.035), *Eubacterium xylanophilum* (γ=-0.409, p=0.009) and two members belonged to the *Clostridiales vadin* BB60 group (γ=-0.546, p=0.026 and γ=-0.565, p=0.020). For example, an increment of 30 min per day in LPA was associated with a 0.4% increase in *Asteroleplasma* (expected change [CI]: 0.407 [0.132; 0.683]), and lower decreases in the relative abundance of *Agathobacter* (expected change [CI]: -0.142 [-0.238;-0.046]), *Lachnospiraceae* CAG56 (expected change [CI]: -0.026 [-0.044; -0.008]), *Muribaculaceae* (expected change [CI]: -0.108 [-0.191; -0.026]), *Eubacterium xylanophilum* (expected change [CI]: -0.052 [-0.093; -0.011]), and *Clostridiales vadin* BB60 group's bacteria (expected changes [CI]: -0.071 [-0.136; -0.007] and -0.058 [-0.123;0.006]).

Finally, increasing time in SB was significantly associated with higher abundances of two bacteria from *Clostridiales vadin* BB60 group (γ=0.415, p=0.037) and *Izimaplasmatales* order (γ=0.636, p=0.028), while demonstrating an inverse association with *Asteroleplasma* (γ=-2.058, p=0.016), *Eubacterum ventriosum* (γ=-0.134, p=0.033) and *Intestinibacter* (γ=-0.151, p=0.040). Prediction analysis from reallocating 30 min per day from LPA or MVPA to SB reported slightly higher abundances of *Clostridiales vadin* BB60 group (0.004 [-0.038; 0.047]) and *Izimaplasmatales* (0.031 [-0.030; 0.094]), while 0.05% and 0.01% decreases in *Asteroleplasma* (0.047 [-0.135; 0.229]), *Eubacterum ventriosum* (-0.014 [-0.027; -0.001]) and *Intestinibacter* (-0.014 [-0.030; 0.002]).

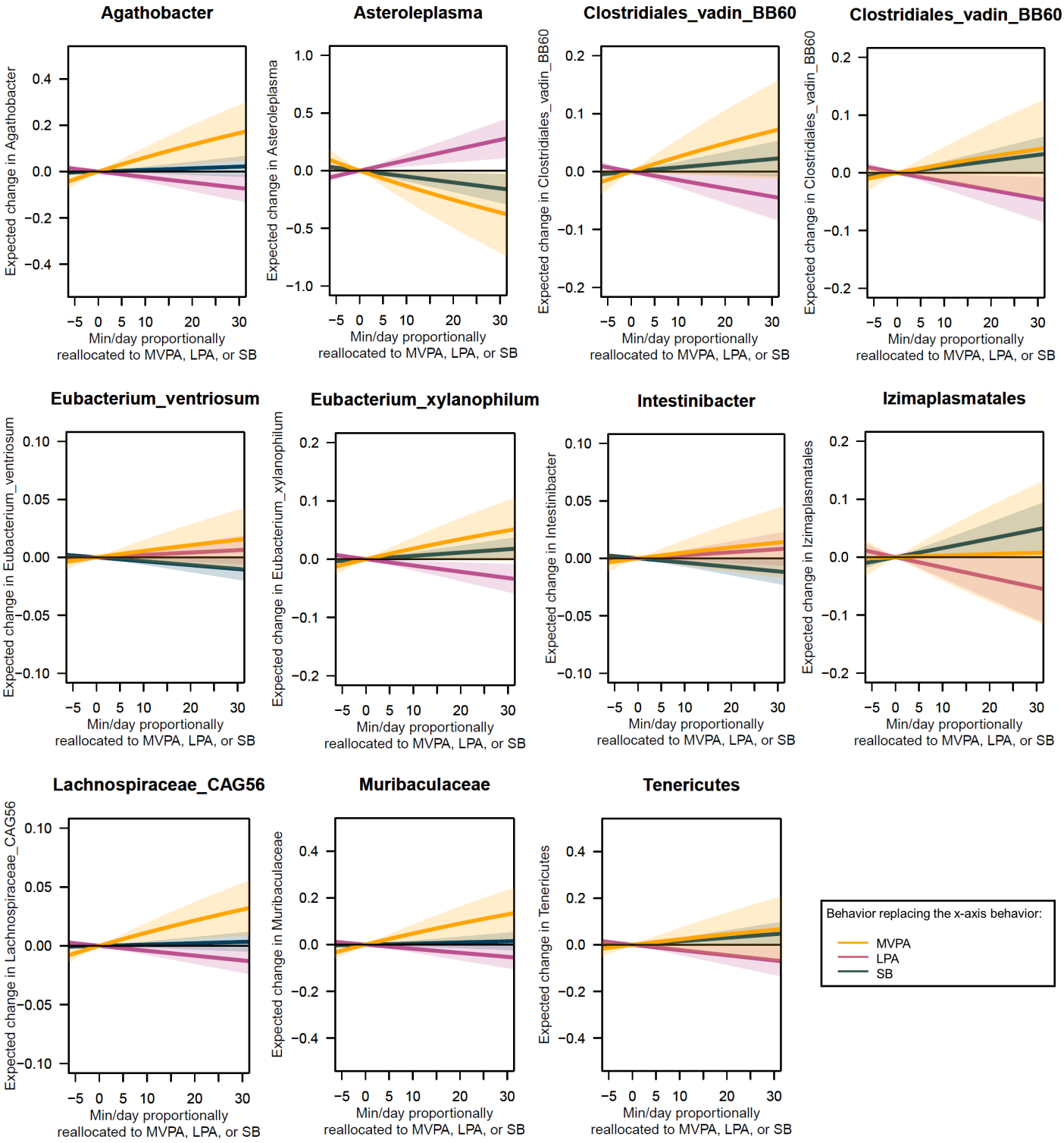

Figure 4. Joint associations of the movement behavior (i.e., MVPA, LPA and SB) composition with bacteria with a relative abundance >0.01%. Only statistically significant models ($p<0.05$) are shown. The models are adjusted by BMI, PCOS diagnosis, accelerometer wear time and fiber score consumption. Each line represents time in a behavior while proportionally reducing the others. Shaded areas represent the 95% confidence intervals.

3.4. Sensitivity analysis

A sensitivity analysis excluding those women who have been diagnosed with PCOS was performed to corroborate the obtained results. A total of 190 control women had valid accelerometry data and were included. The results are in line with those of the whole sample analyses, with no statistically differences detected in the microbial alpha- (Shannon: MVPA groups: p=0.430; SB groups: p=0.591; Richness: MVPA groups: p=0.640; SB groups: p=0.923) and beta-diversities (PERMANOVA test, MVPA: R^2=0.005, p=0.605; SB: R^2=0.006; p=0.265), after adjustment by BMI and fiber score (**Supplementary Figure S1**). Additionally, we did not detect any significantly different abundant taxon after correcting by multiple comparisons (**Supplementary Tables S4** and **S5**).

Concerning sensitivity analyses on compositional data, the previous findings were confirmed with no significant associations between accelerometer assessed time of MVPA, LPA or SB and alpha-diversity metrics (p-values>0.05, **Supplementary Figure S3**). Results from analyzing the association of movement behaviors with relative abundances of specific bacteria although, in agreement, were attenuated. Specifically, *Agathobacter*, *E. ventriosum*, *E. xylanophylum*, *Intestinibacter*, *Lachnospiraceae* CAG56 and the unidentified member of *Izimaplasmatales* order showed similar expected-change curves compared to the main analysis, but the associations did not reach the statistical significance. Additionally, increasing SB time (while reducing the other behaviors) was significantly associated with higher abundance of *Bifidobacterium* (**Supplementary Figure S4**).

4. DISCUSSION

To our knowledge, this is the first study investigating the joint associations of MVPA, LPA or SB (relative to the others behaviors) with the gut microbiome outcomes. CoDA represents a more real approach of the time spent in each behavior during the entire day, since increasing time in one activity means commensurately reducing time in other behaviors. Our findings support that PA of different intensities and SB, although they did not seem to affect the general microbial diversity, have an impact on the abundance of specific gut microbes.

In our first analysis, we characterized and compared the gut microbiome of women with different MVPA and SB levels. A greater alpha-diversity has been related to an overall gut stability and health, while a low diversity has been linked to several diseases such as obesity, diabetes, bowel diseases or colon cancer, among others [40]. We did not detect any significant association of MVPA and SB with the gut microbiome diversity in the middle-aged women. Also, a previous study failed to find any significant differences in the alpha- or beta-diversities when compared the gut microbiome profile of 40 active and sedentary women categorized according to WHO recommendations (i.e., active were those who performed at least 3 hours of PA per week, while sedentary were those who did not practice at least 3 days of PA per week for 30 minutes at a moderate intensity) [41]. While, they found significant correlations between the accelerometer assessed sedentary parameters (i.e., sedentary time and breaks) and alpha-diversity metrics (Shannon diversity and Simpson indexes) [41]. Similarly, numerous studies did not find any associations between the alpha-diversity and PA levels of individuals of different gender, age and health conditions [41–50], while other studies described an increased gut microbiome diversity among participants with higher PA levels [39,51–55].

It is noteworthy to mention that most of the current cross-sectional studies have used self-reported PA data. Self-reported questionnaires generally rely on subjective information, lack of detail when assessing behaviors (since they do not usually differentiate PA of different intensities, e.g., LPA and MVPA), and do not allow to perform analyses on a continuous scale. These studies are forced to analyze groups of different PA levels, while there is no consensus about which standard and valid criteria to use for categorizing participants into one or the other group. As a consequence, researchers face challenges to make right decisions of determining the appropriate criteria for specific populations which turns difficult to obtain conclusive results. Contrastingly, few studies have analyzed the association between the accelerometer assessed-PA and the gut microbiome [45,56,57]. Carter *et al*. recorded PA patterns over a 10-day period by a hip-worn triaxial accelerometer in 37 breast cancer survivors [56]. They quantified MVPA (min/day) to perform multiple regression analysis and examined the associations between MVPA and alpha-diversity. Their results showed that cardiorespiratory fitness (a genetic component modifiable by PA), while not MVPA, correlated positively with gut microbiome diversity [56]. These results are in accordance to those obtained by Zhong *et al.*, where they analyzed whether different accelerometer movement behaviors (i.e., SB, LPA and MVPA) were associated with microbiome outcomes in 100 older female and male participants. Multiple regression analysis did not detect any relationship between physical behaviors and alpha-diversity, however, associations were identified for MVPA and beta-diversity and the relative abundance of specific gut microbes [57]. In our study, where we analyzed 304 women at the same age and considered BMI matching together with fiber score and accelerometer wear time as confounders, no gut microbial diversity indices associated with PA and SB.

In our cohort, we integrated SB, LPA and MVPA as intrinsically codependent behaviors by using CoDA. Traditionally, researchers have analyzed movement behaviors as isolated components which leads to an unreal approach of the time distribution during a 24-hour day [58]. To our knowledge, this is the first CoDA study investigating the inter-relationships between physical behaviors and the gut microbiome. We did not find enough evidence of a significant association of MVPA, LPA or SB with any alpha-diversity metrics (Shannon diversity index and richness), which is in line with previous studies. It has been argued whether the type, dose and duration of PA is determining to produce considerable influence on the gut microbiome [59]. Therefore, there is a need for clarifying how much and what type of PA would be sufficient to increase microbial diversity in the gut [41,60]. Specially, several studies including participants from sports which demand high intensity level of exercise and usually require considerable fitness and dietary requirements, generally report a more diverse gut microbiome changes in athletes compared to non-athletes [61–64]. In this context, a recent meta-analysis of the metagenomics sequencing data of 207 fecal samples aimed to compare the gut microbiome between athletes and individuals with reduced PA, where athletes showed significantly more diverse microbiome compared to non-athletes [60].

Our differential abundance analysis detected statically significant bacteria associated with MVPA, LPA and SB. Particularly, *Agathobacter* and *Lachnospiraceae* CAG56 (both belong to *Lachnospiraceae* family) were positively associated with MVPA (relative to the other behaviors). Since there is a discrepancy in the taxonomic annotation of *Agathobacter*, *Roseburia* and *Eubacterium rectale*, the *Agathobacter* levels in our study may correspond to the *Roseburia* or *E. rectale* levels in others [65,66]. *Lachnospiraceae* family members are among the main producers of short-chain fatty acids (SCFAs), particularly acetate and butyrate, in the gut [67]. SCFAs are metabolic

products originated from dietary non-digestible carbohydrates and have been linked to human physiology, being substrates for energy metabolism and important signaling molecules implicated in the gut-microbiome axis, the regulation of the immune response and the skeletal muscle lipid metabolism [68–70]. In fact, a decrease in the relative abundance of *Lachnospiraceae* has negative health consequences due to the loss of relevant beneficial functions performed by members of this family [67], such as colonization resistance or butyrate-conducted pleiotropic beneficial effects for the host metabolism and immune regulation [71,72]. In line with our study results, numerous human observational [43,51,52,54,57] and experimental [73–79] studies have associated higher abundances of SCFAs-producing gut bacteria from *Lachnospiraceae* family, such as *Roseburia*, *Coprococcus*, *Lachnospira* and *Blautia*, among others, with higher PA levels or PA interventions ranging from 2 to 34 weeks. For example, Whisner *et al*. pointed to *Lachnospiraceae* and *Lachnospira* as important microbial markers for college students with greater MVPA [43]. Similarly, Zhong *et al*. reported four unclassified bacteria from *Lachnospiraceae* family to be positively associated with accelerometer-assessed MVPA in older adults [57].

Asteroleplasma was the only microbe associated with the three PA components, being positively associated with LPA while negatively with MVPA and SB. Several studies have been linked *Asteroleplasma* to chronic conditions, such as type-2 diabetes and tumor metastasis [80,81], although its biological role on human physiology has not been elucidated. Neither are the functional roles of *Clostridiales vadin* BB60 group and *Izimaplasmatales* in human health well-known, which we found to increase when reallocating time from MVPA or LPA to SB. Interestingly, the butyrate-producers *Eubacterium ventriosum* and *Eubacterium xylanophilum* showed a negative association with SB and LPA, respectively. *E. ventriosum* has been described to be important for the gut health and proposed as a biomarker of low risk of colorectal cancer [82,83].

Limitations and strengths

The impact of PA on the gut microbiome independently from host and lifestyle factors such as gender, age or diet, among others, is still unclear since the current knowledge rely on heterogeneous studies [28]. The performed studies are hardly comparable due to disparity of the participants (i.e., age, gender, health status), wide use of self-reported PA questionnaires, lack of standardized criteria to define which PA level categorizes the study population as sufficiently active and relevant covariates missing, especially diet. Thus, studies on bigger sample size, with rigorous PA measurements (objectively obtained by accelerometry) and dietary well-controlled studies are warranted to study the real influence of PA on the gut microbial ecosystem. The strength of this study include a homogenous female population of representative sample size. A major strength is the use of accelerometers which enable to objectively register PA of different intensities and SB. Notably, PA behaviors were codependently analyzed (on a continuous scale) using CoDA by considering different time reallocations. CoDA mirrors more precisely "real life" of study participants, in contrast with the traditional univariate approach that analyzes movement behaviors as independent domains.

A limitation in our CoDA was that we did not have available information on sleep time and, therefore, it was not included in the analyses. Therefore, we were not able to analyze the entire 24-h day, but just the waking hours of the day. While accelerometry is the best and most reliable measuring method, wrist-worn accelerometers have several limitations. For example, they do not capture all types of PA precisely, with an overestimation of upper-body movements while underestimating others activities such as cycling [35]. Regarding microbiome analysis, fecal samples were analyzed by marker gene sequencing. This approach is a DNA-based method that identifies the microbes present in an environment through the analysis of a sequence variation (i.e., hypervariable region)

of a single ubiquitous gene (e.g. 16S rRNA gene). While the whole genome or RNA sequencing have several advantages: 1) They provide a much greater specific identification of the taxa at the species level, compared to marker gene analysis in which classification is normally limited to the genus level; 2) they are able to detect viral and eukaryotic genetic material, while 16S rRNA gene sequencing only identifies bacteria and archaea; 3) they provide information about the functional capability of the identified microorganisms. Hence, more research on metagenomics and meta-transcriptomics is needed to completely understand the molecular mechanisms underlying the relationship between PA, SB and the gut microbiome.

5. CONCLUSIONS

Our findings using CoDA do not support a significant association of PA on the gut microbial diversity. On the other hand, our data indicate that more time in MVPA (relative to LPA and SB) was associated with increase in the relative abundance of beneficial SCFAs-producing microbes. More studies on compositional data including sleep time are warranted to precisely analyze the entire 24-hour day and confirm our results.

SUPPLEMENTARY MATERIAL

Supplementary material can be downloaded in this link:

https://osf.io/bepqc/

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SECTION II. The microbiome in female reproductive health

Study III: The female upper reproductive tract harbors endogenous microbial profiles

ABSTRACT

Introduction. The female reproductive tract harbors unique microbial communities (known as microbiota) which have been associated with reproductive functions in health and disease. While endometrial microbiome studies have shown that the uterus possesses higher bacterial diversity and richness compared to the vagina, the knowledge regarding the composition of the Fallopian tubes (FT) is lacking, especially in fertile women without any underlying conditions.

Methods. To address this gap, our study included 19 patients who underwent abdominal hysterectomy for benign uterine pathology, and 5 women who underwent tubal ligation as a permanent contraceptive method at Hospital Clínico Universitario Virgen de la Arrixaca de Murcia (HCUVA). We analyzed the microbiome of samples collected from the FT and endometrium using 16S rRNA gene sequencing.

Results. Our findings revealed distinct microbiome profiles in the endometrial and FT samples, indicating that the upper reproductive tract harbors an endogenous microbiome. However, these two sites also shared some similarities, with 69% of the detected taxa being common to both. Interestingly, we identified seventeen bacterial taxa exclusively present in the FT samples, including the genera *Enhydrobacter, Granulicatella, Haemophilus, Rhizobium, Alistipes,* and *Paracoccus,* among others. On the other hand, 10 bacterial taxa were only found in the endometrium, including the genera *Klebsiella, Olsenella, Oscillibacter and Veillonella* (FDR <0.05). Furthermore, our study highlighted the influence of the endometrial collection method on the findings. Samples obtained transcervically showed a dominance of the genus *Lactobacillus,* which may indicate potential vaginal contamination. In contrast, uterine samples obtained through hysterescopy revealed higher abundance of the genera *Acinetobacter, Arthrobacter,*

Coprococcus, Methylobacterium, Prevotella, Roseburia, Staphylococcus, and *Streptococcus.*

Discussion. Although the upper reproductive tract appears to have a low microbial biomass, our results suggest that the endometrial and FT microbiome is unique to each individual. In fact, samples obtained from the same individual showed more microbial similarity between the endometrium and FT compared to samples from different women. Understanding the composition of the female upper reproductive microbiome provides valuable insights into the natural microenvironment where processes such as oocyte fertilization, embryo development and implantation occur. This knowledge can improve *in vitro* fertilization and embryo culture conditions for the treatment of infertility.

1. INTRODUCTION

As our understanding of the human microbiota continues to expand, it becomes increasingly evident that it is ubiquitous and exerts significant influence on human physiology and pathophysiology [1–3]. Within the female reproductive tract, a growing body of evidence is associating microbial composition to reproductive functions in both healthy and diseased states [4–7].

While numerous studies corroborate the important role of microbial communities in the female lower reproductive tract (vagina and cervix) in the defense against pathogens, the upper reproductive tract (endometrium, Fallopian tubes -FT, ovaries) was traditionally considered a sterile environment, with the cervix acting as a barrier against bacterial passage [8]. However, with the advent of microbiome studies focusing on the human upper reproductive tract and analysis of microbial genomes, it is now evident that

this region possesses its own distinct microbial communities [7,9,10]. Recent studies have consistently shown that the endometrium harbors greater bacterial diversity and richness compared to the lower reproductive tract. These microbial communities are mainly composed of bacteria belonging to the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. The dominance of *Lactobacillus* in the uterus has been associated with a higher probability of live births, while the presence of *Gardnerella* or *Streptococcus* has been linked to early pregnancy loss or implantation failure in IVF treatment [1,11]. However, due to differences in study design and the absence of proper negative and positive controls, there is a lack of consensus among studies examining the upper reproductive tract microbiota [9,12].

The microbial composition of the FT is less studied, primarily due to challenges associated with sample collection which may affect future fertility. The characterization of the endogenous microbiome of the FT is of particular interest because this microenvironment provides a stable temperature, optimal pH and dynamic fluid secretions that support oocyte fertilization and the early stages of embryo development [13–15]. The limited studies analyzing samples from women with benign diseases or for prophylactic purposes suggest that the FT does indeed harbor an endogenous microbiome. Predominant bacterial taxa identified in these studies include *Firmicutes* (especially *Staphylococcus* spp., *Enterococcus* spp., and *Lactobacillus* spp.), *Pseudomonas* spp. *Burkholderia* spp., *Propionibacterium* spp. and *Prevotella* spp. [15–18]. However, there is ongoing debate regarding whether the FT truly harbors an endogenous microbiome and to what extent it impacts oocyte fertilisation and the initial stages of embryo development.

Given the anatomical connection between the uterus and the FT, with the intramural portion of the uterine tube preventing a complete physical separation between the two

sites, it is reasonable to hypothesize that the microbiome of the FT may be similar to that of the uterus [19–21]. Therefore, comparative studies analyzing uterine and FT samples collected simultaneously from the same donor are necessary to evaluate whether the organs comprising the female upper reproductive tract possess specific endogenous microbial profiles. In the current study, we aimed to analyze the 16S rRNA gene V2-4 and V6-9 regions of endometrial and FT samples obtained from fertile women, with the objective of identifying the microbiome of the female upper reproductive tract in disease-free individuals.

2. MATERIAL AND METHODS

2.1. Study population

This prospective study was conducted at the Service of Obstetrics and Gynaecology of the HCUVA in Murcia, Spain. Patients who underwent a planned laparoscopic hysterectomy with bilateral salpingo-oophorectomy or laparoscopic tubal ligation from January 2016 until June 2018 were recruited to participate in the study. Inclusion criteria were as follows: Caucasian women who had not received hormonal treatment for three months prior to surgery, regular menstrual cycles, and no history of fertility problems, endometriosis or other adnexal pathology detected by transvaginal ultrasound analysis and confirmed through histological examination. Nineteen participants underwent total laparoscopic hysterectomy with bilateral salpingo-oophorectomy to remove the uterus, cervix, ovaries, and FT due to the presence of uterine fibroids and associated abnormal bleeding (see **Figure 1** for the study design). Additionally, five participants underwent tubal ligation to remove the FTs as a permanent

contraception/sterilization measure. This study was approved by the Ethics Research Committee (CEIC) of HCUVA, Murcia, Spain (Approval No. EST: 04/16) and all participants provided written informed consent. Patient data and samples included in this study were registered, stored, and processed by the Biobanco en Red de la Región de Murcia, BIOBANC-MUR (registered on the Registro Nacional de Biobancos – ISCIII, no. B.0000859).

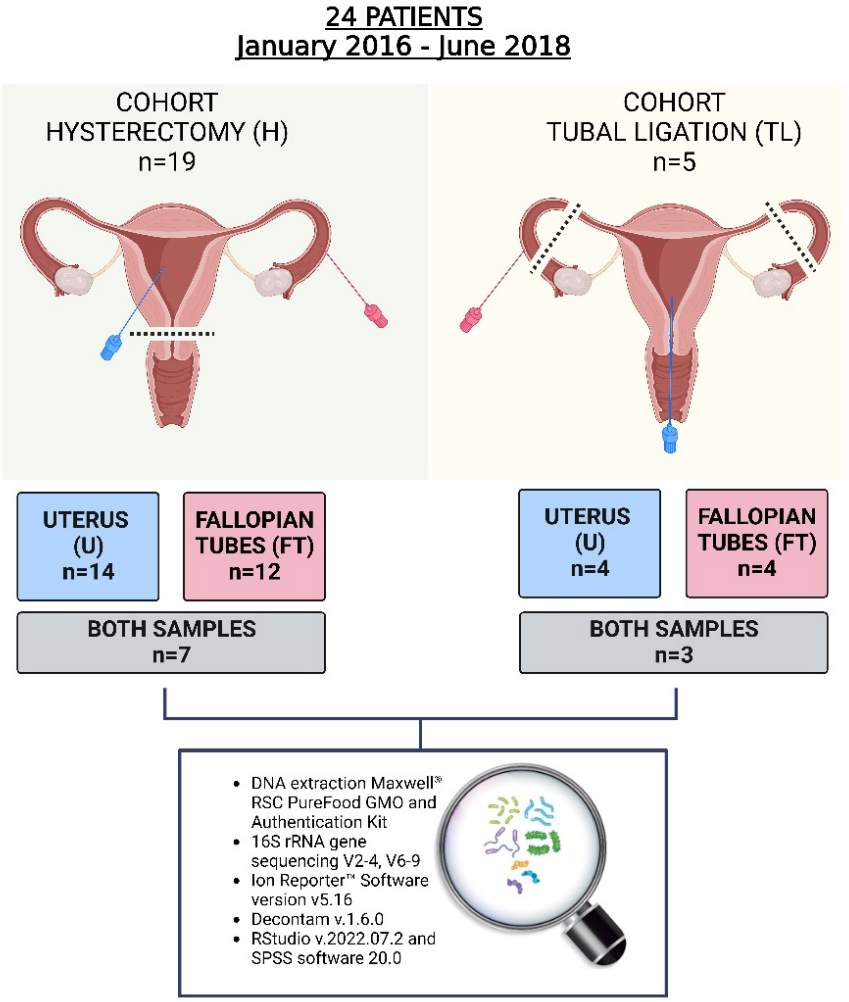

Figure 1. Study design. In total 24 women participated in the study and 34 samples from the upper reproductive tract were retrieved. In the hysterectomy cohort (H), seven women provided both endometrial and Fallopian tube (FT) samples, and in the tubal ligation cohort (TL), three women provided both samples. The rest of the participants provided only one of the samples due to the tissue damage during laparoscopic procedure, non-sterile condition, or blood contamination.

2.2. Collection of FT and endometrial samples

The collection method for the FT samples was standardized for all patients who underwent laparoscopic hysterectomy with bilateral salpingo-oophorectomy or laparoscopic tubal ligation. After the laparoscopic procedure, FTs were removed and transferred to ice-cold Petri dishes. Once dissected, FTs were clamped at both opposite ends to avoid sample waste. Manual mechanical pressure was applied between the extremities, the FT content that accumulated at the upper portion of the ampulla was aspirated through a sterile Mucat device (CDD Laboratorie, France). This class I medical device, complying with Directive 93/42/EEC, indicated for direct exocervical or endocervical aspiration and Hühner test, was adapted to be easily introduced into the tubes. Once introduced, aspiration of the content was performed with the integrated plunger, which slides up and down when pushed by a flexible acetal resin shaft, without a syringe. The content was immediately aliquoted in 1,5 ml Eppendorf Safe-Lock® Tubes, frozen in liquid nitrogen until further analysis.

For the endometrial samples, the collection method varied depending on the type of surgery. During hysterectomy, when the entire upper reproductive tract was removed, direct access to the uterus was achieved using a sterile Mucat device (CDD Laboratorie, France). The device was carefully maneuvered to avoid sampling uterine fibroid tissue (clearly identified visually), as well as potential microbial contamination from the vagina or the cervix. In contrast, for patients undergoing tubal ligation without uterus removal, a speculum was inserted to gently separate the vagina, allowing visualization of the cervix. The cervix was cleaned with sterile saline solution and then the sterile Mucat device (CDD Laboratorie, France) was inserted into the cervix to reach the interior of the uterus. The aspiration of the uterine content was performed with the integrated plunger as

previously described [22]. The collected content was stored in 1,5ml Eppendorf Safe-Lock® Tubes, and frozen in liquid nitrogen until further analysis.

2.3. DNA extraction, amplification, library preparation, and sequencing

DNA extraction from the stored samples was performed using the Maxwell® RSC PureFood GMO and Authentication Kit and Maxwell® RSC Equipment (Promega, USA). A NanoDrop spectrophotometer was used to determine the DNA yield (A260) and purity (A260/A280 ratio) (**Supplementary Table S1**).

Bacterial identification was performed by Genomics Unit from Institute for Biomedical Research of Murcia IMIB-Arrixaca. The multiplex PCR using Ion Torrent 16S Metagenomics kit (Thermo Fisher Scientific Inc., USA) was used to amplify the 16S rRNA gene. Two sets of primers to target the regions V2, V4, V8, and V3, V6-7, V9 (**Supplementary Table S2**). Amplification was performed in a SimpliAmp thermal cycler (Applied Biosystems, USA) following the program: denaturation at 95°C for 10 min, followed by a cyclic 3-step stage consisting of 25 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 20 s; at the end of this stage, the program concluded with an additional extension period at 72°C for 7 min and the reaction was stopped by cooling at 4°C. The resulting amplicons were tested by electrophoresis using 2% agarose gel in tris-acetate-EDTA (TAE) buffer, purified with AMPure® XP Beads (Beckman Coulter Inc., USA), and quantified using QubitTM dsDNA HS Assay Kit in a Qubit 3 fluorometer (Invitrogen, Thermo Fisher Scientific Inc., USA). Afterwards, the Ion Plus Fragment Library Kit (Thermo Fisher Scientific Inc., USA) was used to generate a library from each sample. Each library was indexed by ligating Ion Xpress ™ Barcode Adapters (Thermo Fisher Scientific Inc. USA) to the amplicons. Libraries were purified with AMPure® XP Beads and quantified using the

Ion Universal Library Quantitation Kit (Thermo Fisher Scientific Inc., USA) in a QuantStudio 5 Real-Time PCR Instrument (Applied Biosystems, USA).

Next, the libraries were pooled and clonally amplified onto Ion Sphere Particles (ISPs) by emulsion PCR in an Ion OneTouch™ 2 System (Thermo Fisher Scientific Inc., USA) according to the manufacturer´s instructions. Sequencing of the amplicon libraries was carried out on an Ion 530™ Kit (Thermo Fisher Scientific Inc. USA) on an Ion S5™ System (Thermo Fisher Scientific Inc., USA).

2.4. Data processing

After sequencing, the individual sequence reads were filtered by the Torrent Suite ™ Software v5.12.1 to remove the low quality and polyclonal sequences. The quality filtered data were analyzed using Ion Reporter™ Software version v5.16. Clustering into operational taxonomic units (OTUs) and taxonomic assignment were performed based on the Basic Local Alignment Search Tool (BLAST) using two reference libraries, MicroSEQ® 16S Reference Library v2013.1 and the Greengenes v13.5 database (Life Technologies Corporation, USA). For an OTU to be accepted as valid, at least ten reads with an alignment coverage ≥90% between the hit and query were required. Identifications were accepted at the genus level with sequence identity >97%.

Given that characterization of the low microbial biomass site like the upper reproductive tract requires meticulous contamination control, *in-silico* decontamination approach using Decontam v.1.6.0 [23,24] was applied to discern between the true bacterial sequences and potential contaminants. To use this method, a table of the relative abundances of OTUs (columns) in each sample (rows) was created from the raw data. Next, we included DNA concentration of each sample in the model (from **Supplementary Table S1**). The Decontam score threshold was set to 0.1 as a default

setting to define contaminating phylotypes [23]. The relative abundance of the considered contaminant phylotypes was set to zero as described previously [24]. Furthermore, for diversity and abundance analyses we additionally filtered out those taxa that were detected in less than 30% of the remaining samples, as previously described [25].

2.5. Statistical analyses

Statistical analyses were performed using the R statistical software v.4.2.1 under RStudio v.2022.07.2 and SPSS software 20.0 (SPSS, USA). Microbiome data were aggregated to genus level for diversity and abundance comparisons. All relative abundances were expressed as median and first and third quartiles (q1, q3). Normal distribution of the variables was tested by using the Shapiro-Wilk test. Relative abundances of identified genera did not meet normality and were analyzed using the nonparametric Mann-Whitney *U* test. Furthermore, the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) was performed to validate our results. Benjamini-Hochberg method (false discovery rate [FDR]) was used to obtain adjusted p-values in multiple comparisons. Differences were considered statistically significant between groups when $p<0.05$. Alpha-diversity indices (Shannon diversity index and OTUs number [i.e., richness]) were calculated using the diversity function of the *vegan* R package, both in FT and endometrial samples. Differences among the groups of samples' diversity indices were tested using Mann-Whitney *U* test. Additionally, alpha-diversity was compared between women with both types of samples using a Wilcoxon signed-rank test for paired data. Bray-Curtis dissimilarity was calculated using *vegdist* R function and Permutational Analysis of Variance (PERMANOVA) was performed to analyze beta-diversity using *adonis* R function.

3. RESULTS

3.1. Samples

A total of 34 samples were collected from 24 enrolled patients. The patients' characteristics are presented in **Table 1** and **Supplementary Table S3**. As indicated in **Figure 1**, from the group that underwent laparoscopic tubal ligation, four FT samples and four transcervical endometrial samples were collected. In the hysterectomy group, which involved the extraction of the upper reproductive tract, 12 FT samples and 14 endometrial samples were obtained from the uterus, avoiding uterine fibroid tissue. It was not always possible to collect both types of samples from each patient because some anatomical pieces were damaged after being removed by laparoscopic techniques, and due to the impossibility of collecting some samples with the required sterile conditions and without blood contamination. Both FT and endometrial samples were successfully collected from seven out of 19 patients of the hysterectomy cohort, while three out of the five patients in the tubal ligation cohort provided both samples (**Figure 1**).

Table 1. Demographic characteristics (age, body mass index -BMI and parity) of the study population and collected samples from two groups of patients: patients who underwent a total laparoscopic hysterectomy with bilateral salpingo-oophorectomy and patients who submitted to a laparoscopic tubal ligation.

Study Population Groups	Hysterectomy N=19	Tubal ligation N=5
Age (years)	45±3	37±4
BMI	28,5±4,7	28,3±4
Parity	1,8±0,9	2,2±0,5
Fallopian tube samples	12	4
Endometrial samples	14	4
Both tissue samples	7	3

3.2. Data processing

A total of 245 and 252 bacterial genera were identified in the endometrial and FT samples, respectively. The average number of reads per FT sample was

25241,44±10845,46 (mean±SD). For the endometrial samples, the average number of reads per sample was 30845±18702,56 (mean±SD). Applying the decontamination method using Decontam, two genera, *Aerococcus* in FT samples and *Acidovorax* in the endometrial samples, were identified as contaminant phylotypes and removed from the analysis. Furthermore, to ensure the identification of the "core" microbiome of both sites, an additional filtering step was applied, eliminating bacterial taxa present in less than 30% of the participants, as previously described [25]. As a result, a total of 77 bacterial genera were identified in the FT samples (**Supplementary Table S4**), and 70 bacterial genera were identified in the endometrial samples (**Supplementary Table S5**).

3.3. Microbial profiles of FT samples

The microbial composition at the genus level in FT samples exhibited variability across different samples (**Figure 2**, **Supplementary Figure S1** at family taxonomic level). The most abundant genera among all samples were *Lactobacillus* (relative abundance=14.3 [3.48;24.4]), *Prevotella* (relative abundance=9.29 [0.31;12.7]), *Acinetobacter* (relative abundance=3.20 [1.36;11.7]), *Propionibacterium* (relative abundance=3.09 [2.45;5.86]) and *Faecalibacterium* (relative abundance=3.09 [0.68;4.97]) (**Supplementary Table S4**).

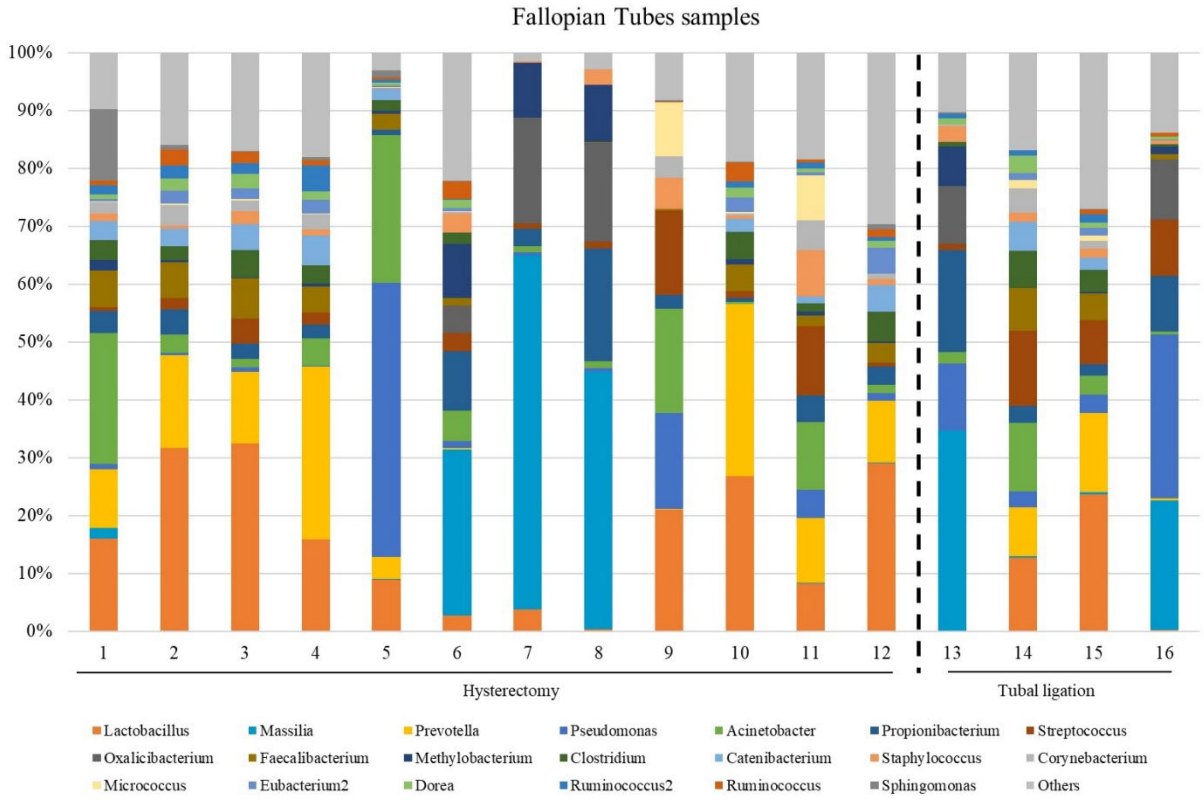

Figure 2. The most abundant genera detected in the Fallopian tubes (FT) samples from patients underwent a total laparoscopic hysterectomy with bilateral salpingo-oophorectomy (patients 1, 2, 3, 5, 9, 10, 11, 12, 13, 15, 16 and 17) or laparoscopic tubal ligation (patients 20, 21, 23 and 24). Percent-stacked barchart of those genera whose mean relative abundances were higher than 1% are represented.

Since the fertile women undergoing tubal ligation had no associated pathology, while women undergoing hysterectomy were diagnosed with benign uterine fibroids, a comparative microbiome analysis was performed to investigate any potential influence of uterine fibroids on the microbial microenvironment in the tubes. No significant differences were revealed in microbial diversity, or in the differential abundance analysis between the two groups (**Supplementary Table S6**).

3.4. Microbial profiles of endometrial samples

The microbiome composition revealed heterogeneity among the endometrial samples. The genus *Lactobacillus* showed the highest average abundance (relative abundance=23.0 [6.89;49.8]), followed by *Prevotella* (relative abundance=4.13

[0.85;13.7]), *Faecalibacterium* (relative abundance=2.18 [0.24;4.12]), and *Clostridum* (relative abundance=2.08 [0.32;5.06]) (**Figure 3**, **Supplementary Table S5**, and **Supplementary Figure S2** indicating family taxonomic level).

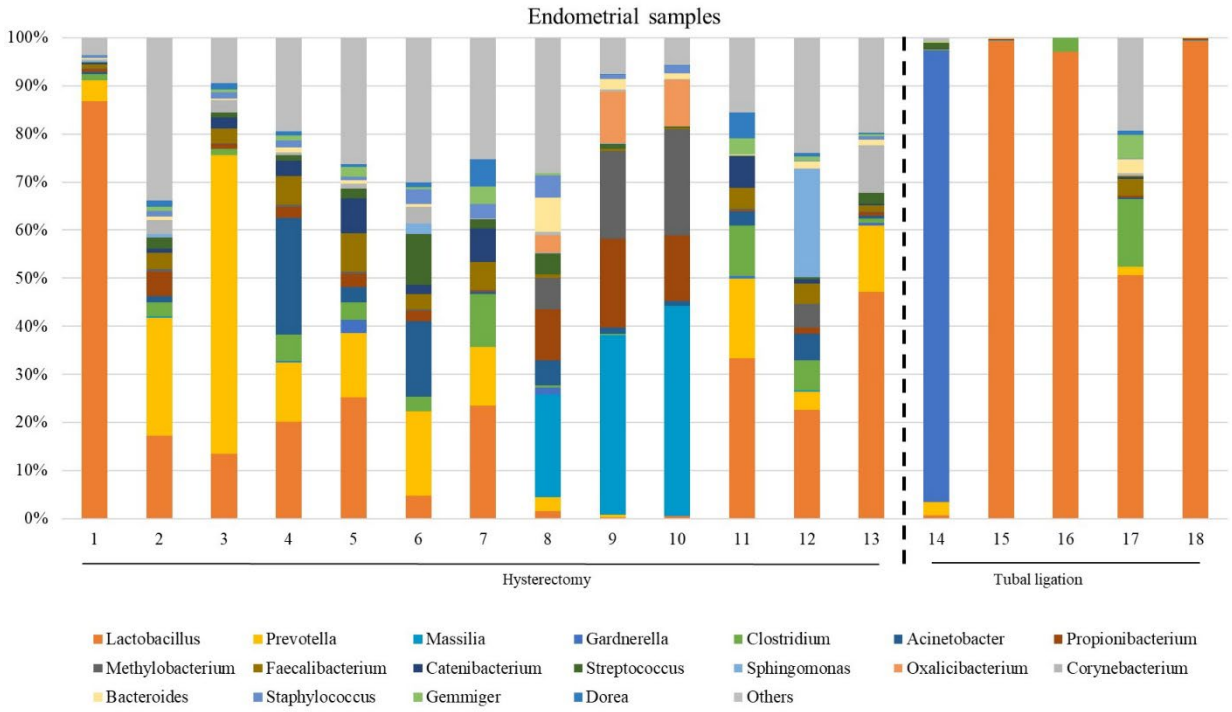

Figure 3. The most abundant genera detected in the endometrial samples from patients undergoing a total laparoscopic hysterectomy with bilateral salpingo-oophorectomy (patients 1, 2, 3, 4, 6, 7, 8, 10, 12, 13, 14, 15, 18 and 19) or laparoscopic tubal ligation (patients 20, 22, 23 and 24). Percent-stacked barchart of those genera whose mean relative abundances were higher than 1% are represented.

Unlike FT samples, the collection method for endometrial samples varied depending on the surgical procedure. In patients undergoing hysterectomy for benign uterine conditions, the entire upper reproductive tract was extracted, allowing direct access to the uterine cavity without passing through the vaginal and cervical canal. The fibroid tissue was visually identified and biopsied, focusing on tissue that presented unaltered morphological characteristics. On the other hand, in women undergoing to tubal ligation for contraceptive purposes and without underlying disease, endometrial biopsy was obtained transcervically. Therefore, we aimed to compare whether the uterine microenvironment could be influenced by the fibroids and whether the sampling method

via cervix (high bacterial contamination risk) could have an impact on the microbial composition in the endometrial samples. When comparing the microbiome of the two sampling techniques, 20 genera presented significantly different abundance (**Supplementary Table S7**). When applying the multiple testing correction, nine genera remained as marginally different between the groups, where *Lactobacillus* was more abundant while *Acinetobacter, Arthrobacter, Coprococcus, Methylobacterium, Prevotella, Roseburia, Staphylococcus, Streptococcus* were less abundant in samples obtained transcervically (**Figure 4**, **Supplementary Table S7**).

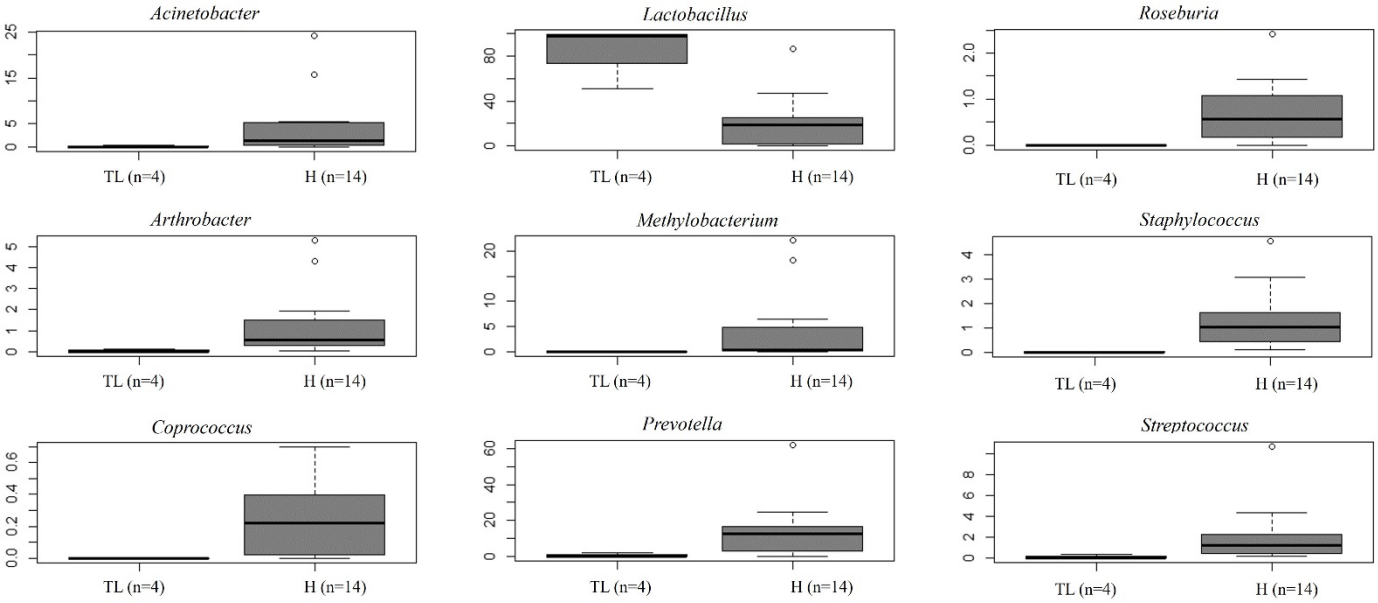

Figure 4. Relative abundance of nine bacterial genera between samples obtained directly from the uterus (hysterectomy, H) (fertile women with fibroids) and transcervically when undergoing tubal ligation (TL) (fertile women without the disease). After multiple testing correction adjustment, the difference remained marginal (FDR=0.083 for all plots).

3.5. Microbiome composition between endometrial and FT samples

When comparing microbial composition between the endometrium and FT, the endometrial samples from the tubal ligation group were excluded from the analysis. This decision was made due to significant microbiome differences, potentially indicating vaginal or cervical contamination characterized by a high abundance of *Lactobacillus*).

Thus, 16 FT samples and 14 endometrial samples were compared. A considerable portion of the detected taxa (60 genera) was found in both sites, indicating shared microbial composition. Additionally, 17 bacterial genera were exclusively detected in the FT samples, while 10 genera were considered endometrial-specific (**Figure 5**, **Table 2**). Out of these detected genera (**Supplementary Table S8**), the relative abundance of 11 genera was significantly between uterine and FT samples, as confirmed by both the Mann-Whitney *U* test and ANCOM-BC analysis. Specifically, *Gardnerella* (p=0.002; FDR=0.042), *Klebsiella* (p=0.004; FDR=0.042), *Olsenella* (p=0.004; FDR=0.042), *Oscillibacter* (p=0.004; FDR=0.042) and *Veillonella* (p=0.004; FDR=0.042) were found to be more prevalent in the endometrium. Conversely, *Enhydrobacter* (p=0.001; FDR=0.042), *Granulicatella* (p=0.001; FDR=0.042), *Haemophilus* (p=0.003; FDR=0.042), *Rhizobium* (p=0.003; FDR=0.042), *Alistipes* (p=0.006; FDR=0.048) and *Paracoccus* (p=0.006; FDR=0.048) were more abundant in FT samples (p-values obtained from the strict Mann-Whitney *U* test analysis).

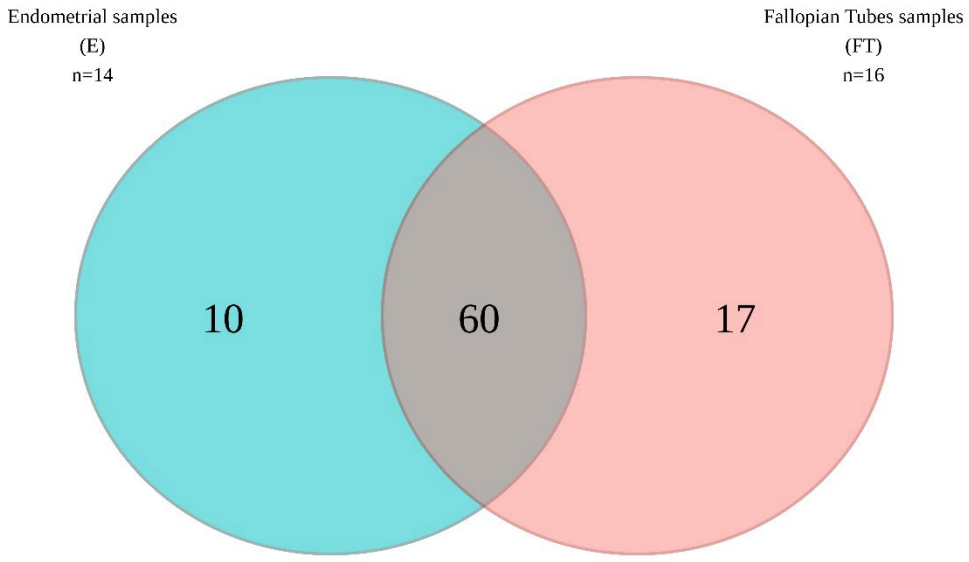

Figure 5. Venn diagram illustrating the bacterial genera present in the upper reproductive tract.

Table 2. Microbial composition of the endometrial and Fallopian tube (FT) samples. The asterisks (*) represent the differentially abundant microbial taxa between uterine and FT samples analyzed by the non-parametric Mann-Whitney *U* test ($p<0.05$). The crosses (†) represent the differentially abundant microbial taxa between the endometrial and FT samples analyzed by the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) ($p<0.05$). P-values were adjusted for the multiple testing correction (False Discovery Rate, FDR).

Fallopian tubes	**Fallopian tubes and Endometrium**			**Endometrium**
Aeromonas †	*Acinetobacter*	*Actinomyces*	*Anaerococcus*	*Barnesiella* †
Alistipes†*	*Arthrobacter*	*Bacillus*	*Bacteroides*	*Brachymonas*†
Bifidobacterium †	*Bilophila*	*Blautia*	*Butyricimonas*	*Chryseobacterium*†
Brachyspira†	*Campylobacter*	*Catenibacterium*	*Cloacibacterium*	*Gardnerella*†*
Brevundimonas†	*Clostridium*	*Collinsella*	*Coprococcus*	*Klebsiella*†*
Burkholderia †	*Corynebacterium*	*Desulfovibrio*	*Dialister*	*Olsenella*†*
Comamonas †	*Dolosigranulum*	*Dorea*	*Enterococcus*	*Oscillibacter*†*
Enhydrobacter†*	*Eubacterium*	*Eubacterium2*	*Faecalibacterium*	*Serratia* †
Flavonifractor †	*Finegoldia*	*Gemella*	*Gemmiger*	*Veillonella*†*
Fusobacterium †	*Helicobacter*	*Herbaspirillum*	*Kocuria*	*Vibrio*†
Granulicatella† *	*Lachnoclostridium*	*Lactobacillus*	*Lactococcus*	
Haemophilus†*	*Massilia*	*Megasphaera*	*Methylobacterium*	
Paracoccus†*	*Microbacterium*	*Micrococcus*	*Mitsuokella*	
Parasutterella †	*Moraxella*	*Neisseria*	*Oxalicibacterium*	
Rhizobium†*	*Parabacteroides*	*Pelomonas*	*Phascolarctobacterium*†	
Shewanella †	*Porphyromonas*	*Prevotella*	*Propionibacterium*	
Sutterella †	*Pseudoflavonifractor*	*Pseudomonas*	*Ralstonia*	
	Roseburia	*Rothia*	*Ruminiclostridium*	
	Ruminococcus	*Ruminococcus2*	*Sphingomonas*	
	Staphylococcus	*Streptococcus*	*Subdoligranulum*	

No significant differences were detected between the endometrial and FT samples in alpha-diversity metrics when comparing the microbiome diversity of endometrial and FT samples (i.e., Shannon, OTUs number [richness]) (**Figure 6A**). Also, beta-diversity represented by PCoA blot based on Bray-Curtis distances did not show any significant dissimilarities between the microbiome composition between the two sample types (**Figure 6B**).

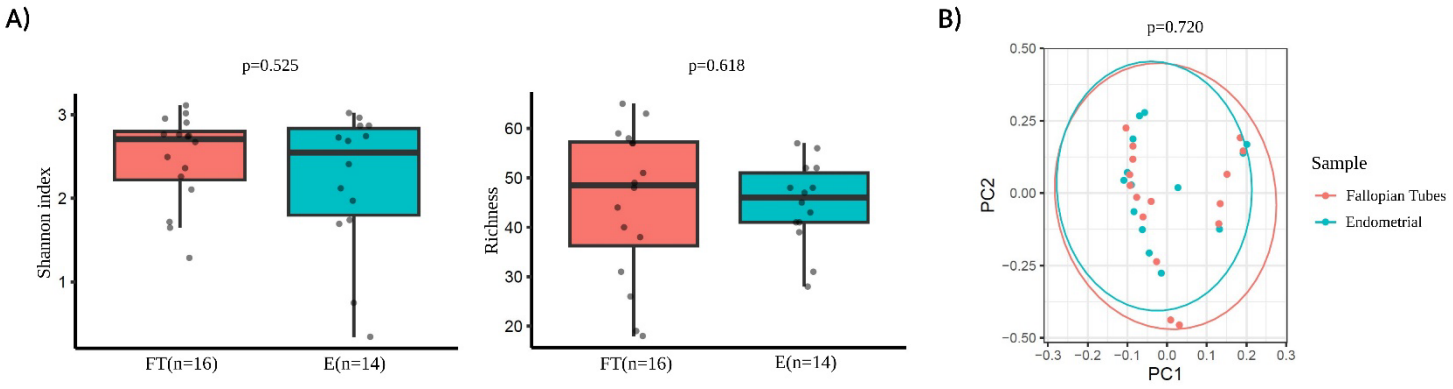

Figure 6. Diversity indices in Fallopian tubes (FT) and endometrial (E) samples. A) Alpha-diversity metrics (i.e., Shannon, OTUs number [richness]) of endometrial and FT samples. B) Beta-diversity represented by principal coordinate analysis (PCoA) based on Bray-Curtis distances (PERMANOVA, R^2=0.024, p=0.720) between endometrial and FT samples.

3.6. Sensitivity analysis in paired endometrial and FT samples

A sensitivity analysis was performed using samples exclusively from patients who underwent hysterectomy (N=7) and had valid samples from both tissues (endometrium and FT) (**Figure 1**, **Supplementary Table S3**). This approach aimed to avoid the possible contamination effect from cervical bacteria.

The comparison of microbial diversity between endometrial and FT samples revealed no significant differences in alpha- (**Figure 7A**) and beta- (**Figure 7B**) diversity metrics. (p >0,05).

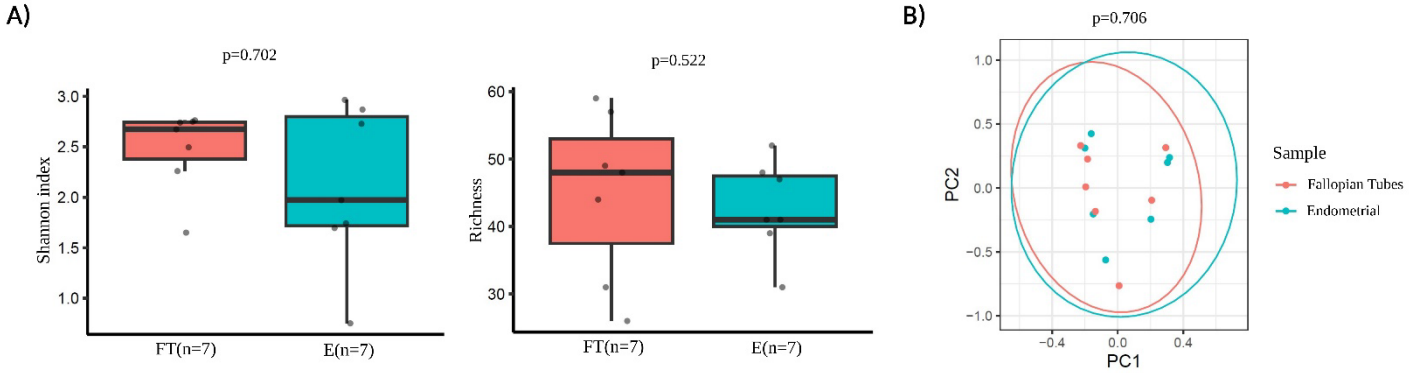

Figure 7. Diversity indices in paired endometrial and FT samples. A) Alpha-diversity metrics (i.e., Shannon, OTUs number [richness]) of endometrial and FT samples when the restricted group of patients with paired samples was selected. B) Beta-diversity represented by principal

coordinate analysis (PCoA) based on Bray-Curtis distances of patients with paired samples (PERMANOVA, R^2=0.048, p=0.706).

In this more restricted subset of samples, the previously observed statistical differences in the relative abundances of the 11 genera (*Gardnerella, Klebsiella, Olsenella, Oscillibacter, Veillonella, Enhydrobacter, Granulicatella, Haemophilus, Rhizobium, Alistipes and Paracoccus*) between endometrial and FT samples did not remain statistically significant after adjusting for the multiple testing correction (FDR) (**Supplementary Table S9**).

As a next step, we performed an additional comparison considering each pair of samples from the same patient. Alpha-diversity analysis did not detect any statistically significant differences when comparing the paired tissue samples of each patient (Shannon diversity index and OTUs number with p>0.05; **Supplementary Table S10** and **S11**, respectively) (**Figure 8A**). However, beta-diversity analysis revealed a significant dissimilarity when comparing the paired samples from the same woman (PERMANOVA, p=0.044) (**Figure 8B**). This finding suggests that the microbiome within an individual, even from two different tissue types (endometrium and FT), is more similar than the same tissue type (e.g. endometrium) between different individuals.

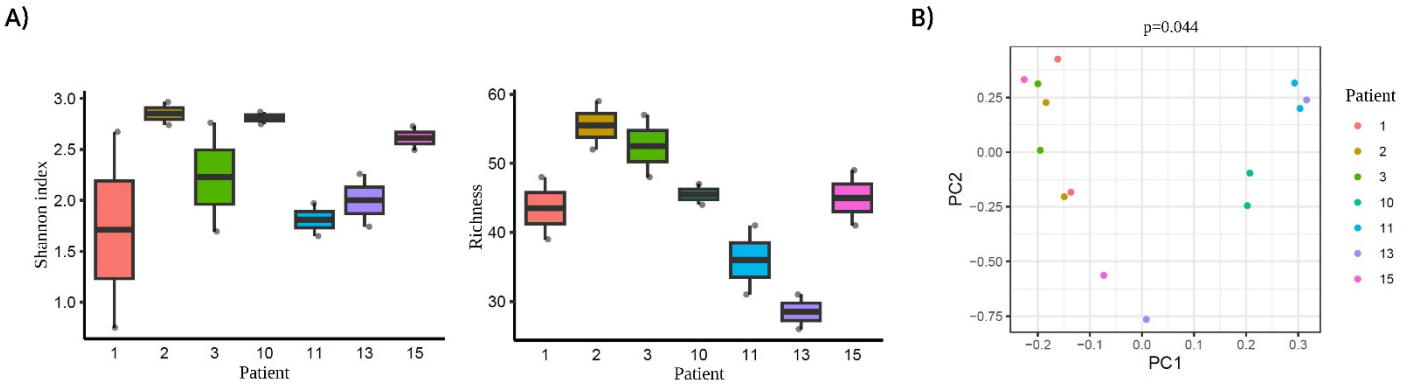

Figure 8. Diversity indices each pair of the tissue samples corresponding to their respective patient. A) Alpha-diversity metrics (i.e., Shannon, OTUs number) of paired endometrium and Fallopian tube (FT) samples from the same women (N=7), all values p>0.05. Each label indicates a patient (e.g. 1). B) Beta-diversity represented by principal coordinate analysis (PCoA) based on Bray-Curtis distances of patients with paired samples (PERMANOVA, R^2=0.622, p=0.044). Each

patient is indicated with one colour, where the two dots of the same colour represent individuals' endometrial and FT samples.

4. DISCUSSION

The female upper reproductive tract plays a critical role in oocyte fertilisation, early embryo development, and embryo implantation. Understanding the detailed microenvironment in the FT and endometrium is essential for manipulating and improving conditions in assisted reproduction technologies. Over 20% of couples at reproductive age suffer infertility, and with the socioeconomic situation where couples delay family planning and have children later in life, the demand for infertility treatment continues to rise worldwide [26].

There is a growing awareness that the microbes colonizing our body are involved in various pathological processes. Therefore, studying the microbiome of female reproductive tract has become a hot topic in order to understand its role in crucial events such as embryo development and pregnancy establishment [6]. Imbalances in the uterine cavity microbiome have been associated with implantation failure, decreased success of assisted reproductive technologies, as well as conditions like endometriosis, endometritis, polyps, and endometrial cancer [10,27,28]. However, very few studies have analysed FT microbiome due to ethical and technical challenges associated with obtaining FT sample without compromising future fertility. As a result, there is currently no consensus on the core microbial composition of the upper reproductive tract, whether in healthy or pathological conditions [10,12,29–31], and further research is needed.

The current study analysed the microbial composition of the upper reproductive tract in women with confirmed fertility. We examined FT and endometrial samples from

patients diagnosed with benign uterine pathology or without the disease. Our findings revealed a shared (~70%) endogenous microbial community present in both sites of the upper reproductive tract, whith *Lactobacillus*, *Prevotella*, and *Faecalibacterium* being the most prevalent taxa. Considering that the intramural portion of the uterine tube in humans does not allow for physical separation between the FT and uterine environments, it is reasonable to assume that there is smooth communication between these anatomical regions, resulting in similar microbiomes. We detected 60 bacterial genera common to both tissues, while 17 bacterial genera were FT-specific and 10 were uniquely present in the endometrium. *Gardnerella*, *Klebsiella*, *Olsenella*, *Oscillibacter,* and *Veillonella* were significantly associated with the endometrial samples, while *Enhydrobacter*, *Granulicatella*, *Haemophilus*, *Rhizobium*, *Alistipes,* and *Paracoccus* were more abundant in FT samples. Although the presence of these genera in the upper reproductive tract has been previously described [8,16,32], the site specificity demonstrated in our results has not been reported before.

When comparing the FT and endometrial samples obtained from the same women, although the sample size was limited, it seems that the two distinct tissue microbiomes were more similar within an individual than the same tissue sample between different individuals. These data support the hypothesis that each person has their own "microbial fingerprint", with microbial residents tailored to their environmental conditions – namely their genetics, diet, and developmental history. These residents persist over time and help to defend against invaders [33]. So, it is expected that there would be more microbial similarities between different body sites within an individual compared to specific body sites between different individuals. Similar results have been described previously ,although with more heterogenous cohorts [18]. Thus, establishing a 'core' microbiome

becomes challenging, as what might be considered healthy in one person may differ from another, adding complexity to the investigation of the human microbiome.

Our study included fertile women with benign uterine conditions (fibroids) and women without the disease who underwent tubal ligation as a terminal contraceptive method. This led to two different methods for obtaining study material: hysterectomy and tubal ligation. The study evaluated the effect of fibroids-related uterine microenvironment on the FT microbiome. FT samples were obtained in both cohorts using the same method, allowing us to study this effect. Our findings showed no association between the fibroids-free endometrial microbiome from women with uterine fibroids and the microbiome of FT. This suggests that fibroids-related uterine environment does not seem to affect the FT microenvironment.

In contrast, the sampling method for obtaining endometrial samples differed considerably between the two cohorts.: In the hysterectomy cases, the reproductive organs were removed, and the endometrial samples were obtained directly by opening the uterus under sterile conditions. However, in the tubal ligation cases, the endometrial samples were obtained transcervically, posing a higher risk of bacterial contamination from the lower reproductive tract (vagina/cervix). Thus, when analysing the endometrial samples from these two cohorts, we cannot determine whether the significant differences observed in the endometrial microbial composition are due to the fibroids-associated uterine microenvironment or the sampling method itself. After applying multiple testing correction, nine genera remained marginally different between the groups. *Lactobacillus* was more abundant in samples obtained transcervically, while *Acinetobacter, Arthrobacter, Coprococcus, Methylobacterium, Prevotella, Roseburia, Staphylococcus, Streptococcus* were more abundant in hysterectomy samples. The difference in *Lactobacillus* abundance depending on the sampling method has been previously

reported, with lower dominance linked to surgeries carrying a lower contamination risk from the vagina and cervix, such as hysterectomy [30], laparoscopy [8] and/or cesarean section [34] [10]. In line with these studies, the uterine samples collected transcervically in our study showed a clear dominance of *Lactobacillus* (abundance of 98,2%), while samples obtained during hysterectomy showed higher diversity and lower prevalence of *Lactobacillus* (abundance of 18,7%). Based on these findings, we believe that the sampling method had a stronger effect on the endometrial microbiome than the fibroids-free uterine sample. A previous study by Winters *et al*. reported that the endometria of women with a median age of 45, who underwent hysterectomy for fibroids were dominated by *Acinetobacter* (abundance of 60%) [30]. Other studies have suggested that *Acinetobacter* may be associated with a normal (or benign) endometrium, while *Methylobacterium* has been associated with endometrial cancer [35]. In our study, disease-free endometrial samples from women with uterine fibroids showed a small relative abundance of *Acinetobacter* and *Methylobacterium*. These two genera, however, along with *Arthrobacter, Coprococcus, Prevotella, Roseburia, Staphylococcus,* and *Streptococcus,* which showed differential presence in endometrial samples, are considered common contaminant genera [9]. Therefore, further research is required to determine which genera are contaminant and which have a role in uterine health. This could involve enrichment analysis of metabolic pathways using RNAseq analysis or whole metagenomics analysis, as well as investigating the impact of factors like uterine fibroids and other pathologies on the microbial composition. Interestingly, a recent study has associated *Clostridium, Ruminococcus, Blautia* and *Lactobacillus* (which were found in both tissues in our study) with Tryptophan metabolism [12]. This suggests a potential host–microbiota crosstalk in the biosynthesis of serotonin and melatonin, as well as serotonin degradation, where Tryptophan acts as a precursor. Specifically, dysregulation

of melatonin has been linked to altered uterine functions, including endometrial receptivity and recurrent spontaneous abortion [36].

Our study is the first to analyze the endometrial and FT samples together from women with confirmed fertility. Nevertheless, some limitations should be acknowledged. Firstly, the relatively small sample size makes the study results preliminary and highlight the need for confirmation in a larger sample size. Secondly, the analysis focused on older reproductive-aged women, and therefore the results should not be generalized for younger women, as age might influence the microbial composition. Thirdly, the endometrial samples were obtained at different cycle phases, which restricts our ability to examine endometrial receptivity. Fourthly, despite taking utmost care to obtain fibroid-free tissue when sampling endometrial biopsies, the effect of fibroids on uterine microenvironment cannot be ruled out. Lastly, the study design lacked negative controls in the sampling process and separate validation, thus, stringent decontamination tools and strict data processing methods were applied.

In conclusion, our study results corroborate that the female upper reproductive tract harbors an endogenous microbiome, although with low microbial biomass. We observed that a significant portion of the microbial profile is shared between the FT and the endometrium, with approximately ~70% of the detected taxa being shared. Interestingly, women have unique microbial profiles, wherein two distinct tissues (FT and endometrium) displayed greater bacterial similarities than the same tissue sample (e.g. endometrium) between two individuals. Unravelling the female upper reproductive microbiome, helps understanding the natural microenvironment where crucial processes of oocyte fertilisation and embryo development occur. This knowledge can be used to improve *in vitro* fertilisation and embryo culture conditions for the treatment of infertility.

SUPPLEMENTARY MATERIAL

Supplementary material can be found online at journal website:

https://www.frontiersin.org/articles/10.3389/fendo.2023.1096050/full#supplementary-material

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Study IV: The gut microbiome in endometriosis: a cohort study on 1000 individuals

ABSTRACT

Study question: Does the gut microbial composition and functionality differ between women with and without endometriosis?

Summary answer: The gut microbiome diversity and composition (species and microbial pathways) were not significantly different between women with and without endometriosis.

What is known already: Endometriosis, defined as the presence of endometrial-like tissue outside of the uterus, is one of the most prevalent gynecological disorders. Although different theories have been proposed, its pathogenesis is not clear. Novel studies indicate that the gut microbiome may be involved in the etiology of endometriosis, nevertheless, the connection between microbes, its dysbiosis and the development of endometriosis is understudied. This study aims to analyze and compare the gut microbiome profile in women with and without endometriosis in a large cohort to identify microbial targets potentially involved in the development of the disease.

Study design, size, duration: This case-control study included a subsample of 1000 women (age=45.61±10.36 years; BMI=25.67±5.59) of the Estonian Microbiome (EstMB) cohort, a volunteer-based sub-cohort of the Estonian Biobank created in 2017. 136 women with endometriosis and 864 control women who have not been diagnosed with endometriosis or any of its most prevalent comorbidities (systemic lupus erythematosus, rheumatoid arthritis, autoimmune thyroiditis, celiac disease, multiple sclerosis and irritable bowel syndrome) were included in the study.

Participants/materials, setting, methods: Microbial DNA from fecal samples was extracted and sequenced by paired-end metagenomic shotgun sequencing (Illumina Novaseq 6000 platform). Microbial functional pathways were annotated using the Kyoto

Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/). Partitioning around medoids (PAM) algorithm was performed to cluster the microbial profile of the Estonian population. The alpha- and beta-diversity and differential abundance analyses were performed to assess the gut microbiome (species and KEGG orthologies [KO]) in both groups.

Main results and the role of chance: The study population was stratified into two enterotypes: one characterized by a high abundance of *Prevotella copri* while the second presented a high abundance of *Bacteroide*s spp. However, the enterotypes were not associated with the presence/absence of endometriosis. Microbial alpha-diversity (Shannon's index and observed richness) was not significantly different between the women with and without endometriosis (all p-values>0.05). Beta-diversity analyses on the microbial and functional profile (species and KO profile) indicated no significant dissimilarity between the groups (PERMANOVA, both R^2<0.07%, p-values>0.05). No differential species nor KO were detected after multiple testing adjustment (all FDR p-values>0.05). Sensitivity analysis including only reproductive-aged women (<50 years) confirmed our study findings on big cohort.

Limitations, reason for caution: This case-control study did not identify a distinct gut microbial profile in women with endometriosis. A deeper analysis considering different endometriosis subtypes and hormonal treatment is needed to further confirm our results.

Wider implications of the findings: To the best of our knowledge, we present the biggest metagenome study on endometriosis. Our findings do not find evidence to support the existence of a gut microbiome-dependent mechanism directly implicated in the pathogenesis of endometriosis.

1. INTRODUCTION

Endometriosis, defined as the growth of endometrial-like tissue outside of the uterine cavity, is a common gynecologic disease, affecting approximately 5-10% of reproductive-aged women [1]. Endometrial lesions cause a chronic inflammatory condition associated with a wide range of reported symptoms, including dysmenorrhea, pelvic pain, dyspareunia and infertility [2,3]. Because these symptoms are associated with other conditions, endometriosis requires laparoscopic examination with excisional biopsy for definite pathology confirmation, which leads to a long diagnostic delay or common misdiagnosis. Although endometriosis is a widespread and burdening reproductive disorder, it has been historically understudied. Notably, proposed hypotheses such as retrograde menstruation, coelomic metaplasia, and Müllerian remnants do not explain the etiology of all the different phenotypes of endometriosis (i.e., superficial, ovarian and deep infiltrating endometriosis) [4]. Thus, endometriosis emerges as an important public health concern with substantial effects on the quality of life of millions of women globally [5].

The microbiome refers to the collection of genomes of the microorganisms (bacteria, viruses, fungi, protozoa and archaea) that inhabit a particular environment [6]. Particularly, the human gastrointestinal system is the most diverse microbiome within the human body, being colonized by trillions of microbes that play key roles regulating host physiological functions [7,8]. Indeed, a healthy balanced gut microbiome is crucial for nutrient absorption, gut epithelial barrier integrity and immune function [9,10]. Nevertheless, compositional and functional perturbations in the microbiome could lead to an unstable state called dysbiosis, which is linked to the different chronic conditions such as obesity, type-2 diabetes, cancer, inflammatory bowel diseases, neurological and reproductive diseases, among others [11–15].

Extensive research associates the gut microbiome with estrogens circulating levels through the secretion of β-glucuronidase, an enzyme that deconjugates estrogen to its active metabolize form [16]. The estrobolome term encapsulates the gut gene repertoire of microbial origin that is capable of metabolizing estrogens leading to epithelial proliferation stimulation throughout the female reproductive tract. Therefore, estrogen dysregulation has been shown to drive proliferative diseases such as endometriosis as well as comorbidities infertility and pelvic pain [17]. Indeed, the use of estrogen-progestins and progestins is the first-line medical treatment due to their safety, tolerability and cost profile, although they are often ineffective and may produce unwanted side effects [18]. Hence, to date, there is no cure for endometriosis and new non-hormonal therapeutic approaches become increasingly necessary [19].

Given the influence of the gut microbiome on immunomodulation and estrogen metabolism, and considering the estrogen-driven inflammatory state in endometriosis, a potential role of the gut microbiome in the pathogenesis of the disease has been proposed [17,20]. Novel studies suggest that gut dysbiosis that induces an increment in the estrogen circulating levels may contribute to the hyper-estrogenic environment promoting the progression of endometriosis [21]. Nevertheless, the connection between microbes, their dysbiosis and the development of endometriosis remains unexplored. Research on the gut microbiome in endometriosis would enable identification of novel biomarkers for noninvasive diagnostic and therapeutic approaches to identify and treat women with endometriosis earlier [22].

In the present study, we set out to analyze and compare the gut microbiome profiles in women with and without endometriosis in a large cohort with the aim to identify microbial signatures and pathways potentially associated with the development of the disease.

2. MATERIAL AND METHODS

2.1. Study population

This case-control study included a subsample of 1000 women of the Estonian Microbiome (EstMB) cohort (age=45.61±10.36 years; BMI=25.67±5.59), a volunteer-based sub-cohort of the Estonian Biobank (EstBB) created in 2017 with the objective of enriching the previous existing data with microbiome data [23]. All participants included in the EstMB provided informed consent for the data and samples to be used for scientific purposes. This study was approved by the Research Ethics Committee of the University of Tartu (approval No. 266/ T10) and by the Estonian Committee on Bioethics and Human Research (Estonian Ministry of Social Affairs; approval No. 1.1-12/17).

For the present study, we included 136 women with endometriosis and 864 control women who have not been diagnosed with endometriosis. Since endometriosis has been reported to have a high degree of comorbidity with other disorders [24–26], control women who were diagnosed with any of the most prevalent comorbidities of endometriosis (systemic lupus erythematosus, rheumatoid arthritis, autoimmune thyroiditis, celiac disease, multiple sclerosis and irritable bowel syndrome) were excluded. Endometriosis was confirmed by diagnostic laparoscopy. Self-reported data on diseases, medications, medical procedures, health-related behaviors in lifestyle, diet, physical activity, living environment, delivery mode, and stool characteristics (Bristol stool scale) were collected from each participant.

2.2. Sample collection and DNA extraction

The sample collection took place between 2017 and 2019. Fresh stool samples were collected by the participants immediately after defecation with a sterile Pasteur

pipette, placing the samples inside a polypropylene conical 15 ml tube and stored in the fridge (+4°C) until transportation. The sample was subsequently delivered to the study center where it was stored at -80°C until processing.

For genomic DNA isolation, microbial DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen, Germany). Approximately 200 mg of stool was used as starting material for DNA extraction following the manufacturer's instructions. Next, the extracted DNA was quantified using Qubit 2.0 Fluorometer with dsDNA Assay Kit (Thermo Fisher Scientific). Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, United States) following the manufacturer's recommendations. Briefly, 1 μg DNA per sample was used as input material, and index codes were added to attribute sequences to each sample. Each DNA sample was fragmented by sonication to an average size of 350 bp, DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. Finally, PCR products were purified (AMPure XP system) and libraries were analyzed for size distribution by Agilent2100.

2.3. Metagenomics analyses

The shotgun metagenomic paired-end sequencing was performed by Novogene Bioinformatics Technology Co., Ltd. in the Illumina NovaSeq6000 platform, resulting in 4.62 ± 0.44 Gb of data per sample (insert size, 350 bp; read length, 2 × 250 bp). First, the reads were trimmed for quality and adapter sequences. The host reads that aligned to the human genome were removed with SOAP2.21 (parameters: -s 135 -l 30 -v 7 -m 200 -x 400) [27]. Quality controlled data of each sample was then used for metagenomic assembly using SOAPdenovo (v. 2.04, parameters: -d 1 -M 3 -R -u –F) [28]. Next, SOAP2.21 was used to map the clean data of each sample to the assembled scaftigs (i.e., continuous

sequences within scaffolds). Unutilized paired-end reads of each sample were compiled together for mixed assembly. MetaGeneMark (v.3.38) was used to carry out gene prediction (gene length>100 bp) based on the scaftigs (≥500 bp), which were assembled by single and mixed samples. CD-HIT (v.4.6) was used to dereplicate the predicted genes based on 95% identity and 90% coverage to generate the gene catalogues (parameters: -c 0.95, -G 0, -aS 0.9, -g 1, -d 0) [29]. The longest dereplicated gene was defined as the representative gene (i.e., unigene). SoapAligner [30] (v.2.21, parameters: -m 200, -x 400, identity≥ 95%) was then used to map the clean data to the gene catalogues and to calculate the quantity of the genes for each sample. The gene abundance was calculated based on the total number of the mapped reads and the normalized gene length. The taxonomic assignment of the metagenomes was performed by comparing the marker gene homologs to a NCBI nonredundant NCBI-nr (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) database (201810) of taxonomically informative gene families using DIAMOND (v0.9.9.110) [31]. The homologs were annotated based on the sequence or phylogenetic similarity to the database sequences. The abundance of different taxonomic ranks was based on the gene abundance tables. As the last step, microbial functional pathways were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/).

2.4. Microbiome analysis

Microbiome diversity analyses were performed and visualized using phyloseq, vegan, microviz and ggplot2 packages in R. Species and KEGG Orthology groups (KOs) presented in >10% of samples and with 0.01% or higher relative abundance were included in downstream analyses. Alpha-diversity was determined by Shannon diversity index and the observed number of unique species (i.e., observed richness), using the “diversity” and “specnumber” functions from the vegan package. Case-control comparisons were tested

by linear-mixed effect models (LME) to adjust for confounders with the function “aov” from the stats package. Beta-diversity was represented by nonmetric multidimensional scaling (NMDS) ordination, based on the Bray Curtis dissimilarity, and tested for significance by Permutational analysis of variance (PERMANOVA) using the “adonis2” function from vegan package.

To identify the differential microbial species between cases and controls, differential abundance analysis was performed using an Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) from the ancombc2 package. ANCOM-BC models the absolute abundances using a linear regression framework [32]. Herein, absolute abundance for identified species presented in >10% of samples with >0.01% within each phylogenetic domain (e.g., 861 bacteria, 3 archaea, 11 eukaryota and 12 viruses) were included in the differential abundance analysis. 3 taxa were unclassified and removed from the analysis. Additionally, ANCOMBC was used to examine differential KOs between women with endometriosis and controls.

2.5. PAM clustering

Fecal samples were clustered by applying the Partitioning Around Medoids (PAM) algorithm, also simply referred to as k-medoids, using the “pam” function from cluster package. K-medoids consists in partitioning (clustering) the data into k clusters “around medoids”, a more robust version of K-means [33]. The number of clusters that best fits the data was selected by looking at the highest Silhouette Index, since 1 denotes the best meaning that the data point is very compact within the cluster to which it belongs and far away from the other clusters.

2.6. Statistics

Descriptive characteristics of the study participants were reported as median (q1; q3) or frequency, as appropriate. Body mass index (BMI), age, frequency of antibiotics consumption in the last year, gut empting frequency and stool characteristics (Bristol stool scale) were included as potential confounders in our analyses. Five women did not record data for age, 9 for antibiotics, 2 for gut empting frequency and 19 for stool consistency. Hence, we imputed missed data using multiple imputation method in SPSS v.28.0.1.0. For comparing non-parametric continuous data, Mann Whitney *U* test was performed, while categorical data was analyzed by χ^2 test.

Since alterations in the gut microbiome have been widely associated with specific menopausal symptoms [20], a sensitivity analysis excluding those women with age 50 or higher was conducted to corroborate our results.

All statistical analyses were performed in R (v.4.2.1) under RStudio (v.2022.07). Statistical significance was set to 0.05 for all analyses (i.e., p-value or q-value<0.05 for analyses using Benjamini-Hochberg false discovery rate [FDR] for multiple correction).

3. RESULTS

Our study population of 1000 women consisted of a total of 136 women with endometriosis and 864 control women. Descriptive characteristics of study participants are summarized in **Table 1**. Study groups did not significantly differ for any characteristic except for age at sample collection that was significantly higher in women with endometriosis compared to controls (FDR p-value=0.005).

Table 1. Descriptive characteristics of the study participants.

Characteristics	Endometriosis N=136	Control N=864	p-value
Age, *median [q1; q3]*	50.0 [40.8; 57.9]	45.0 [36.0; 54.0]	0.005
BMI, *median [q1; q3]*	25.1 [22.2; 29.5]	24.2 [21.6; 28.6]	0.367
Frequency of antibiotics consumption, *n (%)*			
Not in the last year	79 (58.1%)	555 (64.2%)	
In the last year	26 (19.1%)	139 (16.1%)	
In the last 6 months	23 (16.9%)	128 (14.8%)	
In the last month	7 (5.15%)	33 (3.82%)	
In the last week	1 (0.74%)	9 (1.04%)	0.776
Gut empting frequency, *n (%)*			
More than 2 times a day	21 (15.4%)	135 (15.6%)	
Once a day	76 (55.9%)	495 (57.3%)	
3-6 times a week	29 (21.3%)	168 (19.4%)	
2 times a week	3 (2.21%)	12 (1.39%)	
1-2 times a week	1 (0.74%)	6 (0.69%)	
Less than once a week	0 (0.00%)	2 (0.23%)	
Irregular	6 (4.41%)	46 (5.32%)	0.940
Stool consistency (Bristol scale), *n (%)*			
1	12 (8.82%)	63 (7.29%)	
2	31 (22.8%)	138 (16.0%)	
3	22 (16.2%)	146 (16.9%)	
4	30 (22.1%)	241 (27.9%)	
5	12 (8.82%)	114 (13.2%)	
6	28 (20.6%)	147 (17.0%)	
7	1 (0.74%)	15 (1.74%)	0.367

Note: Data presented as median [q1, q3] and frequency, as appropriate. P-values adjusted by Benjamini-Hochberg false discovery rate (FDR). Abbreviations: BMI: body mass-index

3. 1. Microbial landscape of the study cohort

The microbiome composition and functionality of the Estonian study population was characterized by metagenomics shotgun sequencing as previously described [34,35]. A total of 17158 species and 7869 KOs were detected, with an average of 6942273 species reads and 4913880 KOs reads per sample. After filtering by a prevalence >10% and relative abundance >0.01% resulted, we identified 890 species and 1629 KOs. The

average relative abundance of bacteria was 98.14%, followed by 0.93% for taxa of viral origin, 0.66% for eukaryotic taxa, 0.15% for archaea and 0.13% for unclassified taxa. The most predominant phyla were *Bacteroidetes* (45.15%) and *Firmicutes* (39.86%), followed by *Proteobacteria* (7.07%), *Actinobacteria* (1.53%) and *Verrucomicrobia* (0.82%), among others (**Figure 1A**). The most abundant genera consisted of *Bacteroides*, *Prevotella*, *Clostridium*, *Alistipes* and *Faecalibacterium* (**Figure 1B**). More specifically, 890 species presented >10% prevalence and >0.1% of relative abundance, being *Prevotella copri*, Bacteroides vulgatus, *Faecalibacterium prausnitzii*, *Bacteroides prebeius* and *Alistipes putredinis* the most abundant microbes (**Figure 1C**).

PAM clustering stratified the study population into two enterotypes (**Supplementary Figure S1**), where *P. copri* and *Bacteroides* spp. drove the most significant differences in the gut microbiome (**Figure 2A-B, Supplementary Figure S2**). Seventy-two percent of the samples were within the *Bacteroides* spp. enterotype and the remaining 28% belonged to the *P. copri* enterotype. The identified enterotypes were not correlated with the presence/absence of endometriosis, although presented a negative correlation with BMI and positive with stool consistency (**Figure 2C**; **Supplementary Table S1**).

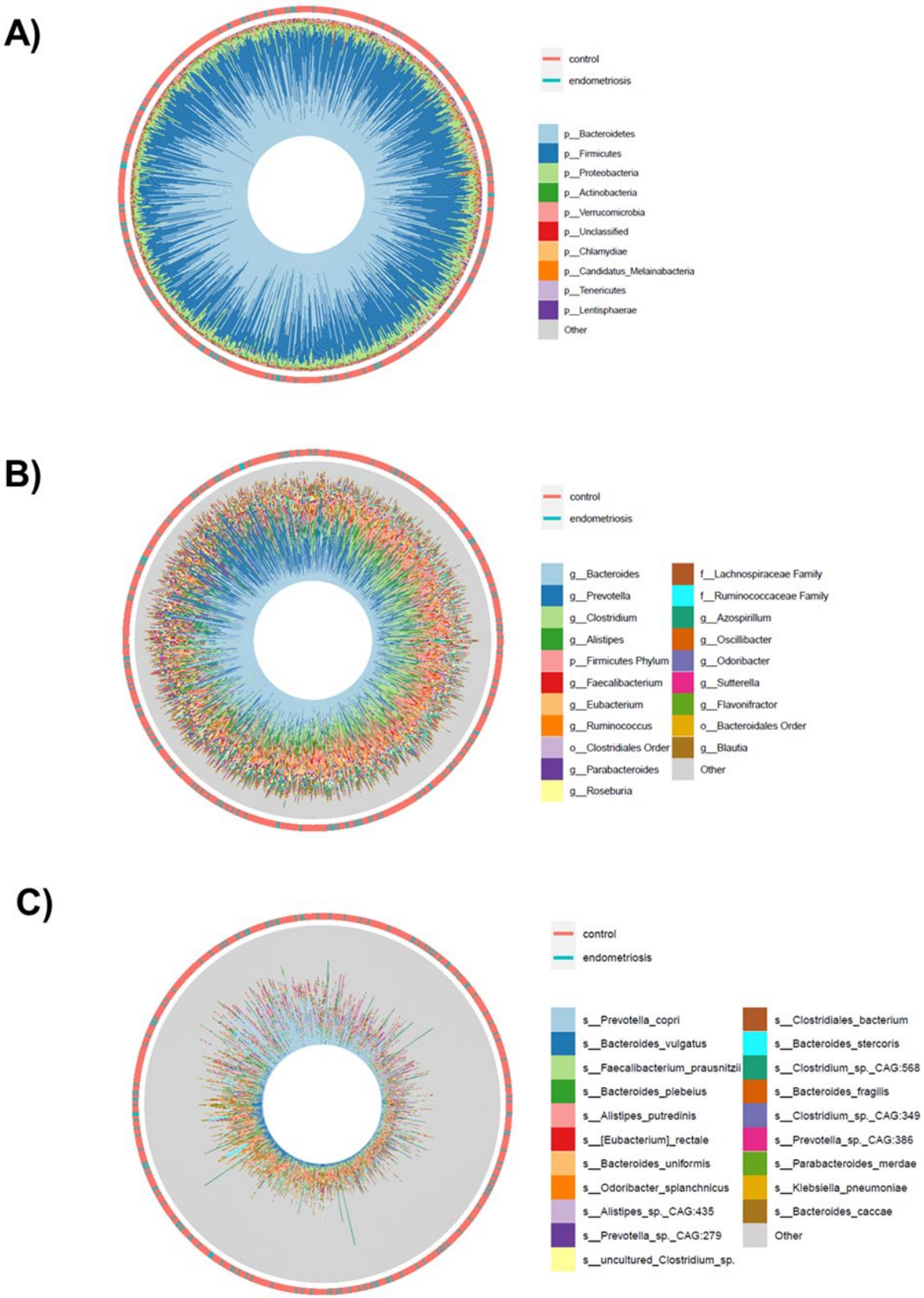

Figure 1. Microbial landscape in the Estonian study population. Circular stacked barplots ("iris plots") show the most relatively abundant phyla (A), genera (B) and species (C) in the study population. The outer bicolor rings indicate the endometriosis and control groups.

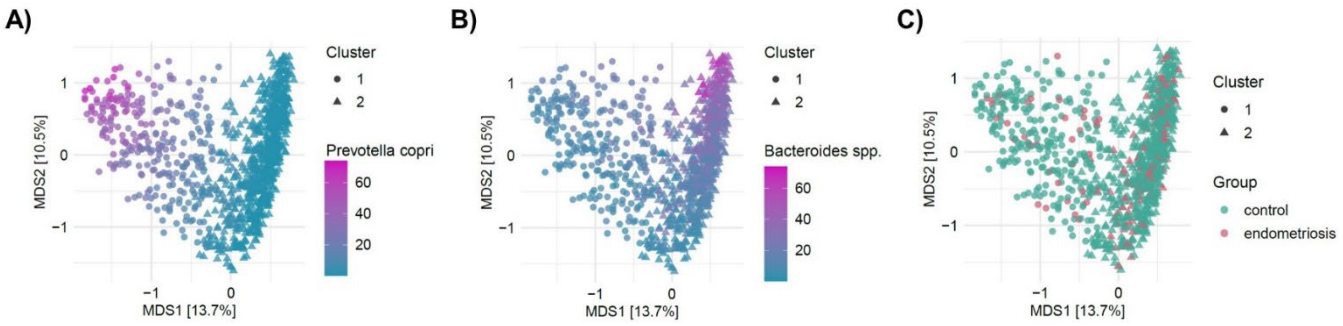

Figure 2. Enterotypes identified in the Estonian study population. (A, B) Relative abundance of *Prevotella copri* and *Bacteroides* spp. within the enterotypes on the nonmetric multidimensional scaling (NMDS) ordination plot of the species-level microbiome profile based on the Bray-Curtis dissimilarity. (C) Distribution of women with and without endometriosis within the enterotypes. The dot's shape indicates the cluster, while the colors highlight the relative abundances (A, B) or the endometriosis and control groups (C).

3.2. Microbial diversity analysis

Next, we aimed to compare the microbial alpha- (characterized by the Shannon diversity index and observed richness) and beta-diversity between women with and without endometriosis. No significant differences between cases and controls were detected in alpha diversity parameters, indicating that species richness was similar between both groups (all p-values>0.05; **Figure 3A-B**). Beta-diversity analyses on the microbial and functional profile (species and KOs profile) indicated no significant dissimilarity between the groups (PERMANOVA, both R^2<0.07%, p-values>0.05; **Figure 3C-D**). Interestingly, the strongest associations with beta-diversity both with species and KOs (all p<0.004), were observed for the stool consistency (evaluated by the Bristol stool scale, both R^2>2%), antibiotics frequency (both R^2>0.5%), BMI (both R^2=0.4%), age (both R^2=0.4%) and gut emptying frequency (both R^2>0.4%).

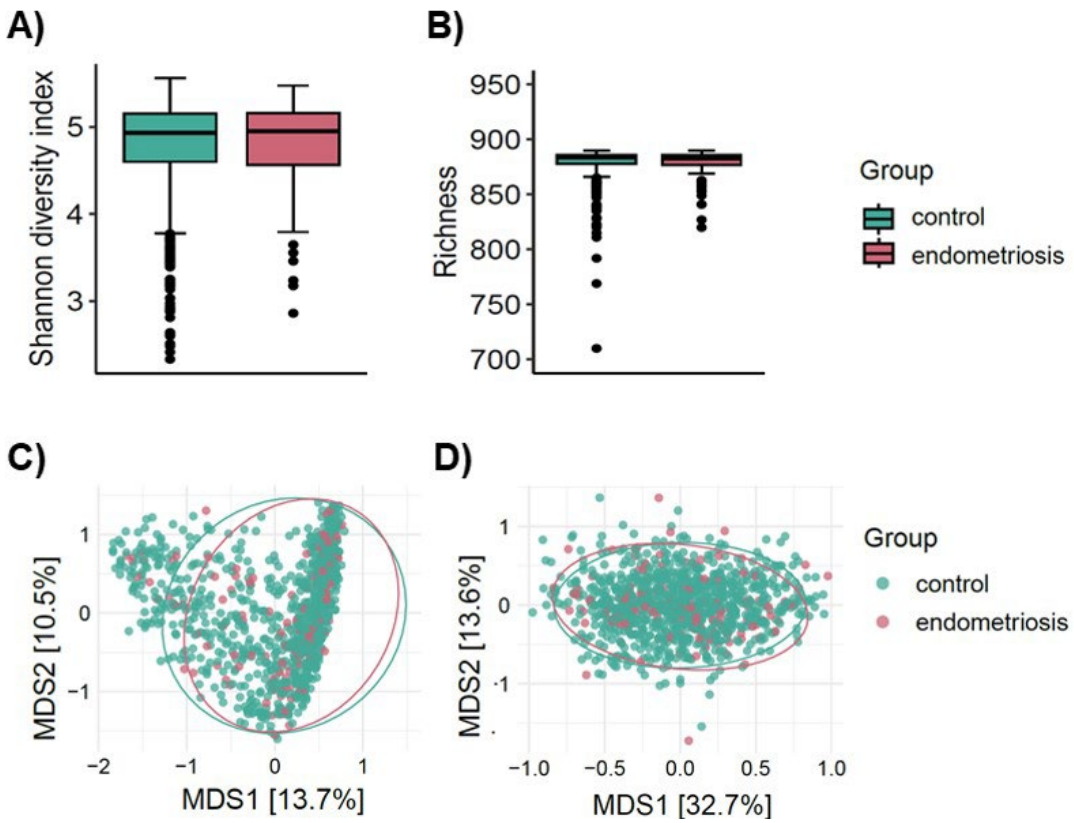

Figure 3. Microbial diversity measures in endometriosis and control groups. (A, B) Alpha-diversity analysis (i.e. Shannon diversity index and observed richness). Groups comparisons indicate no significant differences (Linear-mixed effects: all p-values>0.05). (C, D) Beta-diversity analyses on the nonmetric multidimensional scaling (NMDS) ordination of the species (C) and KOs (D) profile based on the Bray-Curtis dissimilarity (Adonis PERMANOVA, both R^2<0.07%, both p-values>0.05).

3.3. Differential abundance analysis of microbial species and KOs

To detect specific species or microbial pathways that could be potentially involved in the pathogenesis of the disease, an ANCOMBC analysis was performed on the identified species and KOs. Overall, 34 bacteria seemed to be differentially abundant between groups, for example, *Clostridium* sp. CAG:307 (logFC=0.679, p=0.006) and *Acinetobacter* sp. CAG:196 (logFC=0.756, p=0.013) were enriched in the endometriosis group, whereas *Ruminococcus* sp. CAG:177 (logFC=-0.398, p=0.026) and *Roseburia* sp. CAG:45 (logFC=-0.324, p=0.011) were decreased compared to controls (**Supplementary Table S2**). Regarding functional analysis, 14 KOs associated with endometriosis, including nitrogen metabolism (logFC=-0.172, p=0.018) or oxidative

phosphorylation (logFC=-0.043, p=0.014) that were downregulated, while 4 KOs including fatty acid biosynthesis (logFC=0.138, p=0.039), aminoacids metabolism (logFC=0.048, p=0.014) and ATP-binding cassette (ABC) transporter system (logFC=0.184, p=0.033) were upregulated in women with endometriosis compared to controls (**Figure 4**). However, no bacteria and KOs remained significantly different after FDR correction (all p-values>0.05) (**Supplementary Table S2**).

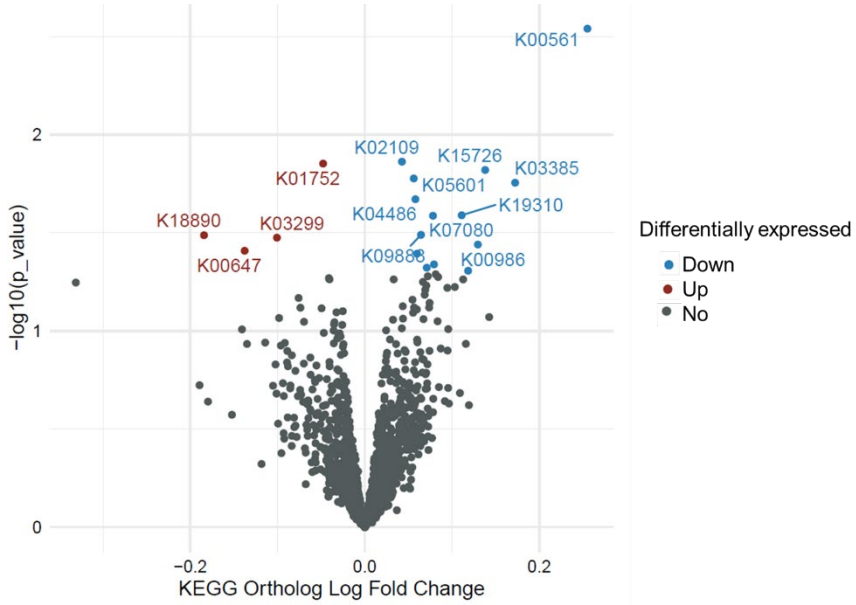

Figure 4. Functional differences in the microbial pathways in endometriosis and control groups. Volcano plot displaying log fold change differences in the expression of KEGG orthologs derived from the ANCOM-BC model. Points in blue and yellow represent KEGG orthologs which were downregulated and upregulated in endometriosis and statistically significant (p<0.05). Points in grey represent KEGG orthologs that were not differentially expressed (p>0.05). No KEGG orthologs remained statistically significantly expressed after Benjamini-Hochberg false discovery rate (FDR) correction (all adjusted p-values>0.05).

3.4. Sensitivity analysis

A sensitivity analysis including only women at their reproductive age (<50 years) and excluding women at menopause (>50 years) was performed to corroborate the previous results on whole cohort. A total of 66 women with endometriosis and 525 control women were finally included. The obtained results were similar to the whole cohort results, detecting no statistically significant differences between the groups in microbial

diversity and differential abundance analyses on the species and KOs profiles (**Supplementary Figure S3** and **Table S3**).

4. DISCUSSION

Endometriosis is a widespread gynecological disorder, and regardless of the active research, there is a lack of understanding of the pathogenesis of the disease and its associated symptoms. Scientific evidence supports that estrogen drives the proliferation of endometrial-like lesions, although the reason why some women develop endometriosis and others do not is still unclear. Since the role of the gut microbiome in inflammatory and proliferative conditions as well as in estrogen metabolism is established [17,20], it is rational to propose an involvement of the gut microbiome in the development of the disease. Indeed, novel studies are focusing on the gut microbial communities as important candidates for investigation in reproductive health and several studies are associating uterine microbes with endometriosis [36–39].

To the best of our knowledge, our study is the first whole metagenome study (identifying bacteria, viruses, fungi, protozoa and archaea) performed in women with endometriosis, while all previous studies have exclusively analyzed the 16S rRNA gene region of the bacteria. Our study results did not identify a distinct compositional or functional gut microbial profiles in women with endometriosis compared to controls, which has been observed also in a previous marker gene-based study (16S rRNA gene analysis) [40]. While other marker gene-based studies have associated several gut microbes with endometriosis [41,42]. The biggest study conducted up to date, analyzed the gut microbiome profile of 66 women with endometriosis and 198 control women [41], where a higher abundance of *Parabacteroides* genus and lower *Paraprevotella* in endometriosis

patients compared to controls were detected. In our study of 1000 participants, we detected decrease in *Paraprevotella clara* and *Parabacteroides* sp. D26 in women with endometriosis, although these differences disappeared after multiple testing correction. A recent study compared the gut microbiome in 12 patients with stage 3/4 endometriosis and 12 healthy women [42]. Although they did not describe any statistically significant differences in alpha-diversity, several genera such as *Blautia*, *Bifidobacterium*, *Dorea* and *Streptococcus*, were significantly increased in the endometriosis group compared to controls, while *Lachnospira* and *Eubacterium eligens* group showed a decreased abundance in women with endometriosis. Another study built classification models with machine-learning on the vaginal and gut microbial composition to predict rASRM stages 1–2 vs. rASRM stages 3–4 endometriosis, and found that the microbe that contributing the most to this prediction was *Anaerococcus* genus [43]. In our study, species from the *Anaerococcus* genus, however, were not detected. Nonetheless, current studies are hardly comparable due to the different sample size and microbiome detection methods, proving contradicting and inconclusive results. Importantly, contrastingly to our study where we analyzed species level by shotgun sequencing, the previous studies performed a 16S rRNA gene analysis, which limits a reliable taxonomic assignment to genus level.

Recently, a higher frequency of *Fusobacterium* in both the endometria and ovarian endometriotic tissues from 79 patients with endometriosis were detected when compared to endometria from 76 control women [44]. Hence, they investigated further the pathogenic role of this bacteria in the development of endometriosis. Interestingly, we detected a higher relative abundance of *Fusobacterium* sp. CAG:815 in the gut in women with endometriosis, although the differences did not remain significant after adjustment for multiple comparisons.

While evidence supporting the role of the endometrial transcriptome in endometriosis development is accumulating [45,46], a new debate is whether there are microbial pathways involved in the pathogenesis of the disease. In this context, our study identified several KOs possibly dysregulated in the presence of endometriosis. We noted that a KO related to ABC transporters was enriched in women with endometriosis. Given the high regenerative capacity of the human endometrium at eutopic and ectopic sites, scientific evidence links the origin of endometriosis to stem cells [47] and supports the existence of endometrial cell subpopulations as candidate endometrial stem cells based on the side population phenotype [48]. This characteristic is due to the differential potential of cells to efflux the Hoechst dye via the ABC family of transporter proteins expressed within the cell membrane [49]. The ATP-binding cassette transporter G2 (ABCG2) expression analysis in samples of endometrium from patients with and without endometriosis found that ABCG2 was highly expressed in the endothelial cells of microvessels of eutopic endometria, and reduced in those of ectopic endometria except in cases of deep infiltrating endometriosis, suggesting that ABCG2+ microvessels may be crucial for the pathophysiology of deep infiltrating endometriosis [50]. Our results are in line with this hypothesis, nevertheless, further research considering the different stages of endometriosis is warranted to analyze potential alterations of the gut microbes and microbial pathways that could be hidden in early endometriosis stages.

Another KO of interest in endometriosis is the long-chain saturated fatty acids biosynthesis, a metabolic pathway catalyzed by fatty acid synthase (FASN). We detected a highly expressed KO related to long-chain saturated fatty acids biosynthesis in women with endometriosis. In some cancer cell lines, FASN has been found to be fused with estrogen receptor, and its overexpression is a common molecular feature in hormone-sensitive cells and is regulated by both estradiol and progesterone [51]. During the menstrual

cycle, FASN expression appears to be linked to endometrial cell proliferation [52,53]. Thus, inhibiting fatty acid synthase has been proposed as a therapy targeting estrogen receptor signaling in breast and endometrial cancer [54]. In fact, several studies associate the high prevalence of endometriosis with excessive lipid intake or a lipid intake imbalance and propose novel lipid metabolism-targeted approaches for the treatment of endometriosis due to the proliferative and inflammatory state of the disease [55].

Our study provides pioneering results about the gut microbiome composition and association with endometriosis on a large-scale study population, however, it has several limitations that should be highlighted. First, the detection power in our study might have been influenced by including different subtypes of endometriosis. Endometriosis is defined as a heterogeneous disease broadly characterized into three phenotypes with different grade of severity: from superficial peritoneal as the least severe form, to ovarian and deep infiltrating endometriosis, the last being the most severe phenotype [4]. Since the inclusion of the three phenotypes could mask the presence of microbial alterations in the most severe forms, additional analyses on the different subtypes are needed to confirm our results. Furthermore, hormonal imbalance has been demonstrated to have a negative impact on the gut microbiome, while it has been reported that hormonal treatment reverses the gut microbiome dysbiosis in reproductive disorders [56]. Since the use of estrogen-progestins and progestins is the first-line medical treatment in endometriosis [18], patients with hormonal treatment may present similar gut microbial profiles than those without the disease. Hence, more studies on women with active endometriosis and no hormonal treatment are warranted to unravel the complex bidirectional relationship between the gut microbiome and endometriosis.

5. CONCLUSIONS

The molecular mechanisms underlying the pathogenesis of endometriosis are not yet fully understood, making endometriosis a challenge to diagnose and treat. In this context, the gut microbiome emerges as a potential diagnostic tool and therapeutic target. We present the biggest whole metagenome study on endometriosis so far, and our study findings do not provide enough evidence to support the existence of a gut microbiome-dependent mechanism implicated in the pathogenesis of endometriosis. More research on large-scale study populations with active endometriosis and no hormonal treatment are needed to provide better understanding of the endometriosis-associated microbiome, and to unravel its potential for diagnosis and treatment approaches.

SUPPLEMENTARY MATERIAL

Supplementary material can be downloaded in this link:

https://osf.io/bepqc/

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5. GENERAL DISCUSSION

5.1. Main contributions of this Doctoral Thesis

The present Doctoral Thesis aimed to advance the current knowledge of the microbiome composition in female population, with a focus on studying the associations of microbial communities with PA/SB and female reproductive health. The current Thesis provides novel insights into the host-microbe associations using meta-omics approaches at marker gene and metagenomics level. First, we provide a comprehensive overview of the relationship between PA, SB and human microbiome, and the first meta-analysis of the microbiome data in association with PA, where the current evidence by meta-analyzing the data of over 2600 individuals is presented (**Study I**). Next, we perform a large study on the association of PA and SB and the gut microbial composition in middle-aged women and investigate the joint association of PA on different intensities (LPA, MVPA) and SB using the novel compositional data approach (CoDA) in this homogeneous female cohort (**Study II**). This approach mirrors "real life" by considering differing time reallocations across movement behaviors, considering that a day has 24 hours and, therefore, increasing one behavior means automatically a reduction in other activity, providing thereby more reliable knowledge of the PA/SB associations with the gut microbiome. Thirdly, we characterize the microbiome of the female reproductive tract in low microbial biomass tissues such as endometrium and FT in the absence of a disease (**Study III**). Ultimately, we performed the first metagenomics study (shotgun metagenomics) in the biggest endometriosis-controls cohort so far (1000 women), in order to identify microbial features (species, pathways) potentially involved in the development of the disease (**Study IV**).

In **section I** of this Doctoral thesis, we identified several microbial taxa significantly associated with higher PA levels. To date, most studies support that PA alters

the microbiome composition (while evidence of SB on microbiome is scarce), mainly increasing the relative abundance of short-chain fatty acids (SCFA)-producing bacteria with health benefits (e.g., *Lachnospiraceae*, *Erysipelotrichaceae*, *Roseburia*, *Coprococcus*, *Veillonella*, *Akkermansia municiphila* and *Faecalibacterium prausnitzii*) [1–7], while PA influence on microbiome diversity is unclear (**Study I**). Similarly, our meta-analysis uniting 2632 participants indicated no consistent impact of PA on microbial diversity. However, at high sport performance level, our meta-analysis on athletes *vs*. non-athletes showed marginally higher gut microbial richness in athletes, as has been reported in previous studies and in a recent meta-analysis on athletes' metagenomics data [8–12]. Therefore, there is a need for clarifying how much and what type of PA would be sufficient to increase microbial diversity in the gut. Additionally, our systematic review and meta-analyses provide future directions to increase homogeneity and comparability between the studies and obtain conclusive findings. We also highlight the need for more studies based on objectively measured PA data and the assessment of SB effects, standardized pipelines for microbiome analysis and inclusion of relevant covariates such as diet, which is often missed in statistical analyses. In this context, our CoDA of the objectively measured PA and SB in association with 16S rRNA gene sequencing (i.e. microbiome analysis) enhanced the study of the inter-relationships between the gut microbiome and accelerometer-measured LPA, MVPA and SB in a sub-cohort of 289 women (**Study II**). Since the study group included women with diagnosed PCOS, a sensitivity analysis, where we excluded these patients, corroborated our results. After adjustments for different covariates (including body mass index –BMI, PCOS diagnosis, accelerometer wear time and fiber intake), we detected significantly positive associations between the MVPA (relative to the other behaviors) and the relative abundance of butyrate-producing bacteria such as *Agathobacter* and *Lachnospiraceae* CAG56 (both

taxa belong to *Lachnospiraceae* family). We did not find any significant associations between the PA and SB behaviors and microbial diversity, being in line with other cross-sectional studies in women [3,13]. Butyrate, among others SCFAs like acetate or propionate, has been related to better human metabolic health [14]. Notably, a published meta-omics analysis proposed a microbiome-encoded enzymatic mechanism via lactate metabolism that could partially explain how microbiome and its metabolites (i.e., SCFAs) contribute to enhance athletic performance, linking *Veillonella atypica* with improved exercise performance in a mouse model [15]. A more recent study discovered a microbiome-dependent mechanism in mice that enhances athletic performance by increasing dopamine signaling during PA [16]. These findings indicate that gut microbial communities may also impact exercise performance, which suggest a bidirectional relationship between the PA and the host microbiome. Our findings, collectively with previous studies, indicate SCFAs-producing bacteria as the possible link between the PA and the gut microbiome. Nevertheless, more studies on compositional data analysis including sleep time are warranted to precisely analyze the entire 24-hour day in the PA/SB-microbiome interaction. Furthermore, future research on individual and combined effects of PA and diet on the gut microbiome is required to fully unravel the complex bidirectional associations between the PA and the gut microbiome.

Section II of this Doctoral Thesis firstly aimed to characterize and compare the microbial profiles in the upper reproductive tract of the low microbial biomass sites as is endometrium and FT by applying 16S rRNA gene analysis approach (**Study III**). There seems to be a consensus among endometrial microbiome studies that uterus is a higher diverse microenvironment than is the vagina, although current knowledge of the FT microbial composition is limited. Since the first events of oocyte fertilization, embryo development and implantation take place in the female upper reproductive tract,

deciphering the microbial microenvironment in the FT and uterus would provide valuable knowledge to improve *in vitro* conditions in assisted reproductive technologies (ART) and manage infertility. We analyzed 24 women with confirmed fertility (submitted to abdominal hysterectomy due to benign uterine pathology or tubal ligation), corroborated the existence of an endogenous microbiome in the upper reproductive tract, and found that around 70% of the identified bacterial taxa is shared between both body sites, probably due to proximity and smooth communication of these sites. Further, endometrial-specific (e.g., *Olsenella*, *Klebsiella*, *Veillonella*) and FT-specific bacteria (e.g., *Enhydrobacter*, *Haemophilus*, *Alistipes*, *Paracoccus*) were identified. Previous studies have also detected the presence of microbes in the upper reproductive tract [17–19], however the microbial site-specificity shown in our results has not been reported before. Our study also demonstrated how the sampling method greatly affects microbiome composition in this low biomass sites, showing a *Lactobacillus* dominance in those samples that were obtained transcervically *vs.* the samples obtained at the hysterectomy. Further, our study results indicate intra-individual microbial similarity rather than inter-individual tissue similarities, suggesting that endometrial and FT microbiome is unique for each individual. These findings support the existence of a "microbial fingerprint" in which co-existing microbial communities are shaped into our genetics and other environmental and lifestyle factors. Lastly, **study IV** analyzed the potential involvement of the gut microbiome in endometriosis in a sub-cohort of 1000 women by shotgun metagenomics. Our study did not report significant compositional or functional microbial differences between the women with endometriosis and women without the disease. Thus, our study does not provide enough evidence about the existence of a microbiome-mediated mechanism in the pathogenesis of the disease on a cohort level. Since endometriosis is a heterogeneous gynecological condition, microbial alterations may

appear only in advanced stages or the most severe forms (i.e., deep infiltrating endometriosis). Indeed, a case-control study described a *Shigella/Escherichia* dominant gut microbiome in women with advanced stages 3/4 endometriosis [21]. Other study has linked pathogenic role of *Fusobacterium* in the formation of ovarian endometriosis [22]. Interestingly, we detected a higher relative abundance of *Fusobacterium* sp. CAG:815 in the gut among women with endometriosis, although it did not remain significant after adjustment for multiple comparisons. Regarding to functional composition, we detected an enrichment of fatty acids synthase (FASN)- and ATP-binding cassette transporters (ABC transporters)-related pathways among endometriosis patients, however, no significant differences remained after multiple correction. ABC transporters have been found highly expressed in eutopic endometrium, and linked to high proliferative capacity of endometrium [23]. Previous evidence reported an increased in ABC transporters only in deep infiltrating endometriosis, suggesting a crucial role for the pathophysiology of this subtype in particular. Altogether, these findings point to the presence of microbial features that could be involved in the pathogenesis of specific subtypes of endometriosis, nevertheless a deeper analysis considering the endometriosis severity stage and disease management strategies are warranted.

5.2. Overall limitations and strengths

There are some limitations in this Doctoral Thesis that should be highlighted:

- Our meta-analysis was performed on the analyzed data from the included studies due to the lack of available raw data (**Study I**). Meta-analysis approach re-analyzing raw data through the same analysis pipeline would reduce potential biases introduced by different methodologies.

- In **study II**, sleep time was not included as a component in our CoDA. Therefore, we were not able to analyze the entire 24-hour day, but yes the composition of movement behaviors during walking hours.
- The sample size was considerably small and we lacked of negative and positive controls in the **Study III** of this Doctoral Thesis, therefore the study results should be interpreted with caution.
- **Study IV** should integrate the analysis of different subtypes of endometriosis. A sub-analysis on women with deep infiltrating endometriosis and no hormonal treatment would provide deeper knowledge of the microbiome-endometriosis associations.

Despite these limitations, this Doctoral thesis presents several strengths that should be stated:

- In **study I**, we present the most comprehensive systematic review in the PA-microbiome field, including 91 studies in healthy (e.g. non-athletes and professional athletes), unhealthy populations, different stages of life (i.e., children, young and older adults), and different body niches (e.g. gut, saliva, vaginal, etc.). Additionally, we quantify the available evidence using meta-analytics methods, following a previous validated methodology [24,25].
- In **study II**, a novel approach (CoDA) was used to revisit the association of PA and SB with the gut microbiome, analyzing the inter-relationship of the accelerometry-assessed behaviors on a continuous scale and in a homogenous study population. This approach provides pioneering results in the microbiome field, adding to the existing evidence primarily based on self-reported PA data, which is subject to assessment bias.

- Despite a small sample size, **study III** provides valuable knowledge to the existence of an endogenous microbiome in the hard-to-obtain body site such as FT and its microbial relationship with the proximal site, endometrium.
- To our knowledge, **study IV** is the biggest microbiome study performed on endometriosis to date. Furthermore, the shotgun metagenomics analysis allowed us to analyze, for the first time, those species and microbial pathways that could be contributing to this widespread reproductive disorder, which is still a challenge to diagnose and treat.

5.3. Future perspectives

There are some points to take into consideration for future research:

- In the PA research field, more studies performing CoDA that integrate co-dependent accelerometer-measured behaviors are warranted to provide a holistic view of the molecular mechanism underlying the PA interactions with different health outcomes, including the gut microbial landscape.
- The influence of SB on microbial composition is understudied, which might have even stronger and long-lasting effects on human health.
- In regard to the reproductive health research, future investigation in bigger study populations of the low biomass regions along the female reproductive tract together with stringent negative controls are needed for identifying the "core" microbiome in health and disease. This knowledge is indispensable before developing any new therapeutic strategies targeted to modulate the microbiome (antibiotics, pro- and pre-biotic solutions) for diagnosing and treating gynecological diseases.

- Overall, future microbiome studies based on appropriate and standardized methodologies, including experimental design, choice of microbiome detection method and unified pipelines for data analysis, would provide well-designed and performed studies, and thereby would increase homogeneity and ensure reproducibility and comparability between studies.
- Using additional meta-omics technologies, such as meta-transcriptomics, meta-proteomics and meta-metabolomics, would provide new information critical for a better insight into microbial homeostasis and, therefore, provide an integrative view of the microbes-host interactions. Thus, advancing microbiome research using multi-omics approaches would enhance our understanding of the crucial crosstalk between the microbes and host and lifestyle factors, which would provide relevant knowledge for improving human health.

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6. CONCLUSIONS

Overall conclusion

This Doctoral Thesis provides new insights into the compositional and functionality profiles of microbes in female health by increasing the current evidence of the associations between microbiome, PA and reproductive health. Through meta-omics analyses, altogether these findings provide step towards in the concept of microorganisms as important allies for female health.

Specific conclusions

SECTION I. Physical activity and microbial composition

Study I. There is a general consensus that points to higher abundances of SCFAs-producing bacteria in physically more active individuals or after a PA intervention. Athletes seem to have a richer microbiome compared to non-athletes. Accelerometry-based observational studies and well-controlled trials are needed to face high heterogeneity between studies and obtain conclusive information on the role of PA in microbial composition.

Study II. No associations were detected between the objectively measured PA and SB with the gut microbial diversity among middle-aged women. Also CoDA does not seem to support compositional effects on the gut microbial diversity. Butyrate-producing microbes such as *Agathobacter* and *Lachnospiraceae CAG56* were significantly more abundant when reallocating time from LPA or SB to MVPA. Integrating sleep measurement into the CoDA would provide a holistic understanding into the PA/SB effects on microbial composition in human.

SECTION II. The microbiome in female reproductive health

Study III. The endometrium and FT seem to harbor endogenous microbial profiles. For the close proximity, the two sites shared around 70% of microbes, while endometrial-specific and FT-specific bacteria were also detected. Further, endometrial and FT samples obtained from the same individual demonstrated more microbial similarity than for the same tissue (endometrium or FT) from two different women. Nevertheless, the study results should be considered as preliminary due to the small sample size.

Study IV. The gut microbial diversity was similar between women with and without endometriosis. No specific microbial features (species and pathways) were statistically different between cases and controls. Our findings do not find enough evidence to support the existence of a gut microbiome imbalance directly implicated in the pathogenesis of endometriosis. However, a deeper analysis of endometriosis severity scale and treatment/management strategies should be integrated into the analysis to gain a broader understanding of the microbiome involvement in the disease.