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UNVEILING IMPLICATIONS OF FECAL MICROBIOTA ON HUMAN'S CARDIOMETABOLIC HEALTH: ROLE OF PHYSICAL ACTIVITY AND FITNESS

REVELANDO LAS IMPLICACIONES DE LA MICROBIOTA FECAL EN LA SALUD CARDIOMETABÓLICA HUMANA: EL PAPEL DE LA ACTIVIDAD Y LA CONDICIÓN FÍSICA

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LOURDES ORTIZ ÁLVAREZ

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“Un pájaro posado en un árbol nunca tiene miedo de que la rama se rompa, porque su confianza no está en la rama, sino en sus propias alas.”

Anónimo

❖

A mi familia, especialmente a mis padres, y a mi prometido por darme esa confianza para creer en mí misma y conseguir todo lo que me proponga.

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LIST OF ABBREVIATIONS

2-AG: 2-arachidonoylglycerol

2-LG: 2-linoleoylglycerol

2-OG: 2-oleoylglycerol

^{18}F-FDG: ^{18}F-fluorodeoxyglucose

AA: arachidonic acid

ACTIBATE: activating brown adipose tissue thought exercise

AEA: anandamide

Alpha LEA: alpha-Linolenoyl ethanolamide

BAT: Brown adipose tissue

BMI: Body mass index

CB_1: Cannabinoid receptors 1

CB_2: Cannabinoid receptors 2

DGLEA: dihomo-gamma-linolenoyl ethanolamide

DHEA: docosahexaenoyl ethanolamide

DNA: deoxyribonucleic acid

EFICCOM (spanish acronim): Efecto del ejercicio FÍsico supervisado a nivel Cerebral, COgnitivo y Metabolómico

END*:* endurance trained group

ENMO: Euclidean Norm Minus One

EU: endotoxins units

FDR: False Discovery Rate

FFM: fat free mass

FMI: fat mass index

GLP-1: glucagon-like peptide 1

GPR: G-protein-coupled receptor

HDL-C: high-density lipoprotein cholesterol

HOMA index: homeostatic model assessment index

HU: Hounsfield Units

LAL: limulus amebocyte lysate

LBM: lean body mass

LDL-C: low-density lipoprotein cholesterol

LEA: linoleoyl ethanolamide

LMI: lean mass index

LPS: lipopolysaccharide

m*g*: mili-gravitational units

NAEs: N-acyl ethanolamines

OEA: oleoyl ethanolamine

PA: physical activity

PCR: polymerase chain reaction

PDEA: pentadecanoyl ethanolamide

PEA: palmitoyl ethanolamide

PERMANOVA: permutational multivariate analysis of variance

PET/CT: positron emission tomography-computed tomography scan

POEA: palmitoleoyl ethanolamide

RES: resistance trained group

RDP: Ribosomal Data Project

ROIs: regions of interest

SCFAs: short-chain fatty acids

SEA: stearoyl ethanolamide

SED: sedentary group

SUV: standardized uptake values

UC: unclassified

UCP1: uncoupling protein-1

VO_{2max}: maximum oxygen volume

WAT: white adipose tissue

ABSTRACT

The microorganisms that colonize the gastrointestinal tract are known as gut microbiota, being bacteria the microorganisms that are found in higher abundance. Bacteria communities regulate host metabolism by producing metabolites that interact with specific receptors on different tissues modulating the host's health. In fact, the alteration of these bacteria communities within the intestine has been observed in individuals with cardiometabolic disease. Thus, strategies aiming to modify the relative abundance of these gut bacteria have been suggested as novel therapies for the prevention and treatment of cardiometabolic diseases. One of the most promising strategies for modulating gut microbiota is increasing levels of physical activity and fitness, yet the scientific evidence is limited.

The present International Doctoral Thesis aims to determine the relationship of novel cardiometabolic risk markers (i.e., endocannabinoids and brown adipose tissue) with fecal microbiota diversity and composition and to study the association of physical activity and fitness levels with fecal microbiota diversity and composition in humans. To unravel these aims, we conducted four cross-sectional studies (***Studies I, II, III*** and ***V***) and one case-control study (***Study IV***) in humans.

The results show that plasma levels of endocannabinoids and their analogues positively correlate with the relative abundance of bacteria belonging to *Firmicutes* and *Verrucomicrobia* phyla which are involved in the regulation of gut barrier integrity (***Study I***). Interestingly, these bacteria communities negatively correlate with brown adipose tissue volume and activity (***Study II***). Higher physical activity levels, especially of vigorous intensity, are associated with higher fecal diversity and relative abundance of certain fecal bacteria (***Study III***). Particularly, we found differences in fecal microbiota composition between resistance and endurance athletes as well as with sedentary individuals (***Study IV***). Lastly, higher physical fitness levels are associated with a higher relative abundance of certain fecal bacteria in both young and older adults (**Study V**).

The present International Doctoral Thesis provides new insights into the understanding of human gut microbiota, and show that endocannabinoids may improve the gut barrier integrity through certain fecal bacteria. Interestingly, these bacteria communities may be involved in the regulation of human brown fat metabolism. Moreover, the observed association of physical activity and fitness with fecal microbiota diversity and composition provide hope for future intervention strategies aiming to potentially modify gut microbiota in humans.

RESUMEN

Los microorganismos que colonizan el tracto gastrointestinal se conocen como microbiota intestinal, siendo las bacterias los microorganismos que se encuentran en mayor abundancia. Las comunidades bacterianas regulan el metabolismo del huésped al producir metabolitos que interactúan con receptores específicos en diferentes tejidos modulando así la salud del huésped. De hecho, se ha observado una alteración de estas comunidades bacterianas dentro del intestino en individuos con enfermedades cardiometabólicas. Por lo tanto, se han sugerido estrategias destinadas a modificar la abundancia relativa de estas bacterias intestinales como nuevas terapias para la prevención y el tratamiento de las enfermedades cardiometabólicas. Una de las estrategias más prometedoras para modular la microbiota intestinal es incrementar los niveles de actividad física y condición física, sin embargo la evidencia al respecto es escasa.

La presente Tesis Doctoral Internacional tiene como objetivos determinar la relación de nuevos marcadores de riesgo cardiometabólico (es decir, endocannabinoides y tejido adiposo pardo) con la diversidad y composición de la microbiota fecal y estudiar la asociación de los niveles de actividad física y condición física con la diversidad y composición de la microbiota fecal en humanos. Para dar respuesta a estos objetivos, llevamos a cabo cuatro estudios transversales (**Estudios I, II, III** y **V**) y un estudio de casos y controles (**Estudio IV**).

Los resultados muestran que los niveles plasmáticos de los endocannabinoides y sus análogos se correlacionan positivamente con la abundancia relativa de bacterias pertenecientes a los filos *Firmicutes* y *Verrucomicrobia* involucradas en la regulación de la integridad de la barrera intestinal (**Estudio I**). Curiosamente, estas comunidades de bacterias se correlacionan negativamente con el volumen y la actividad del tejido adiposo pardo (**Estudio II**). Mayores niveles de actividad física, especialmente de intensidad vigorosa, están asociados con una mayor diversidad fecal y abundancia relativa de ciertas bacterias fecales (**Estudio III**). De hecho, los datos muestran diferencias en la composición de la microbiota fecal entre los atletas de fuerza y resistencia, así como entre los individuos sedentarios (**Estudio IV**). Por último, mayores niveles de condición física están asociados con una mayor abundancia relativa de ciertas bacterias fecales tanto en adultos jóvenes como mayores (**Estudio V**).

La presente Tesis Doctoral Internacional proporciona nuevos conocimientos sobre la comprensión de la microbiota intestinal humana y muestra que los endocannabinoides parecen mejorar la integridad de la barrera intestinal a través de ciertas bacterias fecales. Curiosamente, estas comunidades de bacterias pueden estar involucradas en la regulación del metabolismo de la grasa parda en humanos. Además, la asociación observada de la actividad física y la condición física con la diversidad y composición de la microbiota fecal aporta esperanza para futuras estrategias de intervención que apunten a modificar potencialmente la microbiota intestinal humana.

GENERAL INTRODUCTION

CARDIOMETABOLIC DISEASES: A GLOBAL HEALTH ISSUE

Cardiometabolic diseases refer to a combination or clustering of metabolic abnormalities that include increased insulin resistance, hyperglycemia, visceral adiposity, non-alcoholic fatty liver, dyslipidemia, and hypertension[1]. These abnormalities are becoming a significant global healthcare problem because of the increased risk of obesity, and closely interrelated diseases, such as type 2 diabetes, cardiovascular diseases[2]. Currently, they are the leading cause of death and disability in the world[2]. In fact, the World Obesity and Diabetes Federations have predicted that one billion people globally will suffer from obesity, 578 million people with type 2 diabetes, and 17.4 million hypertension in 2030[3,4].

Cardiometabolic diseases can begin by insufficient physical activity and high caloric intake[2]. This high caloric intake results in obesity and low-grade inflammation, which often precedes metabolic syndrome, type 2 diabetes, and cardiovascular disease[5,6]. Obesity is defined by excessive or abnormal fat accumulation, which contributes the insulin resistance and inflammatory response in the body marked by low-grade inflammation[7]. This process is associated with progressing to type 2 diabetes and cardiovascular disease[5,6]. Although the development of cardiometabolic diseases is multifactorial, they have risk factors and characteristics similar that could help treatment options.

Over the past few decades, substantial efforts have been made to treat cardiometabolic diseases, which have not been very successful[4]. Thus, a critical need exists to identify novel potential targets for adequate prevention and treatment of cardiometabolic diseases.

THE HUMAN MICROBIOME AS A THERAPEUTIC TARGET

The term “microorganism” refers to any too-small living thing to be seen with the naked eye[8]. The human body harbors approximately $10^{(13)}$-$10^{(14)}$ microbial cells, with around a 1.3:1 microbial cells to human cells ratio[9]; we are almost equal parts humans to the microbe. It represents a mass of about 0.2 kg (in an adult body)[9],comparable to the weight of the adult human spleen[10].The aggregate of microorganisms that include protozoa, archaea, viruses, fungi, and bacteria occurring on or within humans constitute microbiota[11,12]. Often the term microbiota is used interchangeably with microbiome, but both terms have subtle differences: microbiota refers to the microorganisms associated with humans, while the collection of genes and genomes of members of microbiota is the microbiome[11,12].

Characterization of the microbiota: what is in there?

Of the three recognized domains of life, bacteria and archaea consist exclusively of microorganisms, which are prokaryotic cells whose main characteristic is the lack of nuclear membrane around their genetic material[8].

The third domain of life, Eukaryotes, is distinguished by their genetic material is contained within a membrane-enclosed nucleus and contains all of the macroscopic, multi-celled organisms that we recognize as plants and animals, but it also includes many microorganisms, such as protoctists and fungi[8].

All living organisms, including the microorganisms, are classified based on their mutual similarity or evolutionary relatedness in different taxonomic ranks, being in descending order: domain, phyla, classes, orders, families, genera, and species[13] (**Fig.1**). These are nested ranks, with each successively lower level contained within the one above[14]. Domains are the highest rank and contain groups of phyla[13]. Classes contain groups of orders, orders contain families, families contain genera, and genera contain species[13]. Species is the most specific category and is defined as a group of closely related organisms that will breed among themselves in eukaryotes, whereas in the case of prokaryotes it can be defined simply as a population of cells with similar characteristics[14].

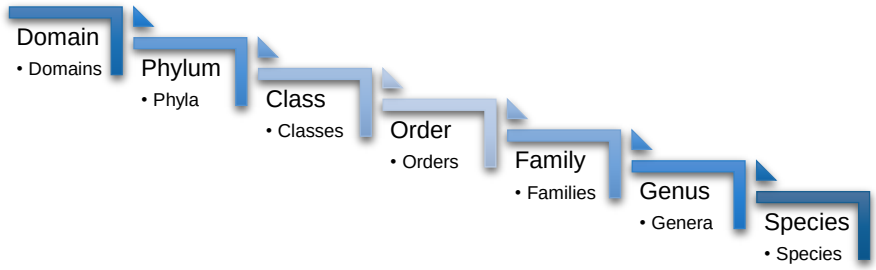

Figure 1. Taxonomy classification of living organisms.

Since bacteria are the most representative microorganisms of the human microbiota[15], we will focus on them in this International Doctoral Thesis. Bacteria are prokaryotic cells, that are defined as unicellular organisms whose genome is mainly composed of a single double-stranded DNA molecule with more minor DNA elements known as plasmids[16]. The cell is surrounded by the plasma membrane, which regulates the transport of nutrients and waste substances[17]. This plasma membrane is covered by a wall with different characteristics, distinguishing Gram-negative and Gram-positive bacteria[17]. Gram-negative bacteria are enclosed by a thin peptidoglycan plasma membrane, which is surrounded by an outer wall[17]. The outer wall acts as a barrier against the diffusion of many compounds, making the cell more resistant to toxic substances[18]. This outer wall mainly contains lipopolysaccharides (LPS), a large amphipathic glycoconjugated which occupies the extracellular space and consists of a lipid A, an oligosaccharide core, and a distal polysaccharide[19]. LPS promotes the secretion of the pro-inflammatory cytokine, stimulating the response of the host immune[20]. Their endotoxic properties reside in the lipid A component[19]. For this reason, LPS has been considered an “alarm molecule” by the body’s innate immunity, warning of a potential threat of invasion by pathogens. Plasma levels of LPS are frequently correlated with conditions associated with low-grade inflammation, being a pro-inflammatory marker in clinical studies[21]. In fact, individuals with metabolic syndrome or type 2 diabetes present higher plasma levels of LPS in comparison with healthy

people[22]. On the other hand, Gram-positive bacteria are surrounded by thicker peptidoglycan layer, lacking an outer wall[17]. The peptidoglycan layer is fundamental in Gram-positive bacteria since maintains a sustained turgor of plasma membrane, preserving the integrity and counteracting the osmotic pressure of the cell[17]. According to the types of reactions bacteria employ to generate energy for growth and other activities, we can find anaerobic, aerobic, or facultative bacteria[23]. Aerobic bacteria require molecular oxygen and cannot grow in its absence[23]. However, anaerobic bacteria cannot grow in the presence of oxygen, and they reduce available organic compounds to various end products such as organic acids and alcohol[23]. Finally, we can find facultative bacteria, they are the most versatile because they preferentially utilize oxygen but also can reduce other compounds in its absence[23]. Therefore, these bacteria can be aerobic or anaerobic, dependent on environmental conditions.

Equilibrium and diversity: a pivotal point in microbiota health

Bacteria are localized in the human body occupying habitats including the oral cavity, respiratory tract, genital organs, skin, and gastrointestinal tract (**Fig. 2**)[11]. Each habitats contains bacteria communities that living symbiotically in a balance status with the host, known as eubiosis[24]. In contrast, dysbiosis is defined as a compositional and functional alteration of these bacteria communities in individuals with disease compared with healthy subjects[24] (**Fig.2**). Thus, eubiosis has been related to a healthier host profile[24], whereas dysbiosis has been linked to disease status[25–27]. In fact, in mice models the *Porphyromonas gingivalis* species contributed to the development of periodontitis in the mouth, intervening the host oral microbiota homeostasis[28]. In asthmatic patients, it has been identified that *Proteobacteria* phylum inside lungs was present in higher proportion (30% vs. 15%), and the rest of bacteria in minor proportion compared to healthy individuals[29,30]. Since *Proteobacteria* phylum is characterized by containing pathogenic bacteria[31,32], this lung microbiota composition might be an accurate predictor of this disease[29,30]. Regarding genital microbiota, lactic acid produced by bacteria belonging to *Lactobacilli* genus in the vaginal epithelium of women provides protection against infectious diseases since the acidifying (i.e., decreasing the pH levels) of the vaginal medium prevents the growth of potential pathogens[33]. Hence, the decrease in *Lactobacilli* genus predominance and the overgrowth of opportunistic pathogens is the reason behind recurrent vulvovaginal infections pathogenesis[33].

Therefore, dysbiosis can feature a loss of beneficial bacteria, an expansion of potentially harmful bacteria, and/or a loss of overall bacteria diversity. For this reason, measuring species' diversity in bacteria communities has been proposed as a marker of microbiota health[34]. Alpha and beta diversities measurements are employed statistical measures based on the identified microbial communities to assess the diversity. Alpha diversity evaluates the richness and evenness of a single sample, considering the number of different phylotypes and their relative abundances[35], whereas beta diversity assesses the differences between samples[36]. Alpha diversity usually includes four indexes: richness Chao, Shannon, inverse Simpson, and evenness Camargo[35].

The richness Chao index estimates the diversity according to the number of different phylotypes in the community[37]; the Shannon diversity increases as both the richness and the evenness of the community increase[38]; the inverse of Simpson diversity is derived from the classical Simpson diversity and indicates the richness in a community with uniform evenness[39]; and the evenness Camargo index determines the equitability of phylotypes frequencies in a community[40]. Interestingly, a reduced alpha diversity in lung or vagina has been associated with respiratory and vulvovaginal infections, respectively[29,30,33]. Thus, a greater alpha diversity of bacteria communities in the different habitats of our body seem to be related to a healthier profile.

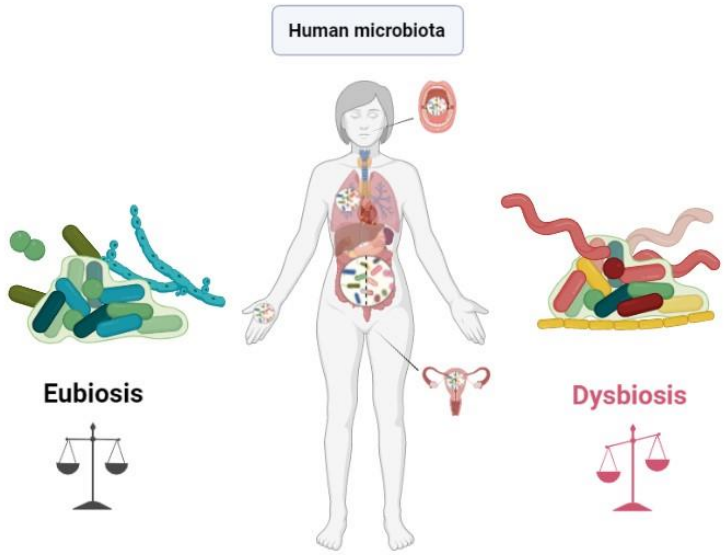

Figure 2. Eubiosis and dysbiosis: two sides of the same coin.

The gut microbiota: the most relevant microbiota among the human body

The human gastrointestinal tract is a series of hollow organs that are connected to each other, belonging to the digestive system, that starts from the oral cavity, continues through the esophagus, the stomach, the gut, and finally ends at the anus[41]. Gastrointestinal tract is responsible for food processing and allowing the survival of commensal, symbiotic bacteria while eliminating pathogens[42]. This protector character is due to epithelial cells providing a physical barrier being the primary line of defense[42,43]. Epithelial cells work in concert with immune and stromal cells limiting the direct contact of pathogens with the epithelium[42,43]. Epithelial cells in the gut produce antimicrobial peptides and secret mucins that lubricate and protect the epithelial intestinal surface[44,45]. Another essential component of the gut barrier is tight junction proteins, which create a seal between neighboring epithelial cells and prevent the entry of pathogens while regulating permeability to water, ions, and nutrients[46].

The epithelial and mucosa are covered by bacteria communities that colonize the gastrointestinal tract called gut microbiota[47]. It is considered the most

significant one in maintaining our health[48], since is involved in several beneficial functions, such as vitamin production, protection against pathogens, histological development, enhancement of the immune system, and the fermentation of "non-digestible foods" to short chain fatty acids (SCFAs), and specific proteins to amino acids[49,50]. The symbiotic relationship between the gut microbiota and the host is potentially mediated by synthesized metabolites, whose are released to portal blood and are metabolized by the liver and other peripheral tissues[51], leading to link the gut with other organ systems[52].

The gut microbiota development begins in the fetus[53] and is completed mainly during the first three years of life[54]. Early life exposures, such as birth delivery route, infant diet, and antibiotic exposure, play a critical role in gut bacteria colonization[55]. Moreover, the gut microbiota composition varies at different time points (i.e., infant, adulthood, and old age) in the same individual and among different people. Therefore, despite similarities in the general composition of human gut microbiota[56], their variations lead to interindividual differences[57].

The composition of gut microbiota at the phylum level is characterized by *Firmicutes* (51-76%) and *Bacteroidetes* (16-42%) in higher abundance compared to *Actinobacteria* (2-20%), *Verrucomicrobia* (2%), and *Proteobacteria* (1%)[58]. However, the density and composition of bacteria vary change along the gastrointestinal tract due to the different environments[59]: in the small intestine, the transit time is short, and bile concentration is high, while in the colon, there are slower flow rates and milder pH[59,60]. Thus, we can find smaller bacteria communities in the stomach and larger bacteria communities, especially anaerobic types, in the colon[61].

- **Role gut microbiota in human metabolism**

After a food intake, food passes through the gastrointestinal tract and the absorption of nutrients take place in the small intestine. However, many food components cannot be digested in the small intestine and are passed into the colon, in which they are fermented by bacteria[49,62]. Acetate, propionate, and butyrate are the primary SCFAs produced in the approximate molar ratio of 60:20:20 in humans[63]. Butyrate is a significant energy source for colonocytes, almost entirely consumed by them[51], whereas acetate and propionate are found in portal blood and are metabolized by the liver and other peripheral tissues[51]. SCFAs play a role in intestinal homeostasis and energy metabolism through binding to specific G-protein-coupled receptors (GPR), such as GPR41, GPR43, and GPR109a, on the epithelium, endocrine, and immune cells[64]. They might reduce the secretion of pro-inflammatory cytokines, reducing local macrophage infiltration[64,65]. Concretely, butyrate activates the β-oxidation in the mitochondria in colonic cells contributing to the control of the anaerobic condition in the colon[48]. This affords anaerobic bacteria to remain close to the epithelium and distances facultative anaerobes such as bacteria belonging *Proteobacteria* phylum that increase the risk of intestinal inflammation[66]. Butyrate and propionate bind to GPR41 and GPR43 in the colon leading to the production of the gut hormones peptide YY and glucagon-like peptide 1 (GLP-1), thereby affecting satiety and glucose homeostasis[64,65]. Since acetate and

propionate, and a small proportion of butyrate, reach the circulation, they can affect peripheral tissue substrate metabolism and function[51]. Acetate and propionate may increase the lipid buffering capacity and prevent chronic low-grade inflammation in the adipose tissue via GPR41 and GPR43[64,65]. In the pancreas, propionate may improve β-cell function and the others SCFA may affect glucose-stimulated insulin secretion via GPR43.

On the other hand, Gram-negative bacteria release pro-inflammatory components, such as LPS[67]. When there is a gut barrier dysfunction, giving rise to increased gut permeability[68], LPS can cross the gut barrier and translocate from the gut into the bloodstream, leading to metabolic endotoxemia and systemic inflammation, as we mentioned before[67,68]. This fact is due to dysbiosis status characterized by cardiometabolic diseases[68], where the expression of tight junction proteins are dysregulated leading to gut barrier breach and entry of LPS. Interestingly, SCFAs are also involved in the epithelial barrier to maintain gut integrity and prevent this translocation of LPS[69]. They increase the expression of tight junction proteins in the epithelial barrier, reduce mucosal permeability, and inhibit the synthesis of inflammatory cytokines[70]. Therefore, changes in the gut microbiota composition can result in the release of different metabolites, which might influence the host metabolism.

- **Fecal microbiota as a proxy of gut microbiota**

Due to the difficulties associated with collecting samples from the human gut, most studies use fecal samples as surrogates. Moreover, bacteria in the colon account for approximately 70% of all bacteria in the human body[61]. Thus, since the colon is the organ that harbors the densest number of bacteria, sequencing bacterial DNA extracted from fecal samples is the most systematic approach to studying the gut microbiota in humans[71]. Besides, scientific evidence has shown that bacteria communities examined directly in the colon present a composition of bacteria similar to that found in feces[72]. The collection of fecal samples is a non-invasive technique, easily obtained, and can be carried out privately by study participants. The fecal samples are usually stored at -80ºC until DNA extraction, preserving bacteria integrity[73]. After extraction of DNA, targeted sequencing is more common today and typically uses the 16S ribosomal RNA (rRNA) gene[71]. It contains highly conserved regions interspersed with nine hypervariable regions (commonly denoted as V1-V9)[71]. Hence, we can amplify the variable regions using specific primers in the conservative regions and sequence the PCR products to analyze the bacterial composition[74]. The V1-V2 and V3-V4 regions are widely used for sequencing nowadays[34]. This technique allows for a low-cost and rapid way to detect the taxonomic composition of a sample[75]. Therefore, fecal samples represent an excellent surrogate and allow for multiple samples to be obtained over short and long time periods from healthy and non-healthy individuals.

RELATIONSHIP BETWEEN CARDIOMETABOLIC DISEASES AND GUT MICROBIOTA

Over the last few years, most of the gut microbiota-related research has focused on the relationship between gut microbiota composition changes and

various disease states, such as cardiometabolic diseases[76,77]. External changes can affect the balance of bacteria communities, leading to dysregulation of bodily functions and diseases[76,77]. Thus, assessing the gut microbiota composition in clinical studies could aid in understanding in a better manner the mechanisms that give rise to cardiometabolic diseases in humans.

Role of endocannabinoids in cardiometabolic diseases through gut microbiota

The endocannabinoid system is a signaling system distributed throughout our body[78]. It is composed of: (1) cannabinoid receptors 1 and 2 (CB_1 and CB_2)[79], which are two GPR also activated by the psychotropic cannabinoid Δ9-tetrahydrocannabinol[79]; (2) two main endogenous lipids, the endocannabinoids anandamide (AEA), a member of the N-acyl ethanolamine (NAEs) class, and 2-arachidonoylglycerol (2-AG), as part of the acylglycerol class[80]; and (3) metabolic enzymes involved in their synthesis and degradation[81]. Endocannabinoids are synthesized from arachidonic acid (AA) and membrane precursors, phosphatidylethanolamine for AEA and phosphatidylinositol for 2-AG, respectively[82]. In addition to AA and the main endocannabinoids, structural analogues also belong to the endocannabinoid system[83].

Endocannabinoids and their analogues are not stored but are formed "on-demand" in various tissues and organs (e.g., brain, liver, gut, adipose tissue, skeletal muscle, and pancreas)[84]. They can modulate different physiological functions, such as the immune response by pro- or anti-inflammatory actions, among others[85]. The endocannabinoid system is involved in gut physiology[86], like regulating gastric secretion[86], motility[86], and gut permeability[87] by preserving or damaging the gut barrier integrity[88]. Since the gut microbiota is a crucial player in the gastrointestinal tract, recent evidence has shown that the endocannabinoid system can modulate it in mice models[83,86]. Administration of exogenous NAEs in mice modified the gut microbiota composition, reducing obesity and dysmetabolism asssociated[89,90]. The deletion of the N-acyl phosphatidylethanolamine phospholipase D, an enzyme involved in the synthesis of N-acylethanolamines, in adipocytes of mouse model increased body weight and fat mass, inducing dysbiosis status[91]. As mentioned above, dysbiosis induces gut barrier dysfunction[68], leading to the translocation of LPS consequent increase in gut permeability[67]. So, the alteration in the endocannabinoid system is associated with obesity and cardiometabolic diseases[92]. These findings suggest that the endocannabinoid system could change gut microbiota composition, resulting in the modification of gut barrier integrity. Therefore, studying the role between the endocannabinoids and their analogues with fecal microbiota diversity and composition could be a promising way to improve human gut barrier integrity, thereby improving host health.

Role of gut microbiota in brown adipose tissue metabolism

Gut microbiota is involved in host energy homeostasis by the distal organs such as adipose tissue via metabolites and cytokines[93]. Adipose tissue is classified into white adipose tissue (WAT) and brown adipose tissue (BAT). WAT accounts for most of the mass of adipose tissue in our body[94], whereas BAT

represents a small part of the total fat mass and is located predominantly in the interscapulum and supraclavicular regions of adult humans[95]. WAT accumulates energy in the form of triglycerides[94]. BAT can consume both glucose and fatty acids[96–98] and dissipate their energy as heat in a nonshivering thermogenesis process through the action of the uncoupling protein-1 (UCP1)[99]. BAT is involved in energy expenditure and insulin sensitivity thought the dissipation of energy in response to cold exposure or β-adrenergic receptor activators[100]. In fact, BAT seems to be responsible for up to 60% of total energy expenditure in rodents[100], being the main tissue responsible for adaptive thermogenesis[101], whereas it hardly represents 1% in humans[102]. Earlier studies have shown that gut microbiota is an essential endogenous factor that can modulate BAT metabolism[103–107]. Despite the fact that the amount of BAT in humans is proportionally much smaller than in mice, their activation may provide new approaches to treat obesity and related cardiometabolic diseases.

Mice models have shown that gut microbiota composition promoted whole-body thermogenesis during cold exposure, with BAT being the main thermogenic effector[103–107]. Cold temperature and microbiota deletion can decrease adiposity through the induction of WAT browning and BAT activity in mice[103–111]. In contrast with animal models[103–111], and despite scarce evidence in humans, fecal microbiota composition was not associated with BAT activity using magnetic resonance imaging after cold exposure in individuals with non-alcoholic fatty liver disease[112]. However, in individuals with obesity, gut microbiota plays a role in remodeling WAT towards a beige-life phenotype[113–115]. The relative abundance of bacteria belonging to *Firmicutes* phylum was positively associates with the increase of browning markers possibly through circulating acetate[113]. Therefore, the role of gut microbiota composition in human BAT metabolism is controversial. Whether certain gut bacteria involved in obesity and cardiometabolic disease development[116,117] could also modulate BAT metabolism via SCFAs and other metabolites released, remain to be ascertained.

ROLE OF PHYSICAL ACTIVITY AND FITNESS IN GUT MICROBIOTA DIVERSITY AND COMPOSITION

Physical activity and exercise

Physical activity is every physical movement using skeletal muscles that produce an energy expenditure above basal levels[118]. Physical activity is associated with a myriad of physiological adaptations that benefit human health[119,120], reducing the risk for premature mortality and reducing the risk of developing more than 25 chronic medical conditions[121,122]. In fact, physical activity ameliorates the tolerance to glucose and the symptoms of anxiety and depression, and lowers the risk of type 2 diabetes, hypercholesterolemia, hypertension, and obesity[119,120].

Recent evidence suggests that physical activity is considered one of the most significant lifestyle factors able to influence the fecal microbiota diversity and composition in humans[123–126]. People who undertook physical activity daily assessed by self-reported questionnaires had higher fecal microbiota diversity

and relative abundance of *Faecalibacterium prausnitzii* species than those who perform physical activity occasionally[127]. Another cross-sectional studies observed that men and women[128], and premenopausal women[129] meeting the physical activity World Health Organization recommendations had a higher fecal microbiota diversity and relative abundance of *Lachnospiraceae* family, and *Akkermansia* and *Faecalibacterium* genera. *Akkermansia* and *Faecalibacterium* genera are associated with reduced intestinal inflammation and, therefore, may play a role in the prevention of the development of cardiometabolic diseases[130]. Of note is that studies investigating the relationship between physical activity and fecal microbiota diversity and composition in humans have used self-reported questionnaires to assess physical activity[127–129]. However, physical activity levels are difficult to register, quantify and categorize, and therefore, questionnaires are not free of certain biases[131]. Given the limitations of self-report methods, accelerometry (i.e. movement sensors) has become the method of choice for objectively measuring physical activity in free-living conditions[132]. Studies using objective measures of physical activity at different intensities would definitively provide more accurate and reliable information about the physical activity levels and therefore would provide more useful information about the true relationship between physical activity and fecal microbiota composition.

The term "exercise" has been used interchangeably with physical activity[133], but they are not synonymous. Exercise is a subcategory of physical activity, defined as a structured, premeditated, and cyclic physical activity aiming to improve or maintain physical fitness, which refers to a set of either health- or skill-related attributes[118]. Exercise improves the health of individuals by decreasing markers of inflammation[134] and circulating lipid concentrations[135] and improving glucose tolerance and insulin sensitivity[136]. For this reason, exercise is often prescribed as a contributory therapy to prevent and treat cardiometabolic diseases. Recent studies suggest that the beneficial effect of exercise on human's health could be mediated by modifying the gut microbiota diversity and composition[123–125,137,138]. In fact, several research groups[123–126,139,140] and we[141] have recently explored how exercise can modify fecal microbiota diversity and composition in humans. The results showed that exercise increased fecal microbiota diversity and the relative abundance of bacteria belonging to *Firmicutes* and *Bacteroidetes* phyla[123–126,139,140]. However, most intervention studies investigating the effect of exercise on fecal microbiota diversity and composition used endurance exercise training[142–144] or concurrent training (endurance and resistance exercises)[141,145,146] in sedentary people. Upon combining different types of exercise training, we cannot distinguish what effects provokes each one on fecal microbiota diversity and composition. Moreover, fecal microbiota diversity and composition remain relatively stable over time in adulthood[147]; however, after a brief exposure such as exercise interventions, bacteria face a substantial but transient perturbation, followed by partial or total recovery of previous taxonomic composition (before exercise training intervention)[148]. In fact, Allen et al.[143] observed that exercise-induced shifts in the fecal microbiota diversity and composition in their sedentary participants after 6-week exercise intervention disappeared 6-week after finishing the exercise intervention. Thus, these exercise-induced shifts in the fecal microbiota diversity and composition may be transient and likely

dependent on repeated exercise stimuli. For this reason, focusing on athletes who have incorporated exercise training into their life for some years would be the best approach for observing how the different types of exercise training affect the fecal microbiota diversity and composition in humans.

Physical fitness

Conceptually, physical fitness refers to the full range of physical qualities, e.g., cardiorespiratory fitness, muscle strength, speed, agility, coordination, and flexibility, which provides the necessary ability and bases to perform physical activity[118]. It can be understood as an integrated measurement of most, if not all, the body structures and functions (i.e., skeletomuscular, cardiorespiratory, haematocirculatory, psychoneurological and endocrine-metabolic) involved in the performance of physical activity[118]. Thus, being physically fit implies that the response of these functions and structures will be adequate. The primary components of physical fitness are cardiorespiratory fitness and muscular strength[118]. Cardiorespiratory fitness is a direct marker of physiological status and reflects overall capacity of the cardiovascular and respiratory systems[149]. Cardiorespiratory fitness is expressed in terms of maximum oxygen consumption (VO_{2max}) [149]. The VO_{2max} can be expressed with respect to subject weight (ml/kg/min), in absolute terms (L/min) or in metabolic equivalents (METS) (1 MET is the energy expenditure at rest [~3.5 ml/kg/min])[149]. Thus, if a subject has a VO_{2max} of 42 ml/kg/min, he also has an energy expenditure of 12 METS (i.e., he/she is able to increase his/her resting energy expenditure 12 fold). A number of important prospective studies have shown that VO_{2max} is the most important predictor of all-cause mortality, and in particular of cardiovascular death both for healthy people and those with cardiometabolic diseases[150,151]. Researchers have also found positive correlations between cardiorespiratory fitness levels and fecal microbiota diversity and the relative abundance of certain fecal bacteria in young healthy adults[152,153] and premenopausal women[154]. Muscle strength refers to a balanced healthy functioning of the musculoskeletal system and requires that a specific muscle or muscle group be able to generate force[118]. Higher muscular strength levels have been associated with lower risk of developing cardiometabolic diseases, respiratory diseases, cancer, and all-cause mortality across several cohorts, age groups, and countries[155,156]. Despite most cross-sectional studies observing the relationship between physical fitness with fecal microbiota diversity and composition in humans focusing on cardiorespiratory fitness[152–154], scientific evidence suggests an positive association of handgrip strength levels with fecal bacteria belonging to *Faecalibacterium* and *Bifidobacterium* genera[157]. Moreover, we know that age-related changes in diet, physical activity, multi-morbidity and medication use may impact gut microbiota diversity and composition[158]. Therefore, the relationship between physical fitness parameters and fecal microbiota diversity and composition in old adults is unknown.

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HYPOTHESIS AND AIMS

HYPOTHESIS

The main hypothesis of this International Doctoral thesis is that novel cardiometabolic health markers are related to fecal microbiota diversity and composition, which may be different according to the physical activity and fitness levels. Specifically, we hypothesize that: (i) endocannabinoids and BAT, as novel cardiometabolic risk markers, are related to specific fecal bacteria genera, and (ii) physical activity at different intensities, and duration, as well as physical fitness levels are related to different bacteria diversity and composition in the feces of young and older adults.

AIMS

The present International Doctoral Thesis aims to investigate the relationship of novel cardiometabolic risk markers (i.e. endocannabinoids and BAT) with fecal microbiota diversity and composition and to study the association of physical activity and fitness levels with fecal microbiota diversity and composition in humans. These overall aims are addressed by five different specific objectives, which are grouped into two sections:

SECTION I: Relationship between novel cardiometabolic markers and fecal microbiota diversity and composition

- **Specific aim I:** To investigate the association of plasma levels of endocannabinoids and their analogues with fecal microbiota diversity and composition in young adults. (*Study I*).

- **Specific aim II:** To investigate the association of fecal microbiota composition with BAT volume and activity, as determined via cold-induced ^{18}F-Fluorodeoxyglucose (^{18}F-FDG) uptake and mean radiodensity in young adults. (*Study II*).

SECTION II: Role of physical activity and fitness in fecal microbiota diversity and composition

- **Specific aim III:** To investigate the association of the time spent in objectively measured physical activity, at different intensities, with fecal microbiota diversity and composition in young adults. (*Study III).*

- **Specific aim IV:** To study whether resistance and endurance athletes have different fecal microbiota diversity and composition between them and compared to sedentary individuals. (*Study IV*).

- **Specific aim V:** To investigate the association of physical fitness, specifically, muscular strength and cardiorespiratory fitness, with fecal microbiota diversity and composition in young and older adults. (*Study V*).

MATERIAL AND METHODS

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

Study	Design	Cohort	Participant's characteristics*	Independent variable	Dependent variables	Statistical analyses
Study I	Cross-sectional	The ACTIBATE study	N=92 (♀=71%) Age: 22.0 ± 2.0 years BMI: 24.9 ± 4.7 kg/m^2	❖Plasma levels of endocannabinoids and their analogues	❖Fecal microbiota diversity and composition ❖Plasma levels of lipopolysaccharides	❖Partial Spearman correlations ❖One-way PERMANOVA ❖Kruskal Wallis test
Study II	Cross-sectional	The ACTIBATE study	N=82 (♀=71%) Age: 22.8 ± 2.2 years BMI: 24.9 ± 4.8 kg/m^2	❖Fecal microbiota composition	❖BAT volume and activity, and mean radiodensity ❖WAT and skeletal muscles	❖Partial Spearman correlations
Study III	Cross-sectional	The ACTIBATE study	N=88 (♀=73%) Age: 21.7 ± 2.3 years BMI: 23.6 ± 4.8 kg/m^2	❖Time spent in objectively measured physical activity	❖Fecal microbiota diversity and composition	❖Partial Spearman correlations ❖One-way PERMANOVA ❖Kruskal Wallis test ❖Analyses of covariance
Study IV	Case-control	Oh My Gutness! Project	SED: N=14 (♀=79%) Age: 30.3 ± 7.2 years BMI: 23.3 ± 3.2 kg/m^2; RES: N=14 (♀=43%) Age:27.3 ± 4.1 years BMI: 23.8 ± 2.5 kg/m^2; END: N=22 (♀=9%) Age:34.0 ± 9.7 years BMI: 23.0 ± 1.7 kg/m^2	❖Types of exercise training	❖Fecal microbiota diversity and composition	❖Analyses of covariance ❖Two-way PERMANOVA
Study V	Cross-sectional	The ACTIBATE study and EFICCOM study	Young adults: N=90 (♀=71%) Age: 22.0 ± 2.3 years BMI: 24.9 ± 4.7 kg/m^2; Old adults: N=34 (♀=50%) Age: 68.5 ± 2.9 years BMI: 29.6 ± 5.4 kg/m^2	❖Physical fitness: muscular strength and cardiorespiratory fitness	❖Fecal microbiota diversity and composition	❖Partial Spearman correlations ❖One-way PERMANOVA ❖Kruskal Wallis test

*Data are presented as mean ± standard deviation, otherwise stated; ♀: Percentage of women; BAT: brown adipose tissue, BMI: body mass index, END: endurance trained group, PERMANOVA: permutational multivariate analysis of variance**,** RES: resistance trained group, SED: sedentary group, WAT: white adipose tissue.

RESULTS AND DISCUSSION

SECTION I

STUDY I: PLASMA LEVELS OF ENDOCANNABINOIDS AND THEIR ANALOGUES ARE RELATED TO SPECIFIC FECAL BACTERIAL GENERA IN YOUNG ADULTS: ROLE ON GUT BARRIER INTEGRITY

ABSTRACT

Objective: To investigate the association of plasma levels of endocannabinoids and their analogues with fecal microbiota diversity and composition.

Methods: Plasma levels of endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), as well as their eleven analogues, and arachidonic acid (AA), were measured using liquid chromatography-tandem mass spectrometry in 92 young adults. DNA extracted from stool samples was analyzed using 16S rRNA gene sequencing. Lipopolysaccharide levels were measured in plasma samples.

Results: Plasma levels of endocannabinoids and their analogues were not related to beta or alpha diversity indexes. Plasma levels of AEA and related N-acylethanolamines correlated positively with the relative abundance of *Faecalibacterium* genus (all rho≥0.26, P≤0.012) and *Akkermansia* genus (all rho≥0.22, P≤0.036), and negatively with the relative abundance of *Bilophila* genus (all rho≤-0.23, P≤0.031). Moreover, plasma levels of 2-AG and other acylglycerols correlated positively with the relative abundance of *Parasutterella* (all rho≥0.24, P≤0.020) and *Odoribacter* genera (all rho≥0.27, P≤0.011), and negatively with the relative abundance of *Prevotella* genus (all rho≤-0.24, P≤0.023). In participants with high lipopolysaccharide values, the plasma levels of AEA and related N-acylethanolamines, as well as AA and 2-AG, were negatively correlated with plasma levels of lipopolysaccharide (all rho≤-0.24, P≤0.020).

Conclusion: Plasma levels of endocannabinoids and their analogues are correlated to specific fecal bacterial genera involved in maintaining gut barrier integrity in young adults. This suggests that plasma levels of endocannabinoids and their analogues may play a role in the gut barrier integrity in young adults.

INTRODUCTION

The endocannabinoid system is mainly composed of cannabinoid receptors 1 and 2 (CB_1 and CB_2), several endogenous lipids called endocannabinoids (endocannabinoids), and metabolic enzymes involved in their synthesis and degradation[1]. Anandamide (AEA), a member of the N-acylethanolamines class, and 2-arachidonoylglycerol (2-AG), as part of the acylglycerol class, are the two primary endocannabinoids[2]. Structural analogues of these two endocannabinoids, as well as arachidonic acid (AA), which is the downstream metabolite of the primary endocannabinoids AEA and 2-AG, also belong to the endocannabinoid system. endocannabinoids and their analogues are produced "on demand" in the brain, liver, adipose tissue, skeletal muscle, and pancreas[3]. Among other actions, they can and exert either pro- or anti-inflammatory actions and modulate the immune response[4]. Interestingly, the endocannabinoid system seems to be involved in gut physiology[5].

The gastrointestinal tract is colonized by microbial communities called gut microbiota[6], which play a relevant role in regulating the innate and adaptive immune system, gut motility, gut barrier homeostasis, nutrient absorption and fat distribution[7]. Dysbiosis, defined as an imbalance in microbial communities[8], can be induced by multiple factors, such as an unhealthy diet[9] or the use of antibiotics and other drugs[10,11], and is related to obesity and non-communicable chronic diseases[12]. Dysbiosis induces gut barrier dysfunction, which leads to the translocation from the gut into the bloodstream of Gram-negative bacterial components, such as lipopolysaccharide[13]. This fact leads to metabolic endotoxemia and systemic inflammation, characterized by an increase in gut permeability[14]. Therefore, changes in the gut microbiota composition result in gut barrier dysfunction[15].

Recent evidence has shown that the endocannabinoid system can modulate the gut microbiota composition in mice models[5,16]. Conditional adipocyte-specific deficiency in mice of N-acyl phosphatidylethanolamine phospholipase D, an enzyme involved in the synthesis of N-acylethanolamines, increased body weight and fat mass, inducing gut microbiota dysbiosis[17]. Plasma levels of certain N-acylethanolamines were positively correlated with the relative abundance of fecal bacteria in middle adults[18]. Several studies have shown that the endocannabinoid system plays a role in regulating gastric secretion[5], motility[5], and gut permeability[19] by preserving or damaging the gut barrier integrity[20], by yet unknown mechanisms.

To date, there is no scientific evidence in humans on whether plasma levels of endocannabinoids and their analogues are associated with fecal microbiota genera involved in the maintenance of gut barrier integrity. This study aimed to investigate the association of plasma levels of endocannabinoids and their analogues with fecal microbiota diversity and composition in young adults.

MATERIAL AND METHODS

Study design and participants

This is a cross-sectional study that was conducted within the framework of the ACTIBATE study[21], an exercise-based randomized controlled trial (Clinical Trials.gov ID: NCT02365129). The present study included baseline data of 92 healthy young adults (27 men and 65 women, age: 18–25 years old). All assessments were performed in Granada (Spain) between October and November 2016. The included participants (i) were physically inactive and had a sedentary lifestyle (less of 20 min moderate to vigorous physical activity on less of 3 days per week), (ii) had stable body weight during the last 3 months (<3 kg change), (iii) were not smokers, (iv) did not take any medication (including antibiotics) in the last 3 months, (v) did not present any acute neither chronic illness, and (vi) were not pregnant.

The participants signed an informed consent that together with study protocol were performed in accordance with the Declaration of Helsinki, as revised in 2013. Moreover, both were approved by the Human Research Ethics Committee of the University of Granada (n°924) and the Centro de Granada, CEI-Granada.

Anthropometry and body composition assessment

We measured weight and height with a SECA scale and a stadiometer, respectively (model 799, Electronic Column Scale, Hamburg, Germany). Dual Energy X-ray Absorptiometry (DEXA, HOLOGIC, Discovery Wi, Marlborough, MA) was used for determining lean mass, fat mass and visceral adipose tissue mass. Body mass index (BMI), lean mass index and fat mass index were computed as weight, lean body mass and fat body mass (kg), respectively, divided by height squared (m^2). The fat mass percentage was determined as the body fat mass divided by the total body mass and multiplied by 100. We measured the waist circumference (cm) in the minimum perimeter, at the end of a normal expiration, with the arms of the participants were relaxed on both sides of the body. However, if the minimum perimeter could not be detected (i.e., participants with overweight/obesity), it was measured just above the umbilicus, in a horizontal plane. Waist circumference was determined as the average of two measures with a plastic tape measure.

Determination of plasma levels of endocannabinoids and their analogues

Endocannabinoids (i.e., AEA, 2-AG) and their analogues including docosahexaenoylethanolamide (DHEA), dihomo-gamma-linolenoylethanolamide (DGLEA), linoleylethanolamine (LEA), alpha-linolenoylethanolamide (alpha LEA), palmitoylethanolamine (PEA), pentadecanoylethanolamide (PDEA), palmitoleoylethanolamide (POEA), oleoylethanolamine (OEA), and stearoylethanolamine (SEA), which belong to the N-acylethanolamines class, and 2-linoleoylglycerol (2-LG) and

oleoylglycerol (2-OG), which belong to the acylglycerol class[22,23], as well as AA were analyzed in plasma samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The method followed the FDA bioanalytical method validation guidelines[24].

❖ Sample preparation

The sample preparation procedure was carried out on ice to prevent analyte degradation. Endocannabinoids and their analogues were extracted using liquid-liquid extraction. Prior to the extraction, 5 µL of an antioxidant solution composed of 0.4 mg/mL of butylated hydroxytoluene (BHT) and 10 µL of an internal standard solution containing the isotopically-labelled analogues were added to 150 µL of thawed plasma samples. Then, 150 µL of a buffer solution composed of 0.2 M citric acid and 0.1 M disodium hydrogen phosphate at pH 4.5 were added to the samples. Subsequently, 1 mL extraction solution composed of butanol (ButOH) and methyl-tertbutylether (MTBE) in a ratio 50:50 (*v*/*v*) was added prior to agitation for 5 min using a bullet blender and centrifugation at 16,000*g* for 10 min at 4°C. The organic supernatant (900 µL) was collected and evaporated to dryness using a SpeedVac instrument at room temperature. The dry residues were then reconstituted in 50 µL of an ice-cold solution of methanol (MetOH) and acetonitrile (ACN) 70:30 (*v*/*v*), prior to agitation (5 min) and centrifugation at 16,000*g* for 10 min at 4ºC. Finally, 40 µL of the supernatant was transferred into a glass vial for further liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

❖ Liquid chromatography-tandem mass spectrometry analysis

Relative quantitation of endocannabinoids and their analogues were performed using a SCIEX QTRAP® LC-ESI-MS/MS System (SCIEX, Framingham, Massachusetts, USA). The separation was performed using a BEH C18 column (50 mm × 2.1 mm, 1.7 µm) from Waters Technologies (Milford, MA, USA) maintained at 40°C. The mobile phase was composed of 0.1% acetic acid in water (A), ACN/0.1% acetic acid in MeOH (90:10, *v*/*v*) (B), and 0.1% acetic acid in isopropanol (C). The gradient was the following: starting condition 20% B and 1% C; increase of B from 20% to 26% between 0.75 min and 0.95 min; increase of B from 26% to 34% between 0.95 min and 6min; increase of B from 34% to 40% between 6 min and 8 min; increase of B from 40% to 54% between 8 min and 10 min; increase of B from 54% to 56% between 10 min and 12 min; increase of C from 1% to 3% between 11 min to 12 min; increase of B from 56% to 78% between 12 min and 13 min; increase of C from 3% to 6% between 12 min to 13 min; increase of B from 78% to 85% between 13 min and 14 min; increase of C from 6% to 15% between 13 min to 14 min; conditions kept for 0.5 min prior to returning to initial conditions at 14.8 min and re-equilibration for 1.2 min. The flow rate was 0.7 mL/min. The injection volume was 10 µL, preceded by an injection of 20 µL of mobile phase A as stacking solution to improve the peak shape and increase sensitivity. MS acquisition was carried out in positive mode, with the following electrospray ionization (ESI) parameters: source temperature, 600°C; Gas 1 (nebulizer gas), 50 L/min; Gas 2 (heater gas), 50 L/min; curtain gas, 30 L/min; collision gas, medium; ion spray

voltage, 5500 V. Selected reaction monitoring was used for tandem MS experiments.

- **Data pre-processing**

SCIEX OS-MQ Software was used for peak detection and integration. The ratio of the analyte peak area to the peak area of the corresponding isotopically-labelled internal standard, referred to as *peak area ratio*, was used for further data analysis. Quality control samples were prepared using plasma samples from healthy subjects. Quality control samples were prepared simultaneously with the study samples and regularly injected throughout the sequences. The results obtained for quality control samples were used to evaluate the quality of the data, including blank effect, retention time shifts, and peak area ratios, as well as peak area ratios, and correct for between batch variations using the in-house developed mzQuality workflow (available at http://www.mzQuality.nl, (accessed on 1 February 2020))[25]. We calculated relative standard deviations for each internal standard and analyte present in the quality control samples. Metabolites showing relative standard deviations higher than 30% on peak area ratios in quality control samples were excluded, whereas metabolites with relative standard deviations between 15% and 30% were treated with caution (**Table 1**). The acylglycerols are biologically present under two isomeric forms, namely, 1-AG and 2-AG, 1-LG and 2-LG, as well as 1-OG and 2-OG, respectively. For each isomer pair, a baseline separation was obtained between the two isomers with the developed LC-MS/MS method. However, the isomeric conversion due to acyl transformation of the 2-isomer into 1-isomer after sampling is a known mechanism, notably reported for 2-AG[26]. Since this conversion could not be experimentally controlled, we summed the peak areas of both isomers 1-AG and 2-AG before calculating the peak area ratio, and labeled the isomer pair “2-AG”. The same strategy was applied for the isomer pair 1-LG and 2-LG, as well as 1-OG and 2-OG, labeled “2-LG” and “2-OG”, respectively.

Table 1. Descriptive characteristics of the participants.

	N	Mean ± SD
Sex (women, %)	92	71 %
Age (years)	92	22 ± 2
Anthropometry and body composition assessment		
Body mass index (kg/m^2)	90	24.87 ± 4.72
Lean mass (kg)	83	41.13 ± 8.98
Fat mass (kg)	83	24.91 ± 9.09
Lean mass index (kg/m^2)	83	14.43 ± 2.24
Fat mass index (kg/m^2)	83	8.84 ± 3.14
Fat mass percentage (%)	83	35.94 ± 7.88
Visceral adipose tissue (g)	83	321.05 ± 177.19
Waist circumference (cm)	90	80.60 ± 13.97
Plasma levels of endocannabinoids and their analogues (peak area ratio)		
AEA and related N-acylethanolamines		
AEA	88	0.14 ± 0.06
AA	88	65.18 ± 21.75
DHEA*	88	0.08 ± 0.16
DGLEA*	87	0.02 ± 0.01
LEA	88	0.22 ± 0.09
alpha LEA	88	0.008 ± 0.004
PEA	87	1.78 ± 0.28
PDEA*	88	0.03 ± 0.01
POEA	88	0.28 ± 0.22
OEA	88	0.70 ± 0.21
SEA	88	1.33 ± 0.23
2-AG and related acylglycerols		
2-AG*	88	0.02 ± 0.09
2-LG*	88	0.22 ± 1.41
2-OG*	88	0.01 ± 0.06
Fecal microbiota parameters		
Alpha diversity indexes		
Richness Chao	92	430.68 ± 150.99
Shannon diversity	92	4.21 ± 0.37
Inverse Simpson diversity	92	34.20 ± 14.16
Evenness Camargo	92	0.24 ± 0.05
Composition (phylum)		
Actinobacteria (%)	92	1.59 ± 1.55
Bacteroidetes (%)	92	39.80 ± 9.06
Firmicutes (%)	92	48.65 ± 10.10
Proteobacteria (%)	92	6.81 ± 5.29
Verrucomicrobia (%)	92	2.09 ± 4.12
Plasma levels of lipopolysaccharide (EU/mL)	85	0.98 ± 1.10

Data are presented as means ± standard deviations (SD). Abbreviations: 2-AG: 2-arachidonylglycerol; 2-LG: 2-linoleoylglycerol; 2-OG: 2-oleoylglycerol; alpha LEA: alpha-Linolenoyl ethanolamide; AA: arachidonic acid; AEA: anandamide; DGLEA: dihomo-gamma-linolenoyl ethanolamide; DHEA: docosahexaenoyl ethanolamide; EU: endotoxins units; LEA: linoleoyl ethanolamide; OEA: oleoyl ethanolamine; PEA: palmitoyl ethanolamide; PDEA: pentadecanoyl ethanolamide; POEA: palmitoleoyl ethanolamide; SEA: stearoyl ethanolamide. *Analytes to be considered with caution as relative standard deviations between 15% and 30% in quality control samples.

Fecal microbiota analyses

❖ Stool collection and DNA extraction

The participants collected a fecal sample (~50-60 g) in plastic sterile containers, which they transported in a portable cooler to the research center. Fecal samples were stored at -80°C until the extraction of DNA. We used a QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain) to extract DNA, following the manufacturer's instructions, and incubating the samples at 95ºC to ensure lysis of both Gram-positive and Gram-negative bacteria. The quantification of DNA was performed using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, DE, USA). The ratio of absorbance at A260/280nm and A260/230nm was used for measuring DNA purity.

❖ Sequencing analysis

DNA extracted was amplified by PCR by primer pairs, 16S Amplicon PCR Forward Primer: 5'CCTACGGGNGGCWGCAG, and 16S Amplicon PCR Reverse Primer: 5'GACTACHVGGGTATCTAATCC targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene[27]. We performed all PCRs using 25 µL reaction volumes, incorporating 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). The cycle steps were as follows: (i) denaturation at 95ºC for 3 min, (ii) 8 cycles of denaturation at 95ºC for 30 s, (iii) annealing at 55ºC for 30 s, (iv) elongation at 72ºC for 30 s, (v) final extension at 72ºC for 5 min. AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) were used to purify the 16S V3 and V4 amplicons from free primers and primer dimers. The index PCR was performed using the Nextera XT Index Kit (Illumina, San Diego, CA, USA), which attaches dual indices and Illumina sequencing adapters, on a thermal cycler using the requirements previously mentioned. Before quantification, AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) were used for purifying the pooled PCR products. The resulting amplicons were sequenced at MiSeq (Illumina, San Diego, CA, USA), using a paired-end (2x300nt) Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA).

❖ Bioinformatics analysis

FastQ files were analyzed with the "dada2"[28] package in R software[29]. We obtained 11,659,014 paired-ends with an average of 126,728 ± 33,395 reads per sample. All samples surpassed a cut-off of 10,000 reads. Samples were resampled to an equal sequencing depth of 30,982 reads using the "phyloseq"[30] package in R[29], retrieving 11,158 phylotypes.

The taxonomic affiliation of phylotypes was assigned using the "classifier" function from the Ribosomal Database Project (RDP), based on the naive Bayesian classification[31] with a pseudo-bootstrap threshold of 80%. A total of 209 genera that belong to 16 different phyla were obtained. In order to determinate further annotation of the phylotypes to species assignments, the

"seqmatch"[32] function from RDP was performed to define the discriminatory power of each sequence read; we executed annotation according to criteria previously published[33]. Microbial communities were analyzed at different taxonomic levels (phylum to species), calculating the relative abundances, which were expressed as percentages. We performed the analyses using the genera with an average relative abundance of more than 0.5%. Only phylotypes found in at least 50% of the participants were submitted to species taxonomy level assignation.

Beta and alpha diversities were estimated based on the identified microbial communities. Beta diversity shows the differences in microbial community composition between individuals, *i.e.*, the degree to which samples differ from one another[34], whereas the alpha diversity considers the number of different phylotypes and relative abundances within a given individual[35]. The beta diversity was quantitatively measured using permutational multivariate analysis of variance (PERMANOVA) based on the Bray-Curtis[36] similarity. The alpha diversity was assessed using richness Chao, Shannon, inverse Simpson and evenness Camargo indexes with the "microbiome"[37] package using R software[29]. The Richness Chao index estimates the diversity according to the number of different phylotypes in the community[38]; the Shannon diversity increases as both the richness and the evenness of the community increase[39]; the inverse of Simpson diversity is derived from the classical Simpson diversity and indicates the richness in a community with uniform evenness[40]; and the evenness Camargo index determines the equitability of phylotypes frequencies in a community[41].

Determination of plasma levels of lipopolysaccharides

Plasma levels of lipopolysaccharide were determined by a chromogenic limulus amebocyte lysate (LAL) assay according to the manufacturer guidelines (LAL Chromogenic Endpoint Assay; HycultBiotech, Uden, The Netherlands) as previously reported[42]. With the purpose of inactivating endotoxin neutralizing agents, we diluted the plasma samples in pyrogen-free water and heated them to 70 °C for 10 min. All samples were measured in duplicate, and results were accepted when the intra-assay coefficient of variation was <10%. Internal recovery controls were included in the assessment.

Statistical analysis

Data are presented as means ± standard deviations unless otherwise stated. All variables were tested for normality using the D'Agostino & Pearson omnibus test. Since most of the variables did not display a normal distribution, non-parametric tests were used for all analyses. It has been unequivocally demonstrated that human plasma levels of endocannabinoids and their analogues are related to adiposity markers such as BMI and visceral adipose tissue mass[43,44]. Based on that, we performed and presented all the analyses adjusting for BMI and the data from both sexes pooled together since we did not detect any sex interaction (all P>0.05). To calculate beta diversity, plasma levels of endocannabinoids and their analogues were divided into tertiles (low,

intermediate and high groups) and compared using a two-way PERMANOVA with 9,999 permutations for significance testing with the Paleontological Statistics (Past3) software. Partial Spearman correlations were used to investigate the correlation of plasma levels of endocannabinoids and their analogues with fecal microbiota alpha diversity and composition, using the "psych"[45] and "corrplot"[46] packages in R software. Next, we divided the participants into quartiles of plasma levels of lipopolysaccharide and we performed partial Spearman correlations, as described above, between the plasma levels of endocannabinoids and their analogues with plasma levels of lipopolysaccharide in Q1, Q4, and the whole cohort. Kruskal-Wallis tests were performed to investigate whether there were significant differences in body composition, plasma levels of endocannabinoids and their analogues and gut microbiota composition outcomes between the different quartiles of plasma levels of lipopolysaccharide with GraphPad Prism. The level of significance was assumed at $P<0.05$. R software (V.3.6.0; http://www.R-project.org, (accessed on 1 June 2019)) and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA) were used for the statistical analysis and graphical plots.

RESULTS

Characteristics of the participants

Table 1 shows the descriptive characteristics of the participants. AEA and seven of its analogues (*i.e.*, AA, LEA, alpha LEA, PEA, POEA, OEA and SEA) showed relative standard deviations values of ≤15%, whereas 2-AG and five of its analogues (*i.e.*, DHEA, DGLEA, PDEA, 2-LG and 2-OG) showed relative standard deviations values between 15% and 30%.

The plasma levels of endocannabinoids and their analogues are not related to fecal microbiota beta and alpha diversities

The beta diversity was similar across the tertiles of the plasma endocannabinoids and their analogues at both the phylum and genus taxonomic levels (all P≥0.312; **Table 2**). Overall, the plasma levels of endocannabinoids and their analogues were not related to the alpha diversity indexes (**Fig. 1**). However, we observed that plasma levels of DHEA (related N-acylethanolamines) were positively correlated with the richness Chao index (rho=0.23, P=0.032; **Fig. 1**), whereas plasma levels of 2-LG and 2-OG (related acylglycerols) were negatively correlated with the evenness Camargo index (rho= -0.30, P=0.004, and rho= -0.31, P=0.003, respectively; **Fig. 1**). We repeated all the analyses adjusting for sex, BMI and energy intake in separate models and the results remained the same (data not shown).

Table 2. Beta diversity across different tertiles of endocannabinoids and their analogues at phylum and genus taxonomic levels.

Tertiles of the plasma levels of endocannabinoids and their analogues (peak area ratio)	**Phylum**		**Genus**	
	Pseudo-F	*P*	*Pseudo-F*	*P*
AEA and related N-acylethanolamines				
AEA	-6.626	0.383	-6.789	0.336
AA	-5.918	0.348	-6.275	0.397
DHEA	-6.581	0.548	-6.620	0.684
DGLEA	-6.339	0.657	-6.146	0.579
LEA	-6.469	0.405	-6.670	0.418
alpha LEA	-6.211	0.361	-6.854	0.746
PEA	-6.032	0.591	-5.996	0.723
PDEA	-5.818	0.312	-6.370	0.485
POEA	-6.495	0.423	-6.516	0.336
OEA	-6.410	0.372	-6.645	0.369
SEA	-6.460	0.373	-6.797	0.442
2-AG and related acylglycerols				
2-AG	-6.281	0.640	-5.808	0.473
2-LG	-6.449	0.570	-6.326	0.595
2-OG	-7.041	0.578	-6.927	0.629

PERMANOVA using 9,999 permutations for significance testing (p-value<0.05), adjusted for body mass index. Abbreviations: 2-AG: 2-arachidonylglycerol; 2-LG: 2-linoleoylglycerol; 2-OG: 2-oleoylglycerol; alpha LEA: alpha-Linolenoyl ethanolamide; AA: arachidonic acid; AEA: anandamide; DGLEA: dihomo-gamma-linolenoyl ethanolamide; DHEA: docosahexaenoyl ethanolamide; LEA: linoleoyl ethanolamide; OEA: oleoyl ethanolamine; PEA: palmitoyl ethanolamide; PDEA: pentadecanoyl ethanolamide; POEA: palmitoleoyl ethanolamide; Pseudo-F: statistic, larger number indicate greater separation between tertiles of plasma levels of endocannbinoids and their analogues; SEA: stearoyl ethanolamide.

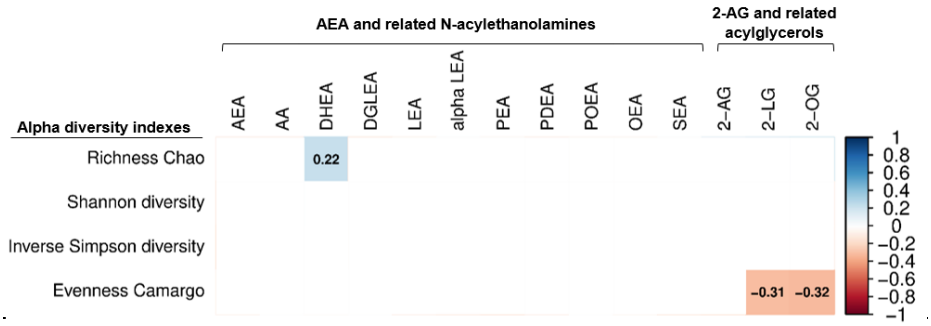

Figure 1. Partial Spearman correlations of plasma levels of endocannabinoids and their analogues with fecal microbiota alpha diversity indexes. *Boxes only represent the statistically significant (P<0.05) correlations and the value within the boxes show the spearman correlation coefficient. Blue boxes indicate positive correlations whereas red boxes indicate negatives correlation. Body mass index was included as confounder in these correlations. Abbreviations: 2-AG: 2-arachidonylglycerol; 2-LG: 2-linoleoylglycerol; 2-OG: 2-oleoylglycerol; alpha LEA: alpha-Linolenoyl ethanolamide; AA: arachidonic acid; AEA: anandamide; DGLEA: dihomo-gamma-linolenoyl ethanolamide; DHEA: docosahexaenoyl ethanolamide; LEA: linoleoyl ethanolamide; OEA: oleoyl ethanolamine; PEA: palmitoyl ethanolamide; PDEA: pentadecanoyl ethanolamide; POEA: palmitoleoyl ethanolamide; SEA: stearoyl ethanolamide.*

The plasma levels of endocannabinoids and their analogues levels are related to the relative abundance of specific fecal microbiota genera

The plasma levels of AEA and related N-acylethanolamines, and AA were positively correlated with the relative abundance of *Verrucomicrobia* and *Firmicutes* phyla, and negatively with the relative abundance of *Proteobacteria* phylum. Similarly, the plasma levels of 2-AG and related acylglycerols were positively correlated with the relative abundance of *Proteobacteria* phylum. Specifically, we observed that the plasma levels of LEA, alpha LEA, PDEA, POEA and OEA levels were positively correlated with the relative abundance of *Akkermansia* genus (*Verrucomicrobia* phylum; all rho≥0.22, P≤0.036; **Fig. 2A**). Additionally, the plasma levels of PEA and SEA were positively correlated with the relative abundance of *Lachnospiraceae incertae sedis* (*Firmicutes* phylum; all rho≥0.26, P≤0.010; **Fig. 2A**) and *Faecalibacterium* genera (*Firmicutes* phylum; all rho≥0.26, P≤0.012; **Fig. 2A**). On the opposite, the plasma levels of AEA and related N-acylethanolamines (*i.e.*, DGLEA, LEA, PDEA and OEA), as well as AA, were negatively correlated with the relative abundance of *Bilophila* genus (*Proteobacteria* phylum; all rho≤-0.23, P≤0.031; **Fig. 2A**). Similarly, the plasma levels of alpha LEA, PEA and SEA were negatively correlated with the relative abundance of *Succinivibrio* genus (*Proteobacteria* phylum; both rho≤-0.23, P≤0.027; **Fig. 2A**). Further, the plasma levels of 2-AG and the related acylglycerols 2-LG and 2-OG were positively correlated with the relative abundance of *Parasutterella* (*Proteobacteria* phylum; all rho≥0.24, P≤0.020; **Fig. 2B**) and *Odoribacter* genera (*Bacteroidetes* phylum; all rho≥0.27, P≤0.011; **Fig. 2B**). In contrast, the plasma levels of 2-AG and 2-LG only were negatively correlated with the relative abundance of *Prevotella* genus (*Bacteroidetes* phylum; rho≤-0.24, P≤0.023; **Fig. 2B**). The results were similar when visceral adipose tissue mass was included as a confounder instead of BMI or physical activity levels (data not shown).

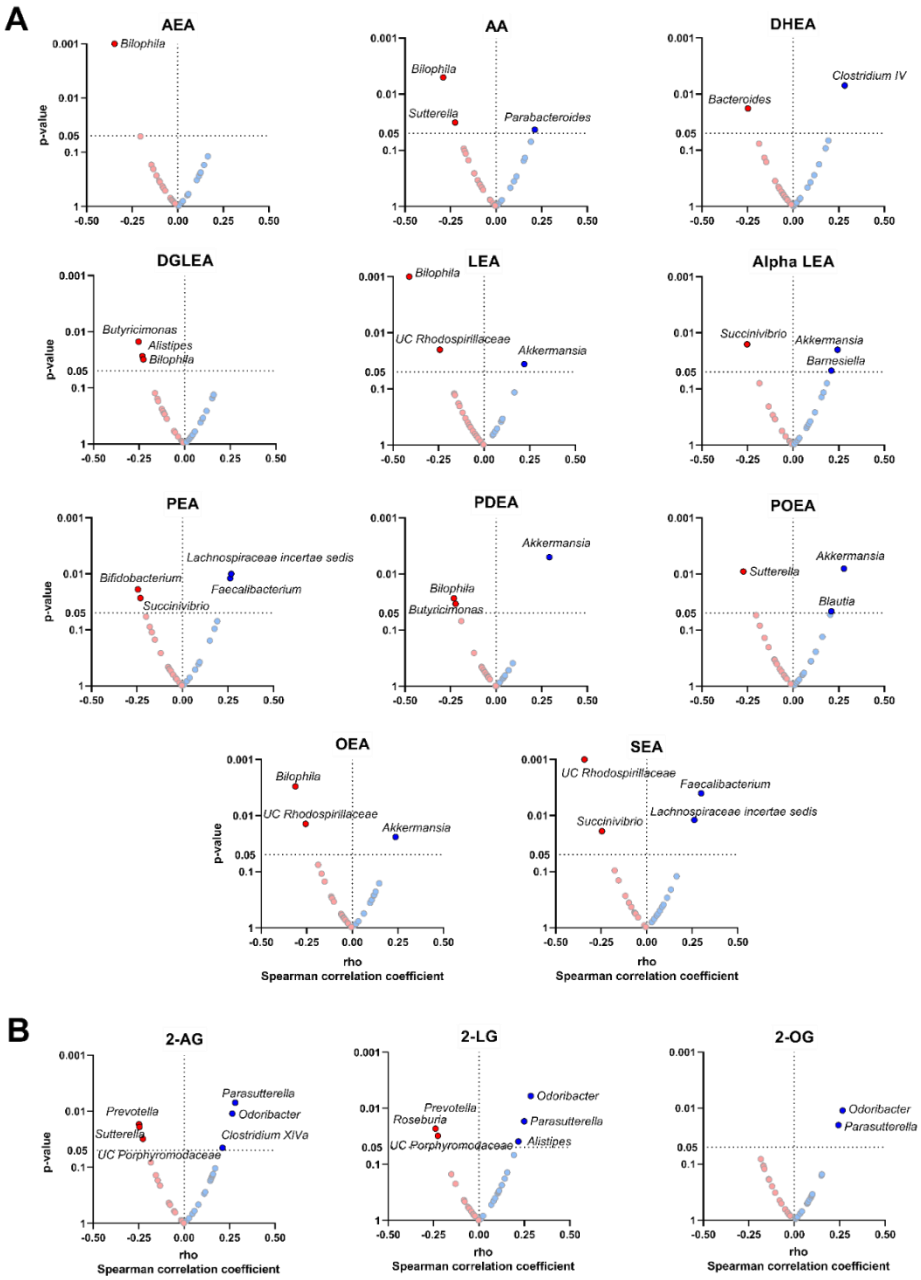

Figure 2. Volcano plots showing partial Spearman correlations of plasma levels of endocannabinoids and their analogues with fecal microbiota composition at genus taxonomic level. *Each volcano plot shows the rho coefficients in the X-axis, whereas the p-values of the correlations are shown on the Y-axis. Panel A shows AEA and related N-acylethanolamines and panel B shows 2-AG and acylglycerols. Red circles indicate negative correlations, whereas blue circles indicate positive correlations. Body mass index was included as a confounder in these correlations. Only those genera with significant p-values (P<0.05) are annotated within the figure. Abbreviations: 2-AG: 2-arachidonylglycerol; 2-LG: 2-linoleoylglycerol; 2-OG: 2-oleoylglycerol;*

alpha LEA: alpha-Linolenoyl ethanolamide; AA: arachidonic acid; AEA: anandamide; DGLEA: dihomo-gamma-linolenoyl ethanolamide; DHEA: docosahexaenoyl ethanolamide; LEA: linoleoyl ethanolamide; OEA: oleoyl ethanolamine; PEA: palmitoyl ethanolamide; PDEA: pentadecanoyl ethanolamide; POEA: palmitoleoyl ethanolamide; SEA: stearoyl ethanolamide; UC: unclassified.

The plasma levels of endocannabinoids and their analogues are related to the relative abundance of specific bacterial species

We investigated whether the relative abundance of specific bacterial species could drive the significant correlations observed between the plasma levels of endocannabinoids and their analogues with the relative abundance of different genera (**Fig. 3**). We found that the plasma levels of AEA, AA, OEA and SEA were positively correlated with the relative abundance of *Bacteroides dorei* (*Bacteroidetes* phylum; all rho≥0.206, P<0.05; **Fig. 3A**), whereas the plasma levels of alpha LEA and POEA were positively associated with the relative abundance of *Parasutterella excrementihominis* (*Proteobacteria* phylum; all rho≥0.236, P≤0.024; **Fig. 3A**). Additionally, the plasma levels of SEA were positively associated with the relative abundance of *Faecalibacterium prausnitzii* (*Firmicutes* phylum; rho=0.245, P=0.019; **Fig. 3A**). Opposite to this, the plasma levels of AEA and DHEA, as well as AA, were negatively correlated with the relative abundance of *Bacteroides vulgatus* (*Bacteroidetes* phylum; all rho≤-0.211, P≤0.045; **Fig. 3A**). Moreover, the plasma levels of AA and OEA, as well as 2-AG and the related acylglycerol 2-LG were negatively correlated with the relative abundance of *Roseburia inulinivorans* (*Firmicutes* phylum; all rho≤-0.219, P≤0.037; **Fig. 3A and 3B**). All associations remained significant when visceral adipose tissue mass was included as a confounder instead of BMI or physical activity levels (data not shown).

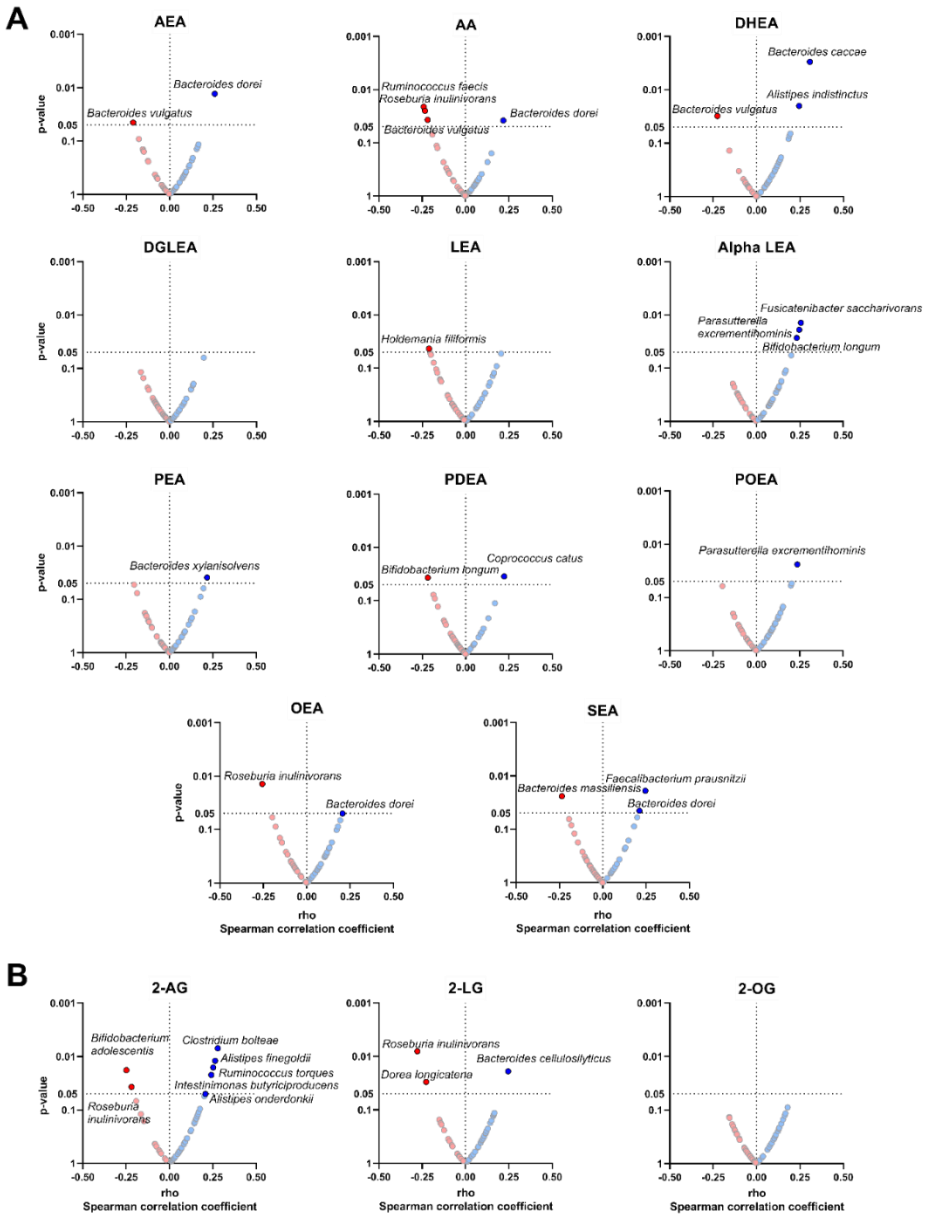

Figure 3. Volcano plots showing partial Spearman correlations of plasma levels of endocannabinoids and their analogues with the relative abundance of certain bacterial species. *Each volcano plot shows the rho coefficients in the X-axis, and p-values of the correlations are shown on the Y-axis. Panel A shows AEA and related N-acylethanolamines, whereas panel B shows 2-AG and related acylglycerols. Red circles indicate negative correlations, whereas blue circles indicate positive correlations. Body mass index was included as a confounder in these correlations. Only those bacterial species with significant p-values (P<0.05) are annotated within the figure. Abbreviations: 2-AG: 2-arachidonylglycerol; 2-LG: 2-linoleoylglycerol; 2-OG: 2-oleoylglycerol; alpha LEA: alpha-Linolenoyl ethanolamide; AA: arachidonic acid; AEA: anandamide; DGLEA: dihomo-gamma-linolenoyl ethanolamide; DHEA: docosahexaenoyl ethanolamide; LEA: linoleoyl ethanolamide; OEA: oleoyl ethanolamine; PEA: palmitoyl*

ethanolamide; PDEA: pentadecanoyl ethanolamide; POEA: palmitoleoyl ethanolamide; SEA: stearoyl ethanolamide.

The plasma levels of endocannabinoids and their analogues are negatively correlated to plasma levels of lipopolysaccharides but only in those participants with high plasma levels of lipopolysaccharides.

We quantified plasma levels of lipopolysaccharide since it is an indirect marker of gut permeability[47]. We observed that plasma levels of endocannabinoids and their analogues were not correlated with the plasma levels of lipopolysaccharide (P≥0.385; **Table 3** and **4**). We then divided the cohort into quartiles of plasma levels of lipopolysaccharide: Q1 (0.14-0.35 EU/mL), Q2 (0.36-0.50 EU/mL), Q3 (0.50-1.22 EU/mL) and Q4 (1.35-5.45 EU/mL). We followed this approach because we wanted to focus in the comparison of extreme values, as well as to explore non-lineal associations. Overall, body composition, plasma levels of endocannabinoids and their analogues, and fecal microbiota composition were similar across the quartiles (**Table 5**). We found that plasma levels of AEA and related N-acylethanolamines (except for DHEA), as well as AA and 2-AG were positively correlated with plasma levels of lipopolysaccharide (all rho≥0.22, P≤0.035; **Table 6** and **7**) only in those participants in Q1. On the opposite, plasma levels of AEA and related N-acylethanolamines (*i.e.*, DGLEA, LEA, PEA, PDEA, POEA and OEA), as well as AA and 2-AG were negatively correlated with plasma levels of lipopolysaccharide (all rho≤-0.24, P≤0.020; **Table 6** and **7**) in participants in Q4. Overall, we found no correlation between the relative abundance of different genera in the feces with the plasma levels of lipopolysaccharide (**Table 8** and **9**).

Table 3. Partial Spearman correlation of plasma levels of AEA and related N-acylethanolamines with plasma levels of lipopolysaccharide adjusted for body mass index.

	AEA and related N-acylethanolamines (peak area ratio)																					
	AEA		AA		DHEA		DGLEA		LEA		Alpha LEA		PEA		PDEA		POEA		OEA		SEA	
	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P
LPS N=85 (EU/mL)	-0.035	0.740	-0.001	0.989	0.050	0.635	0.039	0.716	-0.092	0.385	-0.089	0.401	0.038	0.718	-0.040	0.704	-0.018	0.867	-0.057	0.594	-0.067	0.526

Correlation analyses were performed adjusting for body mass index. Spearman's correlations coefficient (rho) and P-values (P) obtained from univariate Spearman correlation analyses are provided. Abbreviations: alpha LEA: alpha-Linolenoyl ethanolamide; AA: arachidonic acid; AEA: anandamide; DGLEA: dihomo-gamma-linolenoyl ethanolamide; DHEA: docosahexaenoyl ethanolamide; EU: endotoxins units; LEA: linoleoyl ethanolamide; LPS: lipopolysaccharide; OEA: oleoyl ethanolamine; PEA: palmitoyl ethanolamide; PDEA: pentadecanoyl ethanolamide; POEA: palmitoleoyl ethanolamide; SEA: stearoyl ethanolamide.

Table 4. Partial Spearman correlation of plasma levels of 2-AG and related acylglycerols with plasma levels of lipopolysaccharide adjusted for body mass index.

	2-AG and related acylglycerols (peak area ratio)					
	2-AG		2-LG		2-OG	
	rho	P	rho	P	rho	P
LPS N=85 (EU/mL)	0.032	0.765	-0.005	0.966	-0.060	0.575

Correlation analyses were performed adjusting for body mass index. Spearman's correlations coefficient (rho) and P-values (P) obtained from univariate Spearman correlation analyses are provided. Abbreviations: 2-AG: 2-arachidonylglycerol; 2-LG: 2-linoleoylglycerol; 2-OG: 2-oleoylglycerol; EU: endotoxins units; LPS: lipopolysaccharide.

Table 5. Characteristics of the individuals according to quartiles of plasma levels of lipopolysaccharide.

	Quartiles of plasma levels of LPS			
	Q1 (0.14-0.35 EU/mL) n=19	Q2 (0.36-0.50 EU/mL) n=23	Q3 (0.50-1.22 EU/mL) n=22	Q4 (1.35-5.45 EU/mL) n=21	
					P
Age (years)	22 ± 2	22 ± 2	22 ± 2	22 ± 3	0.95
Sex, N					0.24
Men	3	5	7	9	
Women	16	18	15	12	
Body composition parameters					
BMI (kg/m^2)	23.54 ± 5.34	24.08 ± 4.27	25.73 ± 5.11	26.45 ± 4.14	0.05
FMI (kg/m^2)	8.29 ± 3.89	8.69 ± 2.83	9.03 ± 3.07	9.80 ± 2.75	0.33
LMI (kg/m^2)	13.94 ± 1.89	13.90 ± 2.03	15.13 ± 2.60	14.79 ± 2.51	0.25
Plasma levels of endocannabinoids and their analogues (peak area ratio)					
AEA and related N-acylethanolamines					
AEA	0.15 ± 0.09	0.13 ± 0.05	0.14 ± 0.04	0.14 ± 0.05	0.69
AA	69.30 ± 19.65	64.24 ± 21.88	67.53 ± 27.45	66.62 ± 19.62	0.95
DHEA	0.07 ± 0.03	0.12 ± 0.31	0.06 ± 0.02	0.07 ± 0.03	0.62
DGLEA	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.35
LEA	0.25 ± 0.15	0.20 ± 0.06	0.22 ± 0.07	0.21 ± 0.07	0.77
alpha LEA	0.009 ± 0.007	0.008 ± 0.004	0.007 ± 0.003	0.006 ± 0.001	0.75
PEA	1.76 ± 0.32	1.71 ± 0.21	1.81 ± 0.28	1.76 ± 0.22	0.64
PDEA	0.024 ± 0.009	0.024 ± 0.007	0.025 ± 0.009	0.024 ± 0.007	0.99
POEA	0.33 ± 0.36	0.24 ± 0.16	0.28 ± 0.19	0.25 ± 0.16	0.84

	OEA	0.74	±	0.25	0.62	±	0.16	0.70	±	0.21	0.69	±	0.18	0.43
	SEA	1.36	±	0.29	1.23	±	0.17*	1.42	±	0.23*	1.27	±	0.22	**0.04**
2-AG and acylglycerols														
	2-AG	0.008	±	0.007*	0.048	±	0.180	0.028	±	0.032*†	0.008	±	0.006†	**0.01**
	2-LG	0.03	±	0.05	0.62	±	2.73	0.18	±	0.44	0.01	±	0.02	0.07
	2-OG	0.002	±	0.006	0.025	±	0.106	0.012	±	0.034	0.001	±	0.001	0.06
Fecal microbiota composition (genera, %)														
	Bilophila	0.41	±	0.25	0.51	±	0.54	0.53	±	0.36	0.51	±	0.42	0.80
	Akkermansia	3.20	±	4.67	2.75	±	5.56	1.64	±	2.72	1.18	±	2.95	0.30
	Faecalibacterium	5.71	±	3.15	6.68	±	4.69	6.11	±	4.06	5.42	±	2.62	0.90
	Parasutterella	0.72	±	1.16	2.18	±	3.20	1.28	±	2.46	0.46	±	0.70	0.35
	Odoribacter	0.72	±	0.46	0.79	±	0.58	1.14	±	0.70	0.62	±	0.28	**0.04**
	Prevotella	5.17	±	6.60	3.01	±	8.00	4.25	±	7.81	8.11	±	9.87	0.23

Data are presented as means ± standard deviations. Symbols * and † indicate significant differences after correcting for multiple comparisons FDR. Abbreviations: 2-AG: 2-arachidonylglycerol; 2-LG: 2-linoleoylglycerol; 2-OG: 2-oleoylglycerol; alpha LEA: alpha-Linolenoyl ethanolamide; AA: arachidonic acid; AEA: anandamide; BMI: body mass index; DGLEA: dihomo-gamma-linolenoyl ethanolamide; DHEA: docosahexaenoyl ethanolamide; EU: endotoxins units; FMI: fat mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; LEA: linoleoyl ethanolamide; LMI: Lean mass index; LPS: lipopolysaccharide; OEA: oleoyl ethanolamine; PEA: palmitoyl ethanolamide; PDEA: pentadecanoyl ethanolamide; POEA: palmitoleoyl ethanolamide; Q: quartiles; SEA: stearoyl ethanolamide.

Table 6. Partial Spearman correlation of plasma levels of AEA and related N-acylethanolamines with plasma levels of lipopolysaccharide in participants who showed very low and high values (EU/mL) adjusted for body mass index.

	AEA and related N-acylethanolamines (peak area ratio)																			
	AEA		AA		DHEA		DGLEA		LEA		Alpha LEA		PEA		PDEA		POEA		OEA		SEA	
	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P
Q1: Low LPS (N= 19)	0.262	**0.012**	0.228	**0.030**	0.123	0.246	0.221	**0.035**	0.404	**<0.001**	0.428	**<0.001**	0.451	**<0.001**	0.234	**0.026**	0.467	**<0.001**	0.440	**<0.001**	0.428	**<0.001**
Q4: High LPS (N= 21)	-0.464	**<0.001**	-0.389	**<0.001**	-0.001	0.992	-0.472	**<0.001**	-0.300	**0.004**	0.107	0.315	-0.304	**0.003**	-0.281	**0.007**	-0.504	**<0.001**	-0.408	**<0.001**	-0.156	0.139

Correlation analyses were performed adjusting for body mass index. Spearman's correlations coefficient (rho) and P-values (P) obtained from univariate Spearman correlation analyses are provided. The bold indicates p-values <0.05. Abbreviations: alpha LEA: alpha-Linolenoyl ethanolamide; AA: arachidonic acid; AEA: anandamide; DGLEA: dihomo-gamma-linolenoyl ethanolamide; DHEA: docosahexaenoyl ethanolamide; EU: endotoxins units; LEA: linoleoyl ethanolamide; LPS: lipopolysaccharide; OEA: oleoyl ethanolamine; PEA: palmitoyl ethanolamide; PDEA: pentadecanoyl ethanolamide; POEA: palmitoleoyl ethanolamide; Q: quartile; SEA: stearoyl ethanolamide.

Table 7. Partial Spearman correlation of plasma levels of 2-AG and related acylglycerols with plasma levels of lipopolysaccharide in participants who showed very low and high values (EU/mL) adjusted for body mass index.

	2-AG and related acylglycerols (peak area ratio)					
	2-AG		2-LG		2-OG	
	rho	P	rho	P	rho	P
Q1: Low LPS (N=19)	0.374	**<0.001**	0.111	0.295	0.171	0.106
Q4: High LPS (N=21)	-0.243	**0.020**	0.312	**0.003**	0.053	0.615

Correlation analyses were performed adjusting for body mass index. Spearman's correlations coefficient (rho) and P-values (P) obtained from univariate Spearman correlation analyses are provided. The bold indicates p-values <0.05. 2-AG: 2-arachidonylglycerol; 2-LG: 2-linoleoylglycerol; 2-OG: 2-oleoylglycerol; EU: endotoxins units; LPS: lipopolysaccharide; Q: quartile.

Table 8. Spearman correlation of relative abundance of fecal microbiota composition at genera taxonomic level with plasma levels of lipopolysaccharide.

	Genera (%)											
	Bilophila		*Akkermansia*		*Faecalibacterium*		*Parasutterella*		*Odoribacter*		*Prevotella*	
	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P
LPS N=85 (EU/mL)	0.021	0.851	-0.153	0.162	-0.044	0.692	-0.085	0.439	0.043	0.697	0.046	0.675

Spearman's correlations coefficient (rho) and P-values (P) obtained from univariate Spearman correlation analyses are provided. EU: endotoxins units; LPS: lipopolysaccharide.

Table 9. Spearman correlation of relative abundance of fecal microbiota composition at genera taxonomic level with plasma levels of lipopolysaccharide in participants who showed very low and high values (EU/mL).

	Genera (%)											
	Bilophila		*Akkermansia*		*Faecalibacterium*		*Parasutterella*		*Odoribacter*		*Prevotella*	
	rho	P	rho	P	rho	P	rho	P	rho	P	rho	P
Q1: Low LPS (N=19)	-0.679	**≤0.001**	0.320	0.181	0.244	0.313	0.070	0.775	0.236	0.330	-0.278	0.249
Q4: High LPS (N=21)	-0.201	0.383	0.036	0.878	-0.315	0.164	-0.126	0.586	-0.263	0.250	0.003	0.989

Spearman's correlations coefficient (rho) and P-values (P) obtained from univariate Spearman correlation analyses are provided. EU: endotoxins units; LPS: lipopolysaccharide; Q: quartile.

DISCUSSION

The present study shows that the plasma levels of N-acylethanolamines were are positively correlated with the relative abundance of *Akkermansia* and *Faecalibacterium* genera and negatively correlated with the relative abundance of *Bilophila* genus. The plasma levels of acylglycerols were positively correlated with the relative abundance of *Parasutterella* and *Odoribacter* genera and negatively correlated with the relative abundance of *Prevotella* genus. Moreover, we observed a negative and significant correlation between the plasma levels of endocannabinoids and their analogues with the plasma levels of lipopolysaccharide, but only in participants within Q4. These findings suggest that the plasma levels of endocannabinoids and their analogues may be related in the gut barrier integrity through the gut microbiota in young adults[16]. Future studies are needed to ascertain whether endocannabinoids and their analogues could be considered markers of gut barrier integrity in humans.

Role of endocannabinoids and their analogues in gut microbiota diversity

In agreement with previous studies[48], our findings show that the beta diversity was similar across the different plasma endocannabinoids and their analogues tertiles. This could be partially explained by the homogeneity in age and cardiometabolic status of our participants (**Table 1**). Curiously, the plasma levels of DHEA were positive, although weakly, correlated with the richness Chao, an alpha diversity estimator, according to the number of species present in the fecal sample[38]. However, the plasma levels of 2-LG and 2-OG were negatively related to the evenness Camargo, which is a proxy of the equality in the numbers of species within the bacterial community[41]. This might indicate that an increase of plasma levels of 2-LG and 2-OG may be related to the evenness in the bacterial community of young adults, which warrants further investigation.

Role of endocannabinoids and their analogues in the gut barrier integrity

In this study, the plasma levels of LEA, alpha LEA, PDEA, POEA and OEA were positively correlated with the relative abundance of *Akkermansia* genus. *Akkermansia*, as well as other bacteria, generates the short short-chain fatty acids acetate and propionate[49]. These short-chain fatty acids act as a primary energy source for luminal colon cells and nutrients for beneficial commensal microbial promoting gut barrier, as well as also regulating metabolic processes and energy homeostasis[49]. Interestingly, the oral supplementation of OEA for eight weeks caused a significant increase in the relative abundance of *Akkermansia muciniphila* in the colon of individuals with obesity[50]. Furthermore, higher plasma levels of the OEA/PEA ratio showed an increase in the relative abundance of *Akkermansia muciniphila* in the feces of middle-age adults with obesity, which was associated a reduction in systemic inflammation[18]. Interestingly, daily oral gavage administration, for 4 weeks, in the high-fat-diet-

fed mice of *Akkermansia muciniphila* diet-restored the gut mucosal layer and increased gut levels of 2-AG and 2-OG, improving the gut barrier integrity and decreasing metabolic endotoxemia[51]. Therefore, our results suggest that along with OEA, LEA, alpha LEA, PDEA, and POEA could also be contributing to the preservation of the gut barrier integrity by increasing the relative abundance of *Akkermansia* genus.

Our data also show that the plasma levels of PEA and SEA were positively correlated with the relative abundance of *Faecalibacterium* genus, and the plasma level of SEA was positively related to the relative abundance of the *Faecalibacterium prausnitzii* species. *Faecalibacterium* genus and *Faecalibacterium prausnitzii* species are among the most important butyrate-producing bacteria in humans[52]. The SCFA butyrate has anti-inflammatory properties and drives the production of mucin glycoproteins[53], which promote the integrity of gut barrier[14] via secretion of glucagon-like-peptide 1 and 2 by enteroendocrine cells[54]. Similar to butyrate, the PEA present in the membrane of enteroendocrine cells is able to bind to G-protein-coupled receptor 119, reinforcing the gut barrier via secretion of glucagon-like-peptide 1 and 2. This might explain the positive association between PEA and *Faecalibacterium* genus in their protective role of the gut barrier, whereas whether it exists biological meaning of the relationship between plasma levels of SEA and this genus is unknown.

Regarding acylglycerols, we found that the plasma levels of 2-AG, 2-LG and 2-OG were positively associated with the relative abundance of *Parasutterella* and *Odoribacter* genera. Similarly, plasma levels of 2-LG were positively associated with the relative abundance of *Parasutterella* genus in mice fed with high-fat and high-sucrose diets[55]. *Parasutterella* genus influences positively the gut barrier homeostasis, via the production of short-chain fatty acids and hypoxanthine[56], a purine that improves cellular energetics and cytoskeletal function[57]. Furthermore, an increase in the relative abundance of *Odoribacter* genus was linked to an anti-obesity phenotype within the gut in Zucker rats fed with standard-chow diet, via an increase in butyrate production[58]. Altogether, there seems to exist a biological link between endocannabinoids and their analogues with short-chain fatty acids producing bacteria, mediated by actions on host intestinal cells, due to the lack of endocannabinoid receptors in bacteria[59]. However, whether this mechanism can explain how endocannabinoids and their analogues preserve gut barrier integrity should be further investigated.

It is well known that an increasing in plasma levels of lipopolysaccharide is favored by a deterioration of the gut barrier[47]. For that reason, lipopolysaccharide could be considered as a surrogated marker of gut permeability[47]. Lipopolysaccharide is released by several bacteria and individuals with metabolic syndrome or type 2 diabetes[60] present higher circulating levels of lipopolysaccharide in comparison with healthy people. In our study, plasma levels of AEA, DGLEA, LEA, PEA, PDEA, POEA, OEA, AA, and 2-AG, were negatively correlated with the plasma levels of lipopolysaccharide only in participants with high lipopolysaccharide values

(Q4). Of note is that when we repeated these analyses in the group of very low lipopolysaccharide values (Q1), we found opposite directions in these associations. These results suggest that when gut permeability is altered, endocannabinoids and their analogues may be related in the preservation of the gut barrier integrity. This could be explained by the low plasma levels of endocannabinoids and their analogues in participants with higher lipopolysaccharide levels, indicating a possible mobilization of endocannabinoids from the bloodstream to the intestine to reinforce the gut barrier. However, it has been shown that lipopolysaccharide might alter the gene expression of the key enzymes involved in the synthesis of endocannabinoids (N-acyl phosphatidylethanolamine phospholipase D and fatty acid amide hydrolase), and thus lipopolysaccharide, might also modulate plasma levels of endocannabinoids and their analogues[61]. Therefore, it is necessary mechanistic studies to elucidate the role of lipopolysaccharide in the endocannabinoid system synthesis.

Limitations and strengths

The present study suffers the limitation of its cross-sectional design, which precludes the establishment of cause-effect relationships. The study population only included young adults, which does not enable extrapolation of results to older, younger, or unhealthy populations. Moreover, the associations observed were relatively weak. The strengths of this study are that we sequenced using the latest technology (Illumina platform) and annotated with RDP at the level of species taxon. Furthermore, this study is one of the few studies to date that have studied the relationship between endocannabinoids and their analogues with fecal microbiota diversity and composition in young adults.

Conclusions

The plasma levels of LEA, alpha LEA, PDEA, POEA, OEA, PEA, SEA, 2-AG, 2-LG, and 2-OG were positively correlated with the relative abundance of *Akkermansia*, *Faecalibacterium, Parasutterella* and/or *Odoribacter* genera, which may contribute to the preservation of the gut barrier integrity. Moreover, the plasma levels AEA and related N-acylethanolamines (*i.e.,* DGLEA, LEA, PEA, PDEA, POEA and OEA), as well as AA and 2-AG were negatively correlated with the plasma levels of lipopolysaccharide, but only in participants with high levels of lipopolysaccharide. These findings suggest that the plasma levels of endocannabinoids and their analogues are related to specific fecal bacteria genera and may play a role in the gut barrier integrity in young adults.

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SECTION I

STUDY II: FECAL MICROBIOTA COMPOSITION IS RELATED TO BROWN ADIPOSE TISSUE ^{18}F-FLUORODEOXYGLUCOSE UPTAKE IN YOUNG ADULTS

ABSTRACT

Objective: Human brown adipose tissue (BAT) has gained considerable attention as a potential therapeutic target for obesity and its related cardiometabolic diseases; however, whether the gut microbiota might be an efficient stimulus to activate BAT metabolism remains to be ascertained. We aimed to investigate the association of fecal microbiota composition with BAT volume and activity and mean radiodensity in young adults.

Methods: 82 young adults (58 women, 21.8 ± 2.2 years old) participated in this cross-sectional study. DNA was extracted from fecal samples and 16S rRNA sequencing was performed to analyse the fecal microbiota composition. BAT was determined via a static ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography-computed tomography scan (PET/CT) after a 2-h personalized cooling protocol. ^{18}F-FDG uptake was also quantified in white adipose tissue (WAT) and skeletal muscles.

Results: The relative abundance of *Akkermansia*, *Lachnospiraceae sp.* and *Ruminococcus* genera were negatively correlated with BAT volume, BAT SUVmean and BAT SUVpeak (all rho≤-0.232, P≤0.027), whereas the relative abundance of *Bifidobacterium* genus was positively correlated with BAT SUVmean and BAT SUVpeak (all rho≥0.262, P≤0.012). On the other hand, the relative abundance of *Sutterellaceae* and *Bifidobacteriaceae* families were positively correlated with ^{18}F-FDG uptake by WAT and skeletal muscles (all rho≥0.213, P≤0.042). All the analyses were adjusted for the PET/CT scan date as proxy of seasonality.

Conclusion: Our results suggest that fecal microbiota composition is involved in the regulation of BAT, and glucose uptake by other tissues in young adults. Further studies are needed to confirm these findings.

INTRODUCTION

Brown adipose tissue (BAT) is a tissue that dissipates energy through the action of the uncoupling protein-1 (UCP1) in rodents and in humans[1]. Moreover, BAT takes up and oxidizes glucose and lipids, as such working as a nutrient sink, and through its endocrine function may have cardiometabolic benefits[2]. Consequently, BAT activation has been suggested as a potential therapeutic target to combat obesity and its related cardiometabolic diseases[3].

The human gut harbours a vast array of microorganisms as Eukarya, Archaea, fungi, and mainly bacteria[4], commonly known as gut microbiota[5]. Bacteria are classified into five different phyla, of which in humans *Firmicutes* and *Bacteroidetes* are found in higher abundance (>75%) compared to *Proteobacteria*, *Verrucomicrobia*, and *Actinobacteria* (<25%)[6]. Although cold exposure is the main physiological activator of BAT[7], evidence suggests that gut microbiota is an important endogenous factor that can modulate BAT metabolism[8–12]. Indeed, gut microbiota composition can promote whole-body thermogenesis during cold exposure in mice, with BAT being the main thermogenic effector[11,12]. Gut microbiota might be involved in the process of remodelling white adipose tissue (WAT) towards a beige-like phenotype in individuals with obesity[13–15]. However, a recent study showed that fecal microbiota composition was not associated to BAT activity after cold exposure in individuals with non-alcoholic fatty liver disease[16], which appears to be in contrast with previous evidence from rodent models[17–20]. Further research is therefore needed to understand the role of gut microbiota composition in human BAT metabolism.

We hypothesized that the relative abundance of gut bacteria previously reported to improve obesity and cardiometabolic diseases, as *Akkermansia*[21] or *Bifidobacterium*[22] genera, are associated with BAT volume and activity. Thus, the main aim of the present study was to investigate the association of fecal microbiota composition with BAT volume and activity, as determined via cold-induced ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake, and mean radiodensity in young adults. Additionally, we explored the association of fecal microbiota composition with the ^{18}F-FDG uptake by white adipose tissue (WAT) and skeletal muscles.

MATERIAL AND METHODS

Design study and participants

This cross-sectional study was carried out within the framework of the ACTIBATE study[23] (Clinical Trials.gov ID: NCT02365129). A total of 92 young healthy adults (27 men and 65 women, age: 18-25 years old) took part in this study. The assessments were performed in Granada (Spain) between October and November 2016. All participants underwent a comprehensive medical examination and reported themselves to be sedentary (<20 min moderate-vigorous physical activity on <3 days/week), to have a stable body weight over the last 3 months (<3 kg change), not to be exposed to cold regularly, neither

be pregnant, smoking, or taking any regular medication (including antibiotics) that affects the cardiovascular system, or presenting any acute or chronic illness.

The study protocol and the written informed consent were performed in accordance with the Declaration of Helsinki, as revised in 2013, and were approved by the Human Research Ethics Committee of the University of Granada (n°924) and the "Servicio Andaluz de Salud" (Centro de Granada, CEI-Granada).

Body composition assessment

We measured the participants´ weight and height while being barefoot and wearing light clothing, using a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Lean body mass and body fat mass were determined by Dual Energy X-ray Absorptiometry (Hologic Discovery Wi, Marlborough, MA). Body mass index (BMI), lean mass index and fat mass index were calculated as weight, lean body mass and body fat mass in kg divided by height in meters square (m^2).

Fecal microbiota composition analyses

❖ Stool collection and DNA extraction

The participants collected a fecal sample (50-60g) 3±7 days [mean ± standard deviation] prior to the positron emission tomography/computed tomography (PET/CT). They transported the fecal sample in plastic sterile containers inside a portable cooler until arrival at the research centre. Fecal samples were stored at -80°C until extraction of deoxyribonucleic acid (DNA). A QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain) was utilized to extract DNA following the manufacturer's instructions, and samples were incubated at 95ºC to ensure lysis of both Gram-positive and Gram-negative bacteria. The quantification of DNA was performed by using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, DE, USA). We measured absorbance spectrophotometrically at A260/280nm and A260/230nm ratios for determining DNA purity. The A260/280 ratio is used to determine protein contamination [24] whereas the A260/230 ratio indicates the presence of organic contaminants (salt and phenol) in nucleic acids samples[25].

❖ Sequencing Analysis

We amplified DNA extracted by polymerase chain reaction (PCR) with primer pairs, 16S Amplicon PCR Forward Primer: 5'CCTACGGGNGGCWGCAG, and 16S Amplicon PCR Reverse Primer: 5'GACTACHVGGGTATCTAATCC targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene[26]. All PCRs were executed in 25 µL reaction volumes incorporating 12.5 µL 2X KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, MA, USA), 5 µL of each forward and reverse primers (1 µM) and 2.5 µL of extracted DNA (10 ng) following denaturation at 95ºC for 3 min, 8 cycles of denaturation at

95ºC for 30 s, annealing at 55ºC for 30 s, elongation at 72ºC for 30 s, and a final extension at 72ºC for 5 min. PCR clean-up was executed using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) to purify the 16S V3 and V4 amplicon from free primers and primer dimer species. The next step was the PCR index (same condition that before); in this step, we used Nextera XT index kit (Illumina, San Diego, CA, USA) to tagged DNA with sequencing adapters. AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) were used for purifying the pooled PCR products before quantification. The consequential amplicons were sequenced at MiSeq (Illumina, USA), using a paired-end (2x300nt) Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA).

- **Bioinformatics analyses**

"Dada2"[27] R[28] package was used for analysing the generated FastQ files, which retrieved 11,659,014 paired-end with an average of 127±33 x10^3 reads per sample. All samples surpassed a cut-off 10,000 reads. Samples were standardised to equal size of 30,982 reads using "phyloseq"[29] R[28] package, retrieving 11,158 phylotypes.

Taxonomic affiliation of phylotypes was assigned using the "classifier" function from Ribosomal Database Project (RDP), based on the naive Bayesian classification[30] with a pseudo-bootstrap threshold of 80%. We obtained a total of 209 genera that belong to 16 different phyla. Bacterial communities were analysed at different taxonomic levels (phylum to genera), calculating relative abundances in each sample as "(nº reads/total nº reads) ×100 per sample"[31], and expressed as percentages. The analyses were performed using the taxonomic levels with more than 0.5% the relative abundance of average between samples.

Brown adipose tissue measurements

- **Shivering threshold test**

In order to personalize the cooling protocol used to activate human BAT, subjects underwent a cooling test 48-72 hours before the ^{18}F-FDG PET/CT scan, in which their shivering threshold was determined. Briefly, participants arrived in fasted condition (≥6 h), having avoided alcoholic or stimulant beverages within the last 12 h, and having refrained from any moderate physical activity within the last 24 h, and vigorous activity within the last 48 h. They wore standardized clothes (sandals, T-shirt and shorts) and entered a warm room (22.1±1.6 ºC) where they kept seated for 30 min. Later, they entered a cool room (19.8±0.5 °C) and sat down in a chair, where we put them a water-perfused cooling vest (Polar Products Inc., Stow, OH, USA). Each participant's shivering threshold was then determined being seated and following a personalized cooling protocol, as described elsewhere[32,33]. Water temperature started at 16.6°C, and decreased ~ 1.4°C every 10 minutes until shivering occurred (self-reported and visually observed by the researchers). The water temperature at which shivering occurred was considered the shivering

threshold and used for determining the cooling vest water temperature during the personalized cooling protocol before the ^{18}F-FDG-PET/CT scan (4°C above the shivering threshold)[32].

- **Personalized cooling protocol prior to Positron Emission Tomography/Computed Tomography scan**

After 48–72 h of the shivering threshold test, the participants went to the *Hospital Virgen de las Nieves*, Granada (Spain) for assessment of BAT volume, activity and mean radiodensity. They rested in a warm room for 30 min and then they entered a cool room (19.5–20.0°C), where they dressed with the same cooling vest. On this occasion the water temperature was set ~4°C above their shivering threshold. After the first hour of cold exposure, the participants received an intravenous injection of ~185 MBq ^{18}F-FDG while the water temperature was increased by 1°C to prevent shivering. One hour after the injection, the participants underwent a static PET/CT scan, using a Siemens Biograph 16 PET/CT scanner (Siemens, Erlangen, Germany), scanning from the atlas to approximately the mid chest. The date when PET/CT scan was performed was recorded as the day of the year, being January 1st day 1, and December 31st day 366. This date was used as a proxy of seasonal variation.

- **Brown adipose tissue quantification**

All PET/CT images were examined using the Beth Israel plug-in for FIJI software (http://sourceforge.net/projects/bifijiplugins)[34], following a protocol described elsewhere[32,33] and in agreement with current methodological recommendations (BARCIST 1.0)[35]. To determine BAT volume and mean and peak standardized uptake values (SUVmean and SUVpeak), six regions of interest (ROIs) were outlined from the atlas vertebra to thoracic vertebra 4 using a 3D-axial technique. These ROIs comprised the supraclavicular, laterocervical, paravertebral and mediastinal regions. Those voxels with a radiodensity between -190 and -10 Hounsfield Units (HU) and a SUV higher than the individualized SUV threshold, calculated as 1.2/(lean body mass/body mass), were classified as BAT voxels[35]. BAT volume was computed as the sum of the volume of these voxels across all ROIs. BAT SUVmean was determined as the average SUV of all voxels, and SUVpeak as the average of the three voxels presenting the highest SUV within 1cm^3 from the voxel presenting the highest SUV, and meeting the above-mentioned criteria, across all ROIs. All SUV values were expressed relative to lean body mass[36]. BAT mean radiodensity was calculated as the average radiodensity of those voxels meeting the aforementioned criteria in a single region of interest covering the whole body from the atlas to thoracic vertebrae 4, except the mouth. Additionally, we determined the descending aorta (used as a reference tissue) SUVpeak. This was done by drawing a one slice ROI in the descending aorta, at the height of thoracic vertebra 4. We also determined the SUVpeak in the tricipital WAT[37]; as well as in different skeletal muscles, including the cervical, scalene, longus colli, paravertebral, subscapular, sternocleidomastoid, supraspinatus, trapezius, deltoid, pectoralis major, and triceps brachii muscles on both the right and left sides of the body[37]. Then, we obtained the average

for the SUVpeak values of all examined muscles to provide a representative value for all skeletal muscle ^{18}F-FDG uptake.

Statistical analysis

Data are presented as means ± standard deviations unless otherwise stated. All variables were tested for normality using D'Agostino & Pearson omnibus. Most of the variables displayed a non-normal distribution, thus non-parametric tests were used for all analyses. We did not detect any sex interaction across the variables studied (**Fig. S1** and **Table S1**), nor differences between the status of BMI (data not shown), therefore, all main data for men and women, as well as the status of BMI, were pooled together. Partial Spearman correlations were used to investigate the correlation of fecal microbiota composition with BAT volume, ^{18}F-FDG uptake and mean radiodensity by "psych"[38] and "corrplot"[39] R[28] packages. Since seasonality could affect measurements of BAT[40–43], we presented all the analyses adjusting for the PET/CT scan date, that is the natural day. The level of significance was set at $P<0.05$. SPSS (SPSS v. 22.0, IBM SPSS Statistics, IBM Corp. Armonk, NY), R software (V.3.6.0; http://www.R-project.org)[28] and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA) were used for the statistical analysis and graphical plots.

RESULTS

Characteristics of participants

Among the 92 participants with a complete analysis of fecal microbiota composition, only 82 participants had BAT measurements, therefore, these 82 participants were finally included in the analyses. **Table 1** shows the descriptive characteristics of those 82 participants, of whom 70.7% were women. Participants were 21.8 ± 2.2 years old, and their BMI was 24.9 ± 4.8 kg/m^2.

Table 1. Descriptive characteristics of the participants.

	N	**Mean ± SD**
Sex (women %)	82 (70.7%)	
Age (years)	82	21.8 ± 2.2
Body composition variables		
Body mass index (kg/m^2)	82	24.9 ± 4.8
Lean mass index (kg/m^2)	76	14.4 ± 2.3
Fat mass index (kg/m^2)	76	8.9 ± 3.1
Fat mass percentage (%)	76	36.1 ± 7.9
PET/CT variables		
BAT volume (mL)	82	69.0 ± 59.9
BAT SUVmean	82	2.2 ± 1.0
BAT SUVpeak	82	6.4 ± 4.7
BAT Mean radiodensity (HU)	62	-59.0 ± 9.7
Descending aorta SUVpeak	82	0.9 ± 0.2
Subcutaneous WAT Triceps SUVpeak	82	0.10 ± 0.05
All skeletal muscle SUVpeak	82	0.8 ± 0.2

Fecal microbiota variables			
Composition (Phylum)			
	Actinobacteria (%)	82	1.7 ± 1.6
	Bacteroidetes (%)	82	39.9 ± 8.9
	Firmicutes (%)	82	48.3 ± 9.9
	Proteobacteria (%)	82	6.7 ± 5.4
	Verrucomicrobia (%)	82	2.3 ± 4.3

Data are presented as means ± standard deviations (SD). All SUV variables are shown relative to lean body mass. BAT: brown adipose tissue; HU: Hounsfield Units; SUV: standardized uptake value; WAT: white adipose tissue.

Relationship between fecal microbiota composition and cold-induced BAT variables

We observed that the relative abundance of the *Verrucomicrobia* phylum and its lower taxonomic levels (class, order, and family) were negatively correlated with BAT volume (all rho≤-0.229, P≤0.029; **Fig. 1**). In contrast, the relative abundance of the *Actinobacteria* phylum and at lower taxonomic levels (class, order, and family) were positively correlated with BAT SUVmean (all rho≥0.211, P≤0.044; **Fig. 1**) and BAT SUVpeak (all rho≥0.211, P≤0.045; **Fig. 1**).

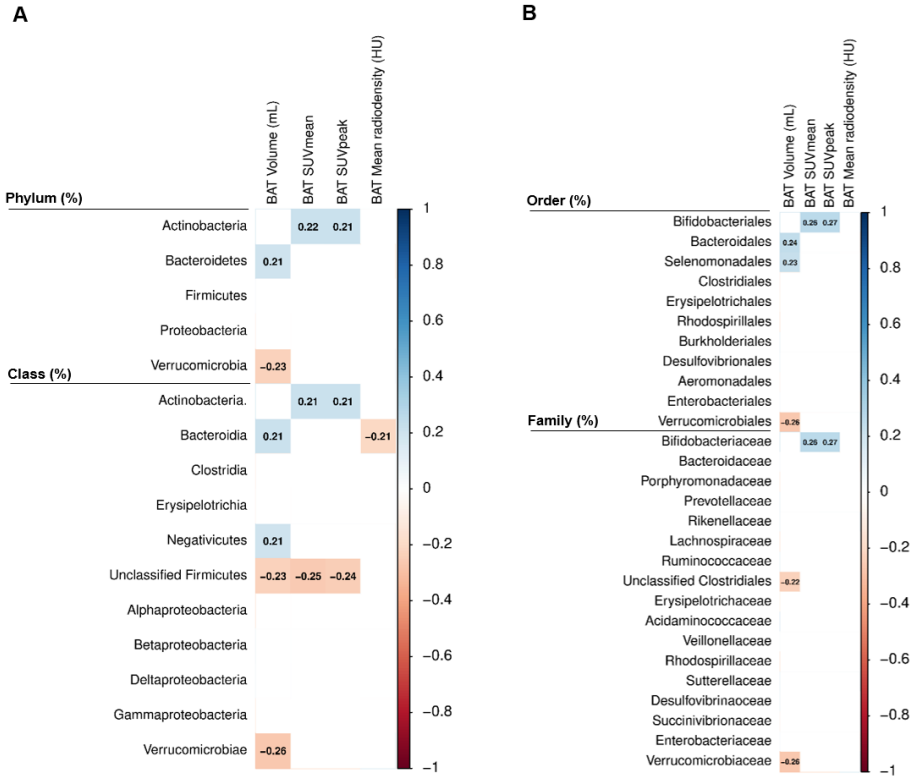

Figure 1. Partial Spearman correlation of fecal microbiota composition with BAT volume, SUVmean, SUVpeak and mean radiodensity adjusted for the PET/CT scan date. *Boxes represent the statistically significant (P<0.05) correlations and the value within the boxes show the partial Spearman correlation coefficient. Blue boxes indicate positive correlation whereas red boxes indicate negative correlation between fecal microbiota composition with cold-induced BAT variables. Panel A shows phylum and class taxonomic levels and panel B indicates order and family taxonomic levels. BAT SUVmean and SUVpeak are shown relative to lean body mass. BAT: brown*

adipose tissue; HU: Hounsfield Units; PET/CT: positron emission tomography/computed tomography; SUV: standardized uptake value.

Next, we investigated whether the relative abundance of specific genera within the above-mentioned taxonomic groups were associated with BAT-related variables (**Fig. 2**). We found that the relative abundance of *Akkermansia* genus (*Verrucomicrobia* phylum) was negatively correlated with BAT volume (rho=-0.262, P=0.012; **Fig. 2**), whereas the relative abundance of *Bifidobacterium* genus (*Actinobacteria* phylum) was positively correlated with BAT SUVmean (rho=0.262, P=0.012; **Fig. 2**) and BAT SUVpeak (rho=0.269, P=0.010; **Fig. 2**). Moreover, the relative abundance of *Lachnospiraceae sp.* and *Ruminococcus* genera (both from *Firmicutes* phylum) were negatively correlated with BAT SUVmean (rho≤-0.268, P≤0.010; **Fig. 2**) and BAT SUVpeak (rho≤-0.232, P≤0.027; **Fig. 2**), although only the relative abundance of *Lachnospiraceae sp.* genus (*Firmicutes* phylum) was negatively correlated with BAT volume (rho=-0.357, P≤0.001; **Fig. 2**). All results persisted when the analyses were divided by sex (**Fig. S1**), and when participants with no detectable/scarce BAT glucose uptake were excluded from the analyses (data not shown).

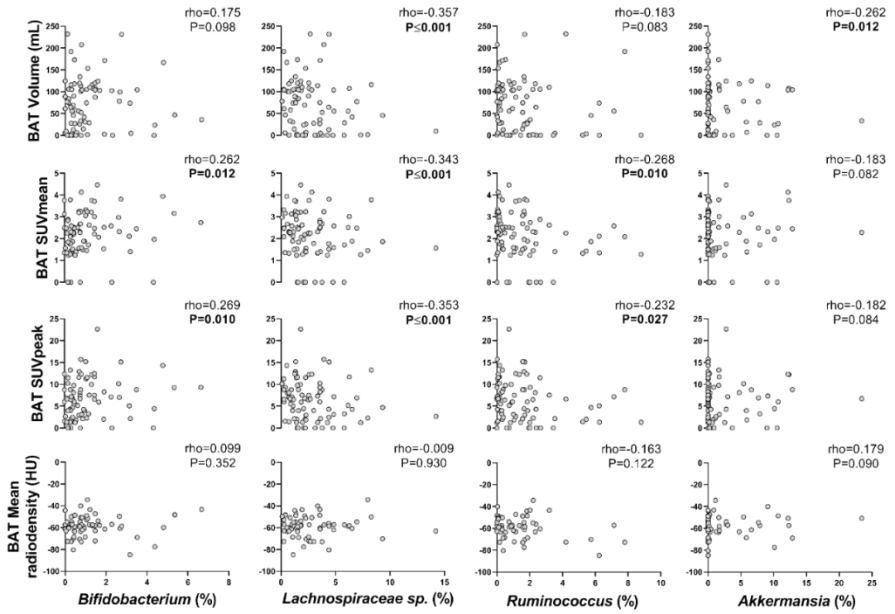

Figure 2. Partial Spearman correlations of relative abundance of Bifidobacterium, Lachnospiraceae sp., Ruminococcus and Akkermansia genera with BAT volume, SUVmean, SUVpeak and mean radiodensity, after adjusting for the PET/CT scan date. *Rho=Partial Spearman's correlations coefficient. P= p-value from univariate partial Spearman correlation. BAT SUVmean and SUVpeak are shown relative to lean body mass. BAT: brown adipose tissue; HU: Hounsfield Units; PET/CT: positron emission tomography/computed tomography; SUV: standardized uptake value.*

Relationship of fecal microbiota composition with cold-induced uptake of ^{18}F-FDG by descending aorta, WAT and skeletal muscles

The relative abundance of *Betaproteobacteria* class, and at lower taxonomic levels (order and family; all *Proteobacteria* phylum) were positively correlated with the SUVpeak of the descending aorta (our reference tissue, all rho≥0.257, P≤0.014; **Fig. 3**) and the subcutaneous WAT triceps (all rho≥0.347, P≤0.001; **Fig. 3**), whereas the relative abundance of *Sutterellaceae* family (*Proteobacteria* phylum) was positively correlated with all skeletal muscles SUVpeak (rho=0.213, P=0.042; **Fig. 3**). In addition, the relative abundance of *Actinobacteria* phylum, and its lower taxonomic levels (class, order, family and genus) were positively correlated with all skeletal muscles SUVpeak (all rho≥0.225, P≤0.032; **Fig. 3**), whereas only the *Bifidobacteriales* order, and its lower taxonomic levels (family and genus) were positively correlated with subcutaneous WAT triceps SUVpeak (all rho≥0.266, P≤0.011; **Fig. 3**).

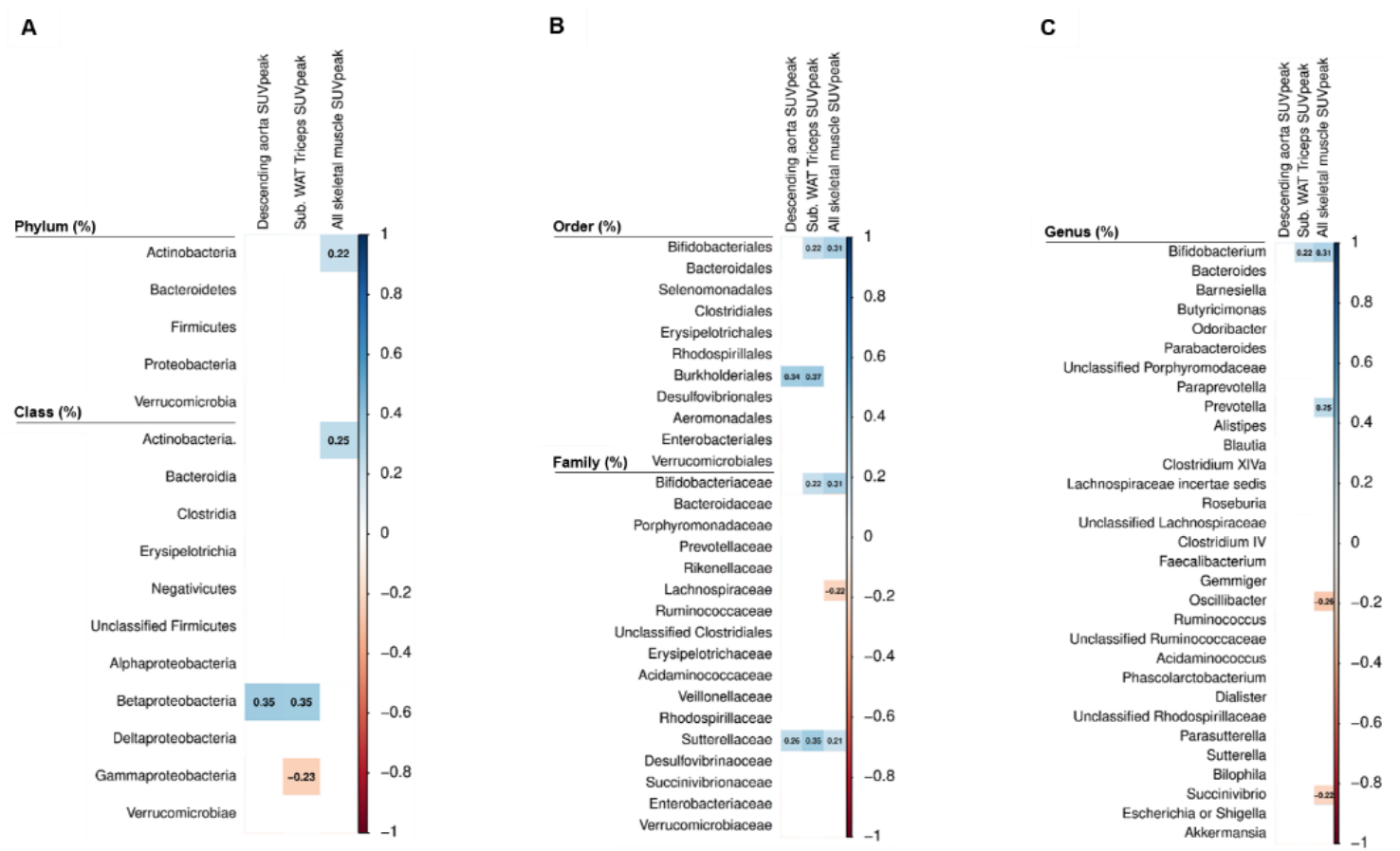
A
Descending aorta SUVpeak
Sub. WAT Triceps SUVpeak
All skeletal muscle SUVpeak
Phylum (%)
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Verrucomicrobia
Class (%)
Actinobacteria.
Bacteroidia
Clostridia
Erysipelotrichia
Negativicutes
Unclassified Firmicutes
Alphaproteobacteria
Betaproteobacteria
Deltaproteobacteria
Gammaproteobacteria
Verrucomicrobiae
0.22
0.25
0.35
0.35
−0.23
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1
B
Descending aorta SUVpeak
Sub. WAT Triceps SUVpeak
All skeletal muscle SUVpeak
Order (%)
Bifidobacteriales
Bacteroidales
Selenomonadales
Clostridiales
Erysipelotrichales
Rhodospirillales
Burkholderiales
Desulfovibrionales
Aeromonadales
Enterobacteriales
Verrucomicrobiales
Family (%)
Bifidobacteriaceae
Bacteroidaceae
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Lachnospiraceae
Ruminococcaceae
Unclassified Clostridiales
Erysipelotrichaceae
Acidaminococcaceae
Veillonellaceae
Rhodospirillaceae
Sutterellaceae
Desulfovibrinaoceae
Succinivibrionaceae
Enterobacteriaceae
Verrucomicrobiaceae
0.22
0.31
0.34
0.37
0.22
0.31
−0.22
0.26
0.35
0.21
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1
C
Descending aorta SUVpeak
Sub. WAT Triceps SUVpeak
All skeletal muscle SUVpeak
Genus (%)
Bifidobacterium
Bacteroides
Barnesiella
Butyricimonas
Odoribacter
Parabacteroides
Unclassified Porphyromodaceae
Paraprevotella
Prevotella
Alistipes
Blautia
Clostridium XlVa
Lachnospiraceae incertae sedis
Roseburia
Unclassified Lachnospiraceae
Clostridium IV
Faecalibacterium
Gemmiger
Oscillibacter
Ruminococcus
Unclassified Ruminococcaceae
Acidaminococcus
Phascolarctobacterium
Dialister
Unclassified Rhodospirillaceae
Parasutterella
Sutterella
Bilophila
Succinivibrio
Escherichia or Shigella
Akkermansia
0.22
0.31
0.25
−0.26
−0.22
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1

Figure 3. Partial Spearman correlation of fecal microbiota composition with tissues related to ^{18}F-FDG uptake adjusted for the PET/CT scan date. *Boxes only represent the statistically significant (P<0.05) correlations and the value within the boxes show the partial Spearman correlation coefficient. Blue boxes indicate positive correlation whereas red boxes indicate negative correlation between fecal microbiota composition with tissues related ^{18}F-FDG uptake adjusted for PET/CT scan date. Panel A shows phylum and class taxonomic levels. Panel B indicates order and family taxonomic levels, and panel C shows genus taxonomic level. All SUV variables are shown relative to lean body mass. ^{18}F-FDG: [^{18}F]fluorodeoxyglucose; PET/CT: positron emission tomography/computed tomography; SUV: standardized uptake value; WAT: white adipose tissue.*

DISCUSSION

This study shows, for the first time, that the relative abundance of *Akkermansia, Lachnospiraceae sp.* and *Ruminococcus* genera negatively correlate with BAT volume and activity (as estimated by ^{18}F-FDG uptake), whereas the relative abundance of *Bifidobacterium* genus positively correlate with BAT activity. Moreover, *Bifidobacteriaceae* and *Sutterellaceae* families positively correlated with ^{18}F-FDG uptake by WAT in the tricipital area and skeletal muscles. These findings suggest that fecal microbiota composition is involved in glucose metabolism by BAT and other tissues including WAT and skeletal muscles in young adults.

The role of gut microbiota composition in BAT activation and metabolism has been investigated in mouce models[17–20], but studies in humans are scarce[16]. In fact, the only study in humans observed that the fecal microbiota composition was not associated to BAT activity after cold-induction in individuals with non-alcoholic fatty liver disease[16]. The present study included a cohort of healthy young adults, and found that the relative abundance of *Akkermansia, Lachnospiraceae sp.* and *Ruminococcus* genera were negatively correlated with BAT volume and activity. It has been previously shown that the bacteria belonging to *Akkermansia* and *Ruminococcus* genera produce short-chain fatty acids (SCFAs), as acetate, to activate BAT thermogenesis and promote WAT browning via triggering the G-protein-coupled receptor (GPR) 43 in mice[17–20]. However, a recent study using single nuclei RNA sequencing in human BAT, demonstrated that BAT is composed of a set of different subpopulations of adipocytes[44]. Indeed, they observed that a rare subpopulation of brown adipocytes inhibited the thermogenic capacity of neighbouring adipocytes via the production of acetate[44]. Accordingly, the same authors showed that local acetate induces BAT thermogenic dysfunction[45]. Therefore, evidence on the relationship between acetate and BAT activation is contradictory. Of note, our data appear to be in line with the former studies in human BAT[44,45], showing that acetate-producing bacteria, such as the *Akkermansia, Lachnospiraceae sp.* and *Ruminococcus* genera, are inversely correlated with BAT ^{18}F-FDG uptake.

We also observed a positive correlation of the relative abundance of *Bifidobacteriaceae* and *Sutterellaceae* families with ^{18}F-FDG uptake by WAT and skeletal muscles, whereas the relative abundance of *Bifidobacterium* genus, and at higher taxonomic levels, were positively correlated with BAT ^{18}F-FDG uptake. Scientific evidence has shown that SCFAs produced by gut microbiota regulate glucose homeostasis and improve insulin sensitivity[46,47].

This might be partially explained because SCFAs increase glucagon-like peptide 1 and 2 secretion from enteroendocrine L-cells by their binding to GPR41 and GPR43[46,47]. This fact leads to an increase in glucose uptake by metabolically active tissues[46,47]. However, all these hypotheses should be confirmed in future experiments investigating whether these bacteria are actually able to increase BAT and other tissues' glucose uptake.

Limitations and strengths

The cross-sectional design of this study precluded us from establishing cause-effect relationships. Our study population includes young adults without relevant disease or comorbidity; thus, our results cannot be extrapolated to older or unhealthier populations. Importantly, SCFAs were not measured and could be of interest for future studies. Further, although BAT takes up glucose from the circulation, intracellular fatty acids are the main substrate of brown adipocytes in humans[48]. Hence, ^{18}F-FDG PET/CT might not estimate accurately the cold-induced BAT metabolic activity, despite being the most widely used technique. Moreover, it will be of scientific interest to investigate whether fecal microbiota composition is related to BAT activity measured at other room temperatures. As for strengths of this study, we should stand out that we sequenced the microbiota composition using the latest technology (Illumina platform) and annotations were made with RDP until genera taxon.

Conclusions

This is the first study showing a negative correlation of the relative abundance of *Akkermansia*, *Lachnospiraceae sp.* and *Ruminococcus* genera with cold-induced BAT volume and activity in young adults. Contrarily, the relative abundance of *Bifidobacterium* genus was positively correlated with BAT activity. Moreover, the relative abundance of *Bifidobacteriaceae* and *Sutterellaceae* families were positively correlated with ^{18}F-FDG uptake by WAT and skeletal muscles. Altogether, these findings suggest that fecal microbiota is involved in the regulation of glucose uptake by human BAT and other metabolic tissues, but future studies are needed to confirm these findings and to elucidate underlying mechanisms.

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SUPPLEMENTARY MATERIALS

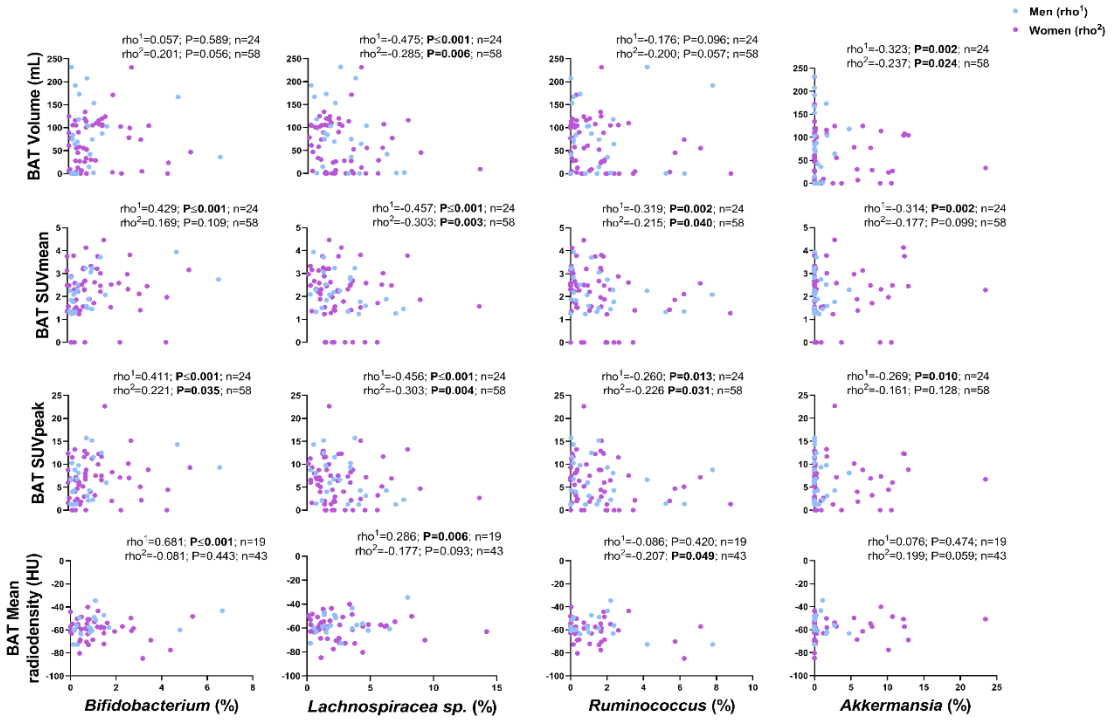

Figure S1. Partial Spearman correlations of the relative abundance of Bifidobacterium, Lachnospiraceae sp., Ruminococcus and Akkermansia genera with cold-induced BAT volume, SUVmean, SUVpeak and mean radiodensity adjusted by sex, after adjusting for the PET/CT scan date. *Blue circles represent men participants and purple circles show women participants. Rho1=Partial Spearman's correlations coefficient for men. Rho2=Partial Spearman's correlations coefficient for women. P= p-value from univariate partial Spearman correlation. N= sample size. BAT SUVmean and SUVpeak are shown relative to lean mass. BAT: brown adipose tissue; HU: Hounsfield Units; PET/CT: positron emission tomography/computed tomography; SUV: standardized uptake value.*

Table S1. Differences in PET/CT and fecal microbiota composition variables between genders.

	Men N=24	**Women N=58**	
	Mean ± SD	*Mean ± SD*	*P*
PET/CT variables			
BAT volume (mL)	83.4 ± 72.8	63.0 ± 53.3	0.370
BAT SUVmean	2.2 ± 0.8	2.1 ± 1.1	0.683
BAT SUVpeak	6.7 ± 4.8	6.3 ± 4.7	0.683
BAT Mean radiodensity (HU)*	-56.8 ± 9.4	-59.9 ± 9.8	0.536
Fecal microbiota variables			
Composition (Phylum)			
Actinobacteria (%)	1.4 ± 1.7	1.8 ± 1.6	0.179
Bacteroidetes (%)	39.9 ± 11.4	39.9 ± 7.8	0.338
Firmicutes (%)	50.5 ± 8.6	47.4 ± 10.3	0.093
Proteobacteria (%)	7.1 ± 4.2	6.5 ± 5.8	0.126
Verrucomicrobia (%)	0.6 ± 1.1	3.0 ± 4.9	0.112

Data are presented as means ± standard deviations (SD). All SUV variables are shown relative to lean body mass. P values were obtained from Mann-Whitney test. *For BAT Mean radiodensity, data are missing for 10 participants (remaining men n= 19 and women n= 43). Abbreviations: BAT: brown adipose tissue; BMI: body mass index; HU: Hounsfield Units; SUV: standardized uptake value.

SECTION II

STUDY III: HIGHER PHYSICAL ACTIVITY LEVELS ARE RELATED TO FECAL MICROBIOTA DIVERSITY AND COMPOSITION IN YOUNG ADULTS

ABSTRACT

Purpose: Increasing physical activity (PA) is recognized as an efficacious approach for preventing and treating cardiometabolic diseases. Recently, the composition of microorganisms living within the gut has been proposed as an important appropriate target for treating these diseases. Whether PA is related to fecal microbiota diversity and composition in humans remains to be ascertained. Thus, we examined the association of the time spent in objectively measured PA with fecal microbiota diversity and composition in young adults.

Methods: A cross-sectional study, enrolled 88 young adults with 22.0 ± 2.3 years (72.7% women), whose the time spent in PA at different intensities was objectively measured with a wrist-worn accelerometer for 7 consecutive days. Fecal microbiota diversity and composition were analysed with hypervariable tag sequencing of the V3–V4 region of the 16S rRNA gene.

Results: Mean the Euclidean Norm of the raw accelerations Minus One (m*g*) during waking time, considerate as overall PA and the time spent in vigorous PA were positively correlated with alpha diversity indexes (all rho≥0.23, P≤0.034). Regarding fecal microbiota composition, participants with low time spent in vigorous PA had higher relative abundance of *Gammaproteobacteria* class (q=0.021, FDR=q-value) compared to the participants with high time spent in vigorous PA, and lower relative abundance of *Porphyromonadaceae* family (q=0.031) and *Alistipes* genus (q=0.015) compared to the individuals with high and intermediate spent in vigorous PA, respectively.

Conclusion: Our results suggest that PA, especially of vigorous intensity, is related to fecal microbiota diversity and *Gammaproteobacteria* class and *Porphyromonadaceae* family in young adults.

INTRODUCTION

Lifestyle physical activity (PA) is associated with a myriad of physiological adaptations that benefit human health; it is one of the most effective strategies to prevent and combat cardiometabolic diseases[1,2], and is related to a 27% decrease in mortality risk[3]. However, the underlying mechanisms that explain how physical activity enhances cardiometabolic health and prevents cardiometabolic disease have yet are remain to be discovered.

Gut microbiota is referred to microbial communities that colonize the gastrointestinal tract[4,5], which plays an indispensable role in host nutrition, metabolic function and immunological response[6]. Dysbiosis consists of a disequilibrium between microbial communities[7], induced by multiples factors such as dietary patterns[8], a sedentary lifestyle[9], and medication[10], which is in turn associated with obesity[11] and cardiometabolic diseases[1]. Recent evidence suggests that PA is considered one of the most significant lifestyle factors able to influence the gut microbiota diversity and composition in humans[12–15]. For instance, case-controlled studies showed that gut microbiota from athletes[16,17] was way more diverse and had a higher proportion of several bacterial taxa in comparison to healthy sedentary individuals. Another cross-sectional study observed that premenopausal women meeting the PA World Health Organization recommendations had a greater relative abundance of *Akkermansia* and *Faecalibacterium* genera than sedentary women[18]. There is evidence indicating that *Akkermansia* and *Faecalibacterium* genera are associated with a reduced inflammation and therefore may play a role in the prevention of the development of cardiometabolic diseases[19]. To date, studies investigating the relationship between PA and gut microbiota composition have used self-reported questionnaires to determine PA levels[16,17,20]. However, these instruments have the disadvantage of misclassifying PA levels and thus compromise the ability to detect true associations[21]. So, new studies using objectively measures of PA at different intensities, according to the time spent in each PA levels, would provide more accurate and reliable information. In the present study we investigated the association of the time spent in objectively measured PA, at different intensities, with fecal microbiota diversity and composition in a cohort of young adults. Based on previous observations with self-reported data, our hypothesis was that higher time spent in PA levels are related to higher fecal microbiota diversity and to a higher relative abundance of healthier bacteria.

MATERIAL AND METHODS

Design study and participants

A total of 92 (65 women) young healthy adults, aged 18-25 years old, were included in the present cross-sectional study. This study was carried out within the framework of the ACTIBATE study[22], an exercise-based randomized controlled trial (Clinical Trials.gov ID: NCT02365129). All assessments were performed in Granada (Spain) between October and November in 2016. Inclusion criteria were: being engaged in less than 20 min moderate-vigorous PA on less than 3 days/week, having a stable body weight over the last 3

months (< 3kg change), not smoking, not taking any medication (including antibiotics in the last 3 months), not presenting any acute or chronic illness and not being pregnant. The study protocol and experimental design were applied in accordance with the last revised ethical guidelines of the Declaration of Helsinki. The study was approved by the Ethics Committee on Human Research of the University of Granada (no. 924) and the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada); all participants signed an informed consent.

Physical activity assessment

PA variables were objectively measured with one accelerometer on the non-dominant wrist (ActiGraph GT3X+, Pensacola, FL), during 7 consecutive days (24h/day)[23]. Detailed information about how to wear the accelerometer was given to participants, including that they would have to remove it in water-based activities.

The sampling frequency of 100 Hz was selected to store the raw accelerations of the accelerometers[24]. We exported and converted the raw accelerations to the ".csv" format using ActiLife v.6.13.3 software (ActiGraph, Pensacola, FL, US). Afterwards, "ggir"[25] package in R software[26] was used for processing the raw ".csv" files. This processing consisted of: (i) local gravity data auto-calibration of accelerations according to the local gravitational acceleration[27], (ii) calculation of the Euclidean Norm of the raw accelerations Minus One *G* with negative values rounded to 0 (ENMO) calculated elsewhere[28], (iii) detection of non-wear time according based on to the raw acceleration of the three axes, (iv) determination of mal detection of sustained functioning of the accelerometer by means of abnormal high accelerations incompatible with human movement (i.e., related to devices malfunctioning), (v) imputation of non-wear time and abnormal high accelerations, (vi) identification of waking and sleeping time based on automatized algorithm guided by the participants' daily reports[29], and (vii) estimation of sedentary time, and the time spent in light PA, moderate PA, vigorous PA, and moderate to vigorous PA using age-specific cut-points in wrist-worn accelerometer, for Euclidean Norm Minus One (ENMO)[30,31]. We measured the mean ENMO (m*g*) during waking time, which is considered an overall indicator of the PA (overall PA). For the analyses, we only included the participants who wore the accelerometers for ≥16 h/day during at least 4 days (including at least 1 weekend day).

Fecal microbiota analyses

❖ Stool collection and DNA extraction

The participants collected approximately 50 g of fecal sample in plastic sterile containers, which was transported in portable coolers to the research center. Fecal samples were stored at -80°C until extraction of DNA. QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain) was used for extraction of DNA, following manufacturer's instructions. The samples were incubated at 95ºC to ensure lysis of both Gram-positive and Gram-negative bacteria. Then, we quantified DNA with a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific,

DE, USA). Finally, DNA purity was determined by measuring the ration of absorbance at A260/280nm (protein contamination[32]) and A260/230nm (salt and phenol contamination[33]).

- **Sequencing Analysis**

DNA extracted was amplified by polymerase chain reaction (PCR) by primer pairs, forward primer (5'CCTACGGGNGGCWGCAG3'), and reverse primer (5'GACTACHVGGGTATCTAATCC3'), targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene[34]. All PCRs were executed in 25 µL reaction volumes incorporating 12.5 µL 2X KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, MA, USA), 5 µL of each forward and reverse primers (1 µM) and 2.5µL of extracted DNA (10 ng) under the following cycling circumstances: (a) denaturation at 95ºC for 3 min, (b) cycles of denaturation at 95ºC for 30s, (c) annealing at 55ºC for 30s, (d) elongation at 72ºC for 30s , (e) a final extension at 72ºC for 5 min. To purify the PCR products from free primers and primer dimers we used AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). Next, the index PCR attached dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina, San Diego, CA, USA), on a thermal cycler using the requirements previously mentioned. After, AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) were used for purifying of the pooled PCR products. The resultant amplicons were sequenced at MiSeq (Illumina, USA), using paired-end (2x300nt) Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA).

Bioinformatics analysis

We analysed the FastQ files with "dada2"[35] package in R software[26], obtaining 11,659,014 paired-ends with an average of 126,728 ± 33,395 reads per sample. Cut-off of 10,000 reads was surpassed for all samples. Samples were resampled to an minimum sequencing depth of 30,982 reads using "phyloseq"[36] package in R[26], returning 11,158 phylotypes.

"classifier" function from the Ribosomal Database Project (RDP) was used for assigning the taxonomic affiliation of phylotypes, based on the naïve Bayesian classification[37] with a pseudo-bootstrap threshold of 80%. We obtained a total of 209 genera belonging to 16 different phyla. "seqmatch" [38] function from RDP was performed to define the discriminatory power of each sequence read with the purpose of annotating species assignments; we executed annotation according to criteria published previously[39]. Microbial communities were analysed at different taxonomic levels (phylum to genus), calculating relative abundances, expressed as percentages. We performed the analyses with those bacteria with more than 0.5% on average in their relative abundance.

Next, alpha and beta diversities were estimated based on the identified microbial communities. Alpha diversity takes into account the number of different phylotypes and relative abundances of a single sample[40], whereas beta diversity shows differences in microbial community composition between individuals, which is the degree to which samples differ from one another[41]. Alpha diversity was assessed based on richness Chao, Shannon, inverse

Simpson and evenness Camargo indexes with "microbiome"[42] package in R software[26]. Richness Chao estimates diversity according to number of different phylotypes in the community[43], that is, higher richness Chao indicates higher diversity in the community. Shannon diversity increases as both the richness and the evenness of the community increase[44], inverse of Simpson diversity is calculated from the classical Simpson diversity and indicates richness in a community with uniform evenness[45], and evenness Camargo determines the equitability of phylotypes frequencies in a community[46]. Beta diversity was measured quantitatively using permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarity[47].

Anthropometric and body composition measurements

Participants' weight and height were measured, without shoes and wearing the standard clothes, using SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Body mass index (BMI) was calculated as weight (kg)/height (m^2). We evaluated body composition by Dual Energy X-ray Absorptiometry (DEXA, HOLOGIC, Discovery Wi, Marlborough, MA). The lean mass index (LMI) and fat mass index (FMI) were calculated as lean body mass and fat body mass, respectively, in kg and divided by height in m^2. The fat mass percentage was determined as the body fat mass divided by the total body mass and multiplied by 100.

Cardiometabolic profile

Fasting blood samples were collected for the assessing cardiometabolic profile. Serum glucose, total cholesterol, high density lipoprotein-cholesterol (HDL-C) and triglycerides were measured following standard methods using an AU5832 automated analyser (Beckman Coulter Inc., Brea CA, USA). Low density lipoprotein-cholesterol (LDL-C) was estimated as: *[total cholesterol – HDL-C – (triglycerides/5)]*, in mg/dL[48]. Serum insulin was measured using the Access Ultrasensitive Insulin chemiluminescent immunoassay kit (Beckman Coulter Inc., Brea CA, USA). Homeostatic model assessment for insulin resistance (HOMA-IR) index was calculated as *(insulin (µU/mL) x glucose (mmol/L)/22.5*[49].

Dietary assessment

Dietary intake was registered using three non-consecutive 24 hours recalls, 2 weekdays and a weekend day. The participants were asked to recall all food consumed during the previous day in a face-to-face interview with dietitians. For improving the accuracy of food quantification, we used coloured photographs of different portion sizes of food during the interviews[50]. All 24 hours recalls were analysed for total energy (kcal), fat, proteins, carbohydrates, and fiber intake (g) by EvalFINUT® software, which is based on the United States Departament of Agriculture (USDA) and "Base de Datos Española de Composición de Alimentos" (BEDCA) databases.

Statistical analysis

Data normality was explored using the D'Agostino & Pearson omnibus, visual histograms and Q-Q plots (data not shown). None of the variables followed a normal distribution, thereby data were presented as median ± interquartile range and non-parametric tests were used for all analyses. Moreover, no sex interaction was detected (all P>0.05), so the both sexes were pooled together. Spearman correlations were performed to investigate the correlation between the physical activity variables and fecal microbiota diversity, using the "psych"[51] and "corrplot"[52] packages in R software[26]. Since fecal microbiota diversity can be modified by several factors as sex[53], BMI[54], and dietary intake[55], we repeated the correlations before mentioned adjusted for sex, BMI, and dietary intake in separated models (data not shown). Moreover, we repeated this analysis by adjusting for accelerometer non-wear time and glucose levels in separated models (data not shown) as possible confounders of PA variables. Overall PA and the time spent in vigorous PA were computed as tertiles according to number of participants with SPSS (SPSS v. 22.0, IBM SPSS Statistics, IBM Corp. Armonk, NY), because they were the only variables with significant correlation with fecal microbiota diversity. The tertiles' values for overall PA were low (13.45-29.44 m*g*), intermediate (30.02-35.41 m*g*), and high (35.49-67.10 m*g*), whereas for the time spent in vigorous PA it were was low (0.02-0.83 min/day), intermediate (0.87-2.67 min/day), and high (2.75-14.40 min/day). Tertiles of overall PA and the time spent in vigorous PA were compared using one-way PERMANOVA with 9,999 permutations for significance testing with the Paleontological Statistics (Past3) software[56] for the calculation of beta diversity. Kruskal-Wallis tests were performed to investigate whether there were significant differences in body composition, dietary intake and cardiometabolic profile as well as fecal microbiota alpha diversity and composition outcomes across tertiles of overall PA and the time spent in vigorous PA. Analysis of covariance was used to compare the relative abundance of genera across tertiles of the time spent in vigorous PA adjusted for protein intake with the data transformed by Blom's formula. All P values were corrected by Benjamini and Hochberg multiple testing for controlling the False Discovery Rate (FDR, shown as q-values)[57]. The level of significance was assumed at P<0.05 and q<0.05. R software (V.3.6.0; http://www.R-project.org)[26] and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA, (http://www.graphpad.com) were executed for the statistical analysis and graphical plots.

RESULTS

Characteristics of participants

A total of 92 participants had data from analysis of fecal microbiota diversity and composition, but only 88 participants had valid physical activity measurements, who were finally included in the analyses. **Table 1** shows the descriptive characteristics of the included participants (age 21.7 (19.8-23.9) years and BMI 23.6 (21.6-28.1) kg/m^2), of whom 72.7% were women. We performed tertiles of overall PA and the time spent in vigorous PA and we observed that generally body composition, dietary intake, and cardiometabolic

profile were similar across them (**Table 2**), with the exception of protein intake and glucose levels (P=0.018 and P=0.003, respectively; **Table 2**).

Table 1. Descriptive characteristics of the participants.

	All (N=88)	**Men (N=24)**	**Women (N=64)**
Age (years)	21.7 (19.8-23.9)	22.0 (19.8-24.5)	21.6 (19.8-23.7)
Body composition			
Body mass index (kg/m^2)	23.6 (21.6-28.1)	25.8 (22.8-32.5)	23.3 (21.0-27.4)
Lean mass index (kg/m^2)	13.9 (12.6-15.7)	17.4 (15.3-18.4)	13.2 (12.5-14.3)
Fat mass index (kg/m^2)	8.7 (6.3-11.5)	7.9 (4.5-11.3)	9.2 (6.5-11.5)
Fat mass (%)	36.7 (31.2-42.6)	32.5 (20.8-37.1)	39.3 (32.9-42.8)
Sedentary and physical activity time			
Non wear time (min/week)	8.6 (0.0-22.2)	8.3 (0.0-28.9)	8.6 (0.0-22.2)
Overall PA (mg)	31.8 (27.2-37.2)	31.0 (24.8-35.3)	32.8 (28.2-37.4)
Sedentary Time (min/day)	785.2 (753.2-820.7)	798.8 (770.0-837.7)	779.2 (752.6-820.2)
Light PA (min/day)	113.6 (99.2-139.0)	109.8 (90.7-124.2)	118.8 (99.7-145.3)
Moderate PA (min/day)	90.0 (67.9-109.5)	84.1 (57.7-103.1)	93.1 (69.7-113.1)
Vigorous PA (min/day)	1.2 (0.7-3.8)	1.3 (0.5-3.3)	1.2 (0.7-4.6)
Moderate to Vigorous PA (min/day)	91.3 (70.5-111.3)	86.3 (58.1-104.6)	95.6 (72.2-116.4)
Dietary intake			
Energy intake (kcal/day)	1847.7 (1580.8-2184.4)	2085.2 (1783.3-2641.3)	1798.2 (1553.6-2090.2)
Fat (g/day)	84.5 (70.0-100.2)	97.8 (71.5-111.8)	82.1 (69.2-96.7)
Proteins (g/day)	72.6 (61.4-90.9)	102.2 (75.3-118.9)	67.0 (59.8-79.4)
Carbohydrates (g/day)	198.2 (159.3-229.6)	200.4 (172.5-244.9)	198.2 (149.2-226.2)
Fiber (g/day)	16.2 (11.7-19.8)	16.3 (13.4-20.5)	16.2 (11.2-19.4)
Cardiometabolic profile			
Glucose (mg/dL)	87.0 (84.0-92.0)	91.0 (85.0-97.0)	87.0 (84.0-92.0)
Insulin (µUI/mL)	6.7 (4.9-10.7)	6.9 (4.8-12.4)	6.7 (4.9-9.5)
HOMA index	1.4 (1.1-2.4)	1.5 (1.0-3.0)	1.4 (1.1-2.2)
Total Cholesterol (mg/dL)	161.0 (141.5-189.0)	153.0 (139.0-174.0)	166.0 (143.0-197.0)
HDL-C (mg/dL)	51.0 (46.0-58.3)	46.0 (40.0-51.0)	53.0 (48.0-63.0)
LDL-C (mg/dL)	94.0 (78.0-114.0)	90.0 (81.0-104.0)	96.0 (78.0-114.0)
Triglycerides (mg/dL)	71.0 (52.8-106.3)	77.0 (60.0-115.0)	68.0 (52.0-98.0)
Fecal microbiota			
Alpha diversity indexes			
Richness Chao	391.6 (339.8-508.1)	374.1 (333.9-448.1)	423.5 (344.1-538.7)
Shannon diversity	4.2 (4.0-4.5)	4.2 (4.0-4.5)	4.3 (4.0-4.5)
Inverse Simpson diversity	31.7 (23.1-42.7)	31.7 (22.5-49.1)	31.7 (23.2-41.5)
Evenness Camargo	0.2 (0.2-0.3)	0.2 (0.2-0.3)	0.2 (0.2-0.3)
Composition (Phylum)			
Actinobacteria (%)	1.1 (0.6-1.9)	1.1 (0.4-1.7)	1.1 (0.6-2.2)
Bacteroidetes (%)	41.3 (34.7-44.8)	41.8 (39.9-45.2)	40.9 (34.3-44.8)
Firmicutes (%)	47.2 (42.1-52.5)	48.7 (43.3-52.0)	45.6 (41.3-53.1)
Proteobacteria (%)	5.1 (3.8-8.2)	6.7 (4.2-8.8)	4.6 (3.5-7.3)
Verrucomicrobia (%)	0.1 (0.0-2.0)	0.0 (0.0-0.8)	0.2 (0.0-3.5)

Data are presented as median (interquartile range). Abbreviations: BMI: body mass index; FMI: fat mass index; HDL-C: high-density lipoprotein cholesterol; HOMA index: homeostatic model assessment index; LDL-C: low-density lipoprotein cholesterol; LMI: Lean mass index; mg: mili-gravitational units; PA: Physical activity.

Table 2. Characteristics of participants according to tertiles of overall PA and the time spent in vigorous PA.

	Overall PA (mg)				**Time spent in vigorous PA (min/day)**			
	Low (13.5-29.4) n=29	Intermediate (30.0-35.4) n=30	High (35.5-67.1) n=29		Low (0.0-0.8) n=29	Intermediate (0.9-2.7) n=30	High (2.8-14.4) n=29	
				P				P
Age (years)	22.3 ± 2.5	22.0 ± 2.0	21.6 ± 2.5	0.461	22.3 ± 2.3	22.2 ± 2.3	21.4 ± 2.2	0.239
Sex, N				0.501				0.555
Men	10	8	6		8	10	6	
Women	19	22	23		21	20	23	
Body composition								
BMI (kg/m^2)	25.6 ± 5.4	24.2 ± 4.7	25.2 ± 4.2	0.548	24.5 ± 5.0	25.7 ± 4.8	24.8 ± 4.6	0.531
LMI (kg/m^2)	14.4 ± 2.5	13.9 ± 2.3	14.9 ± 2.0	0.150	14.0 ± 2.3	14.5 ± 2.2	14.6 ± 2.4	0.380
FMI (kg/m^2)	9.3 ± 3.4	8.7 ± 2.7	8.9 ± 3.3	0.805	9.0 ± 3.5	9.1 ± 3.0	8.8 ± 2.9	0.946
Fat mass (%)	37.1 ± 7.7	36.7 ± 6.5	35.4 ± 8.9	0.823	36.8 ± 8.5	36.6 ± 7.5	35.7 ± 7.3	0.734
Dietary intake								
Energy intake (kcal/day)	1985 ± 597.1	1805 ± 382.1	1992 ± 453.2	0.294	1827 ± 602.5	2055 ± 412.1	1892 ± 411.0	0.080
Fat (g/day)	91.0 ± 36.7	81.4 ± 21.9	89.3 ± 22.8	0.344	82.6 ± 37.2	92.1 ± 23.8	86.6 ± 20.0	0.171
Proteins (g/day)	78.9 ± 27.2	75.2 ±19.6	80.6 ± 23.0	0.597	**71.3 ± 24.6***	**85.2 ± 22.4***	77.9 ± 21.3	**0.018**
Carbohydrates (g/day)	208.6 ± 61.5	188.1 ± 57.5	211.7 ± 67.3	0.215	196.7 ± 67.6	216.0 ± 51.5	194.8 ± 67.0	0.129
Fiber (g/day)	16.9 ± 6.2	16.8 ± 8.0	17.0 ± 5.1	0.529	16.6 ± 7.2	17.7 ± 6.7	16.4 ± 5.6	0.654
Cardiometabolic profile								
Glucose (mg/dL)	89.2 ± 7.5	88.7 ± 6.1	86.8 ± 6.4	0.502	**89.6 ± 6.3***	**90.3 ± 7.0†**	**84.7 ± 5.5*†**	**0.003**

Insulin (µUI/mL)	10.8 ± 9.3	7.4 ± 3.3	7.5 ± 4.3	0.149	9.4 ± 6.2	9.3 ± 8.1	7.0 ± 3.9	0.139
HOMA index	2.5 ± 2.6	1.6 ± 0.8	1.6 ± 1.0	0.132	2.1 ± 1.6	2.2 ± 2.3	1.5 ± 0.9	0.059
Total Cholesterol (mg/dL)	171.8 ± 38.1	167.6 ± 32.6	167.0 ± 42.1	0.757	163.0 ± 32.9	179.9 ± 47.2	163.3 ± 27.4	0.370
HDL-C (mg/dL)	51.4 ± 14.1	54.4 ± 9.3	52.6 ± 12.1	0.241	51.0 ± 13.0	55.2 ± 12.6	52.3 ± 9.8	0.448
LDL-C (mg/dL)	100.8 ± 29.1	97.5 ± 27.0	98.7 ± 29.1	0.893	95.0 ± 25.6	105.4 ± 34.1	96.5 ± 23.2	0.599
Triglycerides (mg/dL)	100.6 ± 65.2	89.6 ± 63.4	80.7 ± 63.0	0.068	84.6 ± 46.9	113.8 ± 91.9	72.3 ± 27.5	0.242

Data are presented as means ± standard deviations. Symbol (*) indicates significant differences between low and high tertiles, whereas symbol (†) shows significant differences between intermediate and high tertiles by means of Kruskal-Wallis. BMI: body mass index; FMI: fat mass index; HDL-C: high-density lipoprotein cholesterol; HOMA index: homeostatic model assessment index; LDL-C: low-density lipoprotein cholesterol; LMI: Lean mass index; m*g*: mili-gravitational units; PA: Physical activity.

Relationship between physical activity and fecal microbiota diversity

Overall PA and the time spent in vigorous PA were positively correlated with alpha diversity indexes, more specifically with Shannon and Inverse Simpson diversity indexes (all rho≥0.23, P≤0.034; **Fig. 1**). Only the time spent in vigorous PA was positively correlated with the Richness Chao index (rho=0.24, P=0.023; **Fig. 1**). However, we did not observe any significant correlation between others PA variables with alpha diversity indexes (all P>0.05; **Fig. 1**). The results were similar when sex, BMI, energy and macronutrients intake, as well as accelerometer non-wear time and glucose levels, were included as a confounder in separated models (data not shown). Moreover, we found that individuals with high time spent in vigorous PA had a higher Richness Chao, Shannon and Inverse Simpson diversity indexes than individuals with low and intermediate time spent in vigorous PA (all P≤0.038; data not shown). However, there were no differences across tertiles of overall PA and the time spent in vigorous PA in the beta diversity at any taxonomic levels (all P≥0.060; **Table 3**).

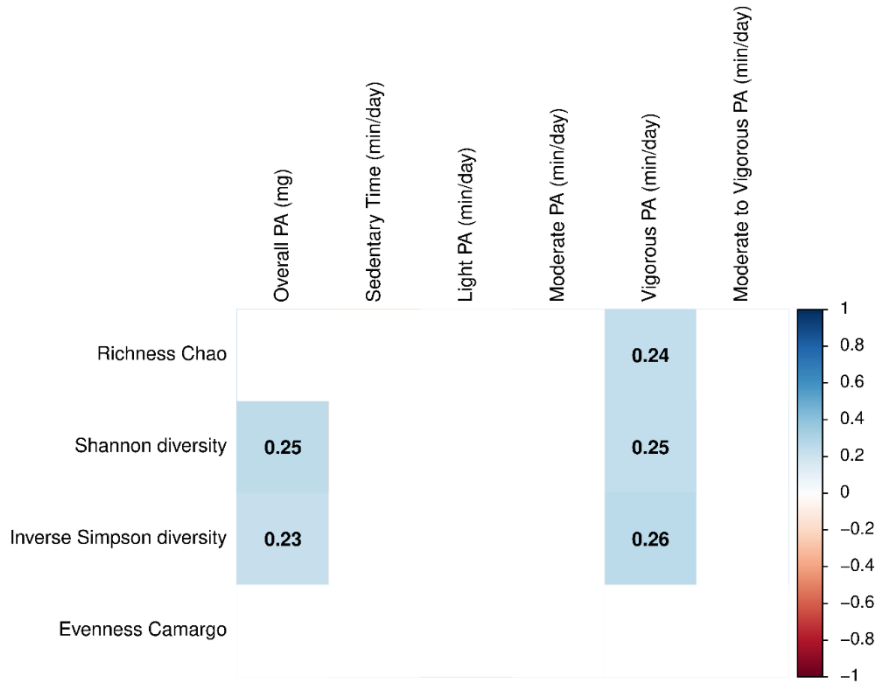

Figure 1. Spearman correlation of physical activity variables with fecal microbiota diversity. *Boxes only represent the statistically significant (P<0.05) correlations and the value within the boxes show the Spearman correlation coefficient. Blue boxes indicate positive correlation whereas red squares indicate negative correlation between physical activity variables with fecal microbiota diversity indexes. mg: mili-gravitational units; PA: Physical activity.*

Table 3. Beta diversity across tertiles of overall PA and the time spent in vigorous PA at all taxonomic levels.

		Tertiles of overall PA (mg)		**Tertiles of the time spent in vigorous PA (min/day)**	
Taxonomic levels		*Pseudo-F*	*P*	*Pseudo-F*	*P*
	Phylum	0.502	0.789	0.967	0.443
	Class	0.926	0.508	1.224	0.262
	Order	0.959	0.475	1.282	0.221
	Family	1.132	0.306	1.476	0.106
	Genus	0.968	0.492	1.524	0.060

PERMANOVA using 9,999 permutations for significance testing (p-value<0.05). mg: mili-gravitational units; PA: physical activity; Pseudo-F: statistic, larger number indicate greater separation[87] across tertiles of total PA and vigorous PA levels.

Relationship between physical activity variables and fecal microbiota composition

We analyzed the differences across tertiles of overall PA and the time spent in vigorous PA on fecal microbiota composition at all taxonomic levels. There were no significant differences across tertiles of overall PA on the relative abundance of all bacteria at the different taxonomic levels (all P>0.05; **Fig. 2**). Similarly, we observed no differences across tertiles of time spent in vigorous PA on the relative abundance of bacteria at phylum taxonomic level (all P≥0.318; **Fig. 3A**). However, we observed that individuals with low time spent in vigorous PA had a higher relative abundance of *Gammaproteobacteria* class (*Proteobacteria* phylum) than individuals with intermediate time spent in vigorous PA (q=0.021, FDR=q-value; **Fig. 3B**). Moreover, individuals with high time spent in vigorous PA had higher relative abundance of Unclassified *Firmicutes* class (*Firmicutes* phylum) and *Porphyromonadaceae* family (*Bacteroidetes* phylum) than individuals with low time spent in vigorous PA (q=0.027, and q=0.031, respectively; **Fig. 3B** and **D**). Finally, we found that individuals with intermediate time spent in vigorous PA had a higher relative abundance of *Alistipes* genus (*Bacteroidetes* phylum) than individuals with low time spent in vigorous PA (q=0.015; **Fig. 3E**). Interestingly, these same participants presented differences in protein intake (q=0.011; **Table 2**). Thus, we repeated the analyses after adjusting for the protein intake and the differences in the relative abundance of *Alistipes* genus disappeared (P=0.080; **Table 4**).

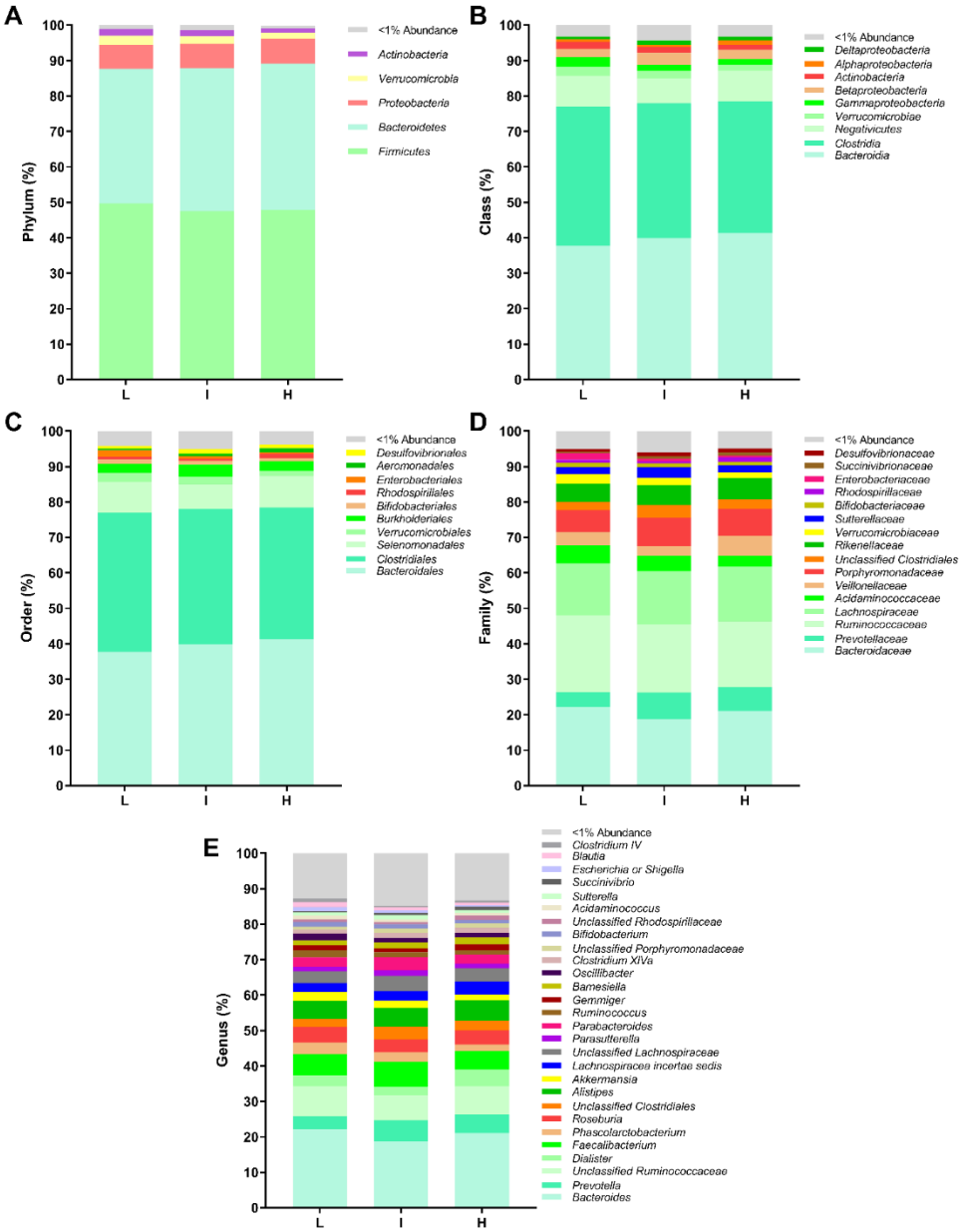

Figure 2. Fecal microbiota composition according to tertiles of overall physical activity (PA) levels *(L: low, 13.45-29.44 mg; I: intermediate, 30.02-35.41 mg; H: high, 35.49-67.10 mg). Panels indicate relative abundance of the fecal microbiota at phylum (A), class (B), order (C), family (D) and genus (E) taxonomic levels according to tertiles of overall PA. Stacked bar represented percentage abundance. Kruskal-Wallis test was used to test for each pairwise comparison, correcting for multiple comparisons FDR (q<0.05) (GraphPad Prism 8.00).*

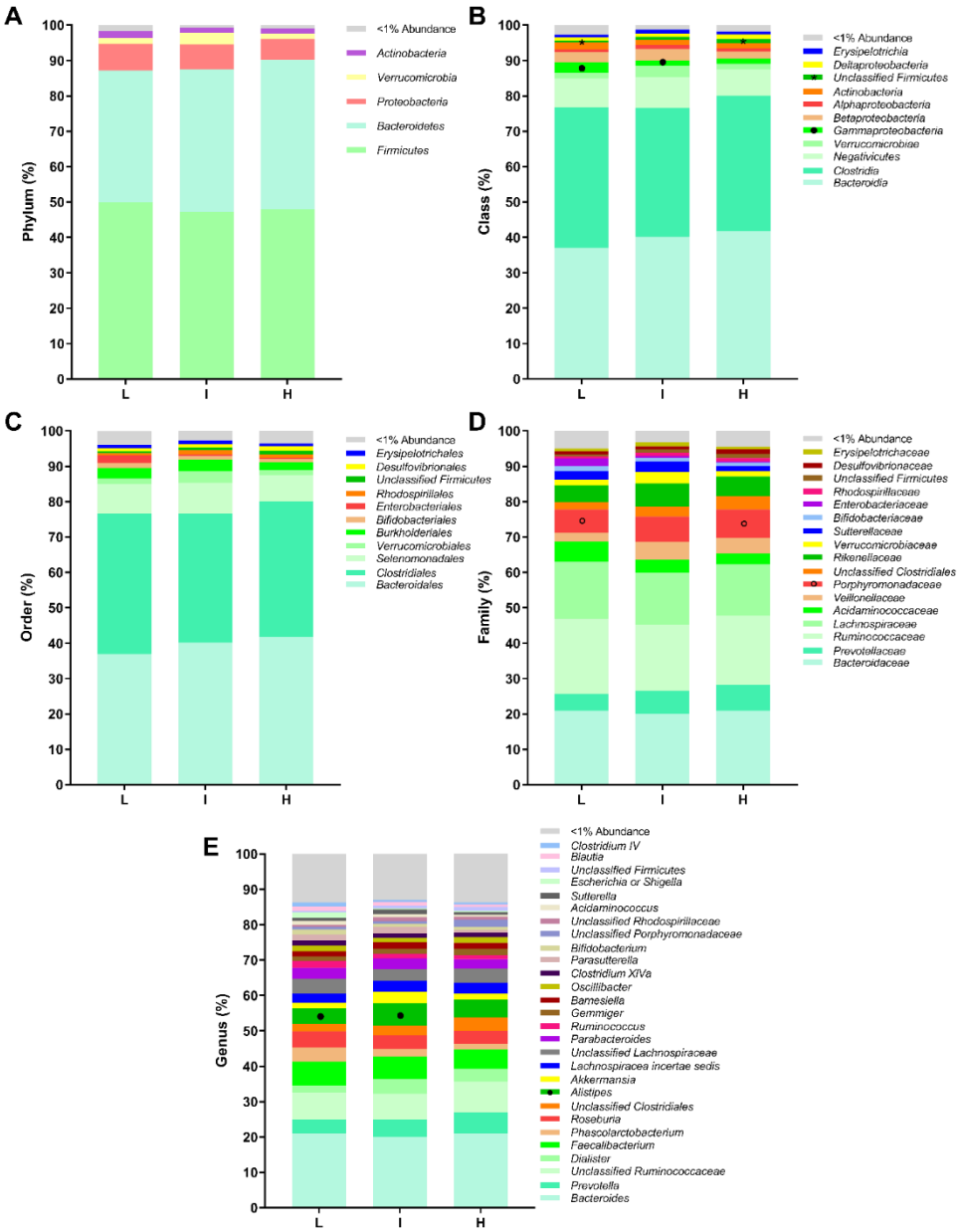

Figure 3. Fecal microbiota composition according to tertiles of the time spent in vigorous physical activity (PA) *(L: low, 0.02-0.83 min/day; I: intermediate, 0.87-2.67 min/day; H: high, 2.75-14.40 min/day). Panels indicate relative abundance of the fecal microbiota at phylum (A), class (B), order (C), family (D) and genus (E) taxonomic levels according to tertiles of the time spent in vigorous PA. Stacked bar represented percentage abundance. Symbols (*) and (○) mean statistical significance differences between low and high time spent in vigorous PA, and symbol (•) represents statistical significance differences between low and intermediate time spent in vigorous PA. Kruskal-Wallis test was used to test for each pairwise comparison, correcting for multiple comparisons FDR (q<0.05) (GraphPad Prism 8.00).*

Table 4. Differences in the relative abundance of *Alistipes* genus across tertiles of the time spent in vigorous PA.

	Time spent in vigorous PA (min/day)				
	Low (0.0-0.8) n=29	Intermediate (0.9-2.7) n=30	High (2.8-14.4) n=29	P for model 1	P for model 2
Alistipes genus (%)	**4.3 ± 2.9***	**6.3 ± 3.3***	5.2 ± 3.0	**0.026**	0.080

Data are presented as means ± standard deviations. Symbol (*) indicates significant differences between low and intermediate tertiles. Model 1: P value from Kruskal Wallis test. Model 2: P values were obtained from one-way analyses of variance adjusted for protein intake (g) with data transformed by Blom's formula. PA: Physical activity.

DISCUSSION

The present study shows that overall PA and the time spent in vigorous PA were positively correlated with alpha diversity indexes in young adults. Moreover, there were differences across the tertiles of time spent in vigorous PA in the relative abundance of *Gammaproteobacteria* class (*Proteobacteria* phylum), *Porphyromonadaceae* family and *Alistipes* genus (both *Bacteroidetes* phylum). These findings indicate that PA may play a role in fecal microbiota diversity and composition in young adults, although further studies are needed to confirm these findings.

Our results showing the positive correlation between PA and alpha diversity are in agreement with previous studies[16,17,58,59]. The mechanisms by which PA may promote a higher fecal microbiota diversity are not known. A possibility could be the changes in the gastrointestinal tract due to intrinsic adaptations of performing PA[60]. Interestingly, from an ecological perspective, microbial diversity may be a key factor in allowing an ecosystem to continue operating properly[61]. In fact, greater species diversity has been associated with a healthy phenotype's host[62]. This is due to the potential effects that the bacteria can exert via metabolites, such SCFAs and neurotransmitters, locally and extra-intestinal tissues in the host[63].

Our data showed that the participants with low time spent in vigorous PA had higher relative abundance of *Gammaproteobacteria* class than individuals with higher time spent in vigorous PA. The relative abundance of *Gammaproteobacteria* class (*Proteobacteria* phylum) has been reported to be increased in obese mice[64] and individuals with obesity[65,66], and disease states such as metabolic diseases[67] and intestinal inflammation[68]. In fact, many common human pathogens, known as sulphur producers[69,70], are found in the *Gammaproteobacteria* class, for example, *Escherichia*, *Shigella*, and *Yersinia* genera[71]. In agreement with our findings, sedentary women and participants with low cardiorespiratory fitness[72] had a higher relative abundance of *Gammaproteobacteria* class in comparison with active women and participants with high cardiorespiratory fitness, respectively. Moreover, several studies have shown that exercise seems to decrease the relative abundance of *Gammaproteobacteria* class[73,74]. Thus, our results suggest that performing less than 1 min/day of vigorous PA, could be related to having a higher relative

abundance of *Gammaproteobacteria* class, bacteria considered health-detrimental.

In contrast, we observed that individuals with high and intermediate time spent in vigorous PA had a higher the relative abundance of *Porphyromonadaceae* family and *Alistipes* genus (both *Bacteroidetes* phylum) than individuals with lower time spent in vigorous PA. Accordingly, in a cross-sectional study of professional martial arts athletes, the relative abundance of *Porphyromonadaceae* family was higher in the higher-level athletes in comparison with the lower-level athletes[75]. Moreover, regular swim training[76] and voluntary wheel running[77], both in mice, were able to increase the relative abundance of *Porphyromonadaceae* family. In fact, it has recently been found that lean individuals had a significantly higher the relative abundance of the *Porphyromonadaceae* and *Rikenellaceae* families than individuals with obesity[54,78]. Of note is that the *Alistipes* genus belongs to the *Rikenellaceae* family. In resistance-trained mice, the relative abundance of *Alistipes* genus was positively correlated with resistance performance[79]. In humans, the relative abundance of *Alistipes* genus is increased after consuming an animal-based diet intake, rich in protein, for 5 days[80]. Certain species that belong to *Alistipes* genus are involved in amino acids metabolism, concretely it can hydrolyse tryptophan to indole[81]. Since tryptophan is an essential amino acid that cannot be produced by animal cells, humans rely on dietary intake, mainly proteins, for incorporating it into the organism[82–84]. In our study, the individuals with intermediate time spent in vigorous PA had higher protein intake than individuals with low time spent in vigorous PA. In fact, when the protein intake was included as confounder, the differences in the relative abundance of *Alistipes* genus between these individuals disappeared. Considering the relationship between *Alistipes* genus with protein metabolism[81], and the results observed in the present study, it is possible to think that these differences were explained by protein intake. Therefore, our data bring light on that spending time on vigorous PA, between 3-14 min/day, could be related to having a higher relative abundance of *Porphyromonadaceae* family, whereas the protein intake seems to modulate the relative abundance of *Alistipes* genus in individuals with intermediate time spent in vigorous PA. Even so, the possible role of time spent in vigorous PA on the relative abundance of *Gammaproteobacteria* class, *Porphyromonadaceae* family and *Alistipes* genus deserves further analysis.

Limitations and strengths

A limitation to consider in the current study follows a cross-sectional design, which prevents a causal interpretation of our results. Well-designed randomized controlled trials should be carried out to elucidate the role of PA in fecal microbiota diversity and composition. In addition, we do not know whether our findings apply to older people or individuals presenting with any metabolic disease. As for strengths of this study, we should stand out that we sequenced the microbiota composition using the latest technology (Illumina platform) and annotations were made with RDP until genus taxon. Moreover, PA was objectively measured by accelerometry during 7 consecutive days (24h/day)[23], and we used a cut-point-free approach to assess overall PA since PA intensities estimated from cut-points might be biased by poor calibration studies[85,86].

Conclusions

Our data showed that overall PA and time spent in vigorous PA are positively correlated with fecal microbiota diversity in young adults. Moreover, the individuals with low time spent in vigorous PA presented higher relative abundance of *Gammaproteobacteria* class, whereas the individuals with high time spent in vigorous PA had higher relative abundance of *Porphyromonadaceae* family. Altogether, these findings suggest that PA, especially of vigorous intensity, is related to fecal microbiota diversity and *Gammaproteobacteria* class and *Porphyromonadaceae* family in young adults. Further studies are needed to confirm this relationship.

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SECTION II

STUDY IV: FECAL MICROBIOTA DIVERSITY AND COMPOSITION IN RESISTANCE AND ENDURANCE ATHLETES: A CASE-CONTROL STUDY

ABSTRACT

Background and aim: Scientific evidence shows that the fecal microbiota diversity and composition of athletes differs from sedentary people, however, it is unclear whether practising different types of exercise (i.e., resistance vs endurance) depicts a different fecal microbiota composition. Thus, we investigated whether resistance and endurance athletes have different fecal microbiota diversity and composition, and compared to sedentary individuals.

Methods: The present case-control study included 50 participants of whom 14 were sedentary (SED), 14 were people experienced in resistance exercise training (RES) and 22 were endurance athletes (END). Body composition, physical fitness and dietary intake were assessed by routine methods. A fecal sample was collected from each participant and DNA extracted was analysed using 16S rRNA gene sequencing.

Results: There were no differences across groups on fecal microbiota beta or alpha diversities. RES participants showed a higher relative abundance of *Pasteurellaceae* family and *Streptococcus* genus compared to END and SED participants (+0.2±0.4%, P≤0.031, and +0.3±0.3%, P≤0.002, respectively). Moreover, END had a higher relative abundance of *Clostridiaceae 1* family and *Clostridium sensu stricto* genus vs. SED participants (+0.3±0.4%, all P=0.039), and a higher relative abundance of *Paraprevotella* genus than RES (+0.99±0.04%, P=0.030). Lastly, SED presented a higher relative abundance of *Intestinimonas* genus vs. RES (+0.2±0.3%, P=0.022).

Conclusion: Our findings indicate fecal microbiota diversity does not differ between resistance, endurance athletes and sedentary people. However, the differences in fecal microbiota composition across groups showed that the type of exercise training may influence on fecal microbiota composition of healthy adults.

INTRODUCTION

The role of the microorganisms that live in the gastrointestinal tract, known as gut microbiota, has become the focus of human health research[1] due to its contribution to many metabolic processes in the host, such as the fermentation of undigested carbohydrates into short-chain fatty acids (SCFAs), lipid metabolism, and vitamin synthesis[2]. Gut microbiota also stimulates the maturation of the immune system and protects against potentially pathogenic microbes[2]. Thus, it is critical to understand factors that impact gut microbiota diversity and composition.

Exercise has well-known effects on metabolism and the immune system[3], but the effects of exercise on the gut microbiota have been less well studied. Intervention studies in humans have investigated the effect of exercise on gut microbiota during endurance exercise training[4–6] or concurrent training (endurance and resistance exercises)[7,8]. Their results have shown that exercise increases fecal microbiota diversity and the relative abundance of bacteria belonging to *Firmicutes* and *Bacteroidetes* phyla[9–13]. However, the concurrent training prevents us to know whether endurance or resistance exercise training provokes different effects on gut microbiota diversity and composition in sedentary people. Moreover, it is well known that after catching up the adulthood, the fecal microbiota diversity and composition keep on relatively stable over time[14]. However, brief exposure to external stimuli, such as exercise, can alter the bacteria communities in a manner substantial but transient, leading to a total or partial recovery of the previous composition when disappearing the external stimuli[15]. Indeed, exercise-induced shifts in the fecal microbiota diversity and composition of sedentary people after six weeks of exercise intervention returned to baseline values six weeks after finishing the exercise intervention[5]. Altogether, these findings indicate that the changes in fecal microbiota diversity and composition may be transient and need repeated exercise stimuli to settle as native bacteria in our gut. For this reason, focusing on athletes who have incorporated exercise training into their life for some years would be the best approach for observing how the different types of exercise training affect the fecal microbiota diversity and composition in humans.

Therefore, the present case-control study aimed to investigate whether resistance and endurance athletes have different fecal microbiota diversity and composition, and compared to sedentary individuals.

METHODS

Study design and participants

A total of 50 adults (38% women) between 18 and 48 years old participated in this case-control study called Oh My Gutness! Project (**Fig. 1**). The participants were recruited from the city of Granada (Spain) through social networks, local media, and posters. The study includes a group of 14 adults that reported to be sedentary, so called sedentary group (SED), a resistance exercise training group (RES; CrossFit or strength training) with 14 athletes and an endurance exercise training group (END; running, cycling or triathlon) with 22 athletes,

according to the main characteristic of the sport modality trained. Inclusion criteria were for the athletes who had to train for ≥3 years and ≥3 days per week, whereas the sedentary people had to do less than 20min/day less 3 days per week of physical activity verified with the International Physical Activity Questionnaire[16]. Moreover, all participants could not participate in a weight loss program and they had to have a stable weight during the last 3 months. Exclusion criteria included diagnostic of gastrointestinal diseases or other medical conditions that can affect the fecal microbiota, being pregnant and consuming antibiotics during the last 3 months, as well as other medication being able to modify the fecal microbiota. The study protocol and informed consent were designed by following the last version of the Declaration of Helsinki. This study was approved by the Ethics Committee on Human Research of the University of Granada (1582/CEIH/2020); all participants gave their written informed consent.

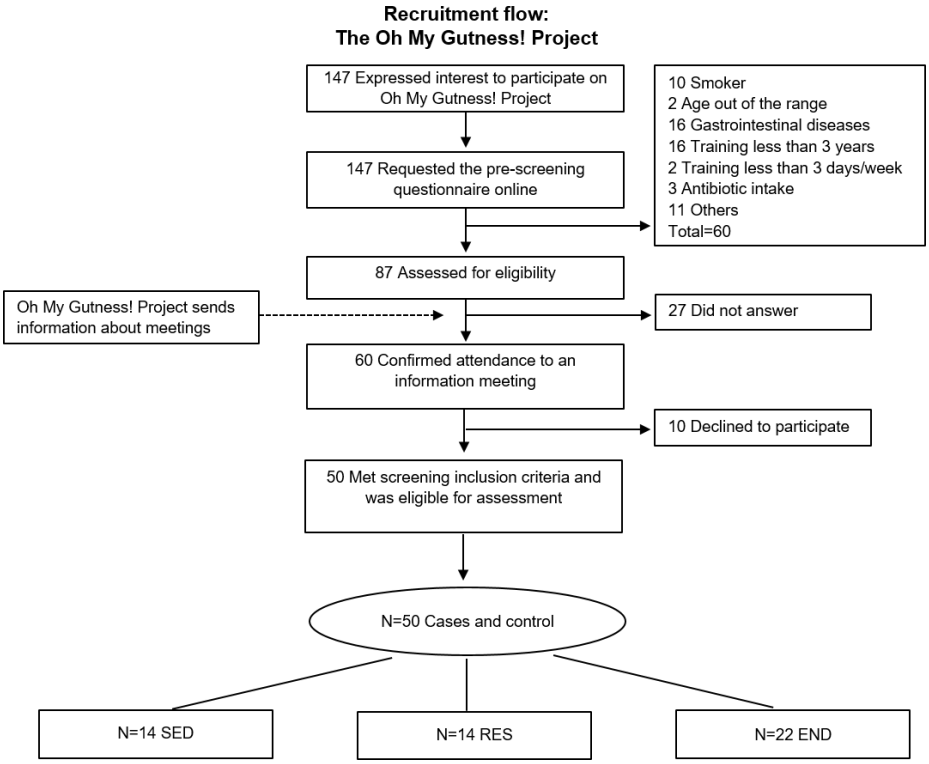

Figure 1. Study participants enrollment from the Oh My Gutness! Project. *Abbreviations: END: endurance trained group; RES: resistance trained group; SED: sedentary group.*

Anthropometric and body composition assessments

The participants were weighted with a SECA scale and we measured their height with a stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Body composition was evaluated by dual-energy X-ray absorptiometry (DEXA, HOLOGIC, Discovery Wi, Marlborough, MA), and obtained data from lean mass (kg), fat free mass (kg), fat mass (kg) and visceral

adipose tissue (g). Body mass index, lean mass index and fat mass index were computed as weight, lean body mass and fat body mass (kg), respectively, divided by height squared (m^2). The fat mass percentage was determined as the body fat mass divided by the total body mass and multiplied by 100.

Physical fitness assessment: Muscular strength

A handgrip strength test was used for measuring muscular strength. It was determined through a Takei 5401 digital Grip-D hand dynamometer (Takei, Tokyo, Japan) following the protocol published elsewhere[17]. Briefly, the participants were asked to squeeze the grip gradually and continuously, and we encouraged them to do their best while performing the tests. It was performed twice, alternating between both hands with a 1 min rest between efforts. Data were expressed in kg in absolute terms and the average of the highest values performed in the test was selected for the analyses.

Dietary intake assessment

Dietary intake was recorded using one 24 h recall through face-to-face interviews performed by qualified and trained dietitians as previously described[18]. The participants were asked to recall all food consumed on the previous day, right the day that they collected the fecal sample. Data recorded in the interviews were introduced independently by two dieticians in the EvalFINUT® software (https://www.finut.org/evalfinut/)[18]. We obtained data from energy (kcal), fat (g), saturated fatty acids (g), monounsaturated fatty acids (g), polyunsaturated fatty acids (g), cholesterol (mg), protein (g), carbohydrates (g), sugar (g), and fibre (g).

Fecal microbiota analyses

A fecal sample was collected for each participant and stored at -80°C until the extraction of DNA with QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain). Next, the quantification of DNA were performed using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, DE, USA). Amplicon libraries were generated as previously described[19] where the V1-V2 region of the 16S rRNA gene was amplified after 20 cycles of PCR reaction using the 27F and 338R primers[20] and sequenced on a MiSeq (2×250 bp, Illumina, Hayward, California, USA). Bioinformatics processing was performed as previously described[21]. FastQ files were analyzed with the "dada2"[22] packages in R software, and the taxonomic affiliation of phylotypes were assigned using the "classifier" function from the Ribosomal Database Project (RDP). Microbial communities were analyzed at different taxonomic levels (phylum to genus), calculating the relative abundances, which were expressed as percentages.

Statistical analyses

Data are presented as means ± standard deviations. Variable normal distribution was verified using the D'Agostino and Pearson omnibus test. None of the fecal microbiota diversity and composition parameters followed a normal distribution, so those values were transformed by Blom's formula[23]. We

conducted all the analyses adjusted for sex. Differences in age, body composition, physical fitness, dietary intake, and fecal microbiota alpha diversity and composition across groups were analyzed by one-way analyses of variance adjusted for sex and correction for multiple comparisons was determined by Bonferroni. We performed two-way PERMANOVA with 9999 permutations for significance testing with the Paleontological Statistics (Past3) software for analyzing the differences across groups in fecal microbiota beta diversity. The level of significance across groups was set at $P < 0.05$. The Statistical Package for the Social Sciences (SPSS) v.25.0 (IBM Corporation, Chicago, IL, USA) was used for all analyses, whereas GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) was used for plots.

RESULTS

Among the initial 147 participants that carried out the pre-screening questionnaire online, 50 participants met screening inclusion criteria and were included in the analysis (**Figure 1**). Anthropometric characteristics were similar across groups (All P≥0.053) (**Table 1**). However, the RES participants had a higher lean mass, lean mass index and fat free mass levels in comparison with SED participants (P≤0.010). Moreover, RES and END participants showed lower fat mass percentages in comparison with SED participants (P=0.010). Physical fitness and dietary intake were similar across groups (P≥0.051).

Table 1. Descriptive characteristics of the participants.

	SED (N=14)	**RES (N=14)**	**END (N=22)**	**P**
Age (years)	30.3 ± 7.2	27.3 ± 4.1●	34.0 ± 9.7●	**0.045**
Sex (% women)	78.6%	42.9%	9.1%	
Years Training		8.5 ± 6.9	11.7 ± 9.4	0.112
Training per week (days)		4.5 ± 0.9	5.3 ± 1.1	0.066
Body composition				
Weight (kg)	64.1 ± 9.7	70.6 ± 10.4	69.3 ± 6.4	0.096
Height (cm)	166.1 ± 9.8	172.1 ± 7.8	173.5 ± 6.9	0.053
Body mass index (kg/m^2)	23.3 ± 3.2	23.8 ± 2.5	23.0 ± 1.7	0.696
Lean mass (kg)	38.0 ± 8.6*	48.9 ± 10.1*	51.1 ± 5.2	**0.008**
Lean mass index (kg/m^2)	13.6 ± 1.6*	16.3 ± 2.5*	17.0 ± 1.2	**0.010**
Fat Free mass (kg)	40.1 ± 8.9*	51.5 ± 10.7*	53.7 ± 5.3	**0.007**
Fat mass (kg)	22.7 ± 6.8	17.8 ± 5.2	14.6 ± 4.0	0.078
Fat mass index (kg/m^2)	8.4 ± 3.0	6.1 ± 1.9	4.9 ± 1.4	0.090
Fat mass (%)	36.2 ± 8.9†*	26.0 ± 7.8*	21.3 ± 5.2†	**0.010**
Visceral adipose tissue (g)	319.6 ± 140.4	249.1 ± 124.4	270.0 ± 94.8	0.060
Physical fitness				
Handgrip (kg)	27.8 ± 11.4	38.5 ± 11.3	40.5 ± 6.6	0.107
Dietary intake				
Energy (kcal)	1929.1 ± 513.6	2503.6 ± 1012.8	2704.6 ± 705.1	0.502
Fat (g)	93.4 ± 35.2	104.8 ± 48.7	119.3 ± 39.1	0.970
SFA (g)	27.5 ± 23.0	31.3 ± 16.6	39.8 ± 16.1	0.494
MUFA (g)	41.3 ± 17.8	41.9 ± 25.4	50.4 ± 19.4	0.664
PUFA (g)	12.3 ± 6.1	14.0 ± 5.4	17.2 ± 8.6	0.896

Cholesterol (mg)	348.1	±	251.4	446.5	±	370.3	364.0	±	215.4	0.343
Protein (g)	82.1	±	25.1	127.6	±	50.4	116.6	±	35.5	0.051
Carbohydrates (g)	186.3	±	78.4	259.8	±	138.1	273.7	±	97.8	0.494
Sugar (g)	5.7	±	7.2	9.7	±	13.4	10.7	±	13.1	0.624
Fibre (g)	35.8	±	26.8	33.9	±	15.9	35.3	±	18.9	0.969

Data are presented as means ± standard deviations. P values were obtained from one-way analyses of variance adjusted for sex. Bonferroni was used to adjust multiple comparisons across groups. ●RES vs. END, *SED vs. RES, and †SED vs. END. *Abbreviations:* END: endurance trained group; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; RES: resistance trained group; SED: sedentary group; SFA: saturated fatty acids.

There were no differences in beta or alpha diversity across groups (P≥0.096; **Fig.2A** and **B**, **Table 2** and **3**). Similarly, we observed no differences across groups in the relative abundance of any bacteria at phylum taxonomic level (all P≥0.174; **Fig. 3A**, and **Table 4**). Interestingly, RES participants showed a higher relative abundance of *Bacilli* class (+0.2±0.1%), *Lactobacillales* order (+0.2±0.1%), *Streptococcaceae* family (+0.3±0.4%) and *Streptococcus* genus (+0.3±0.3%) (all *Firmicutes* phylum) in comparison to SED participants (P≤0.002; **Fig. 3B**, and **Table 4**). They also presented a higher relative abundance of *Pasteurelalles* order, and *Pasteurellaceae* family (both *Proteobacteria* phylum) than END participants (+0.2±0.4%, P≤0.031; **Fig. 3B**, and **Table 4**). Moreover, END participants had a higher relative abundance of *Clostridiaceae 1* family and *Clostridium sensu stricto* genus (both *Firmicutes* phylum) in comparison with SED participants (+0.3±0.4%, P=0.039; **Fig. 3B**, and **Table 4**), and they showed a higher relative abundance of *Paraprevotella* genus (*Bacteroidetes* phylum) than RES participants (+0.99±0.04%, P=0.030; **Fig. 3B**, and **Table 4**). Finally, SED participants presented a higher relative abundance of *Intestinimonas* genus (*Firmicutes* phylum) in comparison with RES participants (+0.2±0.3%, P=0.022; **Fig. 3B**, and **Table 4**).

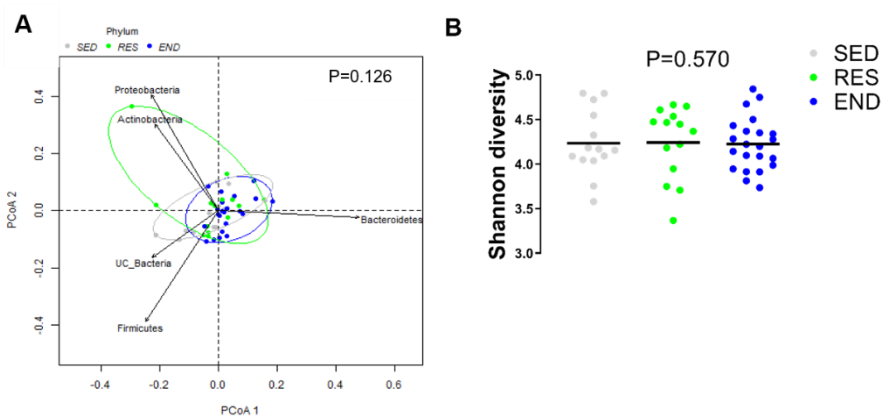

Figure 2. Fecal microbiota diversity of the participants included in the study adjusted for sex. *Panel A shows Principal Coordinate Analysis (PCoA) at phylum taxonomic level. Panel B shows the differences between groups in the Shannon diversity index from alpha diversity. P values (P<0.05) were determined one-way analyses of variance adjusted for sex. Bonferroni was used to adjust multiple comparisons across groups. Abbreviations: END: endurance trained group; RES: resistance trained group; SED: sedentary group; UC: Unclassified*

Table 2. Beta diversity across groups of study at all taxonomic levels adjusted for sex.

	Pseudo-F	*P*
Phylum	-4.904	0.126
Class	-5.204	0.110
Order	-5.145	0.096
Family	-8.188	0.514
Genus	-7.942	0.359

PERMANOVA using 9,999 permutations for significance testing (p-value<0.05). *Abbreviations:* Pseudo-F: statistic, a larger number indicates a greater separation across groups of study.

Table 3. Description of the fecal microbiota alpha diversity of the participants included in the study adjusted for sex

	SED (N=14)			**RES (N=14)**			**END (N=22)**			
	Mean	±	SD	Mean	±	SD	Mean	±	SD	**P**
Diversity indexes										
Richness Chao	191.0	±	46.3	207.0	±	50.3	186.1	±	38.5	0.228
Inverse Simpson diversity	4.2	±	0.4	4.2	±	0.4	4.2	±	0.3	0.512
Evenness Camargo	0.96	±	0.05	0.97	±	0.02	0.97	±	0.03	0.773

Data are presented as mean and standard deviation. P values were obtained from one-way analyses of variance adjusted for sex with data transformed by Blom's formula. Bonferroni was used to adjust multiple comparisons across groups. *Abbreviations:* END: endurance trained group; RES: resistance trained group; SED: sedentary group.

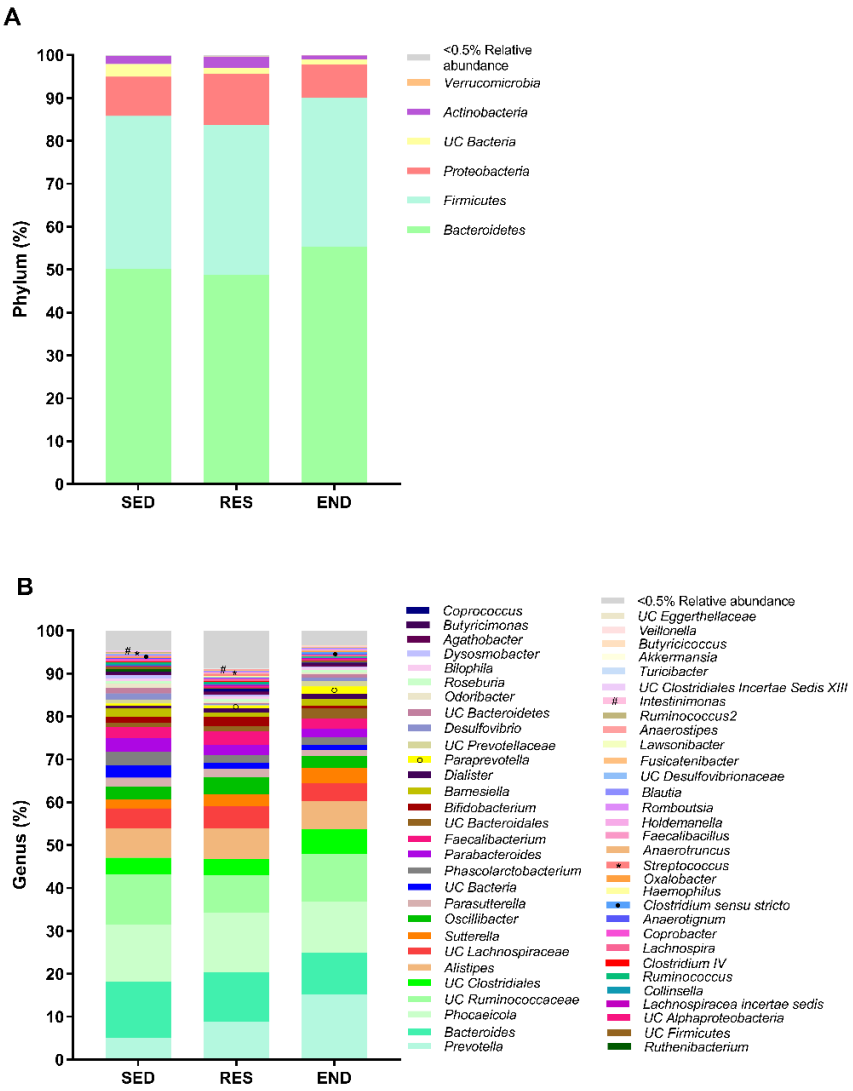

Figure 3. Fecal microbiota composition of the participants included in the study adjusted for sex. *Panels indicate the relative abundance of the fecal microbiota at phylum (panel A) and genus (panel B) taxonomic levels. The stacked bar represented the percentage of relative abundance. Symbols (#) and (*) mean differences between SED and RES, whereas symbol (●) indicates differences between SED and END, and symbol (○) shows differences between RES and END. P values (P<0.05) were determined one-way analyses of variance adjusted for sex. Bonferroni was used to adjust multiple comparisons across groups. Abbreviations: END: endurance trained group; RES: resistance trained group; SED: sedentary group; UC: Unclassified*

Table 4. Description of the fecal microbiota composition of the participants included in the study adjusted for sex

	SED (N=14)			RES (N=14)			END (N=22)			
	Mean	±	SD	Mean	±	SD	Mean	±	SD	P
Phylum (%)										
Actinobacteria	1.9	±	2.0	2.6	±	4.9	0.9	±	0.7	0.309
Bacteroidetes	50.1	±	10.6	48.7	±	13.2	55.3	±	6.2	0.174
Firmicutes	35.7	±	7.8	35.0	±	8.1	34.8	±	6.7	0.988
Proteobacteria	9.2	±	3.9	12.0	±	9.3	7.7	±	4.7	0.302
UC *Bacteria*	2.9	±	3.8	1.3	±	2.0	1.2	±	1.9	0.572
Verrucomicrobia	0.1	±	0.1	0.0	±	0.1	0.0	±	0.1	0.456
Class (%)										
Actinobacteria	1.4	±	1.9	2.2	±	4.3	0.6	±	0.6	0.079
Alphaproteobacteria	0.0	±	0.1	0.4	±	0.8	0.3	±	0.6	0.090
Bacilli	0.1	±	0.3*	0.3	±	0.4*	0.1	±	0.2	**≤0.001**
Bacteroidia	48.9	±	12.5	48.5	±	13.1	54.6	±	6.4	0.337
Betaproteobacteria	6.0	±	2.6	6.5	±	4.4	5.3	±	3.6	0.738
Clostridia	30.7	±	8.7	30.9	±	8.3	30.6	±	7.5	0.871
Coriobacteriia	0.5	±	0.5	0.3	±	0.6	0.3	±	0.3	0.506
Deltaproteobacteria	2.2	±	2.0	0.8	±	0.7	1.3	±	1.6	0.248
Erysipelotrichia	0.4	±	0.3	0.4	±	0.3	0.5	±	0.5	0.822
Gammaproteobacteria	0.5	±	0.7	3.4	±	9.9	0.6	±	1.5	0.101
Negativicutes	4.0	±	2.6	3.2	±	1.6	3.1	±	2.6	0.056
UC *Bacteroidetes*	1.3	±	2.9	0.2	±	0.2	0.7	±	1.9	0.805
UC *Firmicutes*	0.5	±	0.8	0.1	±	0.2●	0.5	±	0.8●	**0.011**
Verrucomicrobiae	0.1	±	0.1	0.0	±	0.1	0.0	±	0.1	0.536
Order (%)										
Acidaminococcales	3.1	±	3.1	2.1	±	1.7	1.8	±	1.6	0.062
Bacteroidales	48.9	±	12.5	48.5	±	13.1	54.6	±	6.4	0.337
Bifidobacteriales	1.4	±	1.9	2.2	±	4.3	0.6	±	0.6	0.088
Burkholderiales	5.4	±	2.7	5.5	±	2.9	5.3	±	3.6	0.994
Clostridiales	30.6	±	8.5	30.8	±	8.3	30.5	±	7.5	0.835
Coriobacteriales	0.4	±	0.4	0.3	±	0.6	0.2	±	0.2	0.339
Desulfovibrionales	2.2	±	2.0	0.8	±	0.7	1.3	±	1.6	0.248
Eggerthellales	0.1	±	0.1	0.1	±	0.1	0.0	±	0.1	0.806
Enterobacterales	0.4	±	0.7	3.0	±	10.0	0.3	±	1.0	0.544
Erysipelotrichales	0.4	±	0.3	0.4	±	0.3	0.5	±	0.5	0.822
Lactobacillales	0.1	±	0.3*	0.3	±	0.4*	0.1	±	0.2	**0.002**
Pasteurellales	0.1	±	0.1	0.3	±	0.5●	0.1	±	0.1●	**0.031**

UC Alphaproteobacteria	0.0	±	0.1	0.3	±	0.8	0.3	±	0.5	0.152
Veillonellales	0.9	±	1.5	1.1	±	1.7	1.2	±	2.0	0.486
Verrucomicrobiales	0.1	±	0.1	0.0	±	0.1	0.0	±	0.1	0.536
Family (%)										
Acidaminococcaceae	3.1	±	3.1	2.1	±	1.7	1.8	±	1.6	0.062
Akkermansiaceae	0.1	±	0.1	0.0	±	0.1	0.0	±	0.1	0.536
Bacteroidaceae	26.4	±	12.0	25.5	±	12.8	21.6	±	12.6	0.675
Barnesiellaceae	2.4	±	2.3	1.1	±	1.1	1.7	±	1.8	0.372
Bifidobacteriaceae	1.4	±	1.9	2.2	±	4.3	0.6	±	0.6	0.088
Clostridiaceae 1	0.0	±	0.1†	0.1	±	0.1	0.3	±	0.5†	**0.039**
Clostridiales Incertae Sedis XIII	0.1	±	0.1	0.1	±	0.1	0.1	±	0.1	0.682
Coriobacteriaceae	0.4	±	0.4	0.3	±	0.6	0.2	±	0.2	0.484
Desulfovibrionaceae	2.2	±	2.0	0.8	±	0.7	1.3	±	1.6	0.248
Eggerthellaceae	0.1	±	0.1	0.1	±	0.1	0.0	±	0.1	0.806
Erysipelotrichaceae	0.4	±	0.3	0.4	±	0.3	0.5	±	0.5	0.822
Lachnospiraceae	6.8	±	5.0	9.0	±	4.3	6.5	±	2.7	0.149
Odoribacteraceae	1.7	±	1.8	0.7	±	0.5	0.8	±	0.6	0.192
Oxalobacteraceae	0.3	±	0.2	0.1	±	0.2	0.1	±	0.2	0.241
Pasteurellaceae	0.1	±	0.1	0.3	±	0.5●	0.1	±	0.1●	**0.031**
Peptostreptococcaceae	0.1	±	0.2	0.2	±	0.2	0.2	±	0.3	0.580
Porphyromonadaceae	3.3	±	1.8	2.5	±	0.8	2.1	±	1.4	0.166
Prevotellaceae	7.1	±	9.4	10.4	±	13.0	19.1	±	15.7	0.196
Rikenellaceae	6.9	±	4.2	7.1	±	4.9	6.5	±	3.3	0.936
Ruminococcaceae	19.6	±	6.4	17.7	±	4.0	17.8	±	4.9	0.902
Streptococcaceae	0.0	±	0.0*	0.3	±	0.4*●	0.1	±	0.2●	**≤0.001**
Sutterellaceae	4.6	±	3.0	4.9	±	2.8	5.1	±	3.7	0.858
UC *Bacteroidales*	0.9	±	2.9†	1.1	±	3.7	2.5	±	4.1†	**0.012**
UC *Clostridiales*	3.9	±	4.0	3.8	±	3.8	5.7	±	5.7	0.339
Veillonellaceae	0.9	±	1.5	1.1	±	1.7	1.2	±	2.0	0.486
Genus (%)										
Agathobacter	0.2	±	0.2	0.6	±	0.6	0.5	±	0.6	0.517
Akkermansia	0.1	±	0.1	0.0	±	0.1	0.0	±	0.1	0.536
Alistipes	6.9	±	4.2	7.1	±	4.9	6.5	±	3.3	0.936
Anaerostipes	0.1	±	0.1	0.1	±	0.2	0.1	±	0.2	0.449
Anaerotignum	0.2	±	0.2	0.2	±	0.5	0.2	±	0.3	0.949
Anaerotruncus	0.0	±	0.0	0.1	±	0.4	0.1	±	0.5	0.124
Bacteroides	13.1	±	9.3	11.5	±	8.9	9.7	±	7.6	0.824
Barnesiella	2.1	±	1.9	1.0	±	1.1	1.6	±	1.8	0.524
Bifidobacterium	1.4	±	1.9	2.2	±	4.3	0.6	±	0.6	0.088

Bilophila	0.7	±	0.4	0.5	±	0.6	0.5	±	0.4	0.840
Blautia	0.1	±	0.1	0.2	±	0.2	0.1	±	0.1	0.507
Butyricicoccus	0.0	±	0.1	0.0	±	0.1	0.1	±	0.1	0.630
Butyricimonas	0.6	±	0.7	0.3	±	0.3	0.3	±	0.3	0.521
Clostridium IV	0.2	±	0.6	0.2	±	0.6	0.1	±	0.4	0.562
Clostridium sensu stricto	0.0	±	0.1 †	0.1	±	0.1	0.3	±	0.5†	**0.039**
Collinsella	0.4	±	0.4	0.3	±	0.6	0.2	±	0.2	0.468
Coprobacter	0.4	±	0.5	0.2	±	0.1	0.1	±	0.2	0.531
Coprococcus	0.1	±	0.1	0.6	±	1.0	0.2	±	0.2	0.151
Desulfovibrio	1.4	±	1.9	0.1	±	0.5	0.8	±	1.6	0.126
Dialister	0.6	±	1.1	1.0	±	1.7	1.1	±	2.1	0.637
Dysosmobacter	0.7	±	0.8	0.4	±	0.4	0.3	±	0.3	0.421
Faecalibacillus	0.2	±	0.3	0.2	±	0.2	0.1	±	0.1	0.663
Faecalibacterium	2.6	±	1.4	3.2	±	1.3	2.2	±	1.1	0.171
Fusicatenibacter	0.2	±	0.2	0.1	±	0.1	0.1	±	0.1	0.556
Haemophilus	0.1	±	0.1	0.3	±	0.5	0.1	±	0.1	**0.026**
Holdemanella	0.1	±	0.2	0.1	±	0.1	0.2	±	0.3	0.653
Intestinimonas	0.2	±	0.3*	0.0	±	0.0*	0.0	±	0.0	**0.022**
Lachnospira	0.2	±	0.3	0.3	±	0.3	0.3	±	0.3	0.263
Lachnospiracea incertae sedis	0.3	±	0.4	0.4	±	0.5	0.2	±	0.3	0.185
Lawsonibacter	0.1	±	0.1	0.1	±	0.1	0.1	±	0.2	0.412
Odoribacter	1.1	±	1.1	0.5	±	0.3	0.5	±	0.5	0.117
Oscillibacter	3.0	±	2.0	4.0	±	3.1	2.8	±	2.3	0.561
Oxalobacter	0.3	±	0.2	0.1	±	0.2	0.1	±	0.2	0.241
Parabacteroides	3.3	±	1.8	2.4	±	0.8	2.1	±	1.4	0.164
Paraprevotella	0.5	±	0.7	0.7	±	1.4●	1.7	±	1.4 ●	**0.030**
Parasutterella	2.0	±	2.6	1.9	±	3.1	1.4	±	3.3	0.636
Phascolarctobacterium	3.0	±	3.1	1.9	±	1.7	1.7	±	1.6	0.080
Phocaeicola	13.3	±	6.7	13.9	±	7.1	11.9	±	7.4	0.685
Prevotella	5.0	±	8.8	8.8	±	12.4	15.2	±	14.0	0.705
Romboutsia	0.0	±	0.1	0.2	±	0.2	0.1	±	0.2	0.325
Roseburia	0.5	±	0.6	0.6	±	0.7	0.6	±	0.6	0.826
Ruminococcus	0.3	±	0.5	0.4	±	0.3	0.2	±	0.2	0.115
Ruminococcus2	0.2	±	0.3	0.0	±	0.0	0.0	±	0.1	0.224
Ruthenibacterium	0.5	±	1.8	0.0	±	0.0	0.1	±	0.1	0.625
Streptococcus	0.0	±	0.0*	0.3	±	0.3*	0.1	±	0.2	**≤0.001**
Sutterella	2.1	±	3.2	2.8	±	2.6	3.6	±	3.2	0.462
Turicibacter	0.0	±	0.1	0.1	±	0.2	0.1	±	0.1	0.499
UC Clostridiales Incertae Sedis XIII	0.1	±	0.1	0.1	±	0.1	0.1	±	0.1	0.792

UC Desulfovibrionaceae	0.2	±	0.3	0.1	±	0.1	0.0	±	0.1	0.340
UC Eggerthellaceae	0.0	±	0.0	0.0	±	0.1	0.0	±	0.0	0.627

Data are presented as mean and standard deviation. P values were obtained from one-way analyses of variance adjusted for sex with data transformed by Blom's formula. Bonferroni was used to adjust multiple comparisons across groups. ●RES vs. END, *SED vs. RES, and †SED vs. END. *Abbreviations:* END: endurance trained group; RES: resistance trained group; SED: sedentary group; UC: Unclassified.

DISCUSSION

The present study shows that fecal microbiota diversity (i.e., beta and alpha) is similar between resistance and endurance athletes, as well as with sedentary participants. However, the study groups presented differences in the relative abundance of certain fecal bacteria, which may indicate that the different types of exercise training influence fecal microbiota composition.

We observed that RES participants had a higher relative abundance of *Streptococcus* genus (*Firmicutes* phylum) and *Pasteurellaceae* family (*Proteobacteria* phylum) in comparison with SED and END participants, respectively. The relative abundance of *Streptococcus* genus and *Pasteurellaceae* family have been associated with an increase in gut inflammation[24] considering that hosting many pro-inflammatory pathogenic species[25]. Hence, these bacteria are commensal parasites on mucous membranes of vertebrate animals, which pathogenic potential becomes manifest under conditions of stress, as high-intensity exercise[26,27]. RES participants were mainly performing CrossFit or strength training. This sport modality is characterized by strength and conditioning program performed at high intensity with little or no rest periods[28]. It has been reported that stress induced by intense exercise increased gut inflammation and the relative abundance of those genera in elite endurance runners[24,29] and men bodybuilders[30]. Even though our athletes did no report gastrointestinal infection or inflammation symptoms, it is plausible that the proliferation of intestinal pathogens species may play a role in the increased incidence of infections in athletes undergoing prolonged and intense physical exercise[31]. Therefore, our data suggest that prolonged high-intensity exercise, as perform our RES participants, may lead to a higher relative abundance of pathogenic fecal bacteria.

We observed that END participants presented a higher relative abundance of *Clostridiaceae 1* family and *Clostridium sensu stricto* genus (both *Firmicutes* phylum) in comparison with SED participants. *Clostridiaceae 1* family and *Clostridium sensu stricto* genus belong to the *Clostridiales* order, which are known to produce SCFAs[32]. SCFAs are key mediators of energy metabolism in the mitochondria of skeletal muscles[33] that in turn help regulating whole-body glucose metabolism[34]. In agreement with our findings, the relative abundance of *Clostridium* genus was increased in cecal samples from free running wheel[35] and forced treadmill running[36] mice models, as well as in fecal samples from elite endurance athletes[24,37], regarding their respective control groups. Moreover, we found that the relative abundance of *Paraprevotella* genus (Bacteroidetes phylum) was more abundant in END vs. RES participants. *Paraprevotella* genus can metabolise xylan and pectin leading to SCFAs

production and the consequent benefits mentioned before[38]. Several studies with athletes[39] and active people[40–42] have also shown a higher relative abundance of *Paraprevotella* genus in comparison with sedentary people. Those athletes, as well as our participants in END are characterized by performing running, cycling or triathlon sports. Curiously, the improvement in cardiorespiratory fitness after 6 weeks of exercise training was associated with the higher relative abundance of *Paraprevotella* genus in individuals with obesity[5]. Thus, these findings bring light that END seems to have better bacterial composition than RES and SED.

Our results also showed that SED participants had a greater relative abundance of *Intestinimonas* genus (*Firmicutes* phylum) in comparison with RES participants. This genus has been associated with an increase in fat intake[43,44] and is more abundant in individuals with overweight or obesity[45]. We also found that an increase in fat mass percentage in SED participants in comparison with RES participants. Considering the relationship between *Intestinimonas* genus with obesity[43–45], it is possible to think that these differences were explained by fat mass percentage.

Limitations and Strengths

The present has some limitations including the case-control design, which does not allow for studying the causal interference. Due to the low sample size, we were not able to perform the analysis by sex, yet all the analysis were controlled for sex. The strengths of this study are that we sequenced using the latest technology (Illumina platform) and annotated with RDP at the level of genera taxon, but further study is necessary to determine functional repertoire including SCFAs production.

Conclusions

The present study shows that fecal microbiota diversity does not differ between resistance, endurance athletes and sedentary people. However, there were differences across groups in the relative abundance of certain bacteria in faeces. All together indicate that the type of exercise training may depict a different fecal microbiota composition, which should be further investigated.

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SECTION II

STUDY V: HIGHER PHYSICAL FITNESS LEVELS ARE POSITIVELY RELATED TO SPECIFIC FECAL BACTERIA IN YOUNG AND OLDER ADULTS

ABSTRACT

Aim: To investigate the relationship between physical fitness (i.e., muscular strength and cardiorespiratory fitness) with fecal microbiota diversity and composition in young and older adults.

Methods: This cross-sectional study involved 90 young adults (71% women; 22.0±2.3y) and 34 older adults (50% women; 68.5±2.9y). Muscular strength was determined by the handgrip strength test, whereas cardiorespiratory fitness was assessed via the maximum treadmill exercise test. Handgrip strength and VO_{2max} absolute levels were computed as tertiles for the young and older cohorts independently. The V1-V2 and V3–V4 regions of the 16S rRNA gene were used for analysing the fecal microbiota diversity and composition. *Bacteria X* refers to a specific genus the name cannot be revealed due to legal patent in elaboration.

Results: Overall, there were no differences across tertiles of handgrip strength nor VO_{2max} absolute levels in alpha and beta diversity in young and older adults. Young and older participants with high handgrip strength levels had a higher relative abundance of *Bacteria X* genus than those with low handgrip strength levels (all P≤0.03). In contrast, only older participants with high handgrip strength levels presented a higher relative abundance of *Faecalibacterium* and *Streptococcus* genera than the other older participants with low handgrip strength levels (all P≤0.029). Young and older participants with high VO_{2max} absolute levels also had a higher relative abundance of *Bacteria X* and *Paraprevotella* genera, respectively, compared to the participants with low VO_{2max} absolute levels (all P≤0.015). In contrast, only the older participants with low VO_{2max} absolute levels showed a higher relative abundance of *Ruminococcus* genus than the older participants with high VO_{2max} absolute levels (P=0.048). Moreover, the species belonging to these significant genera were positively correlated with physical fitness parameters independently of sex and body composition in both cohorts (P<0.05).

Conclusion: The present results suggest that higher physical fitness levels is associated with a higher relative abundance of fecal bacteria, especially those belonging to *Firmicutes* phylum, but more studies are warranted to better understand this relationship.

INTRODUCTION

The human gut contains a vast number of microorganisms called the gut microbiota[1], and their diversity and composition play a significant role in the health and disease of their host[2]. A healthy gut profile known as eubiosis status consists of equilibrium between microorganisms[3], which are involved in the digestion and absorption of nutrients, modulation of the immune system, and protection of enteropathogens[2]. However, an imbalance is followed by a state of dysbiosis[3] and is associated with diseases such as obesity[4] and cardiometabolic diseases[5]. Therefore, modulation of the gut microbiota has become a topic of considerable interest.

Physical fitness is considered a powerful marker of health and a good predictor of morbidity and mortality[6–8]. Physical fitness is a set of attributes that people have or achieve through exercise and are either health- or skill-related[9], whose main components are muscular strength and cardiorespiratory fitness[9]. Recent findings suggest a dynamic relationship between physical fitness levels and gut microbiota diversity and composition, yet the evidence is scarce[10–13]. Cardiorespiratory fitness levels were positively correlated with gut microbiota diversity and composition in young healthy adults[14,15] and premenopausal women[16]. Regarding muscle strength, there is evidence suggesting an association of gut microbiota composition, concretely *Faecalibacterium* and *Bifidobacterium* genera, with handgrip strength in humans[17]. However, most cross-sectional studies measured physical fitness by cardiorespiratory fitness[14–16], ignoring muscular strength, the other main component of physical fitness[9]. Moreover, gut microbiota evolves with age and is characterized by a reduction of diversity with a significant inter-individual variation[18].

Therefore, the present study examined the relationship between physical fitness including muscular strength and cardiorespiratory fitness, with fecal microbiota diversity and composition in two independent groups of young and older adults.

METHODS

Research design and participants

One hundred twenty-four sedentary adults from two independent cohorts participated in this cross-sectional study. A total of 90 young healthy adults (64 women, aged 22.0±2.3y) from ACTIBATE study (ClinicalTrials.gov, ID: NCT02365129)[19] were included in the analyses. They were assessed in Granada (Spain) between October and November 2016. Inclusion criteria were (i) being between 18 and 26 years (ii) practice < 20 min moderate-vigorous physical activity on <3 days/week, (iii) to have had a stable body weight over the last three months (< 3kg change), (iv) and not to have been involved in an exercise training program in the previous three months, (v) not suffering any disease/injury that does not allow doing physical activity. Exclusion criteria were: not to smoke, not to take any medication (including antibiotics in the last three months), not to present any acute or chronic illness, and not to be

pregnant. Moreover, a total of 34 older adults (17 women, aged 68.5±2.9y) from the EFICCOM study (Clinicaltrial.gov. ID: NCT03923712) were included in the analyses. They were undertaken in Cadiz (Spain) between January and February 2020. Their inclusion and exclusion criteria were the same as the ACTIBATE cohort, with an exception to the inclusion criteria of the age: being between 65-75 years.

Participants were informed about the study procedures, and if they met the inclusion criteria and agreed to participate, they signed the informed consent. The study protocol and the written informed consent were performed following the Declaration of Helsinki, as revised in 2013. They were approved by the Human Research Ethics Committee of the University of Granada (n°924) and that of the "Servicio Andaluz de Salud" (Centro de Granada, CEI-Granada), as well as the Human Ethics and Research Committee of the research in Cádiz and the Andalusian Coordinating Committee on Biomedical Research Ethics (codes: 0667-M1-17 and 04/2018, respectively).

Physical fitness assessment

❖ Muscular strength

The handgrip strength test was used for measuring muscular strength. We determined the handgrip strength test through a Takei 5401 digital Grip-D hand dynamometer (Takei, Tokyo, Japan)[20], whose results were expressed in kilograms. The participants were asked to squeeze the grip gradually and continuously, and we encouraged them to do their best while performing the tests. The test was performed twice, alternating between both hands with a 1 min rest between attempts. Men executed the test with the grip span of the dynamometer fixed at 5.5 cm, whereas for women, it was adjusted to the individual's hand size[20]. The mean of the highest values performed in the test was selected for the analyses. Data were represented in absolute terms and relative to lean body mass (LBM).

❖ Cardiorespiratory fitness

Before carrying out the cardiorespiratory fitness test, the participants would have to fulfil specific requirements: fasting for 3-5h, not performing vigorous exercise in the previous 48h or moderate exercise in the previous 24h, and not consuming coffee or tea in the latter period.

Next, a cardiorespiratory fitness test was performed using a maximum treadmill exercise (H/P/Cosmos Pulsar treadmill, H/P/Cosmos Sports & Medical GmbH, Nussdorf-Traunstein, Germany), following the modified Balke protocol[19] in young adults, whereas a treadmill (Lode Valiant, Groningen, Netherlands) using the modified Bruce protocol[21] was performed in older adults.

The warm-up consisted of walking for 1 min at 3km/h, followed by 2 min at 4km/h. The incremental protocol started at the fourth minute, at a speed of 5.3km/h (0% slope). The speed was kept constant while the treadmill slope was

increased by 1% every minute until the participants became exhausted. Finally, they went through a cooling-down period at 4km/h (0% slope) for 5 min. During the test, respiratory gas exchange (oxygen volume (VO_2) and carbon dioxide volume (VCO_2)) was monitored by indirect calorimetry using a CPX Ultima CardioO2 gas exchange analysis system (Medical Graphics Corp, St Paul, MN, USA), with a facemask, model 7400 plastic (Hans Rudolph Inc., Kansas City, MO, USA) and equipped with a preVent™ metabolic flow sensor (Medical graphics Corp, St Paul, MN, USA)[22]. VO_2 was measured using a galvanic fuel cell, whereas VCO_2 used a non-dispersive infrared sensor[22]. The criteria for defining maximum oxygen volume (VO_{2max}) were a respiratory exchange ratio of ≥1.1, a VO_2 plateau (changes of <100 ml/min over three consecutive 10 s intervals), and a heart rate within ten beats/min of the age-predicted maximum (*209–0.73 × age*)[23]. We measured time to exhaustion in seconds. VO_{2max} was expressed in absolute terms and in relative to LBM and relative to fat free mass (FFM).

Body composition assessment

Participants' weight and height were measured without shoes and wearing formal clothes using the SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Body mass index (BMI) was therefore calculated (weight (kg)/ height^2 (m)). In young adults, lean mass and fat mass were evaluated by Dual Energy X-ray Absorptiometry (DEXA, HOLOGIC, Discovery Wi, Marlborough, MA), whereas in older adults, fat mass and fat free mass (FFM) were obtained using a multifrequency bioimpedance (TANITA-MC780MA)[24]. The lean mass index (LMI) was calculated as lean body mass in kg and divided by height in m^2. The fat mass percentage was determined as the body fat mass divided by the total body mass and multiplied by 100.

Dietary intake assessment

Dietary intake was registered using three non-consecutive 24 hours recalls on two weekdays and a weekend day. In a face-to-face interview with dietitians, the participants were asked to recall all food consumed during the previous day. Every 24 hours recalls were analyzed for total energy (kcal), fat (g), protein (g), carbohydrate (g), and fiber (g) intake by EvalFINUT® software in young adults and by DIAL® software in older adults. It is based on the United States Department of Agriculture (USDA) and "Base de Datos Española de Composición de Alimentos" (BEDCA) databases.

Fecal microbiota analyses

The participants collected a fecal sample, and we stored it at -80°C until the extraction of DNA with QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain). We determined the quantification and purity of extracted DNA with a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, DE, USA). Next, for samples of young adults, the V3-V4 region of the 16S rRNA gene was amplified as previously described[25] using the Forward Primer 5′CCTACGGGNGGCWGCAG and Reverse Primer

5′GACTACHVGGGTATCTAATCC. Whereas, for samples of older adults, amplicon libraries were generated as previously described[26], where the V1-V2 region of the 16S rRNA gene was amplified after 20 cycles of PCR reaction using the 27F and 338R primers[27]. All were sequenced on a MiSeq (2×250 bp, Illumina, Hayward, California, USA).

Bioinformatics processing was performed as previously described[25], and the FastQ files obtained were analyzed using the "dada2" [28] packages in R software[29]. The "classifier" and "seqmatch" function from the Ribosomal Database Project (RDP) was used for assigning the taxonomic affiliation of phylotypes. Microbial communities were analyzed at different taxonomic levels from phylum to species, calculating the relative abundances (expressed as percentages).

Statistical analysis

Data are presented as means ± standard deviations unless otherwise stated, including both genders. All variables were tested for whether they followed a normal distribution using the D'Agostino & Pearson omnibus with GraphPad Prism (version 8.0.0). Because none of the fecal microbiota diversity and composition followed a normal distribution, non-parametric tests were used for this analysis. Handgrip strength and VO_{2max} absolute levels were computed as tertiles according to the number of participants in each cohort with SPSS (SPSS v. 22.0, IBM SPSS Statistics, IBM Corp. Armonk, NY). The tertiles' values for handgrip strength were, in young adults, low (19.3-27.5kg), intermediate (27.6-32.2kg), and high (32.6-54.1kg), and in older adults, low (10.4-18.6kg), intermediate (18.9-31.6kg), and high (31.8-46.4kg). The tertiles' values for VO_{2max} absolute were, in young adults, low (1794-2492 mL/min), intermediate (2528-3077 mL/min), and high (3102-4811 mL/min), and in older adults, low (1072-1401 mL/min), intermediate (1404-1897 mL/min), and high (2096-2563 mL/min). Differences in age, body composition, and dietary intake across tertiles were analyzed by one-way analyses of variance adjusted for sex, and Bonferroni determined correction for multiple comparisons. One-way PERMANOVA with 9999 permutations for significance testing with the Paleontological Statistics (Past3) software was used for analyzing the differences across tertiles in fecal microbiota beta diversity. In contrast, to identify the differences across tertiles in fecal microbiota alpha diversity and composition, we performed the Kruskal-Wallis test with GraphPad Prism (version 8.0.0). The P values derived from the Kruskal-Wallis test were corrected by the two-stage step-up method of Benjamini, Krieger, and Yekutieli multiple comparisons by controlling the False Discovery Rate (FDR)[30]. Partial Spearman correlations were conducted to examine the association of the physical fitness and body composition parameters with specific fecal bacteria at genus and species taxonomic levels, adjusted for sex, using "psych" [31] and "corrplot" [32] packages in R[29] software. The level of significance was assumed at $P<0.05$. The Statistical Package for the Social Sciences (SPSS) v.25.0 (IBM Corporation, Chicago, IL, USA), R software (V.3.6.0; http://www.R-project.org)[29] and GraphPad Prism version 8.0.0 for Windows, (GraphPad

Software, San Diego, California, USA, www.graphpad.com) were executed for the statistical analysis and graphical plots.

RESULTS

Characteristics of participants

There were significant differences between men and women in body composition, physical fitness, and dietary intake parameters in both cohorts (all P≤0.029; **Table 1**), whereas there were no significant differences between sex in fecal microbiota analyses in neither cohort (all P≥0.129; **Table 1**). Next, in each cohort, we performed tertiles of handgrip strength (**Table 2**) and VO_{2max} absolute (**Table 3**) levels. We observed that after adjusting for sex, only in young adults, the differences in LMI across tertiles of handgrip strength and VO_{2max} absolute levels remained (all P≤0.002; **Table 2** and **3**).

Table 1. Descriptive characteristics of the participants.

	Young adults										Older adults									
	All (N=90)			Men (N=26)			Women (N=64)			P	All (N=34)			Men (N=17)			Women (N=17)			P
Age (years)	22.0	±	2.3	22.2	±	2.3	21.9	±	2.3	0.579	68.5	±	2.9	68.3	±	2.7	68.8	±	3.1	0.623
Body composition																				
BMI (kg/m^2)	24.9	±	4.7	26.9	±	5.8	24.0	±	4.0	**0.029**	29.6	±	5.4	29.4	±	4.4	29.8	±	6.3	0.840
LMI (kg/m^2)	14.4	±	2.3	17.0	±	2.1	13.5	±	1.4	**≤0.001**										
Fat mass (%)	35.9	±	7.9	29.7	±	9.0	38.3	±	6.0	**≤0.001**	32.5	±	8.4	26.7	±	5.4	38.2	±	6.7	**≤0.001**
FFM (kg in young, % in older)	39.1	±	15.7	46.9	±	21.0	35.8	±	11.6	**0.015**	67.6	±	8.4	73.3	±	5.4	61.8	±	6.7	**≤0.001**
Physical Fitness																				
Handgrip strength (kg)*	31.4	±	7.9	41.1	±	7.0	27.5	±	3.8	**≤0.001**	25.9	±	9.2	33.5	±	5.0	17.7	±	3.8	**≤0.001**
Handgrip strength relative to LBM	0.8	±	0.1	0.8	±	0.1	0.7	±	0.1	0.494										
VO_{2max} absolute (mL/min)*	2887.7	±	683.3	3750.2	±	632.7	2613.9	±	422.7	**≤0.001**	1733.2	±	443.9	2026.8	±	383.1	1439.6	±	276.6	**≤0.001**
VO_{2max} relative to LBM	41.1	±	7.5	44.7	±	8.9	39.9	±	6.7	**0.012**										
VO_{2max} relative to FFM	57.1	±	25.5	46.7	±	31.9	61.4	±	21.1	**0.034**	34.1	±	5.8	33.9	±	6.2	34.2	±	5.7	0.880
Energy and macronutrient intake																				
Energy intake (kcal/day)	1940.9	±	490.9	2160.4	±	523.5	1851.7	±	451.4	**0.006**	1643.4	±	581.1	1779.1	±	650.4	1507.6	±	484.0	0.177
Fat (g/day)	88.3	±	27.4	96.6	±	28.9	84.9	±	26.3	0.066	71.7	±	26.2	78.9	±	31.8	64.5	±	17.1	0.112
Proteins (g/day)	78.2	±	23.0	97.0	±	24.6	70.6	±	17.5	**≤0.001**	52.0	±	23.7	54.7	±	22.8	49.3	±	25.0	0.517
Carbohydrates (g/day)	203.7	±	63.5	220.2	±	66.3	197.0	±	61.6	0.116	148.9	±	71.0	153.2	±	70.4	144.6	±	73.6	0.730
Fiber (g/day)	16.9	±	6.5	18.2	±	6.9	16.4	±	6.2	0.254	71.0	±	40.8	75.3	±	48.3	66.8	±	32.7	0.550
Fecal microbiota																				
Alpha diversity indexes																				
Richness Chao	432.3	±	151.8	388.6	±	98.6	450.1	±	166.1	0.136	180.1	±	35.6	177.8	±	29.4	182.3	±	41.6	0.895
Shannon diversity	4.2	±	0.4	4.2	±	0.3	4.2	±	0.4	0.625	4.2	±	0.3	4.3	±	0.3	4.2	±	0.3	0.568
Composition (Phylum)																				
Actinobacteria (%)	1.6	±	1.6	1.4	±	1.6	1.7	±	1.5	0.194	1.6	±	2.2	0.8	±	0.8	2.4	±	2.8	0.146
Bacteroidetes (%)	40.1	±	8.9	40.3	±	11.0	40.1	±	7.9	0.451	52.6	±	9.6	53.6	±	8.9	51.6	±	10.4	0.909
Firmicutes (%)	48.3	±	9.8	49.7	±	8.7	47.6	±	10.2	0.190	36.2	±	7.2	36.7	±	7.9	35.7	±	6.5	1.000
Proteobacteria (%)	6.8	±	5.3	7.5	±	4.6	6.5	±	5.6	0.147	7.0	±	5.8	7.6	±	7.0	6.5	±	4.5	0.782
Verrucomicrobia (%)	2.1	±	4.2	0.6	±	1.1	2.8	±	4.7	0.129	0.0	±	0.1	0.0	±	0.0	0.1	±	0.1	0.125

Data are presented as means ± standard deviations. P values were obtained from one-way variance analyses and the Mann-Whitney test for fecal microbiota parameters. Abbreviations: BMI: body mass index; FFM: fat free mass; LBM: lean body mass; LMI: lean mass index.
*For handgrip strength, data are missing for one older adult (remaining n= 33), whereas for VO_{2max} absolute, data are missing for five young adults (including n = 85)

Table 2. Characteristics of participants according to tertiles of handgrip strength levels adjusted for sex.

	Young adults				Older adults			
	Low N=30 (19.3-27.5kg)	**Intermediate** N=30 (27.6-32.2kg)	**High** N=30 (32.6-54.1kg)	P	**Low** N=11 (10.4-18.6kg)	**Intermediate** N=11 (18.9-31.6kg)	**High** N=11 (31.8-46.4kg)	P
Age (years)	21.4 ± 2.2	22.2 ± 2.4	22.3 ± 2.2	0.287	68.9 ± 3.5	69.0 ± 2.6	67.4 ± 2.3	0.236
Sex (% women)	100%	93%	20%		100%	45%	0%	
Body composition								
BMI (kg/m^2)	23.1 ± 3.8	24.6 ± 4.1	26.9 ± 5.6	0.189	30.6 ± 3.7	28.1 ± 7.2	30.3 ± 5.1	0.564
LMI (kg/m^2)	12.8 ± 1.1†	14.0 ± 1.5	16.6 ± 2.2†	**0.002**				
Fat mass (%)	38.9 ± 6.8	37.2 ± 6.0	31.4 ± 8.8	0.804	39.7 ± 4.3	29.9 ± 9.5	27.7 ± 5.9	0.369
FFM (kg in young, % in older)	33.1 ± 9.6	37.5 ± 13.7	46.5 ± 19.9	0.265	60.3 ± 4.2	70.1 ± 9.5	72.4 ± 5.9	0.369
Energy and macronutrient intake								
Energy intake (kcal/day)	1753.5 ± 434.9	1932.8 ± 473.4	2136.4 ± 500.5	0.264	1443.7 ± 544.5	1627.6 ± 507.9	1837.2 ± 684.6	0.794
Fat (g/day)	79.4 ± 22.7	89.5 ± 29.5	96.0 ± 27.9	0.298	60.8 ± 17.2	75.7 ± 29.8	77.9 ± 29.6	0.812
Proteins (g/day)	66.1 ± 16.6	75.8 ± 19.4	92.9 ± 24.4	0.226	51.5 ± 29.0	44.1 ± 16.3	59.0 ± 24.7	0.451
Carbohydrates (g/day)	189.9 ± 62.9	201.6 ± 61.5	219.6 ± 64.6	0.617	138.4 ± 74.4	148.5 ± 68.8	157.2 ± 78.2	0.907
Fiber (g/day)	15.1 ± 5.7	17.3 ± 7.0	18.4 ± 6.4	0.250	65.4 ± 38.6	62.4 ± 23.8	83.5 ± 55.9	0.563

Data are presented as means ± standard deviations. P values were obtained from one-way variance analyses adjusted for sex. Bonferroni was used to adjust multiple comparisons across tertiles. Symbol (†) indicates significant differences across tertiles in each cohort. Abbreviations: BMI: body mass index; FFM: fat free mass; LBM: lean body mass; LMI: lean mass index.

Table 3. Characteristics of participants according to tertiles of VO_{2max} absolute levels adjusted for sex.

	Young adults				Older adults			
	Low N=28 (1794-2492 mL/min)	**Intermediate** N=29 (2528-3077 mL/min)	**High** N=28 (3102-4811 mL/min)	P	**Low** N=11 (1072-1401 mL/min)	**Intermediate** N=12 (1404-1897 mL/min)	**High** N=11 (2096-2563 mL/min)	P
Age (years)	21.9 ± 2.1	21.5 ± 2.5	22.5 ± 2.3	0.263	68.6 ± 3.6	69.0 ± 2.1	67.9 ± 2.9	0.717
Sex (% women)	96%	93%	35%		81%	58%	9%	
Body composition								
BMI (kg/m^2)	21.8 ± 3.3†○	25.5 ± 4.1○	27.3 ± 5.0†	**≤0.001**	29.1 ± 7.1	28.8 ± 4.5	31.0 ± 4.5	0.386
LMI (kg/m^2)	12.7 ± 1.1†○	14.3 ± 1.5○	16.3 ± 2.4†	**≤0.001**				
Fat mass (%)	36.0 ± 6.9	38.5 ± 7.9	34.7 ± 8.1	0.089	35.5 ± 9.2	32.7 ± 8.3	29.1 ± 7.0	0.558
FFM (kg in young, % in older)	33.7 ± 7.4	36.0 ± 15.4	48.2 ± 16.0	0.116	64.5 ± 9.2	67.3 ± 8.3	70.9 ± 7.0	0.551
Energy and macronutrient intake								
Energy intake (kcal/day)	1827.0 ± 447.0	1856.2 ± 471.6	1986.5 ± 400.8	0.933	1748.5 ± 650.6	1508.3 ± 446.7	1685.5 ± 659.8	0.332
Fat (g/day)	81.0 ± 20.9	86.5 ± 25.4	86.7 ± 23.2	0.479	76.5 ± 23.1	63.8 ± 24.4	75.5 ± 30.9	0.260
Proteins (g/day)	69.3 ± 17.7	72.1 ± 18.8	84.5 ± 19.5	0.902	57.1 ± 30.5	47.5 ± 16.8	51.8 ± 23.7	0.451
Carbohydrates (g/day)	201.5 ± 73.4	192.7 ± 56.0	211.6 ± 58.8	0.845	155.0 ± 82.3	145.5 ± 77.2	146.5 ± 57.1	0.842
Fiber (g/day)	16.3 ± 6.7	15.4 ± 5.0	18.2 ± 5.4	0.190	83.8 ± 41.6	61.4 ± 23.4	68.8 ± 53.6	0.244

Data are presented as means ± standard deviations. P values were obtained from one-way variance analyses adjusted for sex. Bonferroni was used to adjust multiple comparisons across tertiles. Symbols (†) and (○) indicate significant differences across tertiles in each cohort. Abbreviations: BMI: body mass index; FFM: fat free mass; LBM: lean body mass; LMI: lean mass index.

Relationship between muscular strength and cardiorespiratory fitness with fecal microbiota diversity

Our data showed that there were no differences across tertiles of handgrip strength in alpha (all P≥0.126; **Fig. 1A** and **B**) and beta (all P≥0.256; **Table 4**) diversities in both cohorts. Curiously, the young participants with low VO_{2max} absolute levels had higher Richness Chao index than the young participants with intermediate and high VO_{2max} absolute levels (P=0.016; **Fig. 2A**). However, there were no differences across tertiles of VO_{2max} absolute levels in alpha diversity indexes in older adults (P≥0.086; **Fig. 2B**). Moreover, in both cohorts, we did not find differences across tertiles of VO_{2max} absolute levels in beta diversity at phylum and genus taxonomic levels (P≥0.103; **Table 4**).

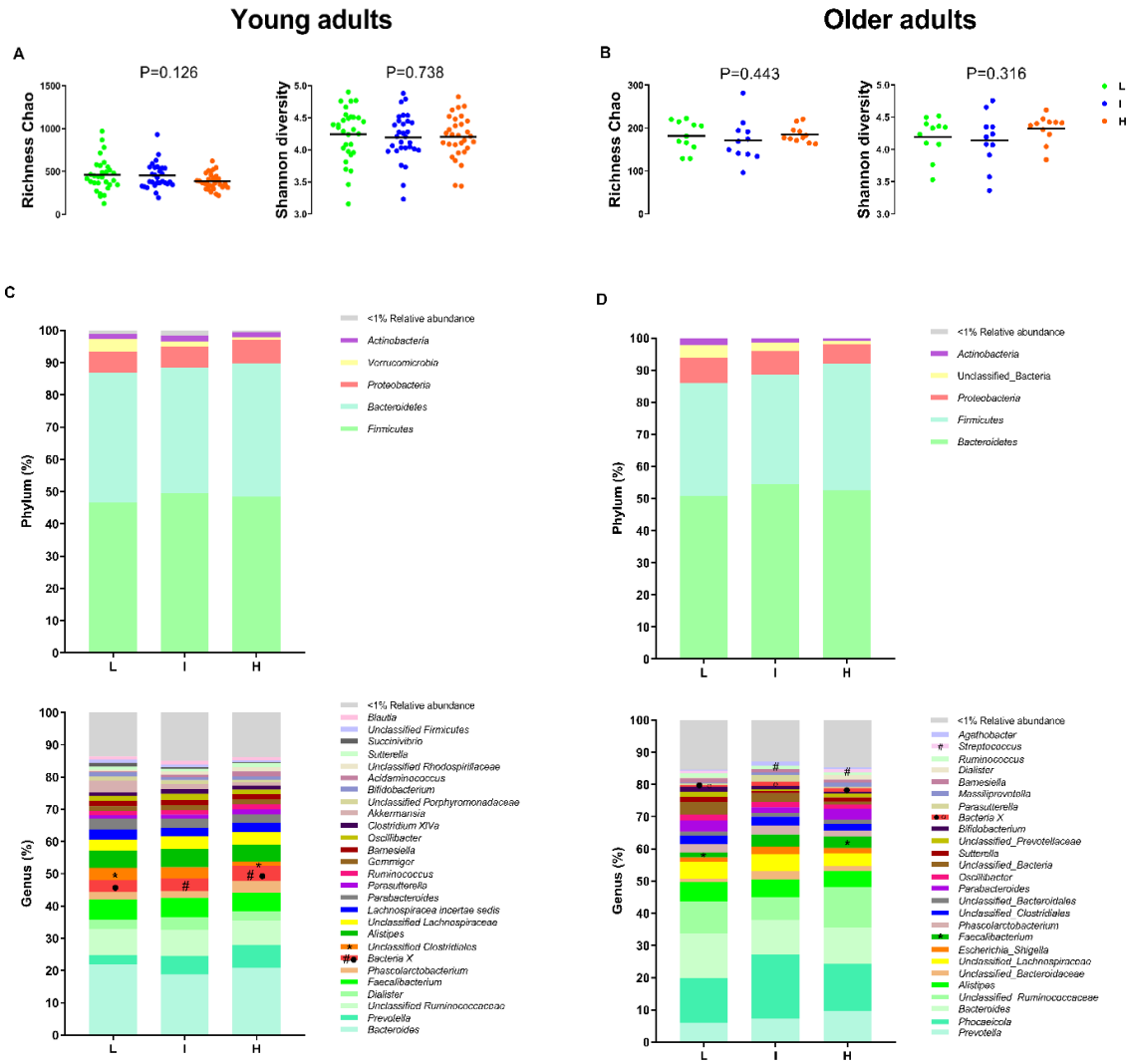

Figure 1. Fecal microbiota diversity and composition according to tertiles of handgrip strength levels in young and older adults *(Young adults: Low (L):19.3-27.5kg, Intermediate (I): 27.6-32.2kg, High (H): 32.6-54.1kg; Older adults: Low (L): 10.4-18.6kg, Intermediate (I): 18.9-31.6kg, High (H): 31.8-46.4kg). Panels A and B show the differences across tertiles of handgrip strength levels in fecal microbiota diversity indexes (richness Chao and Shannon diversity indexes). Panels C and D indicate the relative abundance of the fecal microbiota at phylum and genus taxonomic levels according to tertiles of handgrip strength. The stacked bar represented percentage abundance. Symbols (*) and (•) mean statistical significance differences between Low and High levels, symbol (#) represents statistical significance differences between Intermediate and High levels, whereas symbol (○) indicates statistical significance differences between Low and Intermediate. Kruskal-Wallis test was used to test for each pairwise comparison, correcting for multiple comparisons FDR (P<0.05) (GraphPad Prism 8.00).*

Table 4. Beta diversity across tertiles of handgrip strength and VO_{2max} absolute levels at phylum and genus taxonomic levels.

	Tertiles of handgrip strength levels (kg)				**Tertiles of VO_{2max} absolute levels (mL/min)**			
	Young adults		*Older adults*		*Young adults*		*Older adults*	
Taxonomic levels	*Pseudo-F*	*P*	*Pseudo-F*	*p-value*	*Pseudo-F*	*p-value*	*Pseudo-F*	*P*
Phylum	1.301	0.256	0.622	0.687	0.563	0.742	1.909	0.103
Genus	1.077	0.344	1.034	0.398	1.135	0.278	0.879	0.647

PERMANOVA using 9,999 permutations for significance testing (p-value<0.05). Pseudo-F: statistic, a larger number indicates greater separation[33] across tertiles of handgrip strength and VO_{2max} absolute levels

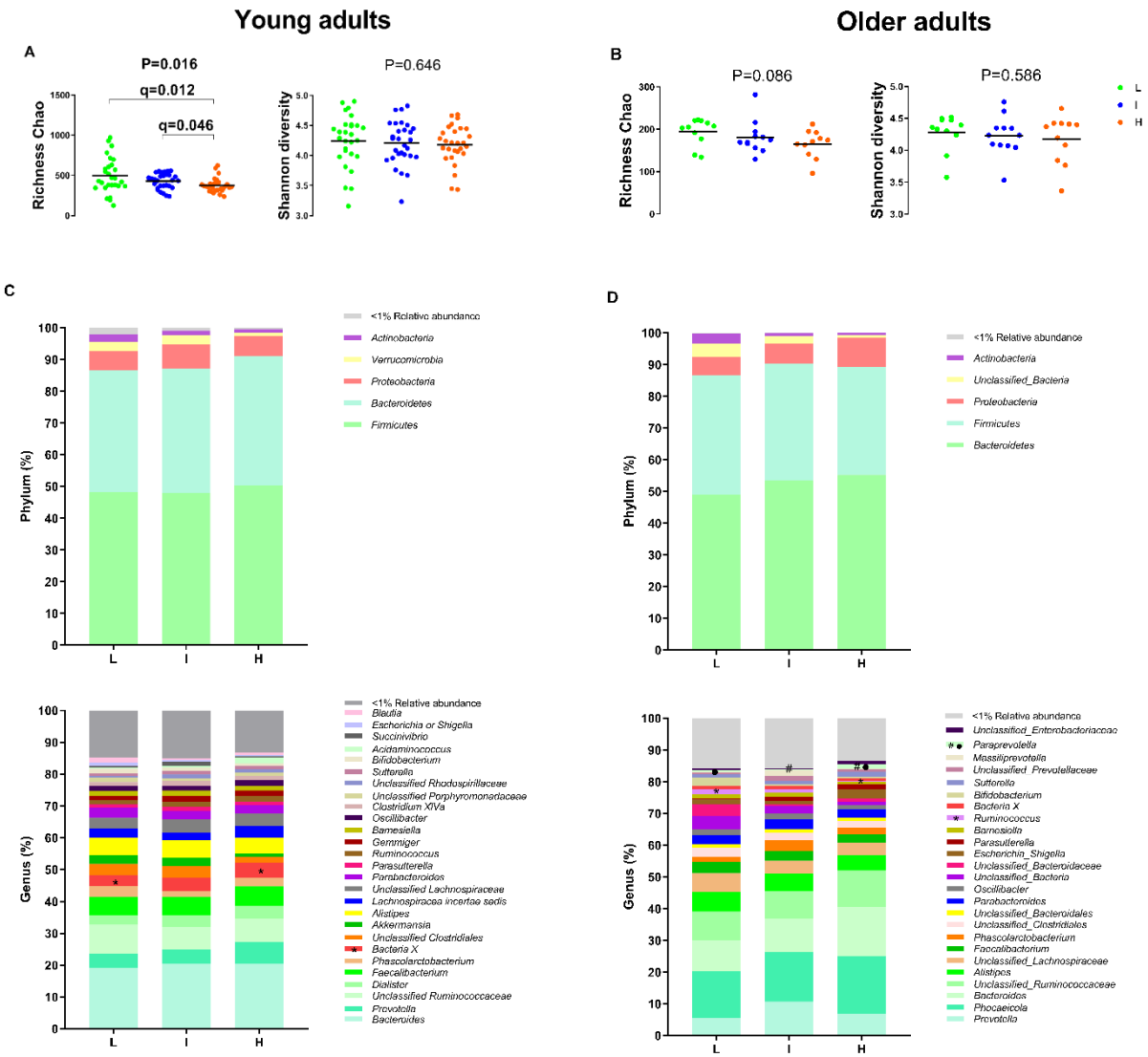

Figure 2. Fecal microbiota diversity and composition according to tertiles of VO_{2max} absolute levels in young and older adults *(Young adults: Low (L): 1794-2492 mL/min, Intermediate (I): 2528-3077 mL/min, High (H): 3102-4811 mL/min; Older adults: Low (L): 1072-1401 mL/min, Intermediate (I): 1404-1897 mL/min, High (H): 2096-2563 mL/min). Panels A and B show the differences across tertiles of VO_{2max} absolute levels in fecal microbiota diversity indexes (richness Chao and Shannon diversity indexes). Panels C and D indicate the relative abundance of the fecal microbiota at phylum and genus taxonomic levels according to tertiles of VO_{2max} absolute. The stacked bar represented percentage abundance. Symbols (*) and (•) mean statistical significance differences between Low and High levels, and symbol (#) represents statistical significance differences between Intermediate and High levels. Kruskal-Wallis test was used to test for each pairwise comparison, correcting for multiple comparisons FDR (P<0.05) (GraphPad Prism 8.00).*

Relationship between muscular strength and cardiorespiratory fitness with fecal microbiota composition

We found no significant differences across tertiles of handgrip strength and VO_{2max} absolute levels in the relative abundance of bacteria at phylum taxonomic level in young and older adults (all P≥0.05; **Fig. 1C** and **D**, and **Fig. 2C** and **D**). At the genus taxonomic level, our results indicated that in both cohorts, the participants with higher handgrip strength levels had a higher relative abundance of *Bacteria X* genus (*Firmicutes* phylum) compared with the other tertiles (all P≤0.03; **Fig. 1C** and **D**). Moreover, the older participants with higher handgrip strength levels presented a higher relative abundance of *Faecalibacterium* and *Streptococcus* genera (both *Firmicutes* phylum) than the older participants with low and intermediate handgrip strength levels, respectively (P=0.029 and P=0.009, respectively; **Fig. 1D**). Curiously, the young participants with high VO_{2max} absolute levels also had a higher relative abundance of *Bacteria X* genus (*Firmicutes* phylum) than the young participants with low VO_{2max} absolute levels (P=0.003; **Fig. 2C**). Moreover, the older participants with high VO_{2max} absolute levels had a higher relative abundance of *Paraprevotella* genus (*Bacteroidetes* phylum) than the older participants with low and intermediate VO_{2max} absolute levels (P=0.015; **Fig. 2D**), whereas they showed a lower relative abundance of *Ruminococcus* genus (*Firmicutes* phylum) than the older participants with low VO_{2max} absolute levels (P=0.048; **Fig. 2D**).

Next, we investigated which bacterial species could be driving the differences observed in the relative abundance of different genera across the different tertiles, using sex as confounders. In young adults, handgrip strength levels were only positively correlated with the relative abundance of *X* species belonging to *Bacteria X* genus (rho=0.21, P=0.044; **Fig. 3A**), whereas VO_{2max} absolute levels were positively correlated with the relative abundance of *Bacteria X* genus, *X*, and *Y* species belonging to *Bacteria X* genus (all rho≥0.29, P≤0.006; **Fig. 3A**). Interestingly, in the older cohort, handgrip strength levels were positively correlated with the relative abundance of *X* species belonging to *Bacteria X* genus, *Faecalibacterium genus,* and *Faecalibacterium prausnitzii* species (all rho≥0.35, P≤0.039; **Fig. 3B**), whereas VO_{2max} absolute levels were negatively correlated with the relative abundance of *Ruminococcus* genus (rho=-0.37, P=0.032; **Fig. 3B**). Since handgrip strength and VO_{2max} absolute levels depend strongly on the muscle mass of the individuals, we repeated the correlations including LMI in young adults, and fat free mass in older adults, as a confounder (**Fig. 3**). The results remained significant, however, the correlation with handgrip strength levels in young adults disappeared (P=0.238; **Fig. 3A**).

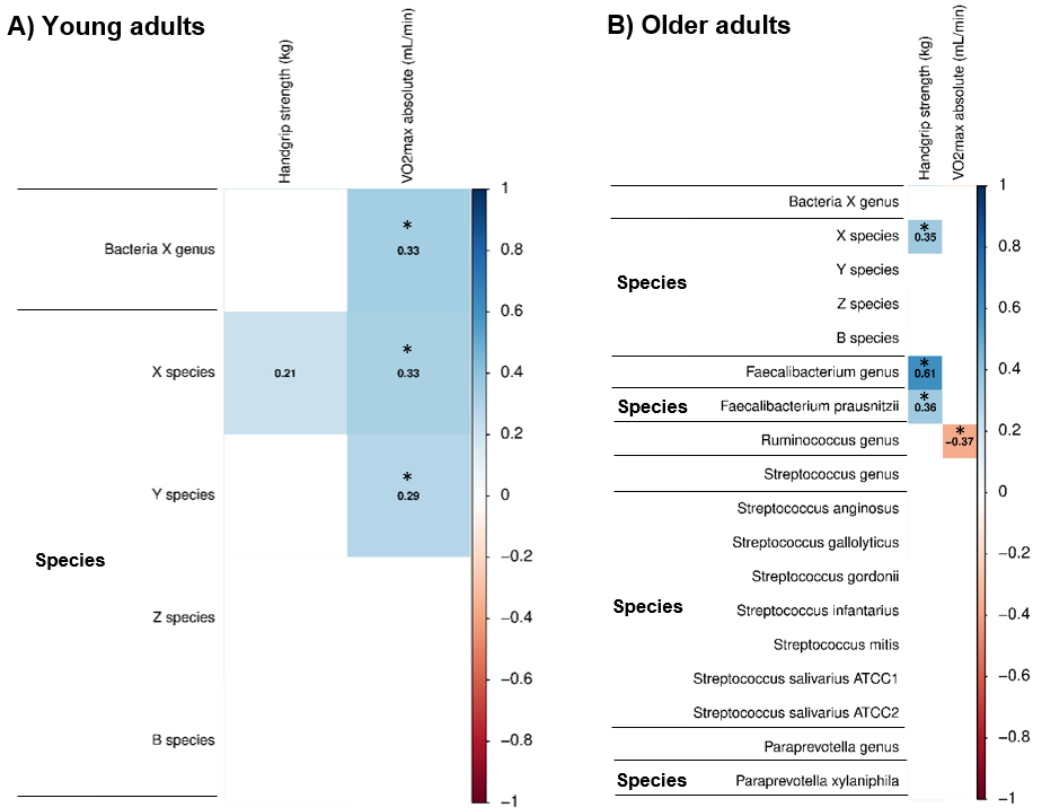

Figure 3. Partial Spearman correlation between Bacteria X, Faecalibacterium, Ruminococcus, Streptococcus, and Paraprevotella genera and species with physical fitness parameters in young and older adults, adjusted for sex. *Boxes only represent the statistically significant (P≤0.05) correlations and the values within the boxes show the partial Spearman correlation coefficient. Blue boxes indicate a positive correlation, whereas red squares indicate a negative correlation between physical fitness and body composition parameters with specific genera and their species adjusted for sex. Symbol (*) indicates that the correlations remain significant after including LMI or fat free mass as a confounder. BMI: Body mass index; LMI: Lean mass index.*

DISCUSSION

The present study shows that fecal microbiota beta or alpha diversity is similar across tertiles of handgrip strength or VO_{2max} absolute levels. Concerning fecal microbiota composition, young and older participants with higher handgrip strength levels had a higher relative abundance of *Bacteria X* genus. Only older participants with higher handgrip strength levels presented a higher relative abundance of *Faecalibacterium* and *Streptococcus* genera. Moreover, the young and older participants with high VO_{2max} absolute levels also had a higher relative abundance of *Bacteria X* and *Paraprevotella* genera, respectively. Still, only the older participants showed a lower relative abundance of *Ruminococcus* genus. Certain species belonging to these significant genera were positively correlated with physical fitness parameters, independently of sex and body composition in both cohorts. Altogether, these findings suggest

that higher physical fitness levels are associated with higher the relative abundance of fecal bacteria, especially those belonging to the *Firmicutes* phylum.

Our results concurs with previous findings where handgrip strength levels were positively correlated with the relative abundance of *Lachnospira, Eubacterium*, and *Ruminococcus* genera (all *Firmicutes* phylum) in older adults[34]. Moreover, they also showed that individuals with lower muscle mass had also lower relative abundance of *Bacteria X* genera (*Firmicutes* phylum)[34]. Curiously, alcohol over-consumers with low handgrip strength levels[35] and sarcopenic older adults[36], characterized by loss of skeletal muscle mass and strength[37], had a low relative abundance of *Faecalibacterium* genus (*Firmicutes* phylum). In agreement with our results, VO_{2max} absolute levels were positively correlated with the relative abundance of *Clostridiales*, *Lachnospiraceae*, and *Erysipelotrichaceae* genera (all *Firmicutes* phylum)[14], and with *Firmicutes*/*Bacteroidetes* ratio[15] in healthy young and older adults. All these genera, which belong to *Firmicutes* phylum, share a common ability to produce short-chain fatty acids (SCFAs)[38]. SCFAs are the primary products of the breakdown of non-digestible carbohydrates by gut bacteria[38]. Butyrate, acetate, and propionate, the most common SCFAs, maintain the integrity of the gut barrier, glucose homeostasis, and lipid metabolism, among other functions[38]. A positive correlation was observed between VO_{2max} absolute levels and butyrate in young, healthy adults[14]. Moreover, *Faecalibacterium* genus and *Faecalibacterium prausnitzii* species are widely known as essential health-promoting gut microbiota components due to butyrate production[39]. Butyrate modulates, among other, skeletal muscle protein synthesis, because it inhibits histone deacetylase, protecting against muscle protein catabolism and preventing age-related muscle mass loss. In our cohort of older adults, handgrip strength levels correlated positively with these bacteria. These results indicate that increased handgrip strength levels are related to a higher relative abundance of fecal bacteria preventing muscle mass loss. Besides, in our cohort of young adults, VO_{2max} absolute levels correlated positively with bacteria belong to *Firmicutes* phylum. Our findings suggest that the butyrate released by these bacteria seems to have a key role in the physical fitness of individuals.

Limitations and strengths

These findings should, however, be taken with caution as some limitations arise. Our study has a cross-sectional design; therefore, no causal interpretation can be established. Well-designed randomized controlled trials should be carried out to elucidate the role of physical fitness in fecal microbiota diversity and composition. As for strengths of this study, our findings apply to young and older people. Moreover, we sequenced the fecal microbiota composition using Illumina platform and annotations were made with RDP until species taxon.

Conclusions

Our data showed that handgrip strength and VO_{2max} absolute levels were no related with fecal microbiota diversity. Young and older participants with higher handgrip strength levels had a higher relative abundance of *Bacteria X* genus, whereas only older participants presented a higher relative abundance of *Faecalibacterium* and *Streptococcus* genera. Likewise, the young and older participants with high VO_{2max} absolute levels also had a higher relative abundance of *Bacteria X* and *Paraprevotella* genera, respectively. Species belonging to these significant genera, members of *Firmicutes* phylum, were positively correlated with physical fitness parameters, independently of sex and body composition in both cohorts. Altogether suggest that higher physical fitness levels can increase the relative abundance of fecal bacteria, especially those belonging to the *Firmicutes* phylum. However, further studies are needed to confirm this relationship.

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GENERAL DISCUSSION

INTEGRATIVE DISCUSSION

The present International Doctoral Thesis aimed (i) to investigate the relationship of novel cardiometabolic markers, such as endocannabinoids and BAT, with fecal microbiota diversity and composition and (ii) to study the association of physical activity and fitness levels with fecal microbiota diversity and composition. The findings from the studies included in **Section I** revealed that the plasma levels of endocannabinoids and their analogues were positively correlated with bacteria belonging to *Firmicutes* and *Verrucomicrobia* phyla (***Study I***). The relative abundance of bacteria belonging to *Firmicutes* and *Verrucomicrobia* phyla were negatively correlated with BAT volume and activity in young adults (***Study II***). Moreover, the results in **Section II** showed that different intensities (***Study III***) and modalities of physical activity (***Study IV***), and physical fitness levels (***Study V***) were related with higher fecal microbiota diversity and with the relative abundance of bacteria belonging to *Firmicutes* and *Bacteroidetes* phyla. Altogether, the results of the present International Doctoral Thesis suggest that endocannabinoids improve the gut barrier integrity through certain fecal bacteria. At the same time, these bacteria can regulate human BAT metabolism. Moreover, the observed associations of physical activity and fitness with fecal microbiota diversity and composition provide scientific evidence showing that different types of exercise may regulate gut microbiota composition differently.

Relationship between novel cardiometabolic markers and fecal microbiota diversity and composition

The increase in the incidence of cardiometabolic diseases is starting to be alarming worldwide[1–3]. Even though cardiometabolic diseases are developed by multiple factors, they are characterized by gut dysbiosis status[4] and low-grade inflammation due to increased gut permeability[4,5]. This situation promotes the necessity to identify novel potential targets for their treatment focusing on gut permeability.

Recently, it was discovered that among the metabolic systems involved in the regulation of the gut barrier function the endocannabinoid system plays a major role[6–8]. The endocannabinoid system is altered in mice models and in humans with obesity and cardiometabolic disease, with an increased abundance of AEA that triggers gut permeability via CB_1-dependent mechanisms on enteroendocrine cells[8–13]. Interestingly, in mice models this modification of the endocannabinoid system was associated with changes in the gut microbiota composition[8,14]. However, human studies evaluating the association between the plasma levels of endocannabinoid and their analogues with gut microbiota diversity and composition are scarce. Findings from *Study I* revealed that endocannabinoids and their analogues were positively correlated with bacteria belonging to *Firmicutes* and *Verrucomicrobia* phyla that are involved in maintaining gut barrier integrity in healthy individuals. SCFAs produced by these bacteria[15,16] bind GPR119 on enteroendocrine cells and stimulate the production of mucin glycoproteins via the secretion of GLP-1 and GLP-2, promoting the integrity of the gut barrier[5,17,18]. Moreover, in our study, plasma levels of endocannabinoids and their analogues were negatively

correlated with the plasma levels of LPS only in participants with high LPS values. Low plasma levels of endocannabinoids and their analogues in participants with higher LPS levels could indicate a possible mobilization of endocannabinoids from the bloodstream to the intestine to reinforce the gut barrier. The mechanism through which endocannabinoids increase the relative abundance of gut bacteria is not well understood. However, it is known that the endocannabinoid system plays a role in regulating the immune system[6–8], and it is thought that this may be one way in which endocannabinoids can influence the gut microbiota. For example, OEA and PEA have been shown to activate the peroxisome proliferator-activated receptor alpha (PPARα) pathway[19]. PPARα increases fatty acid oxidation, decreasing the oxygen bioavailability in the colon, and as a result, the anaerobic milieu in the colon is maintained, which increases the growth of anaerobic strict bacteria[20]. Interestingly, the bacteria belonging to *Firmicutes* and *Verrucomicrobia* phyla, mentioned in our *Study I*, are strictly anaerobic. Moreover, PPARα up-regulates IL-22 production by innate immune cells, which helps to maintain a proper intestinal mucosa function and influences the expression of antimicrobial peptides[20,21]. These peptides are crucial for the host response to fight with the intestinal pathogens[22]. In this context, in PPARα knock-out mice, the production of IL-22 decreased, resulting in decreasing secretion of antimicrobial peptides and, as a consequence, increased pathogenic bacteria leading to dysbiosis status[23]. These results demonstrate that PPARα-mediated IL-22 production by innate immune cells is necessary for maintaining gut microbiota homeostasis. Therefore, altogether our findings indicate that the endocannabinoids and their analogues could be a promising way to improve human gut barrier integrity through changes in fecal microbiota composition, thereby improving host health.

Since endocannabinoids and their analogues are synthesized in various tissues and organs[24], the endocannabinoid system is involved in a broad range of physiological processes. In fact, CB_1 is also expressed in peripheral metabolic tissues, including BAT[25]; thus, it is expected that the endocannabinoid system may regulate energy homeostasis in this thermogenic tissue[26,27]. Upon cold exposure, sympathetic nerve endings in the BAT release norepinephrine that binds to β3-adrenergic receptors on adipocytes and stimulates it[25,28]. This activates adenylyl cyclase, resulting in increased cAMP levels and subsequent activation of protein kinase A[25,28]. Protein kinase A phosphorylates a series of enzymes that enhances intracellular lipolysis, resulting in the liberation of fatty acids from triglycerides[25,28]. These fatty acids liberated undergo β-oxidation within the mitochondrial matrix and activate UCP-1, resulting in increased uncoupled respiration, therefore, results in heat production[25,28]. However, the effects of endocannabinoids and their analogues on BAT is scarce. *In vitro* models showed that the inhibition of CB_1 by rimonabant stimulated thermogenesis in brown adipocytes, evidenced by elevated UCP-1 expression and glycerol release as a measure of intracellular lipolysis[29]. Simultaneously, adrenergic stimulation of brown adipocytes increased the levels of AEA and 2-AG in BAT of mice models, accompanied by elevated expression of enzymes involved in the production of endocannabinoid and their analogues[30]. It indicates that the endocannabinoids and their analogues may act in a negative feedback loop: (a) to inhibit norepinephrine

released by the sympathetic nerves by binding to CB_1 on nerve terminals and (b) to inhibit adenylyl cyclase by binding to CB_1 on brown adipocytes[29], yet there is not enough data on humans. Although cold exposure is the main physiological activator of BAT[31], evidence suggests that gut microbiota is an important endogenous factor that can modulate BAT metabolism[32–36]. For instance, germ-free animals had higher plasma levels of (D)-3-hydroxybutyrate and lower plasma levels of very low-density lipoprotein compared to conventional animals[33]. These data indicated that the gut microbiota modulate the lipid metabolism in BAT, as the absence of gut microbiota stimulated both hepatic and BAT lipolysis while inhibiting lipogenesis[33]. In our *Study II* we observed that the relative abundance of *Ruminococcus* (*Firmicutes* phylum), *Lachnospiraceae sp.* (*Firmicutes* phylum) and *Akkermansia* genera (*Verrucomicrobia* phylum) negatively correlated with BAT volume and activity (as estimated by ^{18}F-FDG uptake) in healthy young adults. Mice models have shown that the acetate released by these bacteria activate BAT thermogenesis via GPR43[37–40]. However, recently it has been demonstrated that a rare subpopulation of brown adipocytes inhibited the thermogenic capacity of neighboring adipocytes via the production of acetate in human BAT[41]. Moreover, we measured the BAT volume and activity via glucose uptake, specifically ^{18}F-FDG uptake, however, BAT consumes both glucose and fatty acids[42,43]. Based on previous observations in our cohort, glucose levels did not correlate with BAT-related outcomes, whereas plasma levels of omega-3 oxylipins were negatively correlated with BAT volume and activity[44]. These findings suggest that human BAT has the ability to switch between oxidative and glycolytic metabolism contingent on substrate availability, although this should be further investigated. Thus, the negative correlations observed in our *Study II* could indicate that BAT consumes acetate, the SCFA released by these bacteria. On the other hand, as we mentioned previously, these SCFAs can stimulate the secretion of GLP-1 by enteroendocrine cells[5,17,18]. GLP-1 controls satiation and glucose metabolism by GLP-1 receptor signaling in vagal afferent neurons[45]. Recently, it has been demonstrated that the activation of the GLP-1 receptor by the binding of GLP-1 derived from the gut reduced BAT thermogenesis in rats[46,47]. This could be another mechanism that explains the inverse association observed between the relative abundance of fecal bacteria and BAT volume and activity in our *Study II*. Interestingly, bacteria belonging to these phyla were positively correlated with the plasma levels of endocannabinoids and their analogues in our *Study I*. Therefore, it is possible to think that the endocannabinoid system could decrease the thermogenic capacity of BAT through fecal microbiota composition (**Fig. 3**). Further research is warranted to unveil whether the plasma levels of endocannabinoids and their

analogues is a contributor to regulating gut barrier integrity and BAT thermogenesis through fecal microbiota composition.

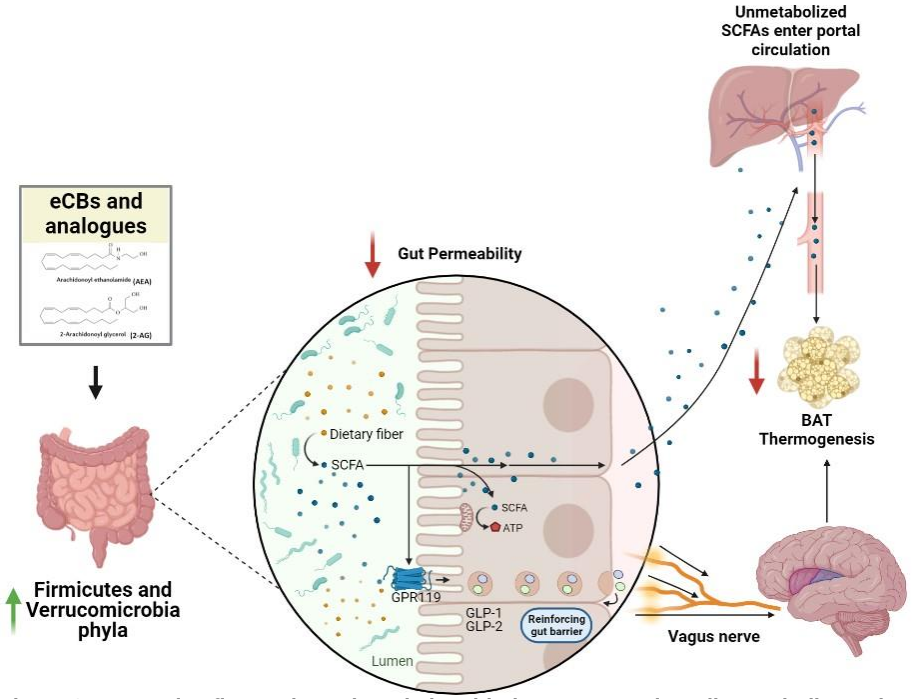

Figure 3. Integrative figure about the relationship between novel cardiometabolic markers and fecal microbiota diversity and composition. *Abbreviation: BAT: brown adipose tissue; eCBs: endocannabinoids; GLP1: glucagon-like peptide 1; GLP2: glucagon-like peptide 2; GPR119: G-protein-coupled receptors 119; SCFAs: short-chain fatty acids.*

Role of physical activity and fitness in fecal microbiota diversity and composition

The benefits of engaging on regular physical activity and performing exercise on cardiometabolic health are well known[48–52]. There is increasing evidence that physical activity and exercise can cause modifications in the gut microbiota[53–58]. It is biologically plausible that these beneficial effects on human health could be mediated by changes in the gut microbiota diversity and composition[53–58]. However, as we mentioned previously, the modification of fecal microbiota diversity and composition may be transient unless participants are exposed to a prolonged stimuli[59]. Thus, focusing on individuals that perform long-term regular physical activity is a good strategy for studying the role of physical activity on autochthonous fecal microbiota diversity and composition in healthy adults.

In *Study III* we observed that overall daily physical activity and the time spent at vigorous intensity were positively correlated with alpha diversity indexes in young sedentary adults. On the other hand, we showed that there were no differences in fecal microbiota diversity between resistance and endurance athletes and sedentary people (*Study IV*). Curiously in *Study III*, young sedentary participants with low time spent in vigorous intensity of

physical activity (less than 1 min/day) had a higher relative abundance of *Gammaproteobacteria* class (*Proteobacteria* phylum), bacteria considered health-detrimental because this class harbour human pathogens species[60–62]. On the other hand, young sedentary participants with high time spent at vigorous intensity physical activity (3-14 min/day) had a higher relative abundance of *Porphyromonadaceae* family (*Bacteroidetes* phylum), bacteria found in high-level athletes[63] and lean individuals[54]. Moreover, in *Study IV*, there were differences across groups (resistance trained, endurance trained and sedentary people) in the relative abundance of certain fecal bacteria belonging to *Firmicutes*, *Bacteroidetes* and *Proteobacteria* phyla. Altogether, these findings indicate that physical activity, especially of vigorous intensity, as well as different type of exercise training, is related to higher fecal microbiota diversity and different fecal microbiota composition in healthy adults, which biological relevance is unknown.

Beyond the role of physical activity in gut microbiota function, the association of physical fitness with gut microbiota function has not been deeply investigated. The results obtained in the *Study V* showed that increased cardiorespiratory fitness and handgrip strength levels correlated positively with bacteria belonging to *Firmicutes* phylum in young and older adults. It is well known that bacteria belonging to the *Firmicutes* phylum can produce butyrate[64]. Butyrate induces skeletal muscle protein synthesis, protects against muscle protein catabolism, and prevents age-related muscle mass loss[65–68]. Thus, the butyrate released by these bacteria seems to have a key role in the physical fitness of individuals.

To date, the mechanisms by which physical activity and exercise may cause changes in human gut microbiota diversity and composition are not fully understood. Probably several mechanisms are involved in the observed associations. For instance, after food intake, the food is moved through the gastrointestinal tract by a series of muscle contractions called peristalsis. Exercise reduces gut transit time because it makes muscle contractions more frequent[69]. The changes in fecal microbiota diversity and composition depending on the gut transit time could be explained by the adaptation of determined fecal bacteria to grow in slow or fast transit time conditions[69]. These conditions could be changes in water activity, fluctuations in nutrient availability, or the ability of own bacteria to attach to colonic tissue or to have a high growth rate[69,70]. On the other hand, during exercise, the skeletal muscle contracts and releases myokines, which exert anti-inflammatory effects in the organism[71]. The myokines are cytokines or other peptides that are produced, expressed and released by muscle fibres[72]. Particularly, interleukin-6 increases fat oxidation and glucose uptake via adenosine monophosphate-activated protein kinase (AMPK) phosphorylation and stimulate the secretion of GLP-1 in the enteroendocrine L cells of the gut during exercise[73,74]. GLP-1 reduces blood glucose, inhibits gastric emptying, and decreases appetite and food intake[75]. Despite myokines playing a role in the gut, whether interleukin-6 or other myokines released from the muscle could impact gut microbiota is unknown. Moreover, exercise increases the production of SCFAs by fecal bacteria[53]. SCFAs are involved in immune modulation, cell proliferation, gut barrier integrity, and intestinal motility modulation and provide anti-inflammatory,

antitumorigenic, and antimicrobial effects[67]. Therefore, the exercise-induced release of SCFAs could be one of the mechanisms by which exercise promotes health.

Altogether these mechanisms support the existence of the muscle-gut axis. However, scientific evidence has also suggested that the gut can communicate with the skeletal muscle, the so-called gut-muscle axis[76–79]. Specifically, it has been shown that the metabolites produced by gut bacteria can impact skeletal muscle physiology[80]. As previously described, gut bacteria can produce SCFAs, which can be consumed by colonocytes or enter portal circulation and be metabolized by the liver and other peripheral tissues, such as skeletal muscle[81]. These SCFAs are capable of activating AMPK in muscle cells, which increases fatty acid uptake and oxidation, glucose uptake and glycogenesis, and inhibits of lipogenesis and glycolysis in the mitochondria of skeletal muscles[80]. This activation of AMPK can take place directly by increasing the AMP/ATP ratio and/or indirectly through the GPR43-leptin pathway[82]. In addition, SCFAs through GPR41 or GPR43 in the colon stimulate the secretion of GLP-1 and peptide YY[82]. Peptide YY is a satiety hormone responsible of reinforcing insulin-mediated disposal of glucose in muscles[83]. Scientific evidence shows that the association between gut-derived SCFAs and skeletal muscle metabolism is further underlined by the emergent role of SCFAs in promoting physical fitness and concretely muscular strength. In fact, mice models supplemented with species belonging to *Lactobacillus* genus (*Firmicutes* phylum) increased by 32% the muscle mass and strength[84,85]. On the other hand, the supplementation of *Bifidobacterium longum* species (*Actinobacteria* phylum), isolated from elite weightlifting athletes, increased around 30% handgrip strength and endurance in mice[86]. In addition, the supplementation of multistrains of bacteria belonging to *Lactobacillus* and *Bifidobacterium* genera as probiotic mixture during 13-week enhanced endurance (decreasing the exhaustion by 48%) and muscular strength (increased by 17%) in older individuals[87]. Bacteria belonging to *Lactobacillus* and *Bifidobacterium* genera produce lactate, which could facilitate the production of acetyl-CoA and butyrate by lactate-utilizing bacteria with the concomitant production of ATP, thus increasing energy availability[88]. Surprisingly, the *X* species belonging to *Bacteria X* genus were positively correlated with handgrip strength and VO_{2max} absolute levels in our cohort of young and older adults, and when transplanted to mice increased by 30% muscular strength in the absence of exercise training. These effects seem to be mediated by the unique capacity of this species to produce intestinal amino acids that modify muscle purine metabolism, probably, increasing muscular strength (data under embargo, patent filled). In this context, where fecal bacteria could improve muscle strength, it will be an interesting therapy for muscle related disorders associated with age, such as sarcopenia (loss of muscle mass[76]). The administration of SCFAs, such as butyrate and acetate, as well as bacteria producers of them, to animal models with muscle wasting, gave rise to in massive improvements in muscle mass[65,66]. The depletion of bacteria producers of butyrate, such as *Faecalibacterium* genus (*Firmicutes* phylum) has been related to the presence of sarcopenia in older adults[89]. Interestingly, our data showed that older adults with higher handgrip strength levels had a higher relative abundance of this bacterium (*Study V*). Therefore,

future studies in this field should aim to study the relationship between the metabolic function of this bacterium and physical fitness in different situations either for improving the performance of athletes or treatment of muscle disorders in older adults.

In summary, the relationship between the skeletal muscle and gut is bidirectional: muscle-gut and gut-muscle axis (**Fig. 4**). Physical activity and exercise shape gut microbiota diversity and composition, and gut microbiota composition influences muscle function, which may substantially improve physical fitness. However, future studies are needed to confirm these findings and to elucidate underlying mechanisms.

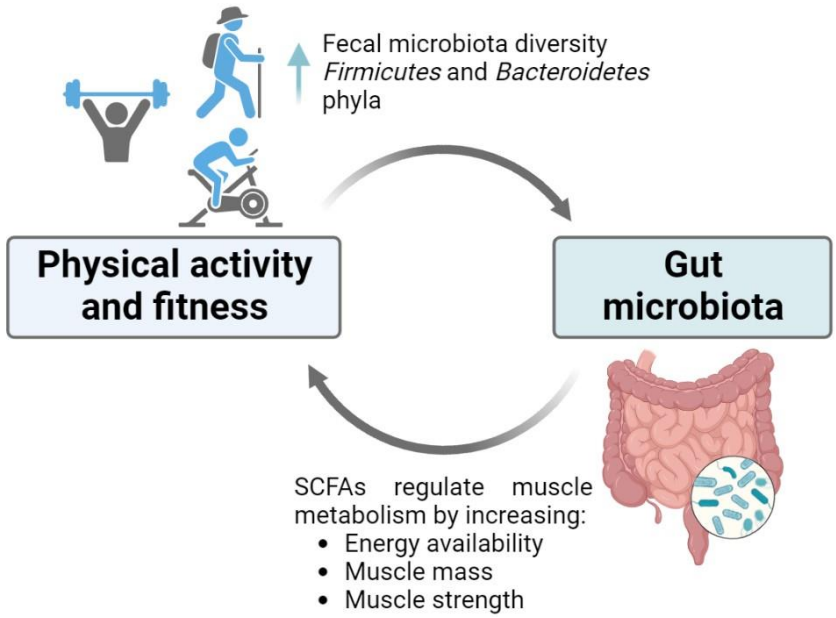

Figure 4. Integrative figure about the interactions between physical activity and fitness with fecal microbiota diversity and composition. *Abbreviation: SCFAs: short-chain fatty acids.*

Interplay between physical activity, endocannabinoids, brown adipose tissue, and gut microbiota in humans

The interplay between physical activity, fitness levels, the endocannabinoid system, brown adipose tissue, and gut bacteria community is complex but involves several common key mechanisms.

Previous observations in our cohort[90] and other human studies have shown that exercise also regulated the plasma levels of endocannabinoids and their analogues[91–94]; however, in contrast to mice models[95], an exercise intervention did not activate BAT in our cohort of young adults[96]. Interestingly, a recent study observed after an exercise intervention that the anti-inflammatory effects of SCFAs produced by gut bacteria were partly mediated by the endocannabinoid system in humans[94]. Thus, it might be plausible that physical activity stimulates the release of endocannabinoids[91–94], which can bind to PPARα in the

enteroendocrine cells[19] and CB_1 on brown adipocytes[25]. PPARα stimulates the increase of gut bacteria by decreasing the oxygen bioavailability in the colon and regulating the immune system[20,21]. SCFAs produced by these bacteria induce the secretion of GLP-1 and GLP-2, which improve gut barrier integrity[5,17,18]. GLP-1 activates the GLP-1 receptor in vagal afferent neurons, reducing BAT thermogenesis[46,47]. Moreover, the activation of CB_1 on brown adipocytes, also inhibit the BAT thermogenesis[29].

Overall, the exact mechanisms by which physical activity leads to improve health in humans are still being studied. However, it is clear that endocannabinoids, gut microbiota, and brown adipose tissue all play a role in this process.

GENERAL LIMITATIONS

The studies included in this International Doctoral Thesis present several limitations that should be acknowledged:

- The cross-sectional design of **Studies I**, **II**, **IV**, and **V** precludes the establishment of cause-effect relationships. Moreover, the case-control design of **Study III** does not allow for studying the causal interference of the presented results.

- Fecal microbiota diversity and composition was assessed by means of taxonomic composition (**Study I-V**). However, the metabolites, as SCFAs, of gut microbiota were not measured in fecal samples and could be of interest for future studies.

- **Studies I**, **II**, and **IV** were carried out in young, sedentary, and relatively healthy adults, which does not enable extrapolation of results to older, middle-aged, or unhealthy populations.

- The BAT assessments of **Study II** were performed using ^{18}F-FDG uptake as marker of BAT activity. Although ^{18}F-FDG uptake is the current gold-standard for BAT quantification, it also has limitations in the assessment of BAT metabolic activity and volume[97].

- Fecal microbiota diversity and composition are highly variable between individuals. Despite of the sample sizes in **Studies I**, **II**, **IV**, and **V** were around 100 individuals, the **Study III** included 50 participants divided into three groups. Due to the low sample size, we were not able to perform the analysis by sex. An increase in sample size would help us to better assess clinically relevant observations.

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CONCLUSIONS

GENERAL CONCLUSION

The present International Doctoral Thesis contributes to the scientific knowledge by developing new insights into the current understanding of the role of human fecal microbiota on cardiometabolic health and the relationship between physical activity and fitness levels with gut bacteria in humans.

SPECIFIC CONCLUSIONS

SECTION I: Relationship between novel cardiometabolic markers and fecal microbiota diversity and composition

- **Specific conclusion I:** The plasma levels of LEA, alpha LEA, PDEA, POEA, OEA, PEA, SEA, 2-AG, 2-LG, and 2-OG were positively correlated with the relative abundance of *Akkermansia, Faecalibacterium, Parasutterella* and/or *Odoribacter* genera, which may contribute to the preservation of the gut barrier integrity. Moreover, the plasma levels AEA and related N-acylethanolamines (*i.e.,* DGLEA, LEA, PEA, PDEA, POEA, and OEA), as well as AA and 2-AG were negatively correlated with the plasma levels of lipopolysaccharide, but only in participants with high levels of lipopolysaccharide. These findings suggest that the plasma levels of endocannabinoids and their analogues are related to specific fecal bacteria genera and may play a role in the gut barrier integrity in young adults. (*Study I*)

- **Specific conclusion II:** The relative abundance of *Akkermansia*, *Lachnospiraceae sp.,* and *Ruminococcus* genera were negatively correlated with cold-induced BAT volume and activity in young adults. Contrarily, the relative abundance of *Bifidobacterium* genus was positively correlated with BAT activity. Moreover, the relative abundance of *Bifidobacteriaceae* and *Sutterellaceae* families were positively correlated with ^{18}F-FDG uptake by WAT and skeletal muscles. Altogether, these findings suggest that fecal microbiota is involved in the regulation of glucose uptake by human BAT and other metabolic tissues, but future studies are needed to elucidate the underlying mechanisms. (*Study II*)

SECTION II: Role of physical activity and fitness in fecal microbiota diversity and composition

- **Specific conclusion III:** Overall physical activity and time spent in vigorous physical activity are positively correlated with fecal microbiota diversity in young adults. Moreover, the individuals with low time spent in vigorous physical activity presented higher relative abundance of *Gammaproteobacteria* class, whereas the individuals with high time spent in vigorous physical activity had higher relative abundance of *Porphyromonadaceae* family. Altogether, these findings suggest that physical activity, especially of vigorous intensity, is related to fecal microbiota diversity and *Gammaproteobacteria* class and *Porphyromonadaceae* family in young adults. (*Study III*).

- **Specific conclusion IV:** Fecal microbiota diversity does not differ between resistance, endurance athletes and sedentary people. However, there were differences across groups in the relative abundance of certain bacteria in faeces. All together, these findings indicate that the type of exercise training may depict a different fecal microbiota composition. (*Study IV*)

- **Specific conclusions V:** Handgrip strength and VO_{2max} absolute levels are no related with fecal microbiota diversity. Young and older participants with higher handgrip strength levels had a higher relative abundance of *Bacteria X* genus. Only older participants with higher handgrip strength presented a higher relative abundance of *Faecalibacterium* and *Streptococcus* genera. Likewise, the young and older participants with higher VO_{2max} absolute levels also had a higher relative abundance of *Bacteria X* and *Paraprevotella* genera, respectively. Species belonging to these significant genera, members of *Firmicutes* phylum, were positively correlated with physical fitness parameters, independently of sex and body composition in both cohorts. Altogether, these findings suggest that higher physical fitness levels are associated with the relative abundance of fecal bacteria, especially those belonging to the *Firmicutes* phylum. (*Study V*)

FUTURE PERSPECTIVES

- The results of the present International Doctoral Thesis shed light on the role of the endocannabinoid system, BAT, and physical activity and fitness in fecal microbiota diversity and composition in humans. With all results, it will be interesting to observe the effect of different types of exercise training on plasma levels of endocannabinoids and their analogues, BAT, and fecal microbiota diversity and composition. Moreover, the effect of endocannabinoid supplementation on exercise, BAT, and fecal microbiota diversity and composition. So, it would be interesting to perform:

    - Clinical trials to examine the effects of endocannabinoid supplementation with and without exercise training intervention, on fecal microbiota diversity and composition, and the activity of brown adipose tissue.

    - Investigation of the effects of different types and intensities of physical activity on endocannabinoid production, modification of fecal microbiota diversity and composition, and the activity of BAT.

- We postulate that the supplementation with endocannabinoids and their analogues decreases gut permeability through the increase in the relative abundance of bacteria involved in the gut barrier integrity. Thus, future intervention studies are needed to investigate whether endocannabinoids and their analogues could be used as new therapies for restoring gut barrier on these diseases. In this context:

    - Studies to determine the optimal dosage and duration of endocannabinoid supplementation for improving gut barrier integrity and reducing the symptoms of diseases associated with increased gut permeability.

    - Clinical trials to evaluate the safety and effectiveness of endocannabinoid supplementation in combination with other therapies, such as probiotics and prebiotics, for the treatment of diseases associated with increased gut permeability.

- BAT consumes both glucose and fatty acids. However, it seems that BAT has the ability to switch between oxidative and glycolytic metabolism contingent on substrate availability. Our results bring to light that BAT consumes acetate. Thus, it would be interesting to perform an intervention study administering acetate to healthy humans and observe if BAT is activated and the changes in fecal microbiota diversity and composition.

- On the other hand, the endocannabinoid system could inhibit BAT thermogenesis via GLP-1 induced by SCFAs. Future research investigating the effects of endocannabinoid supplementation on the levels of GLP-1 and other hormones that regulate BAT activity, as well as the impact of these interventions on the improvement of metabolic health would be interesting.

- Epidemiologic studies measuring physical activity at different intensities by accelerometry should be carried out to elucidate the role of regular physical activity in fecal microbiota diversity and composition in big cohorts of diverse population.

- Studies investigating the differences between athletes of different sports disciplines, together with large sample size and division between sexes will be an interesting approach to knowing the impact of resistance and endurance exercise training on human gut microbiota.

- It would be of great scientific interest to make a commercial probiotic for the therapeutic treatment of muscle-related disorders or reduced muscle strength, as well as for cosmetic purposes such as enhancing athletic ability in healthy individuals.

- Moreover, it will be interesting to investigate whether other bacterial species that were increased in our participants with high handgrip strength and VO_{2max} absolute levels also will improve the muscular strength and cardiorespiratory fitness in pre-clinical studies. In this context, we can make other commercial probiotics for improving the physical fitness in the population.

ANNEXES

MANUSCRIPTS DERIVED FROM THE INTERNATIONAL DOCTORAL THESIS

- **Lourdes Ortiz-Alvarez**, Huiwen Xu, Xinyu Di, Isabelle Kohler, Francisco J. Osuna-Prieto, Francisco M. Acosta, Ramiro Vilchez-Vargas, Alexander Link, Julio Plaza-Díaz, Mario van der Stelt, Thomas Hankemeier, Mercedes Clemente-Postigo, Francisco J. Tinahones, Angel Gil, Patrick C.N. Rensen, Jonatan R. Ruiz, Borja Martinez-Tellez. Plasma levels of endocannabinoids and their analogues are related to specific fecal bacterial genera in young adults: role on gut barrier integrity. *Nutrients*. 2022, 14, 2143. Doi: 10.3390/nu14102143.

- **Lourdes Ortiz-Alvarez**, Francisco M. Acosta, Huiwen Xu, Guillermo Sanchez-Delgado, Ramiro Vilchez-Vargas, Alexander Link, Julio Plaza-Díaz, José M. Llamas, Angel Gil, Idoia Labayen, Patrick C.N. Rensen, Jonatan R. Ruiz, Borja Martinez-Tellez. Fecal microbiota composition is related to brown adipose tissue ^{18}F-Fluorodeoxyglucose uptake in young adults. *Journal of Endocrinological Investigation*. 2022. Doi: 10.1007/s40618-022-01936-x.

- **Lourdes Ortiz-Alvarez**, Huiwen Xu, Francisco M. Acosta, Jairo H. Migueles, Ramiro Vilchez-Vargas, Alexander Link, Julio Plaza-Díaz, Angel Gil, Idoia Labayen, Jonatan R. Ruiz, Borja Martinez-Tellez. Higher physical activity levels are related to fecal microbiota diversity and composition in young adults. Under review in Sports Health-A Multidisciplinary Approach.

- **Lourdes Ortiz-Alvarez**, Huiwen Xu, Ramiro Vilchez-Vargas, Alexander Link, Julio Plaza-Díaz, Angel Gil, Jonatan R. Ruiz, Borja Martinez-Tellez. Fecal microbiota diversity and composition in resistance and endurance athletes: a case-control study**.** In preparation.

- **Lourdes Ortiz-Alvarez**, Huiwen Xu, Julio Plaza-Díaz, Ramiro Vilchez-Vargas, Alexander Link, Angel Gil, David Jimenez-Pavon, Jonatan R. Ruiz, Borja Martinez-Tellez. Higher physical fitness levels are positively related to specific fecal bacteria in young and old adults. In preparation.

SHORT INDUSTRY CURRICULUM VITAE

Lourdes Ortiz Alvarez, PhD

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SUMMARY

- **Biomedical scientist** with a strong **background in human gut microbiota** and inflammatory diseases proved by contributing to a 40,000$ Danone grant funding.
- Strong **builder** of valuable **relationships** and experience organizing weekly research **journal clubs** for 25-50 attendees.
- Extensive **experience communicating complex topics,** evidenced by effectively delivering **Biochemistry lectures** at the University of Granada for 3+ years and **12 conferences** in national and international congresses.

KEY INDUSTRY SKILLS

Biomedicine Knowledge **02/18 – Present**

Gained as Scientist at University of Granada, Spain *(5 years)*

- Highly motivated **biomedicine professional** with experience in **gastroenterology and inflammation diseases** leading to 4 publications as the first author in Q1 journals.
- **Exceptional ability to apply** new procedures quickly proved by acquiring state-of-the-art techniques to **analyze 200+ samples** of human gut microbiota.
- **Highly committed expert** with experience **leading scientific discussions** and providing critical insights in weekly journal clubs for 5+ years.

Networking skills **07/19 – 09/19**

Gained as Visiting Scientist at the University of Magdeburg, Germany (3 months)

- Excellent ability to **liaise with leading scientists** demonstrated by supporting a multidisciplinary project which led to **long-term partnerships** with 2 national and 2 international research institutions.
- Effective **teamwork** skills with proven ability to **build productive collaborations** resulting in 8 scientific publications.

- Active member of the *Profith Group* and successfully **organized symposia** and **engaged** both national and **international expert scientists** to present their research findings which led to 50+ attendees.

Communication skills **09/16 – Present**

Gained as Assistant Scientist at Universidad of Granada, Spain (5 years)

- Excellent **problem-solving** and **leadership** skills proved the successful **coordination of a randomized controlled trial** study with 150 participants, resulting in a new grant funding of >170,000$.
- **Detail-oriented** and **strategic planner** with experience developing impactful **presentations,** proved by 8 abstracts at international conferences.
- Effective **communication and customer-facing** skills as demonstrated by delivering **Biochemistry lectures** which led to pass 100+ students.

SKILLS	ACCOMPLISHMENTS
❖Strong writing communication skills. ❖Excellent research skills in scientific literature as well as industry leaders. ❖Experimental design ❖DNA extraction and PCR ❖Biostatistics analyses: R and SPSS ❖Effective time management and organization skills. ❖Languages: Spanish (Native), English (B1).	❖Giving 5+ workshops based on nutritional education during MSc. ❖Engagement with media for science communication**.** ❖Managing and coordinating 150 participants for 7 different experimental tests**.** ❖Assistant Testing Software

EDUCATION

PhD in Biomedicine **(2018-2023)**

University of Granada, Spain

- Published 10 scientific articles
- 3-months stay in Germany

MSc in Medical Science Liaison **(2021-2022)**

INESEM Business School, Spain

MSc in Genetics & Nutrition **(2016-2017)**

University of Granada, Spain

- Published 1 scientific article
- 1-year stay in Spanish's research center

BSc in Nutrition **(2012-2016)**

University of Granada, Spain

- Awarded as top performer student which granted my PhD funding
- 3-months stay in Spanish's research center

HIGHLIGHTS

- **Founder** of a **Scientific Communication Project** aimed at disseminating nutrition and gut microbiota-related topics to the wider population, called "@lacerezasaludable".
- Extremely organized and effective team player able to **work locally and remotely,** evidenced by engaging with >50 colleagues in >15 different projects.
- **Disposition to travel** and improve the company portfolio by effectively **communicating and collaborating with HCPs,** evidenced by successfully recruiting 200 participants in 3 clinical projects.

SHORT ACADEMIC CURRICULUM VITAE

Lourdes Ortiz Alvarez

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Google Scholar: https://scholar.google.es/citations?user=hC3T4SUAAAAJ&hl=es

LinkedIn: https://www.linkedin.com/in/lourdesortizdn

Education

2018-2023	Ph.D. student in Biomedicine, University of Granada, Spain
2021-2022	Master in Medical Science Liaison, INESEM Business School, Spain
2021-2022	Course “The transition from academy career to industry” online, “Libertad con Ciencia”- Rodrigo Hernando.
2016-2017	Master in Genetic, Nutritional, and Environmental Conditions of Development and Growth, University of Granada, Spain.
2012-2016	Bachelor’s Degree in Human Nutrition and Dietetics, University of Granada, Spain.

International Internship

2019 University Hospital of Magdeburg. Magdeburg, Germany. Department of Gastroenterology, Hepatology and infectious diseases. Prof. Dr. Ali Canbay and Dr. Ramiro Vilchez Vargas. *3-month stay as Ph.D. Student*.

Participation in Research Project

2021-2022 **EXTREME Project.** Efficacy and feasibility of three different 8h time-restricted eating schedules on visceral adipose tissue and cardiometabolic health in Spanish adults with overweight/obesity: a randomized controlled trial. Funded by Junta de Andalucia: ≈30000€.

2020-2021 **Oh my Gutness! Project**. Funded by Unidad Científica de Excelencia: Ejercicio Nutrición y Salud.

2018-2020 **SmartMove project**: Exercise in the prevention and treatment of obesity and insulin resistance: Smart analysis-smart interventions. Funded by the Spanish Ministry of Economy and competitiveness: ≈100000€.

2017-2018 **Ejercicio en la prevención y tratamiento de la obesidad y resistencia a la insulina:** análisis inteligentes intervenciones inteligentes. Funded by the Spanish Ministry of Economy and competitiveness.

2016-2017 **ACTIBATE project**: Activating Brown Adipose Tissue through Exercise. Effects of an exercise intervention on activity and quantity of Brown adipose tissue: A Randomized Controlled Trial. Funded by the Spanish Ministry of Economy and competitiveness among others: ≈600000€.

Publications

- Huiwen Xu, Lucas Jurado-Fasoli, **Lourdes Ortiz-Alvarez**, Francisco J. Osuna-Prieto, Isabelle Kohler, Xinyu Di, Ramiro Vilchez-Vargas, Alexander Link, Julio Plaza-Díaz, Angel Gil, Patrick C. N. Rensen, Jonatan R. Ruiz, Borja Martinez-Tellez. Plasma Levels of Omega-3 and Omega-6 Derived Oxylipins Are Associated with Fecal Microbiota Composition in Young Adults. *Nutrients*. 2022.

  [IF JCR 2021: 6.7. Rank 15/90. Q1. NUTRITION & DIETETICS]

- Lucas Jurado-Fasoli, Xinyu Di, Guillermo Sanchez-Delgado, Wei Yang, Francisco J. Osuna-Prieto, **Lourdes Ortiz-Alvarez**, Elke Krekels, Amy C. Harms, Thomas Hankemeier, Milena Schönke, Concepcion M. Aguilera, Jose M. Llamas-Elvira, Isabelle Kohler,

Patrick C. N. Rensen, Jonatan R. Ruiz, Borja Martinez-Tellez. Acute and long-term exercise differently modulate plasma levels of oxylipins, endocannabinoids, and their analogues in young sedentary adults: A sub-study and secondary analyses from the ACTIBATE randomized controlled-trial. *eBioMedicine*. 2022.

[IF JCR 2021: 11.20. Rank 14/139. Q1. MEDICINE, RESEARCH & EXPERIMENTAL]

- **Lourdes Ortiz-Alvarez**, Francisco M. Acosta, Huiwen Xu, Guillermo Sanchez-Delgado, Ramiro Vilchez-Vargas, Alexander Link, Julio Plaza-Díaz, José M. Llamas, Angel Gil, Idoia Labayen, Patrick C.N. Rensen, Jonatan R. Ruiz, Borja Martinez-Tellez. Fecal microbiota composition is related to brown adipose tissue ^{18}F-Fluorodeoxyglucose uptake in young adults. *J Endocrinol Invest.* 2022.

  [IF JCR 2021: 5.47. Rank 40/146. Q2. ENDOCRINOLOGY&METABOLISM]

- Borja Martinez-Tellez, Guillermo Sanchez-Delgado, Francisco M. Acosta,JuanM.A.Alcantara, Francisco J. Amaro-Gahete, Wendy D. Martinez-Avila, Elisa Merchan-Ramirez, Victoria Muñoz-Hernandez, Francisco J. Osuna-Prieto, Lucas Jurado-Fasoli, Huiwen Xu, **Lourdes Ortiz-Alvarez**, María J. Arias-Tellez, Andrea Mendez-Gutierrez,Idoia Labayen, Francisco B. Ortega, Milena Schönke, Patrick C. N. Rensen, Concepción M. Aguilera, JoséM. Llamas-Elvira, Ángel Gil, Jonatan R. Ruiz. No evidence of brown adipose tissue activation after 24 weeks of supervised exercise training in young sedentary adults in the ACTIBATE randomized controlled trial. *Nat Commun*. 2022; 13 – 1: pp. 5259.

  [IF JCR 2021: 17.6. Rank 6/73. Q1. MULTIDISCIPLINARY SCIENCES]

- **Lourdes Ortiz-Alvarez**, Huiwen Xu, Xinyu Di, Isabelle Kohler, Francisco J. Osuna-Prieto, Francisco M. Acosta, Ramiro Vilchez-Vargas, Alexander Link, Julio Plaza-Díaz, Mario van der Stelt, Thomas Hankemeier, Mercedes Clemente-Postigo, Francisco J. Tinahones, Angel Gil, Patrick C.N. Rensen, Jonatan R. Ruiz, Borja Martinez-Tellez. Plasma levels of endocannabinoids and their analogues are related to specific fecal bacterial genera in young adults: role on gut barrier integrity. *Nutrients.* 2022, 14, 2143.

  [IF JCR 2021: 6.7. Rank 15/90. Q1. NUTRITION & DIETETICS]

- Francisco J Osuna-Prieto, Borja Martinez-Tellez, **Lourdes Ortiz-Alvarez**, Xinyu Di, Lucas Jurado-Fasoli, Huiwen Xu, Victoria Ceperuelo-Mallafré, Catalina Núñez-Roa, Isabelle Kohler, Antonio Segura-Carretero, José V García-Lario, Angel Gil, Concepción M Aguilera, Jose M Llamas-Elvira, Patrick CN Rensen, Joan Vendrell,

Jonatan R Ruiz, Sonia Fernández-Veledo. Elevated plasma succinate levels are linked to higher cardiovascular disease risk factors in young adults. *Cardiovasc Diabetol*. 2021; 20: 151. REVIEWER ACTIVITY

[IF JCR 2021: 8.95. Rank 15/146. Q1. ENDOCRINOLOGY&METABOLISM]

- Lucas Jurado-Fasoli; Elisa Merchan-Ramirez; Borja Martinez-Tellez; Francisco M Acosta; Guillermo Sanchez-Delgado; Francisco J Amaro-Gahete; Victoria Muñoz Hernandez; Wendy D Martinez-Avila**; Lourdes Ortiz-Alvarez**; Huiwen Xu; María José Arias Téllez; María Dolores Ruiz-López; Jose M Llamas-Elvira; Ángel Gil; Idoia Labayen; Jonatan R Ruiz. Association between dietary factors and brown adipose tissue volume/18F-FDG uptake in young adults. *Clin Nutr*. 2021 Apr; 40(4):1997-2008.

  [IF JCR 2021: 7.64. Rank 13/90. Q1. NUTRITION & DIETETICS]

- **Lourdes Ortiz-Alvarez**; Huiwen Xu; Borja Martinez-Téllez. Influence of Exercise on the Human Gut Microbiota of Healthy Adults: A Systematic Review. *Clin Transl Gastroenterol*. 2020 Feb;11(2):e00126.

  [IF JCR 2020: 4.48. Rank 37/92. Q2. GASTROENTEROLOGY & HEPATOLOGY]

- **Lourdes Ortiz-Alvarez**; Borja Martinez-Tellez; Guillermo Sanchez-Delgado; Huiwen Xu; Francisco M.Acosta; Elisa Merchan-Ramirez; Victoria Muñoz-Hernandez; Wendy D.Martinez-Avila; Miguel A. Contreras-Gomez; Angel Gil; Idoia Labayen; Jonatan R Ruiz. Skin temperature response to a liquid meal intake is different in men than in women. *Clin Nutr.* 2019 Jun; 38(3):1339-1347.

  [IF JCR 2019: 6.36. Rank 9/89. Q1. NUTRITION & DIETETICS]

- Guillermo Sanchez-Delgado, Juan M.A. Alcantara, Francisco M. Acosta, Borja Martinez-Tellez, Francisco J. Amaro-Gahete, **Lourdes Ortiz-Alvarez**, Marie Löf, Idoia Labayen, Jonatan R. Ruiz. Estimation of non-shivering thermogenesis and cold-induced nutrient oxidation rates: Impact of method for data selection and analysis. *Clin Nutr*. 2018.

  [IF JCR 2018: 6.40. Rank 6/87. Q1. NUTRITION & DIETETICS]

- Francisco M. Acosta, Jörn Berchem, Borja Martinez-Tellez, Guillermo Sanchez-Delgado, Juan M. A. Alcantara, **Lourdes Ortiz-Alvarez**, Takafumi Hamaoka, Jonatan R. Ruiz. Near-Infrared Spatially Resolved Spectroscopy as an Indirect Technique to Assess Brown Adipose Tissue in Young Women. *Mol Imaging Biol.* 2018.

[IF JCR 2018: 3.34. Rank 31/129. Q1. RADIOLOGY, NUCLEAR MEDICINE AND IMAGING]

- Guillermo Sánchez-Delgado; Juan MA Alcantara; **Lourdes Ortiz-Alvarez**; Huiwen Xu; Borja Martinez-Tellez; Idoia Labayen; Jonatan R Ruiz. Reliability of resting metabolic rate measurements in Young adults: Impact of methods for data analysis. *Clin Nutr.* 2018 Oct;37(5):1618-1624

  [IF JCR 2018: 6.40. Rank 6/87. Q1. NUTRITION & DIETETICS]

Reviewer activity

L Ortiz-Alvarez has served as a reviewer of the journal *Metabolites*

Patent

Title: Application of gut bacterium for improving muscular strength. Leiden University Medical Center

Name of PI: B. Martinez-Tellez. I own 15% of it

Situation: with the attorney, last steps.

Teaching experience

2021-2022	**Biotechnology.** Degree in Pharmacy (1.5 ECTS Credits) University of Granada, Spain.
	Metabolic biochemistry. Degree in Pharmacy (3 ECTS Credits) University of Granada, Spain.
	Metabolic biochemistry. Degree in Human Nutrition and Dietetics (1.5 ECTS Credits) University of Granada, Spain.
2020-2021	**Biotechnology.** Degree in Pharmacy (1.5 ECTS Credits) University of Granada, Spain.
	Structural biochemistry. Degree in Pharmacy (3 ECTS Credits) University of Granada, Spain.
	Structural biochemistry. Degree in Human Nutrition and Dietetics (1.5 ECTS Credits) University of Granada, Spain.
2019-2020	**Structural biochemistry.** Degree in Pharmacy (4.5 ECTS Credits) University of Granada, Spain.

Structural biochemistry. Degree in Human Nutrition and Dietetics (1.5 ECTS Credits) University of Granada, Spain

Congress communications

2022

- **Title:** Niveles plasmáticos de endocannabinoides y análogos están relacionados con la microbiota intestinal en jóvenes adultos. *Poster.*

  Congress: XIII WORKSHOP Sociedad Española de Microbiota, Probioticos y Prebioticos, Valencia, Comunidad Valenciana, Spain.

  Author: Lourdes Ortiz-Alvarez; Huiwen Xu; Xinyu Di; Isabelle Kohler; Francisco J. Osuna-Prieto; Francisco M. Acosta; Ramiro Vilchez-Vargas; Alexander Link; Julio Plaza-Diaz; Mario van der Stelt; Thomas Hankemeier; Mercedes Clemente-Postigo; Francisco J. Tinahones; Angel Gil; Patrick C.N. Rensen; Jonatan R. Ruiz; Borja Martinez-Tellez.

2019

- **Title:** Exercise and human gut microbiota: a systemic review. *Poster.*

  Congress: 13th European Nutrition Conference, Dublin, Ireland

  Author: Lourdes Ortiz Álvarez; Huiwen Xu; Borja Martínez Téllez; Jonatan Ruiz Ruiz.

- **Title:** Physical activity and sedentary time are not associated with meal- and cold-induced thermogenesis in young adults. *Oral communication.*

  Congress: Exercise Metabolism (Cell Symposia), Sitges, Cataluña, Spain.

  Author: Guillermo Sánchez Delgado; Francisco J. Amaro Gahete; Juan Manuel Alcántara Alcántara; Francisco Acosta Manzano; Borja Martínez Téllez; Elisa M. Merchan Ramirez; Lourdes Ortiz Álvarez; Huiwen Xu; Jonatan Ruiz Ruiz.

2018

- **Title:** Brown adipose tissue and cold-induced thermogenesis does not exhibit diurnal variation in human adults. *Poster.*

  Congress: Role of brown adipose tissue in human health international symposium, Granada, Andalucia, Spain.

  Author: Guillermo Sanchez Delgado; Borja Martinez Téllez; Francisco M Acosta Manzano; Juan MA Alcantara Alcantara; Huiwen Xu; Lourdes Ortiz Alvarez; Francisco J. Amaro Gahete; Elisa M Merchan Ramirez; Jose M. Llamas Elvira; Jonatan Ruiz Ruiz.

- **Title:** Skin temperature response to a liquid meal intake in adults: role of sex. *Oral Communication.*

  Congress: V Reunión de Jóvenes Investigadores; XVII Congreso de la Sociedad Española de Nutrición. Barcelona, Cataluña, Spain.

  Author: Lourdes Ortiz Alvarez; Borja Martinez Tellez; Guillermo Sanchez Delgado; Huiwen Xu; Francisco Acosta; Elisa Merchan Ramirez; Jonatan Ruiz Ruiz.

- **Title:** Skin temperature response to a liquid meal intake in adults: role of sex. *Poster.*

  Congress: XVII Congreso de la Sociedad Española de Nutrición. Barcelona, Cataluña, Spain.

  Author: Lourdes Ortiz Alvarez; Borja Martinez Tellez; Guillermo Sanchez Delgado; Huiwen Xu; Francisco Acosta; Elisa Merchan Ramirez; Jonatan Ruiz Ruiz.

- **Title:** Respuesta térmica a la ingesta de una comida en hombres y mujeres. *Oral Communication.*

  Congress: Congreso Jornadas de Investigadores en Formación: Fomentanfo la interdisciplinariedad (JIFFI) 2018, Granada, Andalucia, Spain.

  Author: Lourdes Ortiz Alvarez; Borja Martinez Tellez; Huiwen Xu; Elisa Merchan; Victoria Muñoz Hernandez; Jonatan Ruiz Ruiz.

2017

- **Title:** Objectively measured physical activity and mental health in sedentary young adults. *Oral Communication.*

  Congress: International Symposium Active Brains for all: exercise, cognition and mental health 2017, Granada, Andalucia, Spain.

  Author: Francisco M Acosta; Miguel A Contreras Gomez; Maria Rodriguez Ayllon; Jairo H Migueles; Victoria Muñoz Hernandez; Elisa Merchan; Wendy Daniela Martinez Avila; Huiwen Xu; Lourdes Ortiz Alvarez; Jonatan Ruiz Ruiz.

- **Title:** Brown adipose tissue is not associated with bone mineral density in young healthy adults. *Oral Communication.*

  Congress: EMBO Workshop: "Brown adipose tissue" 2017, Sitges, Cataluña, Spain.

  Author: Guillermo Sanchez Delgado; Borja Martinez Tellez; Yolanda Garcia Rivero; Juanmanuel Alcantara Alcantara; Huiwen Xu; Lourdes Ortiz Alvarez; Francisco M Acosta; Elisa Merchan; Francisco J Amaro Gahete; Josune Olza; Jonatan Ruiz Ruiz.

- **Title:** Diferentes métodos de análisis en pruebas de gasto metabólico basal en adultos jóvenes: efecto en la fiabilidad inter días. *Poster.*

Congress: Congreso Jornadas de Investigadores en Formación: Fomentanfo la interdisciplinariedad (JIFFI) 2017, Granada, Andalucia, Spain.

Author: Francisco J. Amaro Gahete; Guillermo Sanchez Delgado; Juanmanuel Alcantara Alcantara; Lourdes Ortiz Alvarez; Huiwen Xu; Jonatan Ruiz Ruiz.

- **Title:** Evaluación del gasto metabólico basal en adultos jóvenes: impacto de alcanzar el estado estable sobre la fiabilidad de la medida. *Oral Communication.*

  Congress: Congreso Jornadas de Investigadores en Formación: Fomentanfo la interdisciplinariedad (JIFFI) 2017, Granada, Andalucia, Spain.

  Author: Juanmanuel Alcantara Alcantara; Guillermo Sanchez Delgado; Francisco J Amaro Gahete; Huiwen Xu; Lourdes Ortiz Alvarez; Jonatan Ruiz Ruiz.

2016

- **Title:** Efecto de un programa de ejercicio combinado de 6 meses de duración sobre la composición corporal en adultos jóvenes sanos: resultados preliminares del estudio ACTIBATE. *Oral Communication.*

  Congress: Simposio EXERNET. Investigación en Ejercicio, Salud y Bienestar: "Exercise is Medicine" 2016, Cadiz, Andalucia, Spain.

  Author: Guillermo Sanchez Delgado; Borja Martinez Tellez; Juanmanuel Alcantara Alcantara; Francisco M Acosta; Victoria Muñoz Hernandez; Elisa Merchan; Wendy Daniela Martinez Avila; Huiwen Xu; Lourdes Ortiz Alvarez; Ana Luque Peregrin; Jonatan Ruiz Ruiz.

Grant and awards

- **2019-Internship grant:** "Convocatoria de Movilidad de Estudiantes de Doctorado 2018/2019". Plan propio Universidad de Granada ≈2000€

Dissemination news

- **2020-News** about our revision "Influence of Exercise on the Human Gut Microbiota of Healthy Adults: A Systematic Review" in **CanalUGR.**
- **2020-Interview** about our revision "Influence of Exercise on the Human Gut Microbiota of Healthy Adults: A Systematic Review" in "Las Mañanas de Andalucia" **CanalSur Radio.**
- **2018-News** about our article "Skin temperature response to a liquid meal intake is different in men than in women" in **Ideal newspaper.**

Courses

2022

- **Title:** "XIII WORKSHOP de la Sociedad Española de Microbiota, Probióticos y Prebióticos (SEMiPyP)"-10 hours

  Organizer: Sociedad Española de Microbiota, Probióticos y Prebióticos (SEMiPyP)

- **Title:** "Haz que tus presentaciones sean memorables (o, por lo menos, diferentes)"-2 hours

  Organizer: University of Granada

2021

- **Title:** "¿Sabrías contar tu investigación en 3 minutos? Herramientas para ampliar tus habilidades de comunicación científica"-10 hours

  Organizer: University of Granada

- **Title:** "Incorporación de datos ómicos a proyectos de investigación" – 20 hours

  Organizer: University of Granada

- **Title:** "Introducción al procesamiento y análisis molecular de muestras en biomedicina" -20 hours

  Organizer: University of Granada

2020

- **Title:** "Divulgación Científica" -15 hours

  Organizer: University of Granada

- **Title:** "La divulgación científica como herramienta de comunicación y alternativa profesional para estudiantes de doctorado" -2 hours

  Organizer: University of Granada

- **Title:** "Introducción al uso de RStudio (sesión II)" -2 hours

  Organizer: University of Granada

- **Title:** "IV Jornadas de Bioinformática" -10 hours

  Organizer: University of Granada

2019

- **Title:** "Análisis de datos estadísticos con R studio" -6 hours

  Organizer: University of Granada

- **Title:** “Revisión sistemática de estudios. Meta-análisis” -20 hours

  Organizer: University of Granada

2018

- **Title:** “Beneficial and dangerous microorganisms in food: strategies for microbial population control” -15 hours
- **Organizer:** University of Granada

ACKNOWLEDGMENTS

¡POR FIN HA LLEGADO ESTE MOMENTO!

Siempre que había pensado en la escritura de mi tesis, lo primero que se me venía a la mente eran los agradecimientos. Tenía mucha ilusión por escribirlos y ahora que estoy en este punto, ¡dios! ¡Qué difícil es expresar todo lo que me han aportado las personas que han estado conmigo durante esta etapa!

¡GRACIAS A TODOS Y TODAS!

Recuerdo cómo comenzó toda esta aventura… Lo que empezó siendo unas simples prácticas de grado de Nutrición acabó por embarcarme en una tesis doctoral. Quien me iba a decir a mí en febrero de 2016, donde ni siquiera era aún Dietista-Nutricionista, que en febrero de 2023, sería Doctora en Biomedicina. ¡De locos! A la Lourdes del pasado le habría estallado la cabeza.

Pero claro en ese momento, ese febrero de 2016, conocí a un grupo de investigación que me cautivó, mi querido ACTIBATE. En 3 meses consiguieron que quisiera quedarme con ellos y vincular mis prácticas del máster del siguiente curso académico al iMUDS y por tanto al proyecto ACTIBATE.

¡Menudo año! Ahí sí que descubrí que la ciencia no tiene horario ni fecha en el calendario como dice mi querido director Jonatan. Pero a pesar del duro trabajo de ese año la gran familia de amigos que formamos en ese momento fui inmejorable.

Siempre he defendido la idea de que mi director Jonatan me engañó con un café para continuar mis estudios académicos y hacer la tesis doctoral. Pero la realidad es que estaba fascinada por el ambiente que había y lo cómoda que estaba ahí. Es aquí cuando comenzó mi etapa predoctoral y mi camino hacia conseguir el trabajo que hay entre estas páginas. Muchas personas han formado parte de este trabajo, quiero agradeceros a todos el haberme ayudado a llegar hasta aquí, en especial a las personas que voy a mencionar en este apartado.

¡GRACIAS A MI FAMILIA DEL TRABAJO!

En primer lugar, darle las gracias a mi director **Jonatan** que me ayudó a embarcarme en esta aventura. Además me tendió la mano cuando a todo el mundo le concedieron su FPU para hacer la tesis y yo fui la ovejita negra que se quedó en las puertas. Muchas gracias por haberme ayudado en esa etapa y poder ir con fuerzas a la siguiente convocatoria y conseguir finalmente la FPU. Por supuesto tu labor no ha acabado ahí. Aunque yo siempre te he dicho “jefe”, sé a ciencia cierta que eres un líder nato. Siempre nos has escuchado, entendido y ayudado en todo lo que nos ha hecho falta ya sea en lo profesional como en lo personal. Me has hecho ver que lo que se siembra, se recoge, que hay que ir con humildad por la vida y que hay que tener cuidado con lo que se sueña porque se puede cumplir. Esa frase me la dijiste justo el día que eché la segunda convocatoria de la FPU. Sin duda el deseo se cumplió. Aquí estamos hoy con mi tesis doctoral acabada. Muchas gracias de nuevo por haberme

acompañado en este camino y ayudado en convertirme en la profesional que soy a día de hoy.

Bueno ahora viene mi segundo director de tesis **Borja**. Menudo personaje de director me he buscado, mi *furby* investigador. Muchas gracias por ser una olla exprés de ideas brillantes y proporcionarme una perspectiva diferente de la ciencia, así como por abastecerme con un sinfín de copas y *perreos* por Ganivet. Quien me iba a decir a mí cuando te conocí que ibas a ser mi director de tesis…pero mil gracias por serlo, por embarcarte conmigo en este mundo de las "caquitas" en el cual no teníamos ninguno ni idea, sin duda esta tesis no habría salido sin tu ayuda. A pesar de ser mi director siempre te he considerado mi amigo y he estado muy cómoda trabajando contigo. Has apostado por mí, cuando ni yo misma lo hacía en muchas ocasiones (aunque no lo supieras ;), me has dado la fuerza que necesitaba. Has sabido cómo manejar mi poca mano izquierda y hacerme ver que la vida tiene más puntos de vista. Ojalá en un futuro nuestros caminos profesionales se vuelvan a unir. Estoy muy orgullosa de todos tus logros, eres una persona a la que admirar y un ejemplo a seguir, tanto por ser un gran investigador y como por lo gran persona que eres. ¡MIL GRACIAS POR TODO! No te faltarán copas y *perreos* hoy para celebrar el éxito conseguido.

Seguimos con mi equipo ACTIBATE, aquí tenemos a **Guille**, *mi Cabeza o Cervantes*, por sus infinitos discursos donde parecía que se había desayunado una enciclopedia. Razón por la que era temido en los seminarios, los cuales se alargaban siempre que él estaba. Sin embargo, ahora que está fuera se echan de menos jajaja. Admiro mucho todo lo que has conseguido. Aún recuerdo las palabras que me dijiste un día de fiesta "Sigue así, vales para esto", justo antes de tener la FPU y aquí estamos hoy. Muchas gracias por influenciarme con tu filosofía y tu espíritu de ciencia.

Fran Amaro, *mi armario empotrado* jajaja, recuerdo que la primera vez que te conocí pensé: "¿Pero todos esos músculos tenemos en el cuerpo?". Menudo *terminator,* sin duda eres un ejemplo de organización y disciplina. Nunca había conocido a alguien que pudiera llevar tantas cosas a la vez (doctorado, carrera de medicina, entrenamientos a las 5 de la mañana, vida personal), impresionante. Al igual que me sorprendí cuando supe que eras un amante de la bachata como yo. Espero que en la celebración nos echemos unos bailoteos. Gracias por haberme enseñado que si se quiere, se puede.

Juanma, *mi sipo querido*. Menudo tostón fue el conocerme, lo sé, aunque también sé que me quieres con locura. Qué habría sido de ti sin mí en esas mañanas en el coche donde gracias a mí te despertabas. Recuerdo que me "castigabas" con que fuera la chini de copiloto para que así no te taladrara la cabeza jajaja. Fuiste mi primer mentor con esos experimentos con tus queridos analizadores de gases. Te convertiste en mi hermano mayor del iMUDs, con nuestras quedadas a comidas, meriendas, y todo lo que hubiera comida de por medio. Muchas gracias por haberme acompañado en esta etapa, por escucharme y fomentar mi vida fuera del trabajo.

Acosti, *mis chocolatillos*. Como recuerdo las compras del iMUDS, donde llegaban los cereales y varios de chocolate y desaparecían a las horas, quien iba a ser el responsable, mi Acosti jajaja. Sin duda eres una persona excepcional. Gracias por contagiar tu alegría todas las mañanas, con esa sonrisa que iluminaba las duras evaluaciones del ACTIBATE y por endulzarlas ofreciendo chocolatillos a diestro y siniestro (o robándolos en cubículos). Nunca había conocido a alguien con tanta bondad, amabilidad, y positividad como tú. Eres como se dice en mi pueblo "un cachico de pan". Muchas gracias por haberme hecho etapa más fácil gracias a tu actitud, eres un ejemplo de persona a seguir.

A mi equipo nutris, con vosotras comenzó toda esta aventura. **Victoria**, *mi rubia*, hemos tenido nuestros más y nuestros menos, nuestro tira y afloja, jajaja pero hemos logrado solucionarlos. Muchas gracias por haberme enseñado el mundo de la nutrición en el ámbito de la investigación, eres una gran profesional y estoy muy orgullosa de lo que has conseguido. **Daniela**, *mi comadre*, como pasa el tiempo…. Te conocí siendo estudiante de doctorado, bebiendo *Larios Rosé* por Ganivet y mira ahora con tu niña y todo. Has sido una gran amiga y un gran apoyo durante este tiempo. A pesar de que ya no me acompañes con las copichuelas, gracias por todo, siempre me tendrás para cuando te haga falta. **María José**, *mi chilena*, menudo equipo hicimos pasando cuestionarios de nutrición, descifrándote las comidas españolas que no entendías y enseñándome el mundo de la nutrición en Chile. ¡Que buenos ratos! Aunque mejores fueron ese viaje a Sevilla, donde casi te me matas con el golpe en la cabeza, jajajja, pero que es la vida sin aventuras. Gracias por hacerme ver que la vida hay que tomársela con filosofía y tranquilidad. **Eli**, *mi querida Eli*, te has convertido en mi hermana mayor del iMUDS. Muchas gracias por ser la concejala de festejos y una amiga que me ha apoyado y escuchado en todo momento. Esas charlas infinitas, esos cafecitos, esas copas, esos *perreos*, fueron muy importantes para que esta tesis saliera adelante.

Javi, *mi biólogo sabiondo*. Como bien sabes, cuando llegaste al iMUDS preguntando por todo, con esos aires de sabiondo, me caíste algo regular jajaja. Después de ver que detrás de esa fachada de *biólogo sabiondo* se escondía un alcohólico empedernido, ya te quise más. Tienes una mente brillante, y eres una cabra loca, un mix fantástico para ser un profesor e investigador de la UGR. Durante esta etapa me has ayudado a ver que no hay que complicarse, que las cosas salen, que la vida sigue y que no hay que amargarse. Me encanta la manera de ver la vida que tienes, supongo que será por el aire puro que respiras cada dos por tres subiendo a la montaña. Sé que ahora estás lesionado pero estoy segura que tu hiperactividad hará que te recuperes en seguida. Gracias por haber formado parte de esto e influenciarme a ir por el mal camino de vez en cuando.

Luqui, *mi nutri rapado*. Hemos compartido esta aventura desde esas prácticas que hicimos del grado en Nutrición con esta gran familia. Me has ayudado a ver las cosas con otra perspectiva y a sacar a los *furbys* estos de los pubs cuando no había ni dios que los moviera. Eres un profesional increíble, espero que la UGR lo vea y no te deje escapar. No obstante, sé que vas a

llegar lejos y conseguir tus objetivos, vales mucho, suerte en tu etapa final de este viaje.

Manu, *el señor tupé*. He de reconocer que tu semblante de seriedad y de pocos amigos al principio me transmitía pocas esperanzas para una amistad contigo jajaja. Pero al final las primeras impresiones no siempre son buenas pero si le das tiempo te sorprenden y eso me pasó contigo. Eres una persona increíble que tiene un gran potencial. Gracias por abrirte y dejarnos conocer al Manu de fuera del trabajo.

Raquel, *mi pequeña Rachel*. Fuiste como un aire de viento fresco al llegar al iMUDS, te he sentido como mi hermana pequeña desde el minuto 0. Gracias por llegar con esas ganas y esa iniciativa de querer conocer este mundo. Vas a llegar muy lejos, sabes que vales mucho y sólo necesitas creértelo más. Te queda poco para llegar tú también a este punto, y que sepas que voy a estar ahí para verlo. ¡Ánimo que ya te queda nada!

Unai, *o ibai*. Has sido mi entrenador y mi compañero durante esta etapa. Eres un gran profesional y a pesar de que estuvieras con tu empresa externa, siempre estabas ahí para lo que hacía falta. Gracias por haber aceptado todos mis consejos y por haberme puesto más fuerte que el vinagre con tus aparatos de electrocutar jajaja.

Gracias también al resto del equipo que también habéis sido parte de esta aventura y sobretodo de este trabajo, **Mau**, **Andrea**, **Maria Medrano**, y **Almudena** y **Nieves**. A las nuevas generaciones que han llegado como **Antonio** y **Andrés** llegáis pisando fuerte y así os tenéis que coronar en la salida, ánimo que el camino se hace duro pero es muy bonito y apasionante.

También agradecer a mis compañeros de investigación del iMUDS, al equipo PROFITH: **Fran Ortega**, **María**, **Irene**, **Pablo**, **Pepe**, **Pato**, **Cristina Cadenas**, **Jairo**, **Jose Juan**, **Cristina Molina**, **Lucía**, **Fernando**, **Abel**, **Palma Chillón**, **Alex de la O**, **Pedro Acosta**, **Lidia Romero**, **Mireia de Castellón**, **Nuria**, **Carmen Sainz** y **Ana Yara**. Todos sois grandes profesionales, unos ejemplos a seguir. Muchas gracias por haberme aceptado en esta gran familia, donde no solo destacamos por el nivel de ciencia que se hace sino por el nivel de fiestas que organizamos. Tan pronto sacamos “papers” con un nivel de calidad de D1 como “volcamos canoas” que da gusto.

Por supuesto este proyecto no habría sido posible sin otras instalaciones que no fuera el iMUDS, como el querido Hospital. Muchas gracias a **Yolanda** y **Manuel Llamas** por abrirnos las puertas esos domingos infernales y por ayudarnos siempre que ha hecho falta. También el CIBM, gracias a **Ángel Gil** por abrirme las puertas de tu grupo de investigación e introducirme en el fascinante mundo de la microbiota. Siempre han sido muy entrañables y enriquecedoras las charlas contigo. Muchas gracias por ayudarme siempre que ha sido necesario, en cualquier momento, ya sea por teléfono o con correos inmediatos. Al resto de compañeros, **Julio**, me has mostrado como era el CIBM internamente, los lazos existentes, a quien se debía acudir, y como no a darme a conocer. **Chiqui** y **Fran**, mil gracias por estar disponibles siempre,

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¡GRACIAS A LA FAMILIA QUE ELIJO Y ME ELIGE!

Antes de comenzar mi etapa predoctoral, ciertas personas han marcado mi camino y me han ayudado a llegar hasta aquí.

Mis compis de carrera: **Aurora**, **Mariana**, **Sabina** y **Huiwen**. **Aurora**, siempre has sido la hermana mayor del grupo, la *mami* con cariño te decíamos. Me has cuidado desde que nos conocimos y has sido parte del inicio de esta etapa. Gracias a tus enseñanzas echando “pullitas” durante la carrera, pude sobrevivir al “bulling” con cariño de mis queridos compis (“furbys”) durante esta etapa predoctoral. **Mariana**, *mi loquita*, puff qué decirte que no sepas, a pesar de estar lejos siempre he sentido que seguíamos siendo compañeras de piso, de fiestas, de locuras. Eres una gran amiga y un pilar para mí, aunque estés a la distancia. Gracias por siempre sacarme una sonrisa y de fiesta cuando ha hecho falta. **Sabina**, el *conejito*, a pesar de que nos distanciáramos en la carrera, la madurez nos hizo ver que la amistad que teníamos era fuerte y podíamos seguir con nuestros lazos. Muchas gracias por darme tu punto de vista siempre, por ser objetiva, por apoyarme y estar ahí aunque a veces te taladráramos con cosas del trabajo. **Huiwen**, *mi chini*, *mi zape*, en 10 años que nos conocemos hemos pasado mucho juntas. No nos bastaba con hacer la carrera juntas que también tuvimos que hacer el máster y el doctorado, ofú que pesadas somos. Hemos sido un gran equipo de trabajo, nos hemos compaginado perfectamente, hemos sido el equilibrio perfecto. Como cualquier pareja hemos tenido nuestros más y nuestros menos pero siempre hemos sabido solucionarlo. Somos tan contrarias y a la vez tenemos mentalidades muy parecidas que por eso hemos llegado a conseguir nuestros objetivos juntas. Siempre he pensado que eres un orgullo de persona, una amiga inigualable y por supuesto una compañera excelente. Gracias por haber aparecido en mi vida y por haber estado ahí en todo momento, no lo habría conseguido sin ti.

Mis compañeras de piso: **Nazaret**, **Marta** y **Lorena**. Justo cuando comenzó mi FPU, doy con un piso donde casi todas éramos doctorandas también. Desde luego las penas compartidas suenan mucho mejor. Menudas charlas hemos tenido despotricando de los artículos, el doctorado, y todo lo relacionado. Muchas gracias por haberme apoyado y sobretodo aguantado cuando venía a daros la tabarra, y más aún por esas fiestas de desconexión que siempre venían bien.

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¡GRACIAS A MI FAMILIA!

Esta tesis doctoral no solo me ha marcado a nivel profesional sino que gracias a la FPU conocí a la persona que va a compartir el resto de mi vida conmigo: **Marcos**. Mi pareja y prometido, te conocí celebrando mi primer sueldo de FPU y me has acompañado durante estos años. Has estado conmigo en mis buenos y malos momentos. Me has ayudado a relativizar los problemas en esta última etapa donde cualquier cosa me parecía un mundo. Junto con nuestro perrete, **Nobu**, habéis hecho que cada fin de semana fuera un fin de semana de desconexión de verdad, que dejara a un lado el trabajo y disfrutara de la vida. Muchas gracias por aparecer en mi vida, por apoyarme en todo momento y por ayudarme a afrontar esta etapa de la mejor manera posible. Sin duda, esta tesis también es gracias a ti. Ya sabes que te dije que después de la tesis, tocaba organizar la boda, así que la que te espera ahora que tendré tanto tiempo libre. Nos queda mucho por recorrer y por vivir, esta experiencia es solo una de muchas. El sí quiero, está a la vuelta de la esquina, por un futuro juntos, te quiero mucho, chiqui.

Papá y **Mamá**, aún recuerdo la primera vez que os mencioné la palabra doctorado, pensasteis que me iba a hacer médica. A día de hoy, ya al menos conocéis a qué me refiero. Sin duda no habría sido posible llegar hasta aquí sin vuestro apoyo. Esta tesis es gracias al esfuerzo que habéis realizado vosotros por hacer que este aquí hoy. Habéis antepuesto siempre mis necesidades a las vuestras y habéis estado ahí para apoyarme tanto económicamente como moralmente. **Papá**, sé que tu mayor ilusión era que me gustaran las olivas y el campo, pero estoy segura de que estás contento de mi elección y de cómo he afrontado mi carrera profesional. Eres una persona trabajadora, constate, positiva, alegre y fiestero, desde luego mi positividad y ganas de fiesta las he sacado de ti. Nunca has dicho que no a nada de lo que te proponía, que la niña quiere ir de tiendas pues vamos, que necesita hacer mudanza, pues vamos, siempre dispuesto y sin quejarte. Te quiero mucho. **Mama**, mi confidente, siempre has estado detrás de cada llamada de alivio que necesitaba dándome tu punto de vista, quitándole hierro al asunto y apoyándome en todo lo que necesitaba. A pesar de ser una pesada a veces, eres mi mami pesada, que me gusta que este pendiente de mí. Por supuesto mi parte obsesiva, organizadora y cuidadora la he sacado de ti. Te quiero mucho. Estoy muy orgullosa de tener los padres que tengo. Sois excepcionales, no habría podido elegir padres mejores.

Mi hermano, **Jesús**, pequeñajo. No somos unos hermanos normales. La gente normalmente se pelea con sus hermanos, se lleva mal, etc. Pero nosotros somos como la familia de la pradera jajaja. Siempre nos llevamos bien, no necesitamos estar siempre en contacto pero sabemos que podemos contar el uno con el otro cuando nos haga falta. Durante este tiempo has visto

como es hacer una tesis, como consejo de hermana: sigue tu carrera de informático que te irá mejor. Muchas gracias por haberme apoyado y ayudado siempre que lo necesitaba, te quiero mucho pequeñajo.

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En definitiva, MIL GRACIAS A TOD@S Y CADA UNO DE VOSOTR@S, habéis conseguido que esta etapa se cierre de la mejor manera posible. Juntos hemos logrado hacer una tesis excepcional y habéis logrado que crezca como persona de la misma manera.

Así que, sólo puedo deciros una vez más,

GRACIAS, GRACIAS y GRACIAS