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ROLE OF GUT MICROBIOTA IN THE METABOLISM OF YOUNG ADULTS: NOVEL INSIGHTS AND POTENTIAL INTERVENTION STRATEGIES

Papel de la microbiota intestinal en el metabolismo de adultos jóvenes: nuevos avances y posibles estrategias de intervención

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ROLE OF GUT MICROBIOTA IN THE METABOLISM OF YOUNG ADULTS: NOVEL INSIGHTS AND POTENTIAL INTERVENTION STRATEGIES

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A mis padres y mi hermana Elena,

a mis suegros y mi novio Juan,

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"El éxito es aprender a ir de fracaso en fracaso sin desesperarse."

WINSTON LEONARD SPENCER-CHURCHILL
(1874-1965)

TABLE OF CONTENTS

LIST OF ABBREVIATIONS......19
ABSTRACT......21
RESUMEN......22
GENERAL INTRODUCTION......23
The problem: Cardiometabolic diseases......24
The solution might be in the human microbiome!......24
Link between prevalence of cardiometabolic diseases and fecal microbiota composition in humans......28
Potential interventions......31
HYPOTHESIS & AIMS......42
Hypothesis......43
Aims......43
MATERIAL & METHODS......45
RESULTS AND DISCUSION......48
SECTION I......48
Study I: Plasma levels of omega-3 and omega-6 oxylipins are related to the relative abundance of *Proteobacteria* phylum in the feces of young adults......48
STUDY II: Relative abundance of *Firmicutes* and *Bacteroidetes* bacteria is related to plasma levels of bile acids in young adults......69
SECTION II......91
STUDY III: Influence of exercise on the human gut microbiota of healthy adults: a systematic review......91
STUDY IV: A 24-week exercise intervention slightly modifies fecal microbiota composition in young sedentary adults: a randomized controlled trial......139
STUDY V: Impact of an intermittent and localized cooling intervention on skin temperature, sleep quality and energy expenditure in free-living, young, healthy adults.......165
GENERAL DISCUSSION......186
Integrative discussion......187
CONCLUSIONS......195
General conclusion......196
Specific conclusions......196
FUTURE PERSPECTIVES......198
Future perspectives......199
ANNEXES......200
Manuscripts derived from the thesis......201
Short industry curriculum vitae......203
Short academic curriculum vitae......206

LIST OF ABBREVIATIONS

AA: arachidonic acid
ACTIBATE: Activating brown adipose tissue through exercise
AdrA: adrenic acid
ALA: ∝-linolenic acid
ANOVA: analysis of variance
BA: bile acids
BAT: brown adipose tissue
BEDCA: "Base de Datos Española de Composición de Alimentos"
BHT: butylated hydroxytoluene
BMI: Body mass index
BSHs: Bile salt hydrolases
CA: cholic acid
CDCA: chenodeoxycholic acid
CON: control group
COXs: cyclooxygenases
CRP: C-reactive protein
CV: coefficient of variance
CYP450: cytochrome P450
DB: database
DCA: deoxycholic acid
DHA: docosahexaenoic acid
DNA: deoxyribonucleic acid
EPA: eicosapentaenoic acid
FFQ: food frequency questionnaire
FISH: fluorescent in-situ hybridization
FXR: farnesoid X receptor
GCA: glycocholic acid
GCDCA: glycochenodeoxycholic acid
DGCA: glycodeoxycholic acid
DGLA: dihomo-γ-linolenic acid
FDR: False Discovery Rate
GLCA: glycolithocholic acid
GLP-1: glucagon-like peptide-1
GUDCA: glycoursodeoxycholic acid
HDL-C: high-density lipoprotein cholesterol
HOMA: homeostatic model assessment
HRR: heart rate reserve
HRres: heart rate reserve
IAP: intestinal alkaline phosphatase
IL-6: interleukin-6
KEGG: Kyoto Encyclopedia of Genes and Genomes
LA: linoleic acid
LCA: lithocholic acid
LC-MS/MS: liquid chromatography-tandem mass spectrometry

LDL-C: Low-density lipoprotein cholesterol
LOXs: lipoxygenases
LPS: lipopolysaccharide
LTs: leukotrienes
MeSH: Medical Subject Heading
METs: metabolic equivalent of task
MCT4: monocarbosylate transporter 4
MJ: megajoules
MOD-EX: moderate-intensity exercise group
MUFAs: monounsaturated fatty acids
NR: not reported
OTU: operational taxonomic unit
PAST3: Paleontological Statistics Software Package 3.0
PCOA: Principal coordinate analysis
PCR: polymerase chain reaction
PERMANOVA: permutational multivariate analysis of variance
PPARs: peroxisome proliferator-activated receptors
PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analysis
PROSPERO: International Prospective Register of Systematic Reviews
PUFAs: polyunsaturated fatty acids
QC: quality control
qPCR: quantitative polymerase chain reaction
RDP: Ribosomal Database Project
RH: relative humidity
RM: repetition maximum
RSDs: Relative standard deviations
SCFAs: short-chain-fatty-acids
SRM: Selected Reaction Mode
TC: total cholesterol
TLR4: toll-like receptor-4
UC: unclassified
UDCA: ursodeoxycholic acid
USDA: United States Departament of Agriculture
VIG-EX: vigorous-intensity exercise group
VO_{2max}: maximum oxygen consumption
WOS: Web of Science

ABSTRACT

The gut microbiota refers to the microorganisms that colonize the gastrointestinal tract. It has emerged as a pivotal transducer of environmental influences to exert protective or detrimental effects on several host tissues and systems, and as a major player in the maintenance of human health and the pathophysiology of cardiometabolic diseases. Therefore, understanding the role that gut microbiota might play in the development and treatment of cardiometabolic diseases is of great scientific and societal interest. It has been speculated that the potential benefits of exercise and cold therapies on cardiometabolic health can be explained through their impact on the host's gut microbiota.

The aims of this International Doctoral Thesis were to study whether novel markers of cardiometabolic health are related to fecal microbiota composition in young adults (section I); and to investigate the impact of exercise and cold on fecal microbiota and cardiometabolic health, respectively, in young adults (section II). To unravel these aims we conducted two cross-sectional studies (section I), and a systematic review, a 24-week exercise-intervention study and a novel short-term mild cold intervention in young adults (section II).

The results of the present International Doctoral Thesis showed a unique relationship between plasma levels of omega-3 and omega-6 oxylipins and fecal microbiota composition in young adults (**study I**). We also showed that specific bacteria species were related to plasma levels of primary and secondary bile acids (**study II**). We provided the available scientific evidence on the effect of exercise on human fecal microbiota composition (**study III**) and showed that a 24-week moderate-exercise intensity program modified the fecal microbiota composition in young adults (**study IV**). Lastly, we demonstrated that a novel mild cold regimen is able to decrease distal skin temperature in both hands after 3.5 days of activation, yet other metabolic parameters were not affected (**study V**).

Altogether, the results of the present International Doctoral Thesis suggest that novel markers of cardiometabolic health (*i.e.*, oxylipins and bile acids) are associated with fecal microbiota in young adults, and that exercise of moderate intensity is able to modify fecal microbiota composition. Preliminary findings on our cold intervention will pave the way towards new experiments understanding the role of cold exposure in gut microbiota composition in humans.

RESUMEN

La microbiota intestinal se refiere a los microrganismos que colonizan el tracto gastrointestinal. La microbiota intestinal ha surgido como un transductor fundamental de las influencias ambientales para ejercer efectos protectores o perjudiciales en varios tejidos y sistemas del huésped, y como un actor importante en el mantenimiento de la salud humana y la fisiopatología de las enfermedades cardiometabólicas. Por lo tanto, comprender el papel que podría desempeñar la microbiota intestinal en el desarrollo y tratamiento de las enfermedades cardiometabólicas es de gran interés científico y social. Por otro lado, los beneficios potenciales del ejercicio y las terapias basada en frío en la salud cardiometabólica podrían explicarse a través de la modificación en la composición de la microbiota intestinal.

Los objetivos de esta Tesis Doctoral Internacional fueron estudiar si nuevos marcadores de salud cardiometabólica están relacionados con la composición de la microbiota fecal en adultos jóvenes (sección I) e investigar el impacto del ejercicio y el frío en la microbiota fecal y la salud cardiometabólica, respectivamente, en adultos jóvenes (sección II). Para alcanzar estos objetivos, realizamos dos estudios transversales (sección I) y una revisión sistemática, un estudio de intervención de ejercicio durante 24 semanas y un novedoso estudio de frío leve a corto plazo en adultos jóvenes (sección II).

Los resultados de la presente Tesis Doctoral Internacional mostraron que los niveles plasmáticos de oxilipinas omega-3 y omega-6 se relacionan con la composición de la microbiota fecal en adultos jóvenes (**estudio I**). También encontramos que existen algunas bacterias que podrían estar relacionadas con los niveles plasmáticos de ácidos biliares primarios y secundarios (**estudio II**). Además, los resultados de la revisión sistemática pusieron de manifiesto el efecto del ejercicio sobre la composición de la microbiota fecal en humanos (**estudio III**), y encontramos que, tras 24 semanas de ejercicio realizado a intensidad moderada, modificó la composición de la microbiota fecal (**estudio IV**). Por último, demostramos que una intervención novedosa de exposición al frío leve es capaz de disminuir la temperatura de la piel distal en ambas manos después de 3.5 días de activación, sin alterar parámetros metabólicos jóvenes (**estudio V**).

El conjunto de los resultados de esta Tesis Doctoral Internacional sugiere que hay nuevos marcadores de salud cardiometabólica (es decir, oxilipina y ácidos biliares) que están asociados con la microbiota fecal en adultos jóvenes, y que el ejercicio a intensidad moderada, puede modificar la composición de la microbiota fecal. Los resultados de la intervención con frío sirven de base para iniciar hacia nuevos experimentos que ayuden a dilucidar el papel de la exposición al frío en la composición de la microbiota intestinal en humanos.

GENERAL INTRODUCTION

THE PROBLEM: CARDIOMETABOLIC DISEASES

Cardiometabolic diseases are the number-one cause of death in the Western world[1], characterized by obesity, hypertension, diabetes, and dyslipidemia[2]. Smoking, lack of exercise, drinking a lot of alcohol and eating an unhealthy diet are the four main reasons for their rising.

Human obesity has become a global epidemic disease, in developed and developing countries[3], where the food supply is abundant and constantly available and is rich in energy. In addition, physical activity has decreased and sedentary lifestyle dramatically increased[4]. As a result, energy expenditure was diminished and obesity has approximately tripled since 1975[5]. Obesity is defined as a condition of abnormal or excessive fat accumulation in adipose tissue. The distribution of excess fat in the body can be around the waist and trunk (abdominal, central, or android obesity) or peripherally around the body (gynoid obesity)[6]. By the current World Health Organization (WHO) criteria, a body mass index (BMI) higher than 30kg/m^2 is obesity[5]. These patients have an unhealthier cardiometabolic profile and are at increased risk of diabetes and cardiovascular disease compared with normal weight[7,8]. They had high levels of low-density lipoprotein cholesterol (LDL-C), triacylglycerol, increased systolic blood pressure, and low levels of high-density lipoprotein cholesterol (HDL-C)[9].

It is visible that the current strategies used to suppress the prevalence of cardiometabolic diseases are not sufficient, and they have risen[10]. Therefore, the identification of novel markers that will allow clinicians to early identify cardiometabolic diseases is urgently needed.

THE SOLUTION MIGHT BE IN THE HUMAN MICROBIOME!

The human microbiome is a collection of microbial communities and all their genomic elements, whereas microbiota is defined as the community of microorganisms, including eukaryotes, archaea, bacteria, and viruses[11]. When this community of microorganisms is in balance, is called eubiosis, whereas if the community is unbalanced, it is called dysbiosis[12]. The human microbiota varies between individuals[13] and within the same individual over time[14]. The establishment of the infant skin microbiota appears to be mainly influenced by the type of delivery and the subsequent feeding practices[15-17], their interpersonal variation is significantly greater among children than among adults[18]. While growing and being adults, the microbiota is more stable and difficult to modify[18].

Bacteria represent the majority of the microorganisms present in the microbiota. Bacteria are prokaryotic microorganisms, which do not possess either a nucleus or organelles; however, they have a cell

wall with peptidoglycan and a single chromosome and ribosomes in the cytosol. Bacteria have a wide variety of shapes, but the four basic morphologies are spherical (cocci), rod-shaped (bacilli), spiral-shaped (spirilla), and comma-shaped (vibrios), which they depend on the environment[19]. H.C. Gram in 1884 discovered the Gram stain classification, where bacteria are classified as either Gram positive or negative[20]. Gram-positive bacteria have a simple chemical structure, a thick peptidoglycan layer, whereas gram-negative are more complex. In this case, gram-negative bacteria are rich in lipids and the membrane is bilayer[21]. Also, bacteria are organized in taxonomy called bacterial taxonomy levels, which consists of classification, nomenclature, and identification of microorganisms, and the ranking scale limited to phylum, class, order, family, genus, species, and strains[22] (**Fig.1**). Although the bacteria emanate from only a few phyla, there is an immense diversity of the microbiota at lower taxonomic levels, rendering it unique among individuals[23].

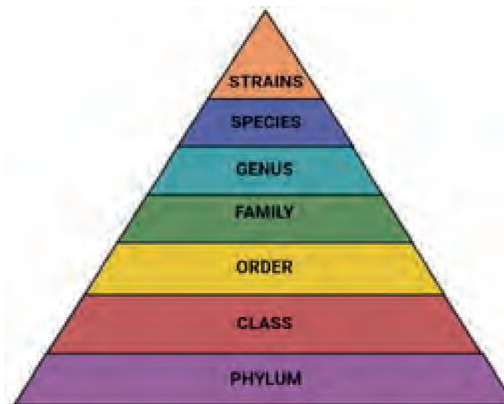

Figure 1: Bacterial taxonomy levels.

Bacteria are living in the body, both external and internal surfaces, such as the gastrointestinal tract, skin, saliva, and vagina[24]. The composition of bacteria varies in the different parts of the body, including along the gastrointestinal tract[25], influenced by PH, aerobic and anaerobic condition, diet composition, antimicrobials intake, or even exercise training[26]. These are commensal bacteria, because co-exist in harmony and are beneficial for the host[27], but under some conditions, these bacteria can be harmful to the host and infections can occur[28]. A marker for microbiota health is alpha diversity, which represents taxonomic richness (i.e., how many species are represented) and evenness (i.e., equitability of the phylotypes frequencies are); individuals with diseases have reported lower alpha diversity relative to healthy control[29,30]. Differences between individuals were significantly greater than intra-individuals differences. To study inter-individual differences, we used beta diversity, which provides measures of the degree to which samples differ from one another[31]. This measure takes into account quantitative sequence abundance and/or phylogeny base[31].

The gut microbiota

The gut microbiota refers to the microorganisms that colonize the gastrointestinal tract[32]. It has emerged as a pivotal transducer of environmental influences to exert protective or detrimental effects on several host tissues and systems, and as a major player in the maintenance of human health and the pathophysiology of cardiometabolic diseases. Bacteria can passage from gastrointestinal to extraintestinal sites, like the liver, kidney, bloodstream, and it is known as bacteria translocation[28]. Bacteria translocation can occur when increased permeability of intestinal mucosal barrier and/or deficiencies in host immune defenses caused by dysbiosis[28].

Microbial density increases from 10 microbial cells per gram in the stomach to 10^{12} microbial cells per gram in the colon[33]. Also bacterial diversity increases in the same axis[34]. The dominant bacteria that compose the human gut microbiota belongs to the *Firmicutes* and *Bacteroidetes* phyla[35] and in fewer proportion *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* phyla[36]. A eubiosis status is characterized by an abundance of species belonging mainly to the *Firmicutes* and *Bacteroides* phyla, whereas dysbiosis status increases *Proteobacteria* phylum[23] (**Fig.2**).

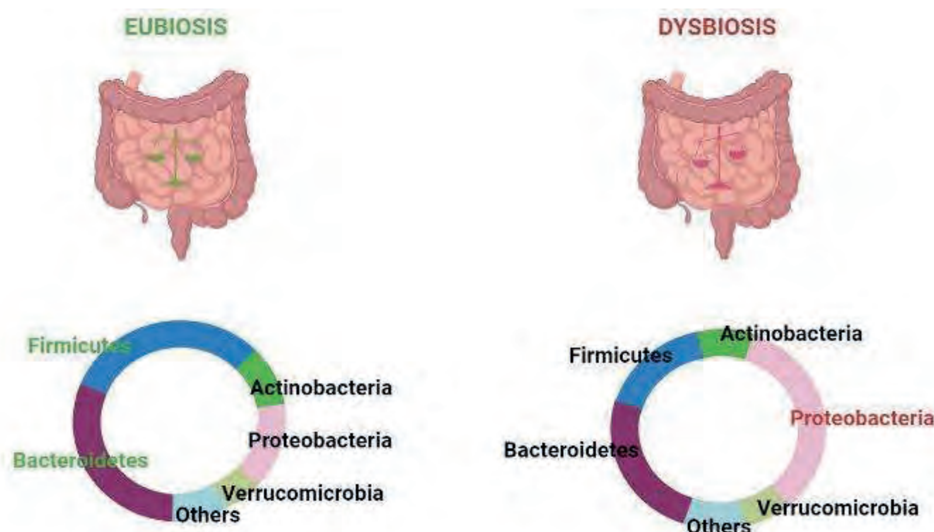

Figure 2: Representative example of the human status of dysbiosis and eubiosis at phylum level

Currently, microbiome research has shown that the gut microbiota is not just a passive bystander[37]. It has been shown a host-gut interaction that may be conducted by metabolite secretion and signaling, which influences host immunity and physiology, including the endocrine, metabolic, and nervous system function in health and disease[38]. The gut microbiome contains more than 5 million genes,

which provides a diverse range of biochemical and metabolic activities to complement host physiology[39].

The gut is the main site of food digestion, in which dietary nutrients are metabolized and absorbed. Besides, fermentation of dietary fibers by anaerobes bacterial allows these bacteria to produce short-chain fatty acids (SCFAs), which include acetate, butyrate, and propionate[40,41] in 3:1:1 proportion[42]. Acetate is mainly produced by anaerobes microbes within the gut, propionate is mainly produced by *Bacteroidetes*, and butyrate by *Firmicutes*[43]. Butyrate is known for being an importunate energy source for colonocytes[44] and can attenuate bacterial translocation and enhance gut barrier function[45]. On the other hand, there are bacteria (i.e., lactic acid bacteria) that are involved in the production of vitamin B12, essential substances benefiting human health but incapable of self-producing[46]. In addition, *Bifidobacteria* are the main producers of folate, a vitamin involved in the host metabolic processes including DNA synthesis and repair[47]. There is an essential symbiotic relationship between gut bacteria and the human host, which plays an important role in the immune system function and inflammatory response[33,45]. Alterations in microbiome-associated metabolite levels and activity are implicated in the pathogenesis of a growing number of illnesses[38]. There is evidence showing that gut microbiome-derived metabolites can reach systemic circulation through the gut barrier[48], and it is recognized by receptors from organs such liver or adipose tissue[49], and fuels metabolic inflammation[48]. Therefore a change in the amounts of these bacteria can develop metabolic diseases such as obesity[50].

Fecal microbiota analysis: A new approach for identifying bacteria within the gut

Fecal samples are frequently used as proxies for gut microbiota because they are naturally collected, non-invasive, and can be easily collected repeatedly[51]. Normally, fresh feces cannot be analyzed immediately, so are stored at -80°C that can preserve microbial integrity[52]. Although, several studies suggest that direct freezing at -20°C in patients´ homes freezers can also be used, even left at room temperature in aerobic condition for up to 24 hours without a dramatic effect on 16S rDNA[53,54].

Accuracy methods are necessary to ensure that the extracted genetic content represents the population of microorganisms. Sample lysis is the first step, we can use bead-beating, physical or chemical methods, and heating at 95°C, before or after, increases lysis efficiency[55] without damaging both Gram-negative and Gram-positive bacteria DNA. Then, the DNA extraction could be manual or semi-automated methods with spin columns or magnetic beads, it depends on the kit. After that, always is necessary for sample purification, in the manual protocol use polyvinylpolypyrrolidone (PVPP)[56], whereas

commercial kits include specific reagents that bind and remove inhibitory compounds, as InhibitEX tablets of QIAamp[57].

The sequencing is based on amplification and sequencing of the 16S, that part is found in all bacteria and has some advantages: highly conserved regions thus use of universal PCR primers, nine highly variable regions to considered and a single bacteria contains many copies of 16S[57]. In the past, Sanger-based technologies is used, but had high costs and only medium throughput. Nowadays, since 2008, high-throughput is available and reduces the cost and time needed for sequencing, allow to research to perform more systematically and with a higher number of samples[57].

After obtaining the DNA reads, the raw data (fastq) have been used to test the performance of bioinformatics tools and data analysis methods, to demonstrate that quality filtering and trimming were been good. The first quality control requires to find and remove low-quality and contaminating reads[58]. After that, the reads can be compared with the reference genomes to evaluate the abundance of these fragments in the original sample, such as the Ribosomal Database Project[59] has been created and can be used to compare sequences (**Fig.3**).

Figure 3: Overview of process from fecal sample collection at home until raw data analysis.

LINK BETWEEN PREVALENCE OF CARDIOMETABOLIC DISEASES AND GUT MICROBIOTA COMPOSITION IN HUMANS

The gut microbiota is now recognized as an important factor in the pathogenesis of cardiometabolic diseases and a target for therapeutic intervention to maintain and improve health[60–62] (**Fig.4**). Several studies indicate that alteration of gut microbiota may play a key role in the development of diseases associated with altered lipid metabolism[63,64]. These communities are important for human physiology, immune system development, and digestion and detoxification reactions. In fact, some of these microorganisms are residing in the gut encode proteins involved in functions important for the host's health, such as enzymes required for the hydrolysis of otherwise indigestible dietary compounds, and the synthesis of vitamins[36,65].

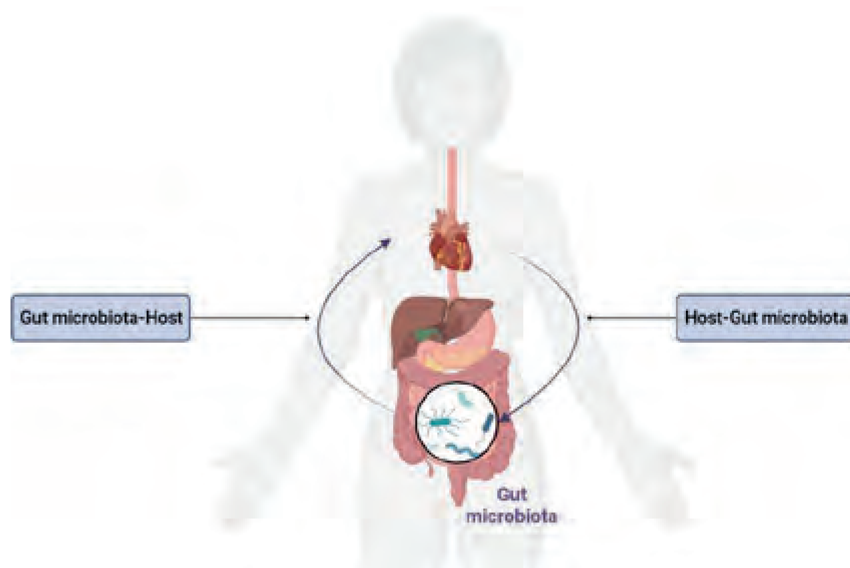

Figure 4: Interactions between gut microbiota and human host.

Oxylipins as source for modifying the gut microbiota composition

Diseases that persist for a long time are known as chronic diseases. Nowadays, they are rising exponentially, and their prevalence correspond with an unbalanced diet, characterized partially by a decrease intake of long-chain omega-3 and an increase intake of omega-6 polyunsaturated fatty acids (PUFAs) [66,67]. To understand the effects of PUFAs, we directly assess oxylipins, which are oxidized lipid mediators[68]. Omega-3 PUFAs are linked directly to anti-inflammatory actions since, both eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), decrease the synthesis of the pro-inflammatory mediators (*i.e.*, prostaglandin E2)[69], while omega-6 PUFAs upregulate inflammation by acting as precursors to pro-inflammatory eicosanoids[70]. For this reason, the diet significantly contributes to the concentration of oxylipins but also depends on oxygenases present for metabolizing these PUFAs and their preference[71]. The oxygenases are lipoxygenases (LOXs), cyclooxygenases (COXs), and cytochrome P450

(CYP450) enzymes family[71], which oxygenate omega-3 and omega-6 PUFAs. These oxylipins are ligands for the peroxisome proliferator-activated receptors (PPARs), as well as for cell-surface G protein-coupled receptors (GPCRs) and Toll-like receptors (TLRs)[72-74] whose dysfunctions can cause inflammatory and immune disorders[73,75].

Recently, Kaliannan et al.[76] showed that FAT-1 mice, which can endogenously convert omega-6 to omega-3 PUFAs, had a higher relative abundance of *Firmicutes*, *Bacteroides*, and *Actinobacteria* phyla[76]. Conversely, the FAT-2 mice, which can transform monounsaturated fatty acids (MUFAs) into omega-6 PUFAs displayed a higher relative abundance of *Proteobacteria* phylum[76]. These results suggest that plasma levels of PUFAs could modulate mice gut microbiota composition and therefore, influence gut microbiota on human metabolism. This effect depends not only on their abundance, but also on the metabolites that they produce[77] (*i.e.,* secondary bile acids)[78].

Gut bacteria play a crucial role in bile acid metabolism

Bile salt hydrolases (BSHs) and 7-α-dehydroxylases are the major bacteria enzymes involved in the metabolism of BA [79]. There are two types of bile acids (BA): primary bile acids and secondary BA. Primary BA cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from cholesterol in hepatocytes, where they are typically conjugated with glycine to produce glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA), respectively, as well as taurine to produce the corresponding tauro-conjugates [80]; glycine-conjugated BA represent 75% of the total pool of conjugated BA [81]. Primary BA are then stored in the gallbladder and released into the intestine to facilitate absorption of dietary lipids and liposoluble vitamins, protect against bacterial overgrowth [82], and eliminate cholesterol [81,83]. Approximately 95% of the BA are reabsorbed in the distal ileum and returned to the liver through the enterohepatic circulation [84]. The remaining 5% of primary BA enter the colon, where they are metabolized into the secondary BA deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) under the action of these bacteria enzymes [85]. BSHs participate in the deconjugation of primary and secondary BA by catalyzing the removal of glycine and taurine [79]. These enzymes are present in certain Gram-positive bacteria of the phylum *Firmicutes* and Gram-negative bacteria of the phylum *Bacteroidetes* [86-88]. Then the action of 7-α-dehydroxylases, which catalyze the removal of the hydroxyl group at the position 7' of the primary BA, producing a secondary BA. The 7-α-dehydroxylases enzymes are expressed by certain anaerobic bacteria, such as Gram-positive bacteria species belonging to the Clostridium genus [86,88].

These secondary BA can either be reabsorbed by colonocytes[89], reach the systemic circulation inducing signaling functions [90,91], reach

the liver via the enterohepatic circulation where they can be conjugated with glycine or taurine[92], or be secreted into the feces[82]. The signaling functions during systemic circulation can regulate glucose and lipid metabolism, predominantly mediated by GPCRs[93]. A few cross-sectional studies have shown that higher levels of circulating BA are linked to an increased risk of obesity and diabetes type 2[94]. Each bile acid interacts with more than one receptor[95], for example, the farnesoid X receptor (FXR) is the main bile acids sensor in the liver and intestine[96–98], and GPBAR1 (TGR5) is mainly expressed in the intestine, which modulates the release of GLP1 and therefore insulin secretion[99,100].

POTENTIAL INTERVENTIONS

Role of exercise as therapy for modifying gut microbiota composition

Exercise is a non-pharmaceutical therapy able to treat many chronic diseases such as obesity and type 2 diabetes [101,102], thanks to increased metabolic activity via the contraction of the skeletal muscles[103]. Several studies have shown that exercise can decrease markers of inflammation, improve overall metabolic health by improving glucose tolerance[104], insulin sensitivity[105], and decreasing circulating lipid concentrations[106]. Although exercise is a great tool to prevent and combat obesity, the molecular mechanisms mediating this protective effect are not well understood.

The exercise ability to enable cross-talk between skeletal muscle and other organs (*e.g.*, gut) has been studied. During exercise, the contraction of skeletal muscle releases molecules called myokines[107]. These myokines can stimulate glucagon-like peptide-1 (GLP-1) receptors in the gut and are involved with host metabolism[108]. Furthermore, exercise increases blood lactate concentration[109], which can cross the gut barrier. As previously mentioned, there are bacteria consuming lactate and releasing SCFAs. This effect of exercise on the gut microbiota composition is largely studied, but the majority of studies are conducted in mice models, where they also showed that exercise seems to restore eubiosis in the gut[110–112]. Whether exercise is able to restore gut eubiosis in humans is not fully elucidated.

Role of cold exposure as therapy for modifying gut microbiota composition

Cold exposure is a very promising therapy able to combat cardiometabolic diseases. Cold exposure activates the sympathetic nervous system, induces a cutaneous vasoconstriction and increases skeletal muscle contractile activity[113]. Overall, cold exposure increases

energy expenditure that leads to a decrease of the body weight improving cardiometabolic health. However, the beneficial effects of cold exposure are not fully explained by an increased in energy expenditure, and several studies have proposed that cold exposure can modify fecal microbiota composition[114,115]. In mice, it is well known that cold exposure increases the intestinal absorption capacity and increased overall thermogenesis through SCFAs production by gut bacteria[114,115]. However, cold exposure is not a feasible long-term therapy since it has some side effects. Therefore, before investigating the beneficial effects of cold exposure on human metabolism, we really need to understand how we can apply long-term cold exposure to humans while minimizing the side effects.

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HYPOTHESIS & AIMS

HYPOTHESIS

The main hypothesis of this International Doctoral thesis is that novel markers of cardiometabolic health are related to fecal microbiota composition and that the latter can be modified through exercise. Specifically, we hypothesize that: (i) oxylipins and bile acids, as novel markers of cardiometabolic health, are related to specific bacteria present in the feces, (ii) exercise, as a therapeutic tool, is able to modify fecal microbiota composition, and iii) mild cold exposure has an impact on body temperature and energy metabolism without side effects in humans.

AIMS

Therefore, the main aim of this International Doctoral thesis is to unravel whether novel markers of cardiometabolic health are related to fecal microbiota composition in young adults and to investigate whether exercise can modify the bacterial composition. This aim is addressed by five different studies, which are grouped into two sections.

SECTION 1: Novel insights: Cardiometabolic markers and fecal microbiota composition

Specific aim 1.1.: To investigate the relationship between plasma levels of omega-3 and omega-6 oxylipins and fecal microbiota composition. (Study I)

Specific aim 1.2.: To investigate the relationship of fecal microbiota diversity and composition with plasma levels of primary and secondary bile acids. (Study II)

SECTION 2: Potential interventions to modify the fecal microbiota composition

Specific aim 2.1.: To summarize the literature on the influence of exercise on the fecal microbiota of healthy adults. (Study III)

Specific aim 2.2.: To investigate the effect of 24-week supervised exercise intervention of moderate and vigorous intensity, on fecal microbiota diversity and composition, and whether those changes are related to exercise-induced cardiometabolic benefits. (Study IV)

Specific aim 2.3.: To determine the effect of an intermittent mild cold stimulus on skin temperature, the perception of skin temperature, sleep quality, resting energy expenditure and the nutrient oxidation rate. (Study V)

MATERIAL & METHODS

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

Study	Design	Participants characteristics*	Independent variable	Dependent variable	Statistical (approach)
Study I	Cross-sectional	N=80 (♀=74%) Age: 21.9 ± 2.2 BMI: 24.7 ± 4.7	Plasma levels of oxylipins	Fecal microbiota composition	Spearman correlation Logarithmic regression
Study II	Cross-sectional	N=80 (♀=74%) Age: 21.9 ± 2.2 BMI: 24.7 ± 4.7	Fecal microbiota composition	Plasma levels of bile acids	Spearman correlation
Study III	Systematic Review	N.A.	Physical activity and exercise	Fecal microbiota composition	Following PRISMA statement1
Study IV	Randomized controlled trial	CON: N= 20 (♀=65%) Age: 22.2 ± 2.2 BMI: 24.1 ± 4.3 MOD-EX: N= 21 (♀=75%) Age: 21.9 ± 2.3 BMI: 26.0 ± 5.3 VIG-EX: N= 20 (♀=70%) Age: 21.5 ± 2.4 BMI: 24.8 ± 4.6	Exercise	Fecal microbiota composition	ANOVA Kruskal wallis Spearman correlation
Study V	Longitudinal	N= 10 (♀=50%) Age: 25.8±3.4 BMI:23.0±3.2	Cold stimulus of 25°C	Skin temperature Perception of skin temperature Sleep quality Resting energy expenditure Nutrient oxidation rate	ANOVA

*Presented as mean ± standard deviation. ♀: Percentage of women, BMI: body mass index (kg/m²), CON: Control group, MOD-EX: moderate-intensity exercise group, N: number of total participants, PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analysis, VIG-EX: vigorous-intensity exercise group.

REFERENCE

1. Liberati A, Altman DG, Tetzlaff J, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. *BMJ*. 2009;339:b2700. Doi:10.1136/bmj.b2700

RESULTS AND DISCUSION

SECTION I

STUDY I: Plasma levels of omega-3 and omega-6 oxylipins are related to the relative abundance of *Proteobacteria* phylum in the feces of young adults

(*In preparation*)

ABSTRACT

Background

Pre-clinical studies suggest that the oxidation products of polyunsaturated fatty acids (PUFAs) (i.e., oxylipins) could influence gut microbiota composition in mice, but nothing is known in humans. Thus, this study aims to investigate the relationship between plasma levels of omega-3 and omega-6 oxylipins and fecal microbiota composition in a cohort of young adults.

Methods

80 young adults (74% women; 21.9 ± 2.2 years old) were included in this cross-sectional study. Plasma levels of oxylipins were measured using liquid chromatography-tandem mass spectrometry, whereas fecal microbiota composition was analyzed with 16S rRNA sequencing.

Results

Plasma levels of omega-3 derived oxylipins were positively correlated with the relative abundance of *Clostridium IV* genus (*Firmicutes* phylum; rho≥0.415, P≤0.009), and negatively correlated with the relative abundance of *Sutterella* genus (*Proteobacteria* phylum; rho≥-0.242, P≤0.04). Similarly, plasma levels of omega-6 derived oxylipins were negatively correlated with the relative abundance of *Sutterella* (*Proteobacteria* phylum; all rho≥-0.263, P≤0.024). Lastly, the omega-6/omega-3 oxylipins ratio was negatively correlated with the relative abundance of *Clostridium IV* genus (*Firmicutes* phylum; rho=-0.334, P=0.004) and *Butyricimonas* genus (*Bacteroidetes* phylum; rho=-0.292, P=0.014).

Conclusions

These results suggest that the plasma levels of omega-3 and omega-6 oxylipins are related to the relative abundance of specific fecal bacteria genus.

BACKGROUND

A disease that persists for a long time is known as chronic disease and is characterized by low-grade chronic inflammation [1-3]. Unfortunately, there are many untreated chronic diseases that are rising exponentially in the modern world [4], and their prevalence are mainly related to an unbalanced diet. This unbalanced diet is characterized partially by a suboptimal intake of omega-3 polyunsaturated fatty acids (PUFAs), and an exacerbated intake of omega-6 PUFAs [5,6].

Omega-3 and omega-6 PUFAs can be oxidized into oxylipins through the action of lipoxygenases (LOXs), cyclooxygenases (COXs), and cytochrome P450 (CYP450) enzymes family [7,8]. These oxylipins are the main mediators of the PUFAs effects in the body through their binding to G protein-coupled or peroxisome proliferator-activate receptors [7,8]. Generally, omega-3 derived oxylipins exert anti-inflammatory actions since, both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), decrease the synthesis of the pro-inflammatory mediators and increase the production of anti-inflammatory mediators [9], while omega-6 derived oxylipins upregulate inflammation by acting as precursors of pro-inflammatory mediators [10].

Gut microbiota is a vast array of microorganisms that colonize the gastrointestinal tract, where bacteria is the most abundant [11]. In humans, it is mainly composed of four phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* [12,13]. The composition and their secreted components are involved in the maintenance of the gut barrier permeability [14,15] and the regulation of the intestinal inflammation status [16,17] and can be modified by diet [18].

A recent study showed that *FAT-1* mice, which can endogenously convert omega-6 to omega-3 PUFAs, had a higher relative abundance of *Firmicutes*, *Bacteroides*, and *Actinobacteria* phyla [19]. In contrast, *FAT-2* mice can transform monounsaturated fatty acids (MUFAs) into omega-6 PUFAs. These mice showed a different gut microbiota composition compared to *FAT-1* mice, particularly depletion of *Bifidobacteriaceae* family (*Actinobacteria* phylum) [20]. In those studies, they demonstrated that omega-6 and omega-3 PUFAs are able to modulate the intestinal permeability, modulate the inflammatory status of the gut that ultimately affects the development of chronic diseases [20]. Based on that, we hypothesize that plasma levels of omega-3 and omega-6 derived oxylipins might be involved in the regulation of certain bacterial communities within the human gut.

In the present study, we aimed to investigate the relationship between plasma levels of omega-3 and omega-6 oxylipins and fecal microbiota composition in young adults.

MATERIAL AND METHODS

Participants

The participants were part of the ACTIBATE study [21], an exercise-based randomized controlled trial (ClinicalTrials.gov ID: NCT02365129) designed to evaluate the effect of exercise training on brown adipose tissue activity. Participants were recruited via advertisements in electronic media and leaflets. All participants reported to be sedentary (less than 20 min of moderate/vigorous physical activity on less than 3 days per week) and had a stable body weight over the preceding three months (<3kg change). The exclusion criteria were being pregnant, smoking, taking any medication or antibiotics, and having an acute or chronic disease. The study protocol was designed following the Declaration of Helsinki. All participants gave their informed consent and were approved by the Ethics Committee on Human Research of the University of Granada (n°.924), and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada).

In the current study, only participants with available baseline data for plasma oxylipins and fecal microbiota samples were included [80 young adults; n=59 women; 18-25 years old].

Determination of plasma levels of oxylipins

Blood samples were collected between 8:00-9:00 AM after 10-h overnight fasting. All blood samples were immediately centrifuged to obtain plasma aliquots (obtained with Vacutainer® Hemogard™ tubes) and stored at -80°C. Plasma levels of omega-3 and omega-6 oxylipins were measured using a targeted metabolomics approach with liquid chromatography-tandem mass spectrometry (LC-MS/MS), using a method validated according to the FDA bioanalytical method validation guidelines [22].

Sample preparation

All preparation steps use liquid-liquid extraction under ice-cold conditions to prevent analyte degradation, except the evaporation step [22]. Before the extraction, 150 µL of plasma sample were transferred into 1.5 mL Eppendorf tubes and mixed with 5 µL of an antioxidant solution composed of 0.4 mg/mL of butylated hydroxytoluene (BHT), and 10 µL of an internal standard mix containing the isotopically-labeled analogs (**Table 1**). Then, 150 µL of buffer solution (0.2 M citric acid and 0.1 M disodium hydrogen phosphate at pH 4.5) was added, followed by the addition of 1000 µL extraction solvent composed of methyl-tertbutylether and butanol (50:50, *v/v*). Then, the samples were mixed for 5 min using a bullet blender (Next Advance, Averill Park, NY), and centrifugated (16,000 g, 10 min, 4 °C). Next, 900 µL of supernatant

was transferred to a new 1.5 mL Eppendorf tube and evaporated to dryness using a SpeedVac system at room temperature. The dry residues were reconstituted in 50 µL of methanol:acetonitrile (70:30, *v/v*), and the reconstituted samples were centrifuged (16,000 g, 10 min, 4 °C). Finally, 40 µL of supernatant was transferred into glass vials for injection in the LC-MS/MS system.

Table 1 List of internal standards used in the LC/MS method.

Abbreviation	Name (International Union of Pure and Applied Chemistry, IUPAC)
Arachidonic Acid-d_8 (C20:4-w6-d_8)	5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid-d_8
Docosahexaenoic Acid-d_5 (C22:6-w3-d_5)	4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid-d_5
Linoleic Acid-d4 (C18:2-w6-d_4)	9Z,12Z-octadecadienoic acid-d_4
d_{11}-5-iPF2a-VI	(8β)-5,9α,11α-trihydroxy-prosta-6*E*,14*Z*-dien-1-oic acid-d_{11}
d_4-8-iso-PGE2	9-oxo-11α,15*S*-dihydroxy-(8β)-prosta-5*Z*,13*E*-dien-1-oic acid-d_4
d_4-8-iso-PGF2a	9α,11α,15*S*-trihydroxy-(8β)-prosta-5*Z*,13*E*-dien-1-oic acid-d_4
d_4-PGD2	9α,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid-d_4
d_4-PGF2a	9*S*,11*R*,15*S*-trihydroxy-5*Z*,13*E*-prostadienoic acid-d_4
d_9-PGE2	9-oxo-11*R*,15*S*-dihydroxy-5*Z*,13*E*-prostadienoic acid-d_9
d_4-iPF2a-IV	(8*S*)-10-[(1*R*,2*S*,3*S*,5*R*)-3,5-Dihydroxy-2-pentylcyclopentyl]-8-hydroxydeca-5,9-dienoic acid-d_4
d_{11}-8,12-iPF2a-VI	(12α)-5,9α,11α-trihydroxy-prosta-6*E*,14*Z*-dien-1-oic acid-d_{11}
d_{17}-10-Nitrooleate	10-nitro,9Z,12*Z*-octadecadienoic acid-d_{17}
d_{11}-14,15-DiHETrE	14,15-dihydroxy-5*Z*,8*Z*,11*Z*-eicosatrienoic acid-d_{11}
d_4-9(S)-HODE	9*S*-hydroxy-10*E*,12*Z*-octadecadienoic acid-d_4
d_4-LTB4	5*S*,12*R*-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraene-1,20-dioic acid-d_4
d_4-TXB2	9*S*,11,15*S*-trihydroxy-thromboxa-5*Z*,13*E*-dien-1-oic acid-d_4
d_6-20-HETE	20-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid-d_6
d_8-12(S)-HETE	12*S*-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid-d_8
d_8-5(S)-HETE	5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid-d_8
d_4-(+/-)12,13-DiHOME	12,13-dihydroxy-9*Z*-octadecenoic acid -d_4

Liquid chromatography - tandem mass spectrometry analysis

LC-MS/MS analysis was performed as previously described. Briefly, the extracted samples were analyzed using a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan), coupled to a SCIEX QTRAP 6500+ mass spectrometer (SCIEX, Framingham, MA). The separation was operated with a BEH C18 column (50 mm × 2.1 mm, 1.7 µm) from Waters Technologies (Milford, MA, USA) kept at 40 °C. The mobile phase consisted of 0.1% acetic acid in water (A), 0.1% acetic acid in acetonitrile/methanol (90:10, *v/v*, B) and 0.1% acetic acid in isopropanol (C). Electrospray ionization was performed using negative mode. For the MS/MS acquisition, a selected reaction mode (SRM) was used. SRM transitions were individually optimized for targeted analytes and respective internal standards using standard solutions.

Table 2. Omega-3 and omega-6 oxylipins abbreviations, complete name and observed variability in area peak ratios.

	Abbreviation	Name	RSD in QC samples
Omega-3 oxylipins			
ALA-Derived	ALA	α-Linolenic acid	9.8%
	9-HOTre	9-hydroxy-octadecatrienoic acid	7.6%
	12,13-DiHODE	12,13-dihydroxy-octadecadienoic acid	5.8%
EPA-Derived	EPA	Eicosapentaenoic acid	8.5%
	5-HEPE	5-hydroxy-eicosapentaenoic acid	13.1%
	12-HEPE	12-hydroxy- eicosapentaenoic acid	12.1%
	14,15-DiHETE	14,15-dihydroxy-eicosatetraenoic acid	8.0%
	17,18-DiHETE	17,18-dihydroxy-eicosatetraenoic acid	9.1%
	DPA	Docosapentaenoic acid	14.0%
DHA-Derived	DHA	Docosahexaenoic acid	9.8%
	4-HDoHE	4-hydroxy-docosahexaenoic acid	14.3%
	8-HDoHE	8-hydroxy-docosahexaenoic acid	19.7%
	11-HDoHE	11-hydroxy-docosahexaenoic acid	17.1%
	13-HDoHE	13-hydroxy-docosahexaenoic acid	12.3%
	14-HDoHE	14-hydroxy-docosahexaenoic acid	14.7%
	16-HDoHE	16-hydroxy-docosahexaenoic acid	15.4%
	17-HDoHE	17-hydroxy-docosahexaenoic acid	9.0%
	20-HDoHE	20-hydroxy-docosahexaenoic acid	23.6%
	19,20-EpDPE	19,20-epoxy-docosapentaenoic acid	13.5%
	19,20-DiHDPA	19,20-dihydroxy-docosapentaenoic acid	7.9%
Omega-6 oxylipins			
LA-Derived	LA	Linoleic acid	10.2%
	9-HODE	9-hydroxy-octadecadienoic acid	7.6%
	13-HODE	13-hydroxy-octadecadienoic acid	7.1%
	9,10,13-TriHOME	9,10,13-trihydroxy-octadecenoic acid	6.9%
	9,12,13-TriHOME	9,12,13-trihydroxy-octadecenoic acid	15.7%
	9,10-EpOME	9,10-epoxy-octadecenoic acid	7.8%
	9,10-DiHOME	9,10-dihydroxy-octadecenoic acid	7.3%
	12,13-EpOME	12,13-epoxy-octadecenoic acid	9.6%
	12,13-DiHOME	12,13-dihydroxy-octadecenoic acid	6.7%
	10-NO2-LA	10-nitro-linolenic acid	13.3%
DGLA-Derived	DGLA	Dihomo-γ-linolenic acid	23.9%
	8-HETrE	8-hydroxy-eicosatrienoic acid	22.8%
	15-HETrE	15-hydroxy-eicosatrienoic acid	13.5%
AA-Derived	AA	Arachidonic acid	13.4%
	PGE2	Prostaglandin E2	36.0%
	TxB2	Thromboxane B2	6.9%
	12-HHTrE	12-hydroxy-heptadecatrienoic acid	8.1%
	8,12-iPF2α	8,12-isoprostane F2alpha	7.2%
	5-HETE	5-hydroxy-eicosatetraenoic acid	10.4%
	11-HETE	11-hydroxy-eicosatetraenoic acid	10.7%
	12-HETE	12-hydroxy-eicosatetraenoic acid	11.4%
	15-HETE	15-hydroxy-eicosatetraenoic acid	9.3%
	20-HETE	20-hydroxy-eicosatetraenoic acid	9.4%

	5,6-DiHETrE	5,6- dihydroxy-eicosatrienoic acid	9.0%
	8,9-DiHETrE	8,9- dihydroxy-eicosatrienoic acid	9.9%
	11,12-DiHETrE	11,12- dihydroxy-eicosatrienoic acid	8.4%
	14,15-EpETrE	14,15-epoxy-eicosatrienoic acid	19.5%
	14,15-DiHETrE	14,15- dihydroxy-eicosatrienoic acid	7.1%
AdrA-Derived	AdrA	Adrenic acid	22.1%
	1a,1b-dihomo-PGF2α	1a,1b-dihomo-9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid	22.1%

Abbreviations: QC: quality control; RSD: relative standard error.

Data pre-processing

For each target compound, SCIEX OS-MQ Software was used to calculate the ratio between its peak area and the peak area of its respective internal standard. The quality of the data was monitored using regular injection of quality control (QC) samples, consisting of blank plasma samples. QC samples were used to correct for between batch variations, using the in-house developed mzQuality workflow (available at http://www.mzQuality.nl) [23]. Relative standard deviations (RSDs) were calculated for the analytes present in the QC samples (**Table 2**).

A targeted metabolomics-based approach was used for the determination of the relative quantitation of oxylipins derived from the conversion of the omega-3 PUFAs α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA); and omega-6 PUFAs linoleic acid (LA), dihomo-γ-linolenic acid (DGLA), arachidonic acid (AA), and adrenic acid (AdrA). All the data from every single omega-3 derived oxylipin species were summed resulting in the sum of omega-3 derived oxylipins. The same was performed for omega-6 derived oxylipins (**Table 2**). The oxylipins detected by this method and their internal standard used are in **Table 1**. Additionally, the omega-6/omega-3 oxylipins ratio was calculated by dividing the sum of omega-6 derived oxylipins by the sum of omega-3 derived oxylipins. The methodology is described in detail in the supplementary material.

Fecal collection and DNA extraction

We used a 60 mL sterile plastic container to store fecal samples at -80°C until DNA extraction. Fecal samples were homogenized in a Stomacher® 400 blender (A. J. Seward and Co. Ltd., London, UK). DNA extraction and purification were performed with a QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain), according to the manufacturer's instructions and the concentration and quality were determined with a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, DE, USA).

Sequencing

DNA was amplified by PCR, using the following primer pairs: 16S Amplicon PCR Forward Primer: 5´CCTACGGGNGGCWGCAG; and 16S Amplicon PCR Reverse Primer: 5′GACTACHVGGGTATCTAATCC targeting the V3 and V4 hypervariable regions. PCR assays were conducted in a final volume of 25 μL including: 12.5 μL of the 2X KAPA HiFi Hotstart prepared mixture (KAPA Biosystems, Woburn, MA, USA), 5 μL of each forward and reverse primer (1 μM), and 2.5 μL of DNA extracted (10 ng). PCR program: (i) denaturation at 95 °C for 3 min, (ii) 8 denaturation cycles at 95 °C for 30 s, (iii) annealing at 55°C for 30 s, (iv) elongation at 72 °C for 30 s, (v) a final extension at 72 °C for 5 min. Next, AMPure XP microspheres (Beckman Coulter, Indianapolis, IN, USA) were used to purify the 16S V3 and V4 amplicon away from free primers and primer-dimer species. For the PCR index step, we used the Nextera XT index kit (Illumina, San Diego, CA, USA) to tag DNA with the sequencing adapters. The pooled PCR products were purified using AMPure XP balls (Beckman Coulter, Indianapolis, IN, USA) before quantification. The amplicons were sequenced in a MiSeq (Illumina, San Diego, CA, USA), using the Illumina MiSeq paired-end sequencing system (2x300nt) (Illumina, San Diego, CA, USA).

Bioinformatics

"*Dada2*" package version 1.10.1 in *R* software [24] was used for merging and filtering raw sequences (FastQ files). All samples that were above the 10,000 reads cut-off point were considered valid for the analyses. The samples were resampled to an equal sequencing depth using the "*Phyloseq*" [25] package in *R* software (30,982 reads), obtaining a total of 11,158 different phylotypes.

Phylotypes were assigned to their specific taxonomic affiliation (from phylum to genus) based on the naive Bayesian classification with a pseudo-bootstrap threshold of 80% [25] using the "*Classifier*" function in Ribosomal Data Project (RDP) [26]. We obtained 16 phyla, which include 209 genera. For the main analysis, we performed the analyses if the average of the sequence abundance was higher than 1% and we used relative abundances (reads percent of each phylotype relative to the total number of reads).

Anthropometry and body composition

The SECA model 799 electronic column scale and stadiometer (SECA, Hamburg, Germany) were used to measure participants´ height and weight, without shoes and wearing standard clothes. A dual-energy X-ray absorptiometry scan (Hologic Discovery Wi Marlborough, MA) was used to measure body composition (lean mass and fat mass). The body mass index (BMI) was calculated as weight/height^2.

Dietary assessment

The dietary assessment has been explained in detail elsewhere [27]. The EvalFINUT® software was used to assess the dietary intake (energy and nutrient intake) from three 24-h dietary recalls [27]. The 24-h dietary recalls were undertaken on 3 separate days (2 weekdays, and one weekend day) face-to-face interviews by qualified and trained dietitians. Two dieticians into the software introduced all data from interviews independently. While food frequency consumption was assessed using a food frequency questionnaire (FFQ)[28]. The participants were answered how often they had consumed each food on average, in commonly used portion size, over the last three months.

Statistical analysis

The descriptive parameters are presented as mean and standard deviation. First, data normality was checked using the D'Agostino & Pearson test, and due to the non-normal distribution of plasma levels of oxylipins and relative abundance of fecal microbiota composition variables, all analyses were conducted using non-parametric tests.

To investigate the relationship between the sum of omega-3, omega-6 derived oxylipins and omega-6/omega-3 oxylipins ratio and fecal microbiota composition (at phylum and genus taxonomy levels), we employed Spearman correlation analysis, using "*psych*" and "*corrplot*" in *R* software. Volcanos plots were used to depict these correlations using GraphPad Prism software (GraphPad Software, San Diego, California, USA, version 8.0.0). Then, to examine the relationship between plasma levels of individual omega-3 and omega-6 oxylipins and specific bacterial microbiota, we used Spearman correlation analysis, using "*psych*" and "*corrplot*" in *R* software. Heatmap plots were used to show these correlations using "*Gplot*" package in *R* software. Lastly, partial Spearman correlations were performed adjusting by BMI, PUFA, and fish intake. The level of significance was set at $P<0.05$.

RESULTS

Characteristics of the study participants

Table 3 shows the descriptive characteristics of the study participants (74% women; 21.9 ± 2.2 years old).

Table 3. Characteristics of the study participants

	N	Mean	±	SD
Sex (women, %)	80	59 (73.8%)		
Age (years)	80	21.9	±	2.2
Body composition				
Lean mass (kg)	80	41.0	±	8.9
Fat mass (kg)	80	36.3	±	7.8
Body mass index (kg/m^2)	80	24.7	±	4.7
Dietary intake				
Energy (kcal/day)	80	1920	±	489
PUFA (g/day)	80	13	±	5
Fish (servings/week)	77	5	±	3
Plasma levels of oxylipins (*area peak ratio*)				
Σ Omega-3	80	172.8	±	65.6
Σ ALA	80	12.0	±	5.1
Σ EPA	80	19.0	±	10.3
Σ DHA	80	141.7	±	54.6
Σ Omega-6	80	99.5	±	27.8
Σ LA	80	28.6	±	10.6
Σ DGLA	80	1.0	±	0.5
Σ AA	80	66.4	±	22.2
Σ AdrA	80	1.0	±	0.6
Omega-6/omega-3 oxylipins ratio	80	0.6	±	0.1
Fecal microbiota composition (phylum, %)				
Actinobacteria	80	1.6	±	1.6
Bacteroidetes	80	39.6	±	9.0
Firmicutes	80	48.8	±	9.7
Proteobacteria	80	6.5	±	4.8
Verrucomicrobia	80	2.3	±	4.3

Data are presented as mean and standard deviation (SD), otherwise stated. Σ: Sum of different oxylipins groups from the individual area peak ratio of each oxylipins group; AA: Arachidonic acid; AdrA: Adrenic acid; ALA: α-Linolenic acid; DHA: Docosahexaenoic acid; DGLA: Dihomo-γ-linolenic acid; EPA: Eicosapentaenoic acid; LA: Linoleic acid; PUFA: polyunsaturated fatty acids.

Plasma levels of omega-3 oxylipins are related to the relative abundance of *Clostridium IV* and *Sutterella* genus

At phylum level, we found no relationship between the sum of plasma omega-3 oxylipins levels with fecal microbiota composition (all P>0.05; **Fig. 1A**, **B**, **C**, and **D**). However, at genus level we identified that the sum of plasma omega-3 and DHA derived oxylipins levels were positively correlated with the relative abundance of *Clostridium IV* (*Firmicutes* phylum; rho≥0.415, P≤0.009; **Fig.1E** and **H**). Moreover, the sum of plasma omega-3, and the ALA, EPA, DHA derived oxylipins levels were negatively correlated with the relative abundance of *Sutterella* genus (*Proteobacteria* phylum; rho≥-0.242, P≤0.04; **Fig. 1E**, **F**, **G**, and **H**).

Interestingly, plasma levels of DPA, DHA, 8-HDoHE, 13-HDoHE and 19,20-DiDHPA (omega-3 oxylipins) were positively correlated with the relative abundance of *Clostridium IV* genus (all rho≥0.314, P≤0.018; **Fig.2A**). Contrary, the plasma levels ALA, EPA, 5-HEPE, DHA, 4-HDoHE and 19,20-DiDHPA were negatively correlated with the relative abundance of *Sutterella* genus (all rho≥-0.338, P≤0.042; **Fig.2A**). Of note, the aforementioned correlations remained significant after adjusting for BMI, PUFA and fish intake (**Table 4** and 5).

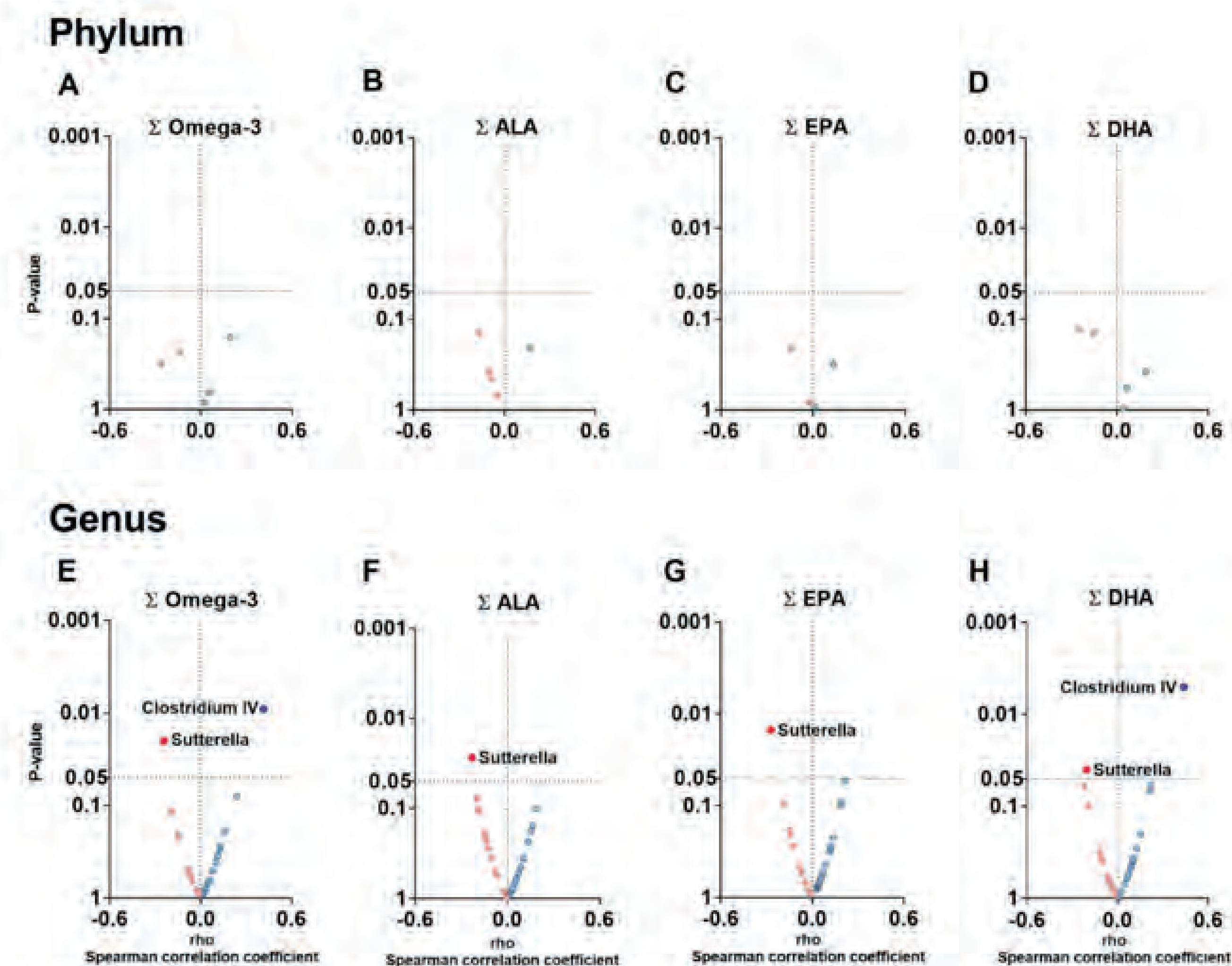

Figure 1. Volcano plots showing the correlations between plasma levels of omega-3 oxylipins and the relative abundance of fecal microbiota composition at phylum and genus levels. The sum (Σ) of omega-3 (A, E), ALA (B, F), EPA (C, G), and DHA (D, H) derived oxylipins were computed from the individual area peak ratio of the derived-oxylipins of each group. The X-axis represents Spearman's correlations coefficients, whereas the Y-axis represents the P values of the correlations. Only significant correlations (P<0.05) were annotated with the name of the phylum or genus. Blue dots represent positive correlations, whereas red dots represent negative correlations. Abbreviations: ALA: α-Linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid.

Figure 2: Spearman correlations between plasma levels of individual omega-3 (Panel A) and omega-6 (Panel B) oxylipins and abundance relative of *Clostridium IV* and *Sutterella* genera. Boxes only represent the statistically significant ($P<0.05$) correlations and the value within the boxes show the spearman correlation coefficient. Blue boxes represent positive correlations whereas red boxes indicate negative correlations.

Table 4. partial spearman correlation between the sum of omega-3 and omega-6 oxylipins and the relative abundance of fecal microbiota composition at genus level.

	Model 1		Model 2	
Omega-3 oxylipins				
	Rho	P	Rho	P
Clostridium IV (%)				
Σ Omega-3	0.286	**0.009**	0.287	**0.010**
Sutterella (%)				
Σ Omega-3	-0.254	**0.022**	-0.255	**0.023**
Omega-6 oxylipins				
	Rho	P	Rho	P
Clostridium IV (%)				
Σ Omega-6	0.114	0.295	0.118	0.296
Sutterella (%)				
Σ Omega-6	-0.265	**0.017**	-0.265	**0.017**
Omega-6/omega-3 ratio				
	Rho	P	Rho	P
Clostridium IV (%)	-0.256	**0.021**	-0.256	**0.022**
Sutterella (%)	0.108	0.338	0.108	0.339

Rho and P values are provided from partial Spearman correlation analyses. Model 1: adjusted for BMI; Model 2: adjusted for BMI, PUFA and fish intake. The sum (Σ) of omega-3 and omega-6-derived oxylipins were computed from the individual area peak ratio of each oxylipins group. Abbreviations: BMI: body mass index; PUFA: polyunsaturated fatty acids.

Table 5. Partial Spearman correlation between the plasma levels of individual omega-3 and omega-6 oxylipins and the relative abundance of fecal microbiota composition at genus level.

		Model 1		Model 2	
Omega-3 oxylipins					
		Rho	P	Rho	P
Clostridium IV (%)					
	DPA	0.332	**0.003**	0.271	**0.003**
	DHA	0.321	**0.004**	0.262	**0.004**
	8-HDoHE	0.339	**0.002**	0.300	**0.002**
	13-HDoHE	0.349	**0.002**	0.328	**0.002**
	19,20-DiHDPA	0.237	**0.035**	0.160	**0.035**
Sutterella (%)					
	ALA	-0.245	**0.029**	-0.224	**0.029**
	EPA	-0.260	**0.020**	-0.241	**0.020**
	5-HEPE	-0.363	**0.001**	-0.348	**0.001**
	DHA	-0.231	**0.039**	-0.196	**0.049**
	4-HDoHE	-0.287	**0.010**	-0.267	**0.010**
	19,20-DiHDPA	-0.228	**0.042**	-0.202	**0.042**
Omega-6 oxylipins					
		Rho	P	Rho	P
Clostridium IV (%)					
	9,10-EpOME	-0.193	0.087	-0.198	0.087
	12,13-EpOME	-0.257	**0.021**	-0.250	**0.021**
	TxB2	0.213	0.058	0.159	0.058
	12-HHTrE	0.216	0.054	0.168	0.054
Sutterella (%)					
	LA	-0.292	**0.009**	-0.278	**0.009**
	DGLA	-0.260	**0.020**	-0.241	**0.020**
	AA	-0.253	**0.024**	-0.233	**0.024**
	5-HETE	-0.366	**0.001**	-0.351	**0.001**
	AdrA	-0.311	**0.005**	-0.294	**0.005**

Rho and P values are provided from partial Spearman correlation analyses. Model 1: adjusted for body mass index; Model 2: adjusted for body mass index, PUFA and fish intake. Abbreviations: BMI: body mass index; PUFA: polyunsaturated fatty acids.

Plasma levels of omega-6 oxylipins are negatively correlated to the relative abundance of *Sutterella* genus

At phylum level, the sum of plasma omega-6, LA and DGLA derived oxylipins levels were negatively correlated with the relative abundances of *Bacteroidetes* (all rho≤-0.245, P≤0.036; **Fig. 3A**, **B** and **C**), whereas the sum of plasma AdrA derived oxylipins levels were negatively correlated with the relative abundance *Proteobacteria* (rho=-0.284, P=0.013; **Fig. 3E**). However, the sum of plasma LA derived oxylipins levels were positively correlated with the relative abundance of *Verrumicrobia* phylum (rho=0.255, P=0.022; **Fig. 3B**). At genus level, we found that the sum of plasma omega-6, LA, and DGLA derived

oxylipins levels were negatively correlated with the relative abundance of *Acidaminococcus* and *Phascolarctobacterium* genera (*Firmicutes* phylum; all rho≥-0.263, P≤0.024; **Fig. 3F, G and H**). We also observed that the sum of omega-6, DGLA, AA, and AdrA derived oxylipins levels were negatively correlated with the relative abundance of *Sutterella*, *Succinivibrio*, and *Gemmiger* genera (*Proteobacteria* phylum; all rho≥-0.263, P≤0.024; **Fig. 3F, G, H, I and J**). Similarly, the sum of plasma DGLA derived oxylipins levels was negatively correlated with the relative abundance of *Odoribacter* genus (*Bacteroidetes* phylum; rho=-0.246, P=0.028; **Fig. 3H**).

Interestingly, plasma levels of LA, DGLA, AA, 5-HETE and AdrA (omega-6 oxylipins) were negatively correlated with the relative abundance of *Sutterella* genus (all rho≥-0.313, P≤0.027; **Fig.2B**). All the aforementioned correlations remained significant after adjusting for BMI, PUFA and fish intake (**Table 4** and **5**).

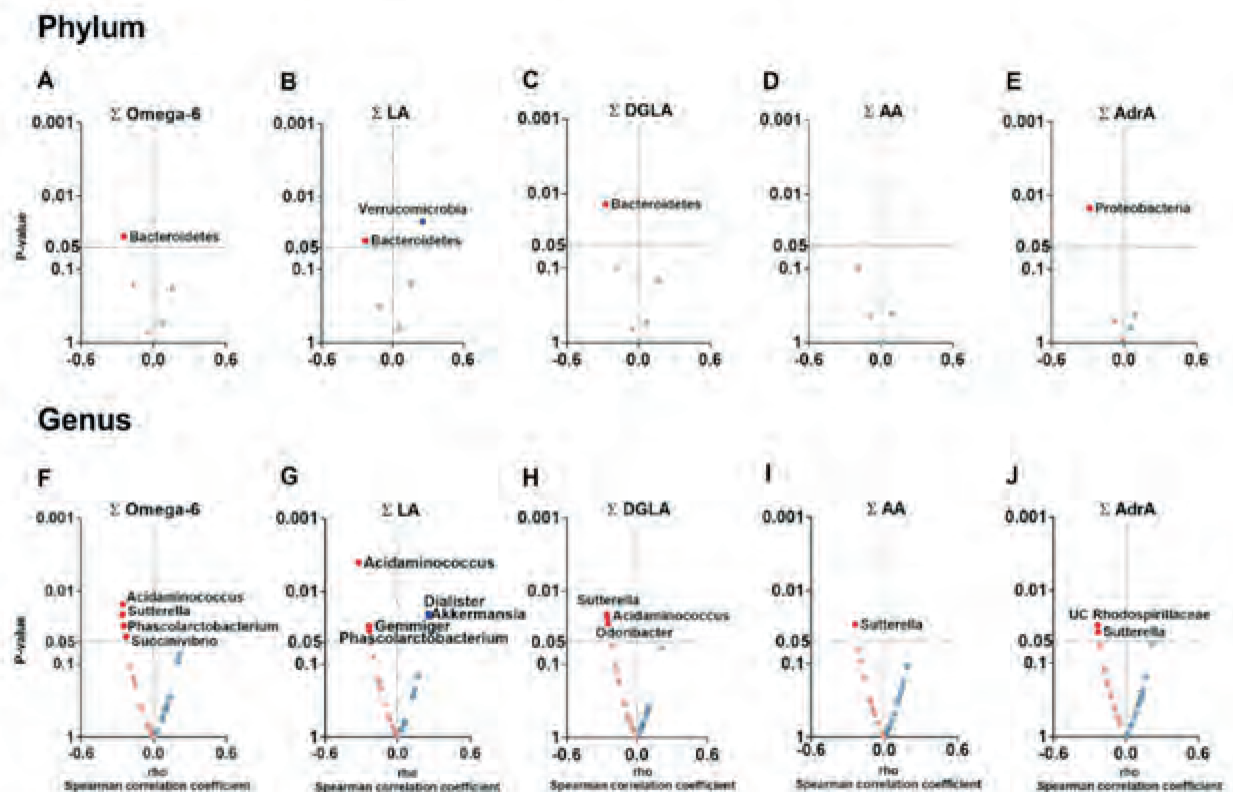

Figure 3. Volcano plots showing the correlations between plasma levels of omega-6 oxylipins and the relative abundance of fecal microbiota composition at phylum and genus levels. The sum (Σ) of omega-6 (A, F), LA (B, G), DGLA (C, H), AA (D, I), and AdrA (D, J) derived oxylipins groups were computed from the individual area peak ratio of each oxylipins group. The X-axis represents Spearman's correlations coefficients, whereas the Y-axis represents the P values of the correlations. Only significant correlations (P<0.05)

Omega-6/omega-3 oxylipins ratio is negatively related to the relative abundance of *Clostridium IV* genus

At phylum level, we found no relationship between the omega-6/omega-3 oxylipins ratio and fecal microbiota composition (all P>0.05; **Fig.4A**). On the other hand, at genus level, the omega-6/omega-3 oxylipins ratio was negatively correlated with the relative abundance of *Clostridium IV* (*Firmicutes* phylum; rho=-0.334, P=0.004; **Fig.4B**) and *Butyricimonas* genera (*Bacteroidetes* phylum; rho=-0.292, P=0.014; **Fig.4B**) independently of BMI, PUFA, and fish intake (**Table 4**).

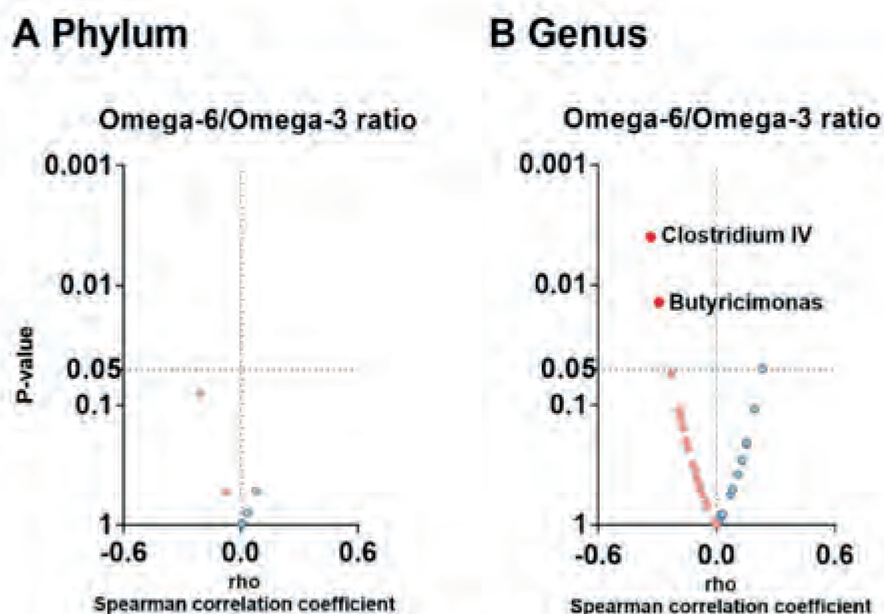

Figure 4. Volcano plots showing the correlations between the omega-6/omega-3 oxylipins ratio and the relative abundance of fecal microbiota composition at phylum and genus levels. The X-axis represents Spearman's correlations coefficients, whereas the Y-axis represents the P values of the correlations. Only significant correlations (P<0.05) were annotated with the name of the phylum or genus. Blue dots represent positive correlations, whereas red dots represent negative correlations.

DISCUSSION

We showed for the first time that plasma levels of omega-3 derived oxylipins are positively correlated with the relative abundance of *Clostridium IV* and negatively correlated with the relative abundance of *Sutterella* genera in young adults. Additionally, plasma levels of omega-6 derived oxylipins are negatively correlated with the relative abundance of *Sutterella* genus, whereas the omega-6/omega-3 oxylipins ratio is negatively correlated with the relative abundance of *Clostridium IV* and *Butirycimonas* genera. These findings suggest that

the plasma levels of omega-3 and omega-6 derived oxylipins might be related to certain gut bacteria genera, and they might be involved in the regulation of the inflammatory status of the gut.

We found that plasma levels of omega-3 derived oxylipins are positively related to relative abundance of *Clostridium IV* genus and the omega-6/omega-3 oxylipins ratio are negatively related to the relative abundance of *Clostridium IV* genus. Similarly, the mouse model capable of transforming omega-6 into omega-3 (i.e., *FAT-1* mice) showed also higher levels of *Clostridium IV* genus compared to wild-type (WT) mice [19]. Bacteria belonging to the *Clostridium IV* genus are well-known bacteria because they exert anti-inflammatory effects and maintain intestinal health via their metabolites, like butyrate [29]. Curiously, both *FAT-1* and WT mice supplemented with omega-3 PUFA (5% corn and 5% fish oils), showed a higher intestinal alkaline phosphatase (IAP) level in the small intestine [19].

Additionally, we identified that plasma levels of both omega-3 and omega-6 oxylipins are negatively correlated with the relative abundance of *Sutterella* genus. In line with these results, female rats which consumed a high-LA diet (omega-6), showed a decrease in the relative abundance of *Sutterella* genus decreased [30], whereas the oral supplementation of omega-3 (i.e., 2g EPA + 2g DHA) for 8 weeks, increased the relative abundance of *Sutterellaceae* family in 22 middle-aged healthy humans [31]. Then, after 8 weeks of a wash-out, the relative abundance returned to baseline levels [31]. These studies clearly show that either supplementation of omega-3 or omega-6 can modulate the relative abundance of this specific bacterial composition. Mechanistically, this relationship might be explained since dietary fatty acids can modify the fatty acid composition of the intestinal brush border membrane increasing the IAP activity through their influence on the membrane fluidity [32] or the local lipid microenvironment [33]. In this sense, it has shown that both omega-3 [19] and omega-6 [34] could increase gut IAP expression, through the modulation of resolvins (last mediators of the anti-inflammatory cascade derived from omega-3 oxylipins) [35] or through the increment in the relative abundance of bacteria producing lipopolysaccharides (LPS), like *Sutterella* [20]. The IAP dephosphorylates LPS [36], prevents the LPS from binding to its receptor (toll-like receptor-4) [36]. Consequently, if the LPS is not bound to its receptor, the inflammatory cascade is not activated [36]. Therefore, we could speculate that the relationship observed between plasma levels of omega-3 and omega-6 and relative abundance of this genus in humans could be partially explained by the modulation of IAP, which in the last instance could protect the host from a pro-inflammatory status.

CONCLUSIONS

Our study reveals that plasma levels of omega-3 oxylipins are positively related to the relative abundance of *Clostridium IV* genus, whereas plasma levels of omega-3 and omega-6 oxylipins are negatively related to the relative abundance of *Sutterella* genus in fecal samples of young adults. These results might suggest that plasma levels of omega-3 and omega-6 oxylipins could modulate human gut microbiota composition modulating the inflammatory status of the gut.

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RESULTS AND DISCUSSION

SECTION I

STUDY II: Relative abundance of *Firmicutes* and *Bacteroidetes* bacteria is related to plasma levels of bile acids in young adults

(Submitted)

ABSTRACT

Background

Gut bacteria play a crucial role in the metabolism of bile acids (BA). However, whether an association exists between the fecal microbiota composition and circulating BA levels in humans is poorly understood. In this study, we aimed to investigate the relationship of fecal microbiota diversity and composition with plasma levels of primary and secondary BA in young adults.

Methods

80 young adults (74% women; 21.9 ± 2.2 years old) were included in this cross-sectional study. Fecal microbiota diversity and composition were analyzed with 16S rRNA sequencing. Plasma levels of BA were measured using liquid chromatography-tandem mass spectrometry.

Results

Fecal microbiota beta (pseudo-F=1.845, P=0.025) and alpha (rho≥0.237, P≤0.033) diversities were positively related to the plasma levels of the secondary BA glycolithocholic acid (GLCA), but were not related to the plasma levels of primary BA (P≥0.338). Moreover, the relative abundance of species *Clostridium bolteae*, *Blautia wexlerae*, and *Clostridium leptum* (*Firmicutes* phylum) was negatively correlated with the plasma levels of primary BA including cholic acid, chenodeoxycholic acid, glycocholic acid, and glycochenodeoxycholic acid (all rho≤-0.234, P≤0.039). The relative abundance of species *Bacteroides ovatus*, *Parabacteroides merdae* and *Bacteroides dorei* (*Bacteroidetes* phylum) were negatively correlated with the plasma levels of secondary BA including deoxycholic acid, glycodeoxycholic acid and GLCA (all rho≤-0.248, P≤0.043).

Conclusions

These results suggest that the relative abundance of specific fecal bacteria species might modulate the plasma levels of BA in young adults.

BACKGROUND

The gut microbiota is composed of a complex set of microorganisms that colonize the gastrointestinal tract, where bacteria are the most abundant [1]. The most abundant bacteria present in the human gut microbiota belong to the *Firmicutes* and *Bacteroidetes* phyla [2], representing more than 70% of the total bacteria [3]. Gut microbiota composition is modulated by extrinsic factors, such as diet [4], exercise [5], and medication [6,7], as well as by intrinsic factors, such as age [8] and host genetics [9]. Recent advances have shown that the metabolites of these bacteria influence several biological processes, such as digestion and absorption of nutrients, as well as the homeostatic maintenance of host immunity and gut barrier permeability [10,11]. For this reason, the impact of gut microbiota on human metabolism depends not only on their abundance, but also on the metabolites that they produce [12].

The primary bile acids (BA) cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesized from cholesterol in hepatocytes, where they are typically conjugated with glycine to produce glycocholic acid (GCA) and glycochenodeoxycholic acid (GCDCA), respectively, as well as taurine to produce the corresponding tauro-conjugates [13]; glycine-conjugated BA represent ~75% of the total pool of conjugated BA [14]. Primary BA are then stored in the gallbladder and released into the duodenum to facilitate absorption of dietary lipids and liposoluble vitamins, to protect against bacterial overgrowth [15], and to eliminate excess cholesterol [14,16]. Approximately 95% of the BA are reabsorbed within the distal ileum and returned to the liver through the enterohepatic circulation [17]. The remaining 5% of primary BA enter the colon, where they are metabolized into the secondary BA deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) upon the action of specific bacterial enzymes [18].

Bile salt hydrolases (BSHs) and 7-α-dehydroxylases are the major bacterial enzymes involved in the metabolism of BA [19]. BSHs participate in the deconjugation of primary and secondary BA by catalyzing the removal of glycine and taurine [19]; these enzymes are present in certain Gram-positive bacteria of the phylum *Firmicutes* and Gram-negative bacteria of the phylum *Bacteroidetes* [20-22]. The 7-α-dehydroxylases enzymes convert primary BA to secondary BA and are expressed by certain anaerobic bacteria, such as Gram-positive bacteria species belonging to the *Clostridium* genus [20,22]. These secondary BA can either be reabsorbed by colonocytes [23], reach the systemic circulation inducing signaling functions [24,25] and reach the liver via the enterohepatic circulation where they can again be conjugated with glycine or taurine [26], or be secreted into the feces [15]. However, it is unknown whether a relationship exists between bacterial species expressing BSHs and 7-α-dehydroxylases and circulating levels of primary and secondary BA in humans.

The aim of the present study was to investigate the relationship of fecal microbiota diversity and composition with plasma levels of primary and secondary BA in a cohort of young adults.

MATERIAL & METHODS

Participants

This study was conducted using baseline data from the ACTIBATE (Activating brown adipose tissue through exercise in young adults) study [27], a randomized controlled trial designed to evaluate the effect of exercise training on brown adipose tissue (BAT) activity (Clinical trials identifier: NCT02365129). The University of Granada recruited participants via advertisements in electronic media and leaflets. The inclusion criteria were: being sedentary, i.e., less than 20 min of moderate/vigorous physical activity on <3 days/week and having a stable body weight over the last 3 months. The exclusion criteria were: being pregnant, smoking, being frequently exposed to cold temperatures, presenting any acute or chronic disease (e.g., hypertension or diabetes) that can interfere with or be aggravated by exercise; taking medication that potentially affects the cardiovascular system and/or its function, and antibiotics. The study protocol and the written informed consent were performed in accordance with the last revised Declaration of Helsinki, and were approved by the Ethics Committee on Human Research of the University of Granada (n°.924), and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada).

We selected participants from the ACTIBATE study with available data for fecal microbiota and plasma samples, which resulted in 80 young adults (59 women, 21 men, age 18–25 years) included in the present study.

Fecal microbiota analysis

A fecal sample (50-60 g) was obtained from each volunteer and introduced in a 60 mL plastic sterile container. The fecal samples were transported in a portable cooler with an ice plate to the research center and stored at -80°C until DNA extraction. Fecal samples were homogenized in a Stomacher® 400 blender (A. J. Seward and Co. Ltd., London, UK). DNA extraction and purification steps were performed with a QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain) according to the manufacturer's instructions. DNA concentration and purity were determined with a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, DE, USA).

Sequencing

Extracted DNA was amplified by PCR targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene using the following primer pairs: 16S Amplicon PCR Forward Primer: 5´CCTACGGGNGGCWGCAG; and 16S Amplicon PCR Reverse Primer: 5′GACTACHVGGGTATCTAATCC. The PCR assays were carried out in a 25 μL final reaction volume, including 12.5 μL of the 2X KAPA HiFi Hotstart prepared mixture (KAPA Biosystems, Woburn, MA, USA), 5 μL of each forward and reverse primer (1 μM), and 2.5 μL of extracted DNA (10 ng). The following PCR program was used: (i) denaturation at 95°C for 3 min, (ii) 8 denaturation cycles at 95°C for 30 s, (iii) annealing at 55°C for 30 s, (iv) elongation at 72°C for 30 s, (v) final extension at 72°C for 5 min. Next, AMPure XP microspheres (Beckman Coulter, Indianapolis, IN, USA) were used to purify the 16S V3 and V4 amplicon away from free primers and primer-dimer species. For the PCR index step, we used the Nextera XT index kit (Illumina, San Diego, CA, USA) to tag DNA with the sequencing adapters. The pooled PCR products were purified using AMPure XP balls (Beckman Coulter, Indianapolis, IN, USA) before quantification. The amplicons were sequenced in a MiSeq (Illumina, San Diego, CA, USA), using the Illumina MiSeq paired-end sequencing system (2x300nt) (Illumina, San Diego, CA, USA).

Bioinformatics analysis

The "*Dada2*" [28] package version 1.10.1 in *R* software [29] was used for analyzing the raw sequences (FastQ files). All samples that were above the 10,000 reads cut-off threshold were considered valid for subsequent analyses. Samples were standardized to an equal sequencing depth of 30,982 reads using the "*Phyloseq*" [30] package in *R* software, leading to a total of 11,158 different phylotypes.

Phylotypes were assigned to their specific taxonomic affiliation (from phylum to genus) based on the naive Bayesian classification with a pseudo-bootstrap threshold of 80% [30] using the "*Classifier*" function in Ribosomal Data Project (RDP) [31]. We obtained a total of 209 genera that belong to 16 different phyla. To determine the species taxonomies, we used the "*Seqmatch*" function in RDP. For the main analysis, we used relative abundances as the read's percentage of each phylotype relative to the total number of reads. We performed the analyses when the average of the relative sequence abundance was higher than 1%. Only those species with ≥ 97% coincidence with the respective representative sequence read and found in at least 50% of the participants were annotated, identifying a total of 50 different species for the analyses.

Beta diversity indicates the number of species shared among the microbial community from the individuals, and was assessed by pseudo-F statistics. Pseudo-F shows the ratio between cluster variance and within-cluster variance [(between-cluster-sum-of-squares/(c-1))/(within-cluster-sum-of-squares/(n-c))], where c is the number of

clusters and n is the number of variables [32]. A pseudo-F value of 1 indicates that the variance between and within-group is similar; if pseudo-F ≥ 1, the between-group variance is higher than the within-group variance. Alpha diversity reflects the number of different phylotypes and the relative abundance of these phylotypes within the same participant. A total of 4 different alpha diversity indexes were calculated: i) *species richness* (number of different phylotypes in the community) [33]; ii) *evenness index* (equitability of the phylotypes frequencies in the community) [34]; iii) *Shannon index* (number and equitability of the phylotypes in the community) [33]; iv) *inverse Simpson index* (derived from the classical Simpson index; richness in a community with uniform evenness) [35].

Determination of plasma levels of bile acids

Blood samples were collected between 8:00-9:00 AM after 10-h overnight fasting. Blood samples were collected in Vacutainer Tubes® and immediately centrifuged. Serum (obtained with Vacutainer® SST™ II Advance tubes) and plasma (obtained with Vacutainer® Hemogard™ tubes) aliquots were stored at -80°C until analyses. Primary (i.e., CA, CDCA, GCA, and GCDCA,) and secondary BA (i.e., DCA, glycodeoxycholic [GDCA], glycolithocholic [GLCA], glycoursodeoxycholic [GUDCA]) were measured in plasma samples, using liquid chromatography-tandem mass spectrometry (LC-MS/MS), using a method validated according to the FDA bioanalytical method validation guidelines[36].

Sample preparation

The sample preparation was performed on ice, except for the evaporation step. BA were extracted using liquid-liquid extraction (34). Before the extraction, 150 µL of plasma sample was transferred into a 1.5 mL Eppendorf tube and mixed with 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-labeled analogs (**Table 1**)[37]. Then, 150 µL of buffer solution (0.2 M citric acid and 0.1 M disodium hydrogen phosphate at pH 4.5) were added, followed by the addition of 1 mL extraction solvent composed of methyl-tertbutylether and butanol (50:50, *v/v*). Samples were then mixed for 5 min using a bullet blender (Next Advance, Averill Park, NY) and centrifugated (16,000 g, 10 min, 4 °C). Next, 900 µL of supernatant were transferred to a new 1.5 mL Eppendorf tube and evaporated to dryness using a SpeedVac system at room temperature. The dry residue was reconstituted in 50 µL of methanol:acetonitrile (70:30, *v/v*), and centrifuged (16,000 g, 10 min, 4°C). Finally, 40 µL of the supernatant was transferred into an autosampler vial and 10 µL was injected into the LC-MS/MS system.

Table 1. List of internal standards.

Abbreviation	Name (International Union of Pure and Applied Chemistry, IUPAC)
d4-CA-ISTD	3α,7α,12α-trihydroxy-5β-cholan-24-oic acid-d4
d4-GCA-ISTD	N-(3α,7α,12α-trihydroxy-5β-cholan-24-oyl)-glycine-d4
d4-DCA-ISTD	3α,12α-dihydroxy-5β-cholan-24-oic acid-d4
d4-GDCA-ISTD	N-(3α,12α-dihydroxy-5β-cholan-24-oyl) glycine-d4
d5-GUDCA-ISTD	N-(3α,7β-dihydroxy-5β-cholan-24-oyl)-glycine-d5

CA: cholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GDCA: glycodeoxycholic acid; GUDCA: glycoursodeoxycholic acid.

Liquid chromatography-tandem mass spectrometry

LC-MS/MS analysis was performed as previously described [38]. Briefly, the extracted samples were analyzed using a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan), coupled to a SCIEX QTRAP 6500+ mass spectrometer (SCIEX, Framingham, MA). The separation was carried out using a BEH C18 column (50 mm × 2.1 mm, 1.7 μm) from Waters Technologies (Milford, MA) kept at 40°C. The mobile phase consisted of 0.1% acetic acid in water (A), 0.1% acetic acid in acetonitrile/methanol (90:10, *v/v*, B) and 0.1% acetic acid in isopropanol (C). The data acquisition was performed using electrospray ionization in negative mode. MS/MS acquisition was carried out using Selected Reaction Mode (SRM). SRM transitions were individually optimized for targeted analytes and their respective internal standards using standard solutions.

Data pre-processing

For each target compound, the ratio between its peak area and the peak area of its respective internal standard was calculated using SCIEX OS-MQ Software and was used for further data analysis. The data quality was monitored using regular injection of quality control (QC) samples, prepared from of blank plasma samples. QC samples were used to correct for between batch variations, using the in-house developed mzQuality workflow (available at http://www.mzQuality.nl) [38]. Relative standard deviations (RSDs) were calculated for each analyte present in the QC samples (**Table 2**)[37]. All analytes showed RSD values in QC samples below 9%, ensuring high data quality.

Anthropometry and body composition

A SECA model 799 electronic column scale and stadiometer (SECA, Hamburg, Germany) were used to measure participants´ height and weight, without shoes and wearing standard clothes. A dual-energy X-ray absorptiometry scan (Hologic Discovery Wi Marlborough, MA) was used to measure body composition (fat mass and lean mass). The body

mass index (BMI) was calculated as weight/height^2 (kg/m^2).

Table 2. Bile acids abbreviations and observed variability in peak area ratios in quality control samples.

Abbreviation	Name (International Union of Pure and Applied Chemistry, IUPAC)	ChEBI ID	RSD of QCs
Primary bile acids			
CA	3α,7α,12α-trihydroxy-5β-cholan-24-oic acid	16359	7.1%
CDCA	3α,7α-dihydroxy-5β-cholan-24-oic acid	16755	7.0%
GCA	N-(3α,7α,12α-trihydroxy-5β-cholan-24-oyl)-glycine	17687	6.6%
GCDCA	N-(3α,7α-dihydroxy-5β-cholan-24-oyl)-glycine	3593	5.8%
Secondary bile acids			
DCA	3α,12α-dihydroxy-5β-cholan-24-oic acid	28834	8.3%
GDCA	N-(3α,12α-dihydroxy-5β-cholan-24-oyl) glycine	27471	6.0%
GLCA	N-[(3α,5β)-3-hydroxy-24-oxocholan-24-yl]-glycine	37998	7.4%
GUDCA	N-(3α,7β-dihydroxy-5β-cholan-24-oyl)-glycine	89929	7.0%

Observed variability is expressed as relative standard deviation of the peak area ratio in the quality control samples. CA: cholic acid; CDCA: chenodeoxycholic acid; ChEBI: Chemical Entities of Biological Interest; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; QC: quality control; RSD: relative standard deviation.

Cardiometabolic parameters profile

Fasting serum glucose, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and triacylglycerols concentrations were measured following standard methods using an AU5832 biochemical analyzer (Beckman Coulter Inc., Brea, CA, USA) with Beckman Coulter reagents OSR6521, OSR6116, OSR60118 and OSR6187, respectively. Low-density lipoprotein cholesterol (LDL-C) was estimated with the Friedewald formula: [TC – HDL-C – (TG/5)], in mg/dL [40]. Insulin was measured using the Access Ultrasensitive Insulin Chemiluminescent Immunoassay Kit (Beckman Coulter Inc., Brea CA, USA). The homeostatic model assessment (HOMA) index was calculated as [insulin (μU/mL) x glucose (mmol/L)/22.5] [41].

Statistical analysis

The descriptive parameters are reported as mean and standard deviation. Since we did not observe sex interaction in our cohort (all P≥0.05), men and women were analyzed together. D'Agostino & Pearson tests revealed that the relative abundance of the different bacterial species within the feces, plasma levels of BA, and serum cardiometabolic profile parameters followed a non-normal distribution. Therefore, all the analyses were conducted using non-parametric tests.

In order to investigate the association between fecal microbiota beta diversity and plasma levels of primary and secondary

BA, we divided into tertiles of the plasma levels of BA (i.e., low, intermediate, and high concentration), and compared across tertiles using a PERMANOVA analysis with 9999 random permutations, based on Bray-Curtis dissimilarity. This analysis was performed with the Paleontological Statistics Software Package 3.0 (Past3) [42]. Four different alpha diversity indexes were calculated using the "*Vegan*" package in *R* software. To investigate the association between alpha diversity indexes and plasma levels of primary and secondary BA, we employed Spearman correlation analyses using "*Psych*" and "*Corrplot*" packages in *R* software. Then, to investigate the association between fecal microbiota composition and plasma levels of primary and secondary BA, we used Spearman correlation and partial Spearman correlations analysis using "*Psych*" and "*Corrplot*" packages in *R* software. The correlations between alpha diversities indexes and plasma levels of primary and secondary BA were represented as heatmap plots using "*Gplot*" package in *R* software. Volcanos plots were used to depict the correlations between fecal microbiota composition (at genus and species taxonomy levels) and plasma levels of primary and secondary BA by using GraphPad Prism software (GraphPad Software, San Diego, California, USA, version 8.0.0). The level of significance was set at $P<0.05$.

RESULTS

Characteristics of participants

Table 3 shows the descriptive characteristics of the participants (74% women; 21.9 ± 2.2 years-old; BMI: 24.7 ± 4.7 kg/m^2).

Fecal microbiota diversity positively correlates with plasma levels of glycolithocholic acid

We found differences in fecal microbiota beta diversity at genus taxonomy levels only among tertiles (low *vs* high) of plasma levels of the secondary BA GLCA (pseudo-F=1.845, P=0.025; **Table 4**). Additionally, we observed positive correlations of *evenness* (rho=0.237, P=0.033), *Shannon* (rho=0.313, P=0.004), and *inverse Simpson* alpha diversity indexes (rho=0.283, P=0.010) with plasma levels of GLCA (**Fig. 1**). No relationships were observed between fecal microbiota beta and alpha diversities and plasma levels of other BA (all P>0.05).

Table 3. Descriptive characteristics of study participants.

		N	Mean ± SD		
Age (years-old)		80	21.9	±	2.2
Sex (women, %)		80	73.7% (n=59)		
Body composition parameters					
Fat mass (kg)		80	25.2	±	9.1
Lean mass (kg)		80	41.0	±	8.9
Body mass index (kg/m2)		80	24.7	±	4.7
Cardiometabolic profile parameters					
Glucose (mg/dL)		79	87.6	±	6.1
Insulin (µUI/mL)		79	8.1	±	4.8
HOMA index		79	1.8	±	1.2
Total cholesterol (mg/dL)		79	167.8	±	36.0
Total triglycerides (mg/dL)		79	85.1	±	50.5
HDL-C (mg/dL)		79	53.5	±	11.7
LDL-C (mg/dL)		79	97.0	±	27.0
Plasma levels of bile acids (expressed as peak area ratio)					
Primary bile acids	CA	80	28.6	±	48.5
	CDCA	80	0.5	±	0.7
	GCA	80	1.9	±	1.7
	GCDCA	78	5.4	±	3.6
Secondary bile acids	DCA	80	17.3	±	13.8
	GDCA	79	1.9	±	1.5
	GLCA	80	4.5	±	4.1
	GUDCA	80	16.9	±	18.2
Fecal microbiota parameters					
Diversity indexes					
Species Richness		80	380.0	±	109.6
Evenness Index		80	0.7	±	0.0
Shannon Index		80	4.2	±	0.4
Inverse Simpson Index		80	35.6	±	14.5
Composition (phylum)					
Actinobacteria (%)		80	1.6	±	1.6
Bacteroidetes (%)		80	39.6	±	9.0
Firmicutes (%)		80	48.8	±	9.7
Proteobacteria (%)		80	6.5	±	4.8
Verrucomicrobia (%)		80	2.3	±	4.3

Data are presented as mean and standard deviation (SD), except for sex. CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

Table 4. Differences in beta diversity calculated by statistic permutational multivariate analysis of variance (PERMANOVA) among tertiles of plasma levels of bile acids (peak area ratio) at phylum and genus taxonomic levels.

	Phylum		Genus	
	Pseudo-F	P-value	Pseudo-F	P-value
Primary bile acids				
CA	0.411	0.838	1.541	0.072
CDCA	0.510	0.767	1.119	0.305
GCA	1.010	0.401	0.800	0.690
GCDCA	0.541	0.762	0.736	0.820
Secondary bile acids				
DCA	1.892	0.102	1.190	0.250
GDCA	0.676	0.618	1.037	0.381
GLCA	0.237	0.937	1.845	**0.025**
GUDCA	0.607	0.685	0.714	0.806

PERMANOVA using 9999 permutations for significance testing (P<0.05). CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid.

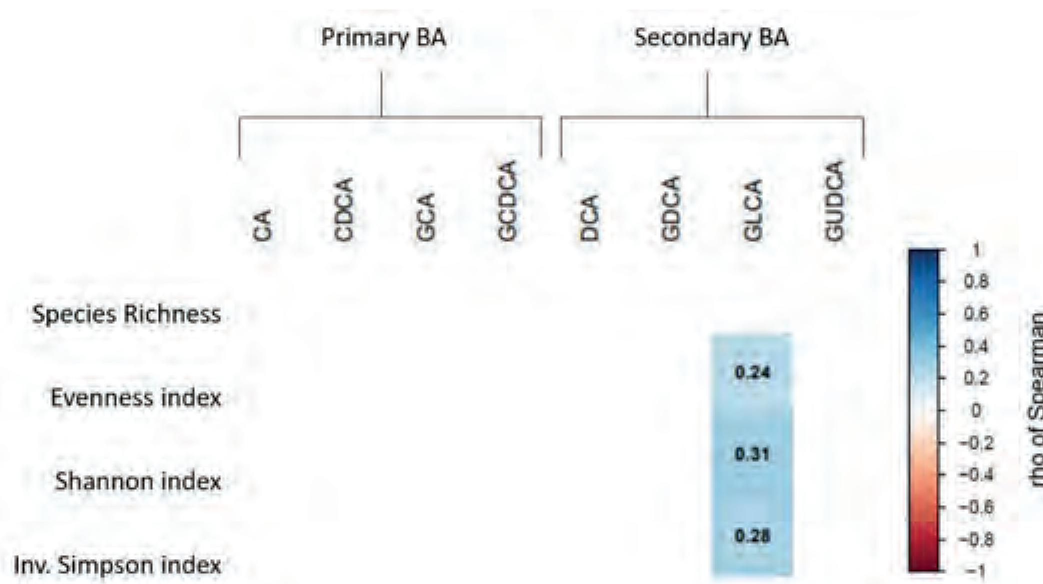

Figure 1: **Spearman correlations between fecal microbiota alpha diversity indexes and plasma level of bile acids.** Blue boxes represent positive and significant correlations (P<0.05), while the value within the boxes shows the Spearman's correlations coefficients. BA: Bile acids; CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; Inv: Inverse.

Relative abundance of genera belonging to the *Firmicutes* and *Bacteroidetes* phyla is positively correlated with plasma levels of glycolithocholic acid

Overall, we found that the relative abundance of the genera belonging to the different phyla found in the feces was not related to the plasma levels of the primary BA CA (**Fig. 2A**), GCA (**Fig. 2C**), and GCDCA (**Fig. 2D**). Nevertheless, we observed a positive correlation between the relative abundance of the *Roseburia* genus (*Firmicutes* phylum) and the plasma levels of CDCA (rho=0.231, P=0.038; **Fig. 2B**). Furthermore, a positive correlation between the relative abundance of *Oscillibacter* genus (*Firmicutes* phylum) and the plasma levels of the secondary BA DCA and GLCA (both rho≥0.221, P≤0.049; **Fig. 2E** and **G**) was found the relative abundance of the *Unclassified Rhodospirillaceae* genus (*Proteobacteria* phylum) was positively correlated with the plasma levels of DCA, GDCA, and GLCA (all rho≥0.236, P≤0.035; **Fig. 2E**, **F** and **G**, respectively). We also found that the relative abundance of *Unclassified Clostridiales*, *Unclassified Firmicutes*, *Ruminococcus*, and *Clostridium IV* genera (*Firmicutes* phylum) were positively correlated with plasma levels of GLCA (all rho≥0.263, P≤0.018; **Fig. 2G**). Additionally, the relative abundance of *Barnesiella* and *Butyricimonas* genera (*Bacteroidetes* phylum) were positively correlated with plasma levels of GLCA (all rho≥0.257, P≤0.023; **Fig. 2G**). The relative abundance of the *Bifidobacterium* genus (*Actinobacteria* phylum) was negatively correlated with plasma levels of DCA (rho=-0.047, P=0.048; **Fig.2E**). Finally, the relative abundance of *Paraprevotella* and *Prevotella* genera (*Bacteroidetes* phylum) was negatively correlated with plasma levels of GDCA and GLCA (all rho≥-0.270, P≤0.045; **Fig. 2F** and **G**). These analyses were repeated after adjusting for BMI, glucose, insulin, HOMA index, and TC, TG, HLD-C and LDL-C serum levels, and the results remained significant (data not shown).

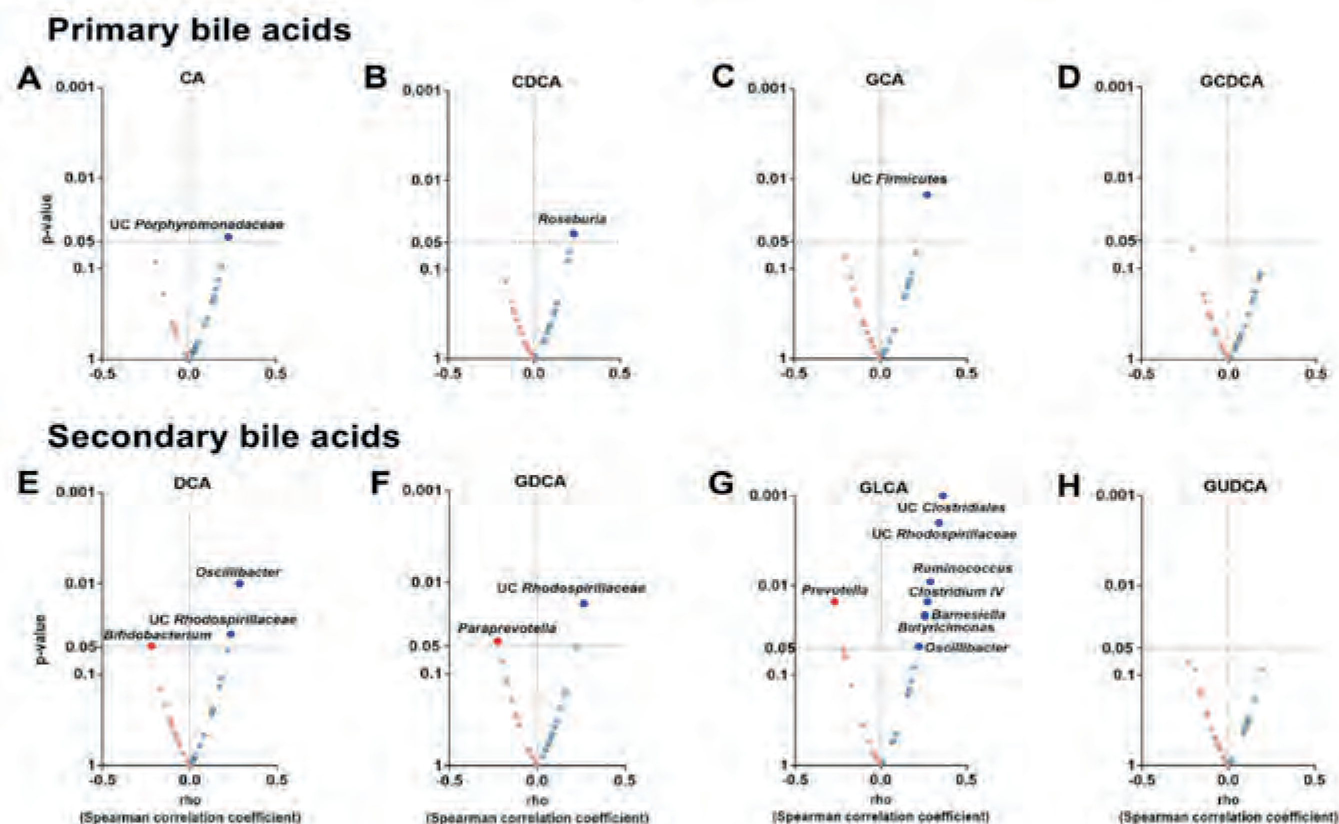

Figura 2: Volcano plots showing Spearman correlations between fecal microbiota composition at genus taxonomic level and plasma level of bile acids. X-axis represents the Spearman's correlations coefficients, whereas y-axis represent the P-value of the correlations. Only those correlations that achieved the significant threshold (P<0.05) were annotated with the name of the genera CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; UC: unclassified.

Relative abundance of species from *Firmicutes* and *Bacteroidetes* phyla is negatively correlated with plasma levels of primary and secondary bile acids

We further investigated whether the relative abundance of specific bacterial species could be driving the significant correlations found in the previous analyses (**Fig. 2**). The relative abundance of C*lostridium bolteae, Clostridium leptum,* and *Blautia wexlerae* species (*Firmicutes* phylum) was negatively correlated with plasma levels of various primary BA (CA, CDCA, GCA and/or GCDA; all rho≤-0.234, P≤0.039; **Fig. 3A**, **B**, **C**, and **D**). Similarly, the relative abundance of *Bacteroides ovatus, Parabacteroides merdae,* and *Bacteroides dorei* species (*Bacteroidetes* phylum) was negatively correlated with the plasma levels of various secondary BA (DCA, GDCA, and GLCA; all rho≤-0.248, P≤0.043; **Fig. 3E**, **F** and **G**). The relative abundance of *Clostridium leptum* genus was negatively correlated with the plasma levels of all

primary BA CA, CDCA, GCA, and GCDCA (all rho≤-0.234, P≤0.038; **Fig. 3A, B, C, and D**), as well as the secondary BA GDCA (Rho≤-0.257, P≤0.022; **Fig. 3F**). Moreover, the relative abundance of *Bacteroides dorei* genus was negatively correlated with the plasma levels of the primary BA GCDCA (Rho=-0.22, P=0.043; **Fig. 3D**), as well as the secondary BA DCA, GDCA, and GLCA (All rho≤-0.22, P≤0.045; **Fig. 3E, F and G**). In contrast, the relative abundance of *Bacteroides vulgatus, Alistipes onderdonkii,* and *Bacteroides xylanisolvens* species (*Bacteroidetes* phylum) was positively correlated with the plasma levels of GLCA (all rho≥0.235, P≤0.036; **Fig. 3G**). These analyses were repeated after adjusting for BMI, glucose, insulin, HOMA index, and TC, TG, HLD-C and LDL-C serum levels, and the results remained significant (data not shown).

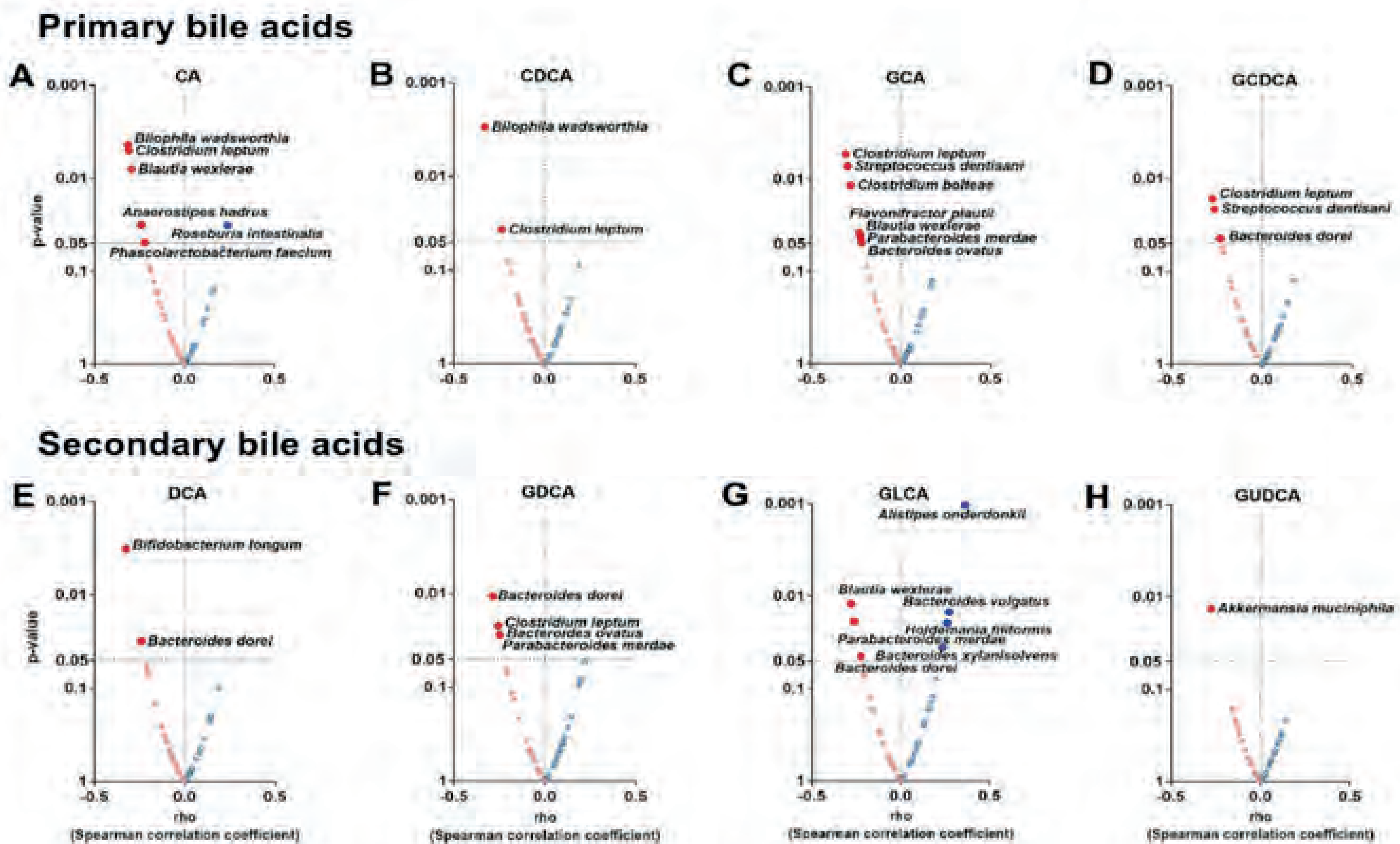

Figure 3: Volcano plots showing Spearman correlation between fecal microbiota composition at species taxonomic level and plasma levels of bile acids. X-axis represents the Spearman's correlations coefficients, whereas y-axis represent the P-value of the correlations. Only those correlations that achieved the significant threshold (P<0.05) were annotated with the name of the species. CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid.

DISCUSSION

In the present study, we show that the fecal microbiota diversity and composition are positively correlated with the plasma levels of the secondary BA GLCA in young adults. In addition, our study reveals that the relative abundance of bacterial species belonging to the *Firmicutes* and *Bacteroidetes* phyla is negatively correlated with the plasma levels of primary BA (i.e., CA, CDCA, GCA, and GCDCA) and secondary BA (i.e., DCA, GDCA, and GLCA) (**Fig. 4**). These findings suggest that the relative abundance of specific gut bacterial species might modulate the plasma levels of BA in young adults.

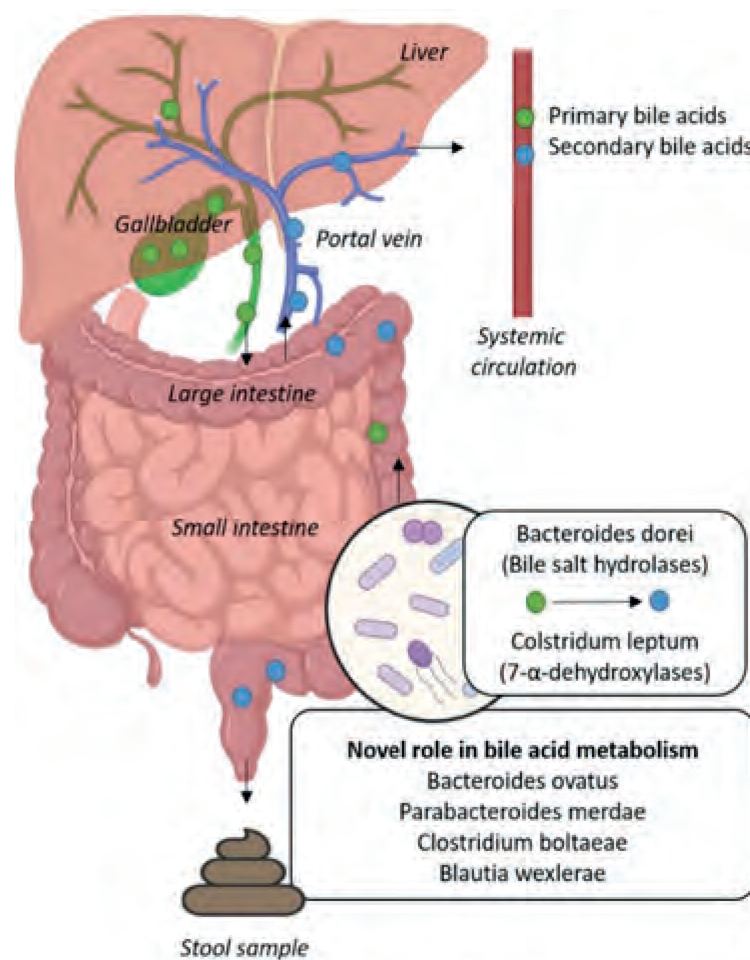

Figure 4: **Overview of the role of gut bacteria species in bile acids metabolism.** Primary BA are synthesized from cholesterol in the liver, where they are conjugated with taurine and glycine. Then, they are stored in the gallbladder and released into the small intestine. In the colon can be metabolized into secondary BA by the action of specific bacterial BSHs and 7-α-dehydroxylases that are released to the systemic circulation. BA: bile acids; BSHs: Bile salt hydrolases. Created with BioRender.com.

Overall, we found no relationship between fecal microbiota diversity and plasma levels of primary and secondary BA, which concurs with the homogeneity of the phenotypic characteristics of our participants. However, we found significant differences in beta diversity between participants with low and high plasma levels of GLCA. Besides, the alpha diversity indexes were positively associated with the plasma levels of GLCA, which suggests that the relative abundance of specific genera and species might be explaining the observed differences whitin the high GLCA concentration tertile.

In vitro experiments have shown that bacterial species belonging to the *Firmicutes* phylum (i.e., *Clostridium leptum*) and *Bacteroidetes* phylum (i.e., *Bacteroides dorei*) express either BSHs or 7-α-dehydroxylases enzymes [43,44]. We observed that the relative abundance of *Clostridium leptum* (*Firmicutes* phylum) was negatively correlated with plasma levels of the primary BA CA, CDCA, GCA, and GCDCA. In agreement with our findings, it has been shown that the relative abundance of *Clostridium leptum* is negatively associated with the content of primary BA CA and CDCA in feces of non-alcoholic steatohepatitis patients [45]. These findings suggest that *Clostridium leptum*, via 7-α-dehydroxylase activity, could be involved in the production of secondary BA.

Additionally, we observed that the relative abundance of *Bacteroides dorei* (*Bacteroidetes* phylum) was negatively correlated with the plasma levels of the primary BA GCDCA, as well as with the plasma levels of the secondary BA DCA, GDCA, and GLCA. In line with these results, it has been shown in middle-aged patients with type 2 diabetes that the relative abundance of *Bacteroides dorei* is positively associated with the plasma levels of the unconjugated BA CA and CDCA [44]. This suggests that *Bacteroides dorei,* via BSH activity, could reduce the plasma levels of the conjugated BA GDCA, GCDCA, and GLCA. From a clinical point of view, the *Bacteroides* genus is known to have an important role in maintaining an eubiosis status [46]. Preclinical studies in diet-induced obese mice showed that the administration of *Bacteroides dorei* increases the expression of the ileal BA transporter in enterocytes [47], which is associated with a significant decrease in body weight and an improved lipid and glucose profile via farnesoid X receptor activation [47,48].

Our study shows that the relative abundance of *Clostridium leptum* (*Firmicutes* phylum) and *Bacteroides dorei* (*Bacteroidetes* phylum) species is related to the plasma levels of primary and secondary BA. However, whether other bacteria species belonging to those same phylum could be involved in the metabolism of BA deserves futher investigation.

CONCLUSION

Our findings support the hypothesis that specific bacterial species, specially those related to the *Firmicutes* and *Bacteroides* phylum, could be modulating the plasma levels of BA in humans. Nonentheless, further research is needed to better understand whether a causal relationship exists between the bacterial species expressing enzymes involved in BA metabolism with the circulating levels of BA in humans.

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RESULTS AND DISCUSSION

SECTION II
STUDY III: Influence of exercise on the human gut microbiota of healthy adults: a systematic review

(Clin Transl Gastroenterol.)

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ABSTRACT

Objectives

To summarize the literature on the influence of exercise on the gut microbiota of healthy adults.

Methods

A systematic and comprehensive search in electronic database, including SciELO, Scopus, PubMed, and Web of Science up to July 5, 2019. Eligibility criterion was original studies conducted on healthy humans including exercise interventions or interventions involving any type of physical activity.

Results

The initial search retrieved 619 articles of which 18 met the inclusion criteria, 9 were observational, 4 reported very short-term exercise interventions, and 5 reported medium/long-term exercise interventions. Higher levels of physical activity or cardiorespiratory fitness were positively associated with fecal bacterial alpha diversity. Contrasting associations were detected between both the level of physical activity and cardiorespiratory fitness and fecal counts for the phyla *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. Higher levels of physical activity and cardiorespiratory fitness were positively associated with the fecal concentration of short-chain fatty acids. Reports on the effects of very short-term and medium/long-term exercise interventions on the composition of the gut microbiota were inconsistent.

Discussion

Higher levels of physical activity and cardiorespiratory fitness are associated with higher fecal bacterial alpha diversity and with the increased representation of some phyla and certain short-chain fatty acids in the feces of healthy adults. Very short-term and medium/long-term exercise interventions seem to influence the fecal counts of some phyla. However, the heterogeneity between studies hampers any strong conclusions from being drawn. Better-designed studies are needed to unravel the possible mechanisms through which exercise might influence the composition and activity of the human gut microbiota.

BACKGROUND

Humans live in symbiosis with different microorganisms present on the skin, in the oral cavity, vagina and gut.[1] These microorganisms affect host nutrition, metabolic function, gut development, and the maturation of the immune system and epithelial cells.[2] The gut microbiota refers to the microorganisms (approximately 100 trillion of them)[3] that colonize the gastrointestinal tract.[4] Five phyla representing ~160 species can be detected in the large intestine alone.[5] The most representative phyla are *Firmicutes* (60%-65%), *Bacteroidetes* (20%-25%) and *Proteobacteria* (5%-10%), although this may vary widely between one person and another.[6]

Eubiosis, which is associated with good health status,[7] requires the intestinal ecosystem be in good microbial equilibrium; dysbiosis is any change in this equilibrium.[7] Dysbiosis has been strongly linked to obesity, type 2 diabetes,[8] inflammatory bowel disease,[9] colon cancer, and autism.[10] Some studies have shown that restoring eubiosis in the gut of obese mice improves their metabolic profile[11-13] and reduces insulin resistance.

Physical activity is characterized by any movement of the skeletal muscles that demands energy expenditure, whereas exercise is a structured, planned and repetitive physical activity, the purpose of which is to improve or maintain physical fitness.[14] Several studies report that increasing the amount of physical activity undertaken improves the physical and mental health of persons of any age.[15] Exercise can be included in the treatment of many chronic diseases.[15,16] In animal models exercise seems to restore eubiosis in the gut,[17-19] although the mechanisms involved remain unknown.[20,21]

The influence of exercise on the gut microbiota of healthy humans is poorly understood.[1,22,23] Three systematic reviews on the subject are available, but they suffer from two major limitations[24-26]: (i) they omit information on several key studies,[27-40] and (ii) they focus on both healthy and unhealthy human subjects alike (since the effect of exercise on the gut microbiota in healthy humans is unclear, studying the effect of exercise on the gut microbiota in unhealthy humans hampers the interpretation of the results; it is impossible to know whether any changes are caused by exercise or the disease itself). The present work focuses on the influence of exercise on the gut microbiota of healthy adults.

MATERIAL AND METHODS

This systematic review was conducted adhering to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement,[41] and was registered with the International Prospective Register of Systematic Reviews (PROSPERO registration number: CRD42018114664).

Search strategy

A literature search was conducted across the Scielo, Scopus, PubMed and the Web of Science (WOS) databases, taking into account reports published up until July 5th 2019. The following search strategies were followed: for Scielo, (gut) AND (microbiota) (see **Table 1**); for Scopus, (gut) AND (microbiota) AND (exercise) AND (human) AND (humans) (see **Table 2**), and for PubMed (((((((((((((((((“Gastrointestinal Microbiome") OR ((("Fecal Microbiota") OR "Cecal Microbiota") OR "Fecal Microbiota")))))) AND (((Exercises) OR Training))))) AND Human) NOT (((((((((((((((((("Mice"[MeSH]) OR "Rats"[MeSH]) OR "Animal Experimentation"[MeSH]) OR "Models, Animal"[MeSH])) OR ("rats" OR "mouse"))) OR "mice")) OR "rat")))))))))))))))) NOT Review. When exploring PubMed, Medical Subject Heading (MeSH) terms were included to increase the power of the search (see **Table 3**). A slightly different search strategy was used for the WOS database since it does not include MeSH terms: ((((((((((((((((((Gut) OR Intestinal) OR Gastrointestinal) OR Fecal) OR Cecal) OR Faecal)) AND (((((Flora) OR Microflora) OR Microbiotas) OR Microbiome) OR Microbiomes)))) AND (((Exercises) OR Training)) AND Human))) NOT (Mice OR Rat* OR (Experiment* AND Animal*) OR (Research* AND Animal*) OR mouse OR (model* AND animal*))))))). For further details see **Table 4**.

Table 1. Search strategy used for **Scielo**, and number of articles found.

Search criterion 1	Search criterion 2	Items found (Combining criteria 1 “AND” 2)
Gut	Microbiota	105
Total items found	All the searches were combined using the operator “OR” so that duplicated articles were excluded from the final number of articles found. The final search term was: (gut AND microbiota). A refinement was made to exclude reviews: (REVIEW)	105

The search recruited articles published until Julye 5th 2019; no lower date limit was set.

Table 2. Search strategy used for Scopus, and number of articles found.

Search criterion 1	Search criterion 2	Search criterion 3	Search criterion 4	Items found (Combining criteria 1 “AND” 2 “AND” 3 “AND” 4)
Gut	Microbiota	Exercises OR Training	Human OR Humans	131

Total items found	All the searches were combined using the operator "OR" so that duplicated articles were excluded from the final number of articles found. The final search term was: (gut AND microbiota AND (exercises OR training) AND human AND humans). A refinement was made to exclude reviews: (REVIEW)	131

The search recruited articles published until July 5th 2019; no lower date limit was set.

Table 3. Search strategy used and number of articles found in PubMed.

Search criterion 1	Search criterion 2	Search criterion 3	Search criterion 4	MeSH Entry Terms for Criteria 4	Search criterion 5	Items found (Combining criteria 1 "AND" 2 "AND" 3 "NOT" 4 "NOT" 5)
"Gastrointestinal Microbiome" OR "Fecal Microbiota" OR "Cecal Microbiota" OR "Fecal Microbiota"	Exercises OR Training	Human	"Mice"[MeSH]	Mus Mouse Mus musculus Mice, House House Mice Mouse, House House Mouse Mus domesticus Mus musculus domesticus domesticus, Mus musculus Mice, Laboratory Laboratory Mice Mouse, Laboratory Laboratory Mouse Mouse, Swiss Swiss Mouse Swiss Mice Mice, Swiss	Review	232
			"Rats"[MeSH]	Rat Rattus Rattus norvegicus Rats, Norway Rats, Laboratory Laboratory Rat Laboratory Rats Rat, Laboratory		
			"Animal Experimentation"[MeSH]	Experimentation, Animal Animal Research Research, Animal Animal Experimental Use Animal Experimental Uses Experimental Use, Animal Experimental Uses, Animal Animal Experiments Animal Experiment Experiment, Animal		

			Experiments, Animal	
		"Models, Animal"[MeSH]	Animal Model Animal Models Model, Animal Laboratory Animal Models Animal Model, Laboratory Animal Models, Laboratory Laboratory Animal Model Model, Laboratory Animal Models, Laboratory Animal Experimental Animal Models Animal Model, Experimental Animal Models, Experimental Experimental Animal Model Model, Experimental Animal Models, Experimental Animal	
		"rats" OR "mouse" OR "mice" OR "rat"		
Total items found	The final search term was: ((((((((((((((((("Gastrointestinal Microbiome") OR ((("Fecal Microbiota") OR "Cecal Microbiota") OR "Faecal Microbiota")))))) AND (((Exercises) OR Training))))) AND Human) NOT (((((((((((((((((("Mice"[MeSH]) OR "Rats"[MeSH]) OR "Animal Experimentation"[MeSH]) OR "Models, Animal"[MeSH])) OR ("rats" OR "mouse"))) OR "mice")) OR "rat"))))))))))))))))) NOT Review			232

The search recruited articles published until July 5th 2019; no lower date limit was set. MeSH (Medical Subject Headings) is the National Library of Medicine controlled vocabulary thesaurus used for indexing articles for PubMed.

Table 4. Search strategy used and number of articles found in Web of Science.

Search criterion 1	Search criterion 2	Search criterion 3	Search criterion 4	Items found (Combining criteria 1 "AND" 2 "AND" 3 "NOT" 4)
((Gut OR Intestinal OR Gastrointestinal OR Fecal OR Cecal OR Fecal) AND (Flora OR Microflora OR Microbiotas OR Microbiome OR Microbiomes))	Exercises OR Training	Human	(Mice OR Rat* OR (Experiment* AND Animal*) OR (Research* AND Animal*) OR mouse OR (model* AND animal*)	151
Total items found	All searches were combined using the operator "OR" so that duplicated articles were excluded from the final number of articles found. The final search term was: ((((((((((((((((((Gut) OR Intestinal) OR Gastrointestinal) OR Fecal) OR Cecal) OR Fecal)) AND (((((Flora) OR Microflora) OR Microbiotas) OR Microbiome) OR Microbiomes)))) AND (((Exercises) OR Training)) AND Human))) NOT (Mice OR Rat* OR (Experiment* AND Animal*) OR (Research* AND Animal*) OR mouse OR (model* AND animal*))))))). Moreover, we refined excluding document types: (REVIEW)			151

The search recruited articles published until July 5th 2019; no lower date limit was set.

Selection criteria

The inclusion criteria were: (i) observational and intervention studies; (ii) studies including exercise interventions (either very short-term or medium/long-term) or interventions involving any type of physical activity; (iii) studies including effects on gut microbiota as an outcome. Case-control studies were included, but only those that reported data for the healthy controls. The exclusion criteria were: (i) studies written in languages other than English or Spanish; (ii) studies including unhealthy people, (iii) reviews, and, (iv) studies in animal models. No restrictions were placed on subject age or body composition.

After removing duplicates, eligibility was finally assessed by: (1) reading the title and abstract; and (2) reading the full text if still potentially eligible.

Data extraction

The following information was collected from each included study: (i) the authors' names and bibliographic references; (ii) number of subjects and their sex, age and body mass index [BMI]); (iii) exercise outcomes; (iv) control diet type (standardized diet and/or adjusting

the results for nutritional intake); (v) fecal sample collection; (vi) technique used for gut microbiota analysis, and (vii) the main findings. Two authors (LOA and HX) conducted the literature search and data extraction independently; disagreements were resolved by consensus. The articles selected were classified according to the type of study (observational, very short-term exercise interventions and medium/long-term exercise interventions).

Table 5. Observational studies that reported a relationship between exercise or physical activity and the gut microbiota of healthy adults.

Study/Year	Subjects	Exercise Outcomes	Control of diet	Fecal sample collection	Gut microbiota analysis techniques	Main Findings
Cross-sectional						
McFadzean et al., 2014(44)	N=1493	Participants self-reported physical activity levels: Never (N) =46 Rarely (R)=45 Occasionally (O)=301 Regularly (r)=577 Daily (D)=309	Standardized diet: No Adjusted for diet: No	1	qPCR: MyseqIllumina Software: QIIME Reference DB: Greengenes Outcome: OTU	Statistical analysis: ANOVA, Bonferroni test ↑ α-diversity in O, r and D groups ($p<0.05$) vs R β-diversity: There were no distinctly different clusters according to physical activity. Comparison between groups: Family: ↑ *Erysipelotrichaceae* (11.776% O) Genus: ↑ *Lachnospira* (3.805% r) ↑ *Coprococccus* (1.665% r) ↑ *Oscillospira* (2.75% D) Species: ↑ *F. prausnitzii* (17.94% D)
Estaki et al., 2016(35)	N=41(22 males/19 females)	Assessment of cardiorespiratory fitness (VO_{2max})	Standardized diet: No	1 after test	qPCR: MySeq Illumina Software: QIIME	Statistical analysis: Spearman correlations

	by cycle test and metabolic cart. Incline: Continuous incremental ramp Power: started at 50 W and increased by 30 W/min End: participant reached self-declared exhaustion or when rpm fell below 50. Participant's group by VO_{2max}: Low (33±4.8 VO_{2max}): N=14 (25.5±3.3 years and 25.5±3.9kg/m^2) Moderate (41.9±4.3 VO_{2max}): N=12 (24.3±3.7 years and 23.5±0.5kg/m^2) High (54.8±5.6 VO_{2max}): N=13	Adjusted for diet: No		Reference DB: Greengenes Outcome: OTU	α-diversity positively correlated with VO_{2max} (R^2=0.204) β-diversity: There were no distinctly different clusters according to cardiorespiratory fitness. Correlation with VO_{2max}: Order: ↑ *Clostridiales* Families: ↑ *Lachnospiraceae* ↑ *Erysipelotrichaceae* Genera: ↑ *Coprococcus* ↑ *Roseburia* ↑ *Adlercreutzia* SCFAs: ↑ Butyrate ↓ Propionate ↓ Acetate

		(26.2±5.5 years and 22.8±5.5kg/m²)				
Yang et al., 2017(36)	N=71 Females, premenopausal	Assessment of cardiorespiratory fitness (VO_{2max}) by cycle test: Power: Increased 25 W each 2 min. End: participant reached self-declared exhaustion Participant's groups by VO_{2max}: Low: N=24 (40.4 years and 31.7kg/m²) Moderate: N=23 (39.7 years and 27.9kg/m²) High: N=24 (30.6 years and 24.6kg/m²)	Standardized diet: No Adjusted for diet: Yes (Fat %, energy from HC, protein, fat and alcohol by 3 days food records)	1	Flow cytometry-FISH	Statistical analysis: ANOVA, Sidak post-hoc test. High vs Moderate and Low: Phylum: ↑ *Bacteroidetes* (High group)
Case and Control						
Clarke et al., 2014(45)	N=86 males Athletes: N=40 (28.8±3.8 years and 29.1±3kg/m²)	Assessment of physical activity by EPIC-Norfolk questionnaire.	Standardized diet: No Adjusted for diet: No	1	qPCR: Roche Software: BLAST and QIIME	Statistical analysis: Kruskal-Wallis and Mann-Whitney tests ↑ α-diversity in athlete group

	High BMI controls: N=23 (30.8±5.6 years) Low BMI controls: N=23 (28.1±5.1 years).				Reference DB: SILVA 16S rRNA Outcome: OTU	Athletes vs high BMI controls: Phylum: ↑ *Firmicutes* ↓ *Bacteroidetes* Family: ↑ *Ruminococcaceae* ↑ *S24-7* ↑ *Succinivibrionaceae* ↑ *Akkermansiaceae* Genus: ↑ *RC9 gut group* ↑ *Succinivibrio* ↑ *Akkermansia* Athletes vs low BMI controls: Family: ↑ *Prevotellaceae* ↑ *Erysipelotrichaceae* ↑ *S24-7* ↑ *Succinivibrionaceae* ↓ *Lactobacillaceae* Genus: ↑ *Prevotella* ↑ *Succinivibrio* ↓ *Lactobacillus* ↓ *Bacteroides*
Bressa et al., 2017(46)	N=40 Females, premenopausal.	7 days of measurement of physical activity	Standardized diet: No	1	qPCR: MySeq Illumina	Statistical analysis: Bivariate correlation, Spearman correlation coefficients.

	Active (A): N=19 (30.7±5.9 years and 24.4±4.5kg/m^2) Sedentary (S): N=21 (32.2±8.7 years and 22.9±3.0kg/m^2)	by accelerometer.	Adjusted for diet: Yes (total energy, protein, carbohydrates, lipids, fiber, ethanol, vegetables, cereals, dairy products, fruits, processed meat, beer and coffee, by food frequency questionnaire)		Software: QIIME Reference DB: Greengenes Outcome: OTU	= α-diversity (A = S) β-diversity: Distinctly different clusters seen according to physical activity. Active vs Sedentary: Families: ↓ *Barnesiellaceae* (0.37±0.35% A; 0.86±0.65% S) ↓ *Odoribacteriaceae* (0.44±0.26%A; 0.66±0.40% S) Genus: ↑ *Bifidobacterium* ↑ *Haemophilus* ↑ *Paraprevotella* ↑ *Coprococcus* ↑ *Ruminococcacear UC 1* ↓ *Desulfovibrionaceae UC* ↓ *Turicibacter* ↓ *Barnesiellaceae UC* ↓ *Odoribacter* ↓ *Ruminococcacear UC2* ↓ *Ruminococcus* Species: ↑ *Faecalibacterium prausnitzii* ↑ *Roseburia hominis*

						↑ *Akkermansia muciniphila*
Petersen et al., 2017(27)	N= 33 cyclists (22 males/11 females; 19-49 years) Professionals: N=22 Amateurs: N=11	Assessment of physical activity levels by questionnaires on the average number of hours of exercise per week (h/wk): 6-10h 11-15h 16-20h >20h	Standardized diet: No Adjusted for diet: Yes (questionnaire NR)	1	qPCR: MySeq Illumina Software: R studio References DB: RDP Outcome: Abundance relative	Statistical analysis: Spearman rank and Fisher correlations No significant differences between professionals and amateurs According to exercise load: >20h/w: Genus: ↑ *Prevotella* (14.75%) 16-20h/w: Genus: ↑ *Prevotella* (12.12%) >11h/w: Genus: ↑ *Prevotella* (≥2.5%) 6-10h/w: Genus: ↑ *Prevotella* (0.15%)
Barton et al., 2018(28)	N=86 males Athletes: N=40 (28.8±3.8 years) Low BMI control: N=22 (28.1±5.1 years) High BMI control: N=24 (30.8±5.6 years)	Assessment of physical activity levels by EPIC-Norfolk questionnaire.	Standardized diet: No Adjusted for diet: No	1	qPCR: HiSeq Illumina Software: HUMAnN2 V.0.5.0 pipeline. References DB: Metacyc Outcome: relative abundance	Statistical analysis: Kruskal-Wallis test ↑ α-diversity in athlete group Athletes vs control group: SCFAs ↑ Propionate ↑ Acetate ↑ Butyrate ↑ Valerate
Durk et al., 2018(33)	N=37 Males: N=20 (25.9±2.7 years	Assessment of cardiorespiratory fitness (VO_{2max}) by treadmill.	Standardized diet: No	1	qPCR: FAST SYBR Green Software: Master Mix	Statistical analysis: VO_{2max} positively correlated with the

	and 24.1±4.0 kg/m² Females: N=17 (25.4±1.8 years and 23.2±3.0 kg/m²)	Incline: increased by 2% every 2 minutes Speed: constant End: participant reached self-declared exhaustion.	Adjusted for diet: No		Reference DB: NR Outcome: NR	*Firmicutes/Bacteroidetes* ratio (r=0.48)
Mörkl et al., 2018(34)	N=88 females Athletes: N=20 (22.15±3.86 years) Normal weight: N=26 (24.93±3.75 years) Overweight: N=22 (25.32±3.98 years) Obese: N=20 (26.9±6.09 years)	Assessment of physical activity levels by IPAQ score.	Standardized 1 diet: No Adjusted for diet: No		qPCR: Ion Torrent Software: QIIME Reference DB: NR Outcome: OTU	Statistical analysis: ANOSIM y Adonis (QIIME test) ↑ α-diversity in athlete group β-diversity: Distinctly different clusters seen according to physical activity.

ANOVA, analysis of variance; BMI, body mass index; DB, database; FISH, fluorescent in-situ hybridization; NR, not reported; OTU, operational taxonomic unit; qPCR, quantitative polymerase chain reaction; RDP, Ribosomal Database Project; RM, repetition maximum; SCFAs, short-chain fatty acids; UC, unclassified; VO_{2max}, maximum volume of oxygen. Standardized diet: participants followed an established standard diet; Adjusted for diet: to statistically adjust the results based on nutritional questionnaires.

Study quality

With the purpose of evaluating quality of the studies included, we used PEDro Methodological Quality Scale[42]. This tool consists of 11 items that assessing the interpretability of studies, internal as well as external validity and it is able to detect potential bias with good reliability[42,43]. Total score was obtained by adding of the scores for items 2 to 11. Scores of ≤ 3 were viewed as describe studies of low methodological quality, 4 to 6 those of moderate quality, and ≥ 7 those of high methodological quality.

RESULTS

General overview

Figure 1 shows the PRISMA consort diagram for the search strategy. A total of 619 studies were found across four of the databases examined (no eligible studies were detected in the Scielo and Scopus databases), 580 of which were excluded after reading the title and abstract. Thus, 18 studies met the inclusion criteria, nine of which were observational (i.e., three cross-sectional [35,36,44] and six case-control studies[27,28,33,34,45,46]) (**Table 5**), four of which were very short-term exercise intervention studies (two was a case-control study[32,37]) (**Table 6**), and five of which were medium/long-term exercise intervention studies (one was a case-control study[47]) (**Table 7**). All studies were published between 2014 and 2019.

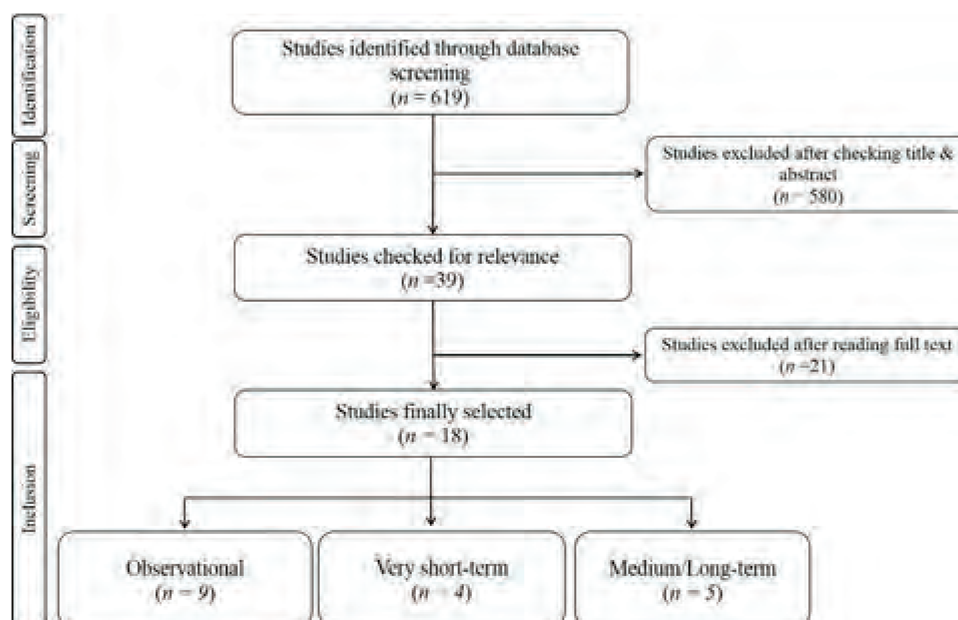

Figure 1: Flowchart showing the literature search and article selection process.

Table 6. Summary of studies that reported very short-term exercise to have an effect on the gut microbiota of healthy adults.

Study/Year	Subjects	Exercise Outcomes	Control Diet	Fecal sample collection	Gut microbiota analysis techniques	Main Findings
Cohort						
Lundgren-Kownacki et al., 2018(38)	N=12 Males: N=6 (27.2±2.8 years and 24.0±2.0 kg/m2) Females: N=6 (24.0±3.7 years and 23.8±1.1 kg/m2)	2 days of physical work: day 1 with water for rehydration and day 2 without rehydration (3h/d-34°C, 60%RH). Activities involved: bricks, stepping, biking and arm crank at rotating intervals every 20 min	Standardized diet: Yes (dinner the evening before each test day and standardized breakfast of an egg sandwich each test day.) Adjusted for diet: No	1 sample after each day	qPCR: NR Software: GeneMapper and R Outcome: 16S rRNA gene copies/g samples	Statistical analysis: Kruskal-Wallis test α-diversity: there were no differences between days Day 1 vs day 2: Family: = *Enterobacteriaceae* Genus: = *Lactobacillus*
Zhao et al., 2018(39)	N=20 runners (16 males; 4 females) (31.3±6.1 years and 22.6±2.1kg/m2)	km of half-marathon	Standardized diet: Yes (same kind of food during the two sample time periods) Adjusted for diet: No	2 samples: before and after marathon	qPCR: HiSeq Illumina Software: QIIME Reference DB: RDP Outcome: OTU	Statistical analysis: linear discriminant analysis, effect size analysis = α-diversity between after and before the marathon After vs before marathon: Phylum: ↑ *Actinobacteria* Class: ↑ *Coriobacteria* Order: ↑ *Coriobacteriales* Family:

						↑ *Coriobacteriaceae* ↑ *Succinivibrionaceae* Genus: ↑ *Actinobacillus* ↑ *Pseudobutyrivibrio* ↑ *Collinsella* ↑ *Mitsuokella* ↑ *Ruminiclostridium* Species: ↑ *Coprococcus_2* ↑ *Ruminococcus bicirculans* ↑ *Collinsella aerofaciens*
Case and control						
Shukla et al., 2015(37)	N=10 (46.5±13 years, 24.6±3.3kg/m2 and 28.2±9.6 VO2max)	Assessment of cardiorespiratory fitness (VO2max) by cycle test: Speed: 60-70 rpm. Power increased 5 W each 20 s. End: participant reached self-declared exhaustion Min: 13.1±3.4	Standardized diet: No Adjusted for diet: No	3 samples (baseline, and 48 and 72h post-exercise)	qPCR: Roche Software: Explicet Reference DB: RDP Outcome: OTU.	Statistical analysis: ANOVA, Fisher test 72 h-post exercise vs baseline: Phylum ↑ *Bacteroidetes* ↑ Others (NR)
Scheiman et al., 2019(32)	Cohort 1, N= 25: Marathoners: N=15 (11females; 27.1 years; 22.4 kg/m2) Controls: N=11(6 females; 29.2	Cohort 1: 42.2 km Boston Marathon (Not for control participants) Cohort 2: Not specify type of exercise	Standardized diet: No Adjusting by diet: Yes (food frequency	Cohort 1: 5 samples collected in the previous 5 days of the marathon, and other 5 samples after	qPCR: MiSeq or HiSeq 2500 system Software: Metagenomic Phylogenetic Analysis Reference DB:	Statistical analysis: Wilcoxon rank-sum test with continuity correction After vs before exercise: Cohort 1: Genus: ↑ *Veillonella* (marathoners) =*Veillonella* (controls)

	years, 22.9 kg/m2) Cohort 2, N= 11 Ultramarathoners and Olympic rowers (6 females).		questionnaire)	the race. Total of 209 samples. Cohort 2: 87 samples baseline and post-exercise	Outcome: Relative abundance	Cohort 2: Genus: ↑ *Veillonella* (ultramarathoners and rowers)

ANOVA, analysis of variance; DB, database; NR, not reported; OTU, operational taxonomic unit; qPCR, quantitative polymerase chain reaction; RDP, Ribosomal Database Project; RH, relative humidity; VO2max: maximum volume of oxygen. Standardized diet: Participants followed an established standard diet; Adjusted for diet: to statistically adjust the results based on nutritional questionnaires.

Table 7. Summary of studies that reported medium/long-term exercise to influence the gut microbiota of healthy adults.

Study/Year	Participants	Exercise Outcomes	Control of diet	Fecal sample collection	Gut microbiota analysis techniques	Main Findings
Cohort						
Cronin et al., 2018(40)	N=25 (11 males; 14 females) (35 years; 27.9 kg/m^2)	3 times per week over 8 weeks of concurrent training (endurance + resistance exercises): Endurance exercise Intensity: moderate (Borg rate scale 5 to 7/10) Min: 18 min until 32 min Increasing 15% to 20%. Resistance exercise: 7 machine-based resistance exercises. Minimum: 3 sets of 8 repetitions in each machine. Maximum: 3 sets of 12 repetitions in each machine. Weight: 70% of the individual´s one-repetition maximum (1RM) value.	Standardized diet: No Adjusted for diet: No	2 samples (baseline, and after exercise period)	qPCR: HiSeq Illumina Software: HUMAnN2 Reference DB: Metacyc database Outcome: NR	Statistical analysis: ANOVA = α-diversity between groups β-diversity: Distinctly different clusters were seen after the intervention.

Munukka et al., 2018(29)	N=17 women (36.8±3.9 years and 31.8±4.4 kg/m^2)	6 weeks of endurance training (cycling) 3d/wk 30-60min/d low-moderate intensity. Weeks 1– 2: 3 sets x40min steady-state cycling at low intensity Weeks 3–4: 3x50min 3x10-min moderate intensity Rest, low intensity Weeks 5–6: 60min 4x10-min at moderate intensity Rest, low intensity	Standardized diet: No Adjusted for diet: Yes (fat %, energy %, fiber %, sucrose % by 3 days food records).	2 samples: baseline and post-exercise training	qPCR: MySeq Illumina Software: QIIME Reference DB: SILVA Outcome: OTU	Statistical analysis: General linear model α-diversity: there was no difference after exercise training ↑ β-diversity after exercise at the genus level After training vs baseline: Phylum ↓ *Proteobacteria* ↑ *Verrucomicrobia* Family ↑ *Verrucomicrobiaceae* Genus ↑ *Akkermansia*
Morita et al., 2019(30)	N=29 women	Non-randomized intervention endurance group N=17 (66-75 years; 18.9-23.1 kg/m^2) 7d/wk 60min/d 12 weeks brisk walking at an intensity of ≥ 3 metabolic equivalents (METs) as measured by accelerometer. Increasing the intensity and duration as much as possible. Resistance group: N=12 (66-77 years; 18.7-24.0 kg/m^2)	Standardized diet: No Adjusted for diet: Yes (food frequency questionnaire)	2 samples: basal and post exercise training	qPCR: Takara Thermal Cycler Dice TP650T Software: GeneMapper Reference DB: Human Fecal Microbiota T-RFLP profiling Outcome: OTU	Statistical analysis: Wilcoxon Signed-rank test, After interventions: 1.Endurance group: Genus: ↑ *Bacteroides* ↓ Others Specie: ↓ *Clostridium subcluster XIVa* 2.Resistance group: Species: ↑ *Clostridium cluster IX*

		1 h/week for 12 weeks: strengthening the trunk muscle Activities involved: arching–swaying, plank, pelvic rotation in the supine position, and bird-dog.				
Keohane et al., 2019(31)	N=3 men (26.5±1.3 years; 24.4±1.4 kg/m²)	3748.91 km east-west Transatlantic rowing race over 33 days and 22 hours; three participants.	Standardized diet: Yes - 21 MJ per day during preparatory period - 33.5 MJ per day during race Adjusted for diet: No	4 samples: baseline, mid-way point, before race finished and at 3 months post-race)	qPCR: Ilumina NextSeq Software: HUMAnN2 V.0.99 Reference DB: - Outcome: Abundance relative	Statistical analysis: - ↑ α-diversity since mid-point until race end Throughout the race for all participants: Genus: ↑ *Subdoligranulum* UC Specie: ↑ *Dorea longicatena* ↑ *Roseburia hominis* ↓ *Bacteroides finegoldii*
Case and control						
Allen et al., 2017(47)	N=32 Normal weight: N=18 (9 males/ 9 females) (25.1±6.5 years). Obese: N=14 (3 males/ 11 females) (31.2±8.5 years).	6 weeks of endurance training (cycling or treadmill [subjects chose]) 3d/wk 30-60 min/d moderate-vigorous intensity. Week 1: 30min at 60% HRR Week 2: lasted 45 min =intensity Week 3: 60 min at 60% HRR	Standardized diet: Yes -7 food records -Evaluators designed a 3-day food menu (with meal of 7	3 samples. (Baseline, after 6 weeks of the intervention and after 6 weeks of	qPCR: MySeq Illumina. Software: QIIME References DB: RDP Outcome: OTU	Statistical analysis: PERMANOVA and ANOVA α-diversity: no difference between groups after exercise or in the washout period β-diversity: No distinct difference between groups after the intervention. After training vs baseline:

	Week 4-6: increased the intensity by 5% HRR each week Week 6: 60 min at 75%HRR	food records) -Participants followed this 3-day food menu prior to each fecal collection. Adjusted for diet: Yes	washout) .		Order: ↑ *Clostridiales* Genus: ↑ *Roseburia* ↑ *Lachnospira* ↑ *Faecalibacterium* ↑ *f_Lachnospiraceae UC* SCFAs in normal weight group: ↑Acetate ↑ Butyrate

ANOVA, analysis of variance, DB, database; HRR, heart rate reserve; MJ, megajoules; METs, metabolic equivalent of task; NR, not reported; OTU, operational taxonomic unit; qPCR, quantitative polymerase chain reaction; RDP, Ribosomal Database Project; SCFAs, short-chain fatty acids; UC, unclassified. Standardized diet: Participants followed an established standard diet; Adjusted for diet: to statistically adjust the results based on nutritional questionnaires.

The sample size of 17 of these 18 studies ranged from 3 to 88 subjects[27-40,45-47] and one had a sample size of 1493 subjects.[44] Five of the 18 studies involved only women,[29,30,34,36,46] three involved only men,[28,31,45] eight involved both men and women,[27,32,33,35,38-40,47] and two studies did not report subject sex.[37,44] Subject age ranged from 18 to 77 years; one study did not report subject age.[44] Four studies included sedentary subjects,[29,30,40,47] three focused on active participants,[27,31,39] and nine involved both sedentary and recreationally active subjects.[28,32-36,44-46] Two studies did not report the levels of physical activity practiced.[37,38] Physical activity was assessed by questionnaire in five studies,[27,28,34,44,45] and using an accelerometry-based method in one.[46] Four studies measured cardiorespiratory fitness via the maximum oxygen consumption (VO_{2max}) test.[33,35-37]

Eight studies did not record the participants' diet before assessments were made,[28,33-35,37,40,44,45] six studies[27,29,30,32,36,46] examined the diet via means of food records or food frequency questionnaires and adjusted the results accordingly. Four studies established a fixed diet for some days before the collection of feces.[31,38,39,47]

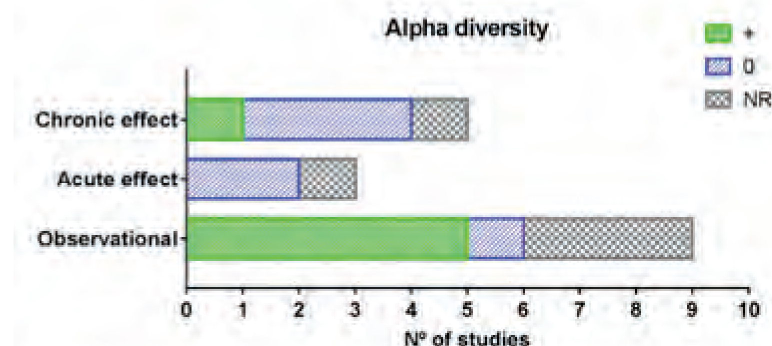

Figure 2: Influence of exercise on alpha diversity by study design. The green bars (+) indicate the number of studies showing a positive association between physical activity or cardiorespiratory fitness and bacterial alpha diversity. The blue bars (0) represent the number of studies showing no influence of physical activity or cardiorespiratory fitness on alpha diversity. The grey bars ("NR") indicate the number of studies that reported no results in this respect.

The bacterial alpha diversity was reported in 12 studies (n=66·6%)[28,29,31,34,35,38-40,44-47] (**Figure 2**, **Tables 5**, **6** and **7**) and beta diversity in seven (n=38·8%)[29,34,35,40,44,46,47] (**Table 5**, **6** and **7**). The studies reported on the gut microbiota data using different taxonomic ranks (**Table 3**, **5**, **6** and **7**); two reported at the phylum level,[36,37] seven at the genus level,[27,29,32,35,38,45,47] and five at the species level.[30,31,39,44,46] Only one study reported the ratio *Firmicutes/Bacteroidetes*,[33] and only three reported the concentration of short-chain-fatty-acids (SCFAs) in the feces[28,35,47] (**Figure 3**). Such heterogeneity hampers comparisons; no meta-analysis performed. However, gut microbiota data reported by the different studies could all be converted to the phylum level,

making comparisons and interpretations somewhat easier (**Figure 4**, 5 and **Table** 8).

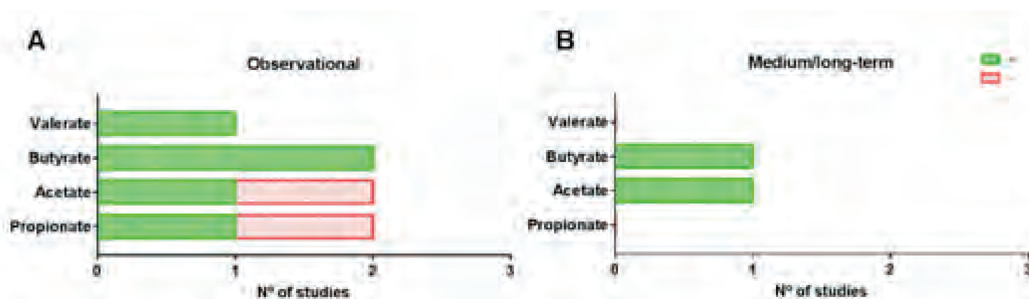

Figure 3: Summary of the main findings of the present review with respect to short-chain fatty acids (SCFAs), by study design. Panel A represents the main results of all observational studies (n=2). Panel B shows the main results of the medium/long-term studies (n=1). The green bars (+) indicate the number of studies showing a positive association between levels of physical activity or cardiorespiratory fitness and SCFAs, or a positive effect for an exercise intervention. The red bars (-) indicate a negative association between levels of physical activity or cardiorespiratory fitness and SCFAs.

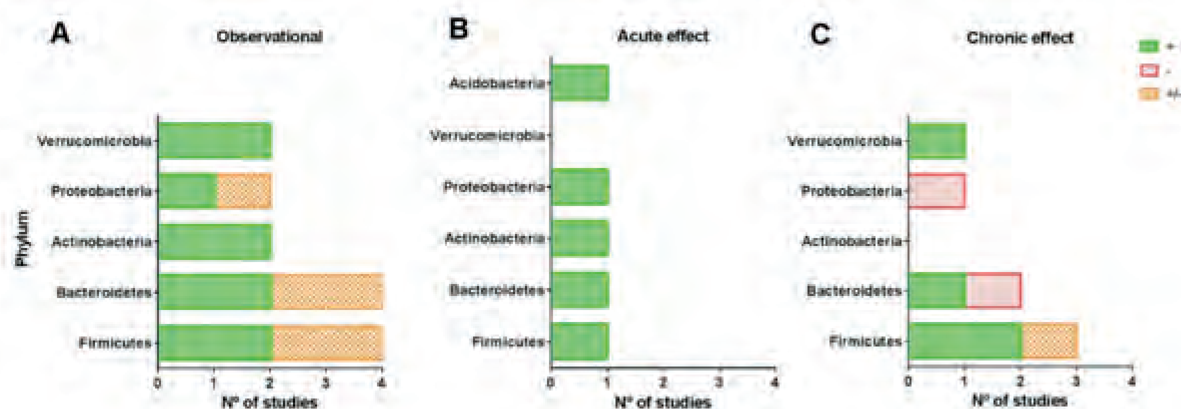

Figure 4: Summary of the main results of the present review by study design. Panel A represents the main findings of all observational studies. Panel B represents the main findings of all very short-term intervention studies. Panel C represents the main findings of all medium/long-term intervention studies. The green bars (+) indicate the number of studies that showed a positive influence of physical activity or cardiorespiratory fitness on the different phyla, or a positive effect for an exercise intervention. The red bars (-) indicate the number of studies that showed a negative effect for an exercise intervention. The orange bars (+/-) represent the number of studies showing positive and negative findings within the same phyla. These findings come from the observational studies of Clarke et al. 2014, [42] and Bressa et al. 2017, [43] and from the medium/long-term exercise intervention study of Morita et al. 2019. [38]

Table 8. Taxonomic ranks used in the studies included in the present review. The first column represents the different taxonomic classifications: phylum, class, order, family, genus and species; the second column represents the different microorganisms belong to those classifications. The numeration indicates the higher-level taxonomic rank to which a microorganism belongs. Each row shows the different articles examined, grouped by study type.

		Observational									Very short-term exercise Interventions				Medium/long-term exercise Interventions				
		McFadzean et al., 2014	Clarke et al., 2014	Estaki et al., 2016	Yang et al., 2017	Bressa et al., 2017	Petersen et al., 2017	Barton et al., 2018	Mörkl et al., 2018	Durk et al., 2018	Shukla et al., 2015	Lundgren-Kownacki et al., 2018	Zhao et al., 2018	Scheiman et al., 2019	Allen et al., 2017	Cronin et al., 2018	Munukka et al., 2018	Morita et al., 2019	Keohane et al., 2019
Alpha diversity		+	+	+	NR	0	NR	+	+	NR	NR	0	0	NR	0	0	0	NR	+
Beta diversity		0	NR	0	NR	0	NR	NR	+	NR	NR	NR	NR	NR	0	+	+	NR	NR
Phylum	1.*Firmicutes*	+	+/-	+	NR	+/-	NR	NR	NR	NR	NR	NR	+	+	+	NR	NR	+/-	+
	2.*Bacteroidetes*	NR	+/-	NR	+	+/-	+	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	+	+/-
	3.*Actinobacteria*	NR	NR	+	NR	+	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	4.*Proteobacteria*	NR	+	NR	NR	+/-	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	-	NR	NR
	5.*Verrucomicrobia*	NR	+	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR
Class	1.1.*Clostridia*	+	+	+	NR	+/-	NR	NR	NR	NR	NR	NR	+	NR	+	NR	NR	+/-	+
	1.2.*Erysipelotrichia*	+	+	+	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.3.*Bacilli*	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.4.*Negativicutes*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	+	NR	NR	NR	NR	NR
	2.1.*Bacteroidia*	NR	+/-	NR	NR	+/-	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	+/-
	3.1.*Actinobacteria*	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	3.2.*Coriobacteriia*	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	4.1.*Deltaproteobacteria*	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	4.2.*Gammaproteobacteria*	NR	+	NR	NR	+	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	5.1.*Verrucomicrobiae*	NR	+	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR
Order	1.1.1.*Clostridiales*	+	+	+	NR	+/-	NR	NR	NR	NR	NR	NR	+	NR	+	NR	NR	+/-	+
	1.2.1.*Erysipelotrichales*	+	+	+	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.3.1.*Lactobacillales*	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.4.1.*Selenomonadales*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	1.4.2.*Veillonellales*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR
	2.1.1.*Bacteroidales*	NR	+/-	NR	NR	+/-	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	+/-
	3.1.1.*Bifidobacteriales*	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	3.2.1.*Coriobacteriales*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	3.2.2.*Eggerthellales*	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

	4.1.1.Desulfovibrionales	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	4.2.1.Aeromonadales	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	4.2.2.Pasteurellales	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	5.1.1.Verrucomicrobiales	NR	+	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR
Family	*1.1.1.1.Hungateiclostridiaceae*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	1.1.1.2.Lachnospiraceae	+	NR	+	NR	+	NR	NR	NR	NR	NR	NR	+	NR	+	NR	NR	-	+
	1.1.1.3.Ruminococcaceae	+	+	NR	NR	+/-	NR	NR	NR	NR	NR	NR	+	NR	+	NR	NR	NR	+
	1.1.1.4.Family UC	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+
	1.2.1.1.Erysipelotrichaceae	+	+	+	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.3.1.1.Lactobacillaceae	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.4.1.1.Selenomonadaceae	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	1.4.2.1.Veillonellaceae	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR
	2.1.1.1.S24-7	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2.1.1.2.Rikenellaceae	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2.1.1.3.Prevotellaceae	NR	+	NR	NR	+	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+
	2.1.1.4.Barnesiellaceae	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2.1.1.5.Odoribacteraceae	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2.1.1.6.Bacteroidaceae	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	-	+
	3.1.1.1.Bifidobacteriaceae	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	3.2.1.1.Coriobacteriaceae	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	3.2.2.1.Eggerthellaceae	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	4.1.1.1.Desulfovibrionaceae	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	4.2.1.1.Succinivibrionaceae	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	4.2.2.1.Pasteurellaceae	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	5.1.1.1.Akkermansiaceae	NR	+	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR
	5.1.1.2.Verrucomicrobiaceae	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR
Genus	*1.1.1.1.1.Ruminiclostridium*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	1.1.1.2.1.Coprococcus	+	NR	+	NR	+	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	1.1.1.2.2.Roseburia	NR	NR	+	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	+
	1.1.1.2.3.Lachnospira	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR
	1.1.1.2.4.Pseudobutyrivibrio	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	1.1.1.2.5.Lachnospiraceae UC	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR
	1.1.1.2.6.Clostridium cluster XIVa	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+
	1.1.1.2.7.Dorea	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+
	1.1.1.3.1.Faecalibacterium	+	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR

	1.1.1.3.2.*Oscillospira*	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.1.1.3.3.*Ruminococcaceae UC1*	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.1.1.3.4.*Ruminococcaceae UC2*	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.1.1.3.5.*Ruminococcus*	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	1.1.1.3.6.*Subdoligranulum UC*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+
	1.1.1.4.1.*Clostridium cluster IX*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+
	1.2.1.1.1.*Turicibacter*	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.3.1.1.1.*Lactobacillus*	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.4.1.1.1.*Mitsuokella*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	1.4.2.1.1.*Veillonella*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR
	2.1.1.1.1.*Rikenellaceae* RC9 gut group	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2.1.1.3.1.*Prevotella*	NR	+	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+
	2.1.1.3.2.*Paraprevotella*	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2.1.1.4.1.*Barnesiellaceae* UC	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2.1.1.5.1.*Odoribacter*	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2.1.1.6.1.*Bacteroides*	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	-
	3.1.1.1.1.*Bifidobacterium*	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	3.2.1.1.1.*Collinsella*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	3.2.2.1.1.*Adlercreutzia*	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	4.1.1.1.1.*Desulfovibrionaceae* UC	NR	NR	NR	NR	-	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	4.2.1.1.1.*Succinivibrio*	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	4.2.2.1.1.*Haemophilus*	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	4.2.2.1.2.*Actinobacillus*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	5.1.1.1.1.*Akkermansia*	NR	+	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR
Species	1.1.1.2.1.1.*Coprococcus_2*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	1.1.1.2.2.1.*Roseburia hominis*	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+
	1.1.1.2.7.1.*Dorea longicatena*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+
	1.1.1.3.1.1.*Faecalibacterium prausnitzii*	+	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	1.1.1.3.5.1.*Ruminococcus bicirculans*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	+
	2.1.1.3.1.1.*Prevotella copri*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	-
	2.1.1.6.1.1.*Bacteroides finegoldii*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	3.2.1.1.1.1.*Collinsella aerofaciens*	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR
	5.1.1.1.1.1.*Akkermansia muciniphila*	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
SCFAs	1.Propionate	NR	NR	-	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR
	2.Acetate	NR	NR	-	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR

3.Butyrate	NR	NR	+	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR
4.Valerate	NR	NR	NR	NR	NR	NR	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

Green cells (+) indicate a positive relationship with, or an effect of exercise or physical activity on, alpha and beta diversities, different microorganism or SCFAs. Red cells (-) indicate a negative relationship with, or null effect of exercise or physical activity on, alpha and beta diversities, different microorganism or SCFAs. Yellow cells (+/-) indicate positive and negative results detected between exercise or physical activity and different microorganism. Symbol (0) indicated no relationship seen between exercise/physical activity and alpha and beta diversities. "NR" indicates that the above types of relationship were not reported. SCFAs: Short-chain fatty acids.

Methodological quality of clinical trials

Based on PEDro scale criteria, all study 100% (n = 18)[27,28,37–40,44–47,29–36] were of medium quality (**Table 9**). Criteria 2 and 3 (regarding allocation) and 5, 6 and 7 (regarding the blinding process) were the least satisfied by the different studies, whereas criteria 4, 8, 9, 10 and 11 (regarding the design of randomization and the data displayed) were the best satisfied.

Table 9. Methodological quality of clinical trials using PEDro Scale

Study/Year	*1	2	3	4	5	6	7	8	9	10	11	Score
Observational studies												
Cross-sectional												
McFadzean et al., 2014 (44)	N	N	N	N	N	N	N	Y	Y	Y	Y	4
Estaki et al., 2016 (35)	Y	N	N	Y	Y	N	N	Y	Y	Y	Y	6
Yang et al., 2017 (36)	Y	N	N	Y	Y	N	N	Y	Y	Y	Y	6
Case and control												
Clarke et al., 2014 (45)	Y	N	N	N	N	N	N	Y	Y	Y	Y	4
Bressa et al., 2017 (46)	Y	N	N	Y	N	N	N	Y	Y	Y	Y	5
Petersen et al., 2017 (27)	Y	N	N	N	N	N	N	Y	Y	Y	Y	4
Barton et al., 2018 (28)	Y	N	N	N	N	N	N	Y	Y	Y	Y	4
Durk et al., 2018 (33)	Y	N	N	N	N	N	N	Y	Y	Y	Y	4
Mörkl et al., 2018 (34)	Y	N	N	N	N	N	N	Y	Y	Y	Y	4
Short-term exercise studies												
Cohort												
Lundgren-Kownacki et al., 2018 (38)	Y	N	N	Y	N	N	N	Y	Y	Y	Y	5
Zhao et al., 2018 (39)	Y	N	N	Y	N	N	N	Y	Y	Y	Y	5
Case and control												
Shukla et al., 2015 (37)	Y	N	N	Y	N	N	N	Y	Y	Y	Y	5
Scheiman et al., 2019 (32)	Y	N	N	Y	N	N	N	Y	Y	Y	Y	5
Medium/long-term exercise studies												
Cohort												
Cronin et al., 2018 (40)	Y	Y	Y	Y	N	N	N	N	Y	Y	Y	6
Munukka et al., 2018 (29)	Y	N	N	Y	N	N	N	Y	Y	Y	Y	5
Morita et al., 2019 (30)	Y	N	N	Y	N	N	N	Y	Y	Y	Y	5
Keohane et al., 2019 (31)	Y	N	N	Y	N	N	N	N	Y	Y	Y	4
Case and control												
Allen et al., 2017 (47)	Y	N	N	Y	N	N	N	Y	Y	Y	Y	5

1: Eligibility criteria specified; 2: Individuals randomly allocated; 3: Allocation concealed; 4: Groups similar at baseline regarding most important prognostic indicators; 5:
"Blinded" participants; 6: "Blinded" therapist; 7: "Blinded" assessors; 8: Measures of at least one key outcome obtained from more than 85% of participants; 9: Data analyzed
by "intention to treat"; 10: Statistical comparisons between groups; 11: Point measures and measures of variation.
* Item number 1 not used to calculate PEDro score because it influenced external validity but not internal or statistical validity of trial.
Y, criteria satisfied; N, criteria not satisfied.

Observational studies - detailed examination

Of the nine observational studies detected, four reported a positive association between the level of physical activity and bacterial alpha diversity,[28,34,44,45] although one study observed no such association.[46] One study reported a positive association between cardiorespiratory fitness and bacterial alpha diversity.[35] Three studies did not report bacterial alpha diversity at all.[27,33,36] Beta diversity was reported by four of the above nine studies,[34,35,44,46] but only one[34] reported physical activity to be associated with it.

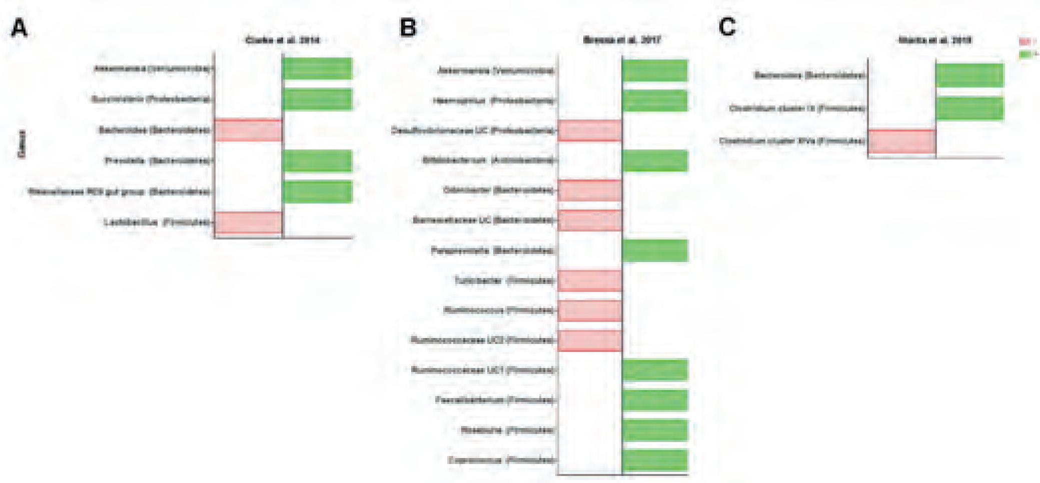

Figure 5: Summary of the main findings of the observational studies of Clarke et al. 2014 [42] (A) and Bressa et al. 2017 [43] (B), and the medium/long-term intervention study of Morita et al. 2019 [38] (C). The green bars (+) represents a positive association between physical activity and the gut microbiota at the genus level, or a positive effect of an exercise intervention. The red bars (-) represent a negative association between physical activity and gut microbiota at the genus level, or a null effect of an exercise intervention.

McFadzean et al.[44] recorded the self-reported physical activity level (undertaken never, rarely, occasionally, regularly or daily) of 1493 people, and observed people who undertook physical activity occasionally, regularly and daily to return higher bacterial alpha diversity values than those who undertook physical activity rarely. They also reported the highest fecal *Faecalibacterium prausnitzii* (*Firmicutes* phylum) counts for those who undertook daily physical activity (**Table 5**). Petersen et al.[27] reported the frequency of training in cyclists to correlate positively with fecal *Prevotella* (phylum *Bacteroidetes*) counts, independent of race category (professional or amateur), and after taking into account nutritional intake (**Table 5**).

Clarke et al.[45], Mörkl et al.[34] and Barton et al.[28] reported bacterial alpha diversity to be higher in athletes compared to healthy, sedentary controls. Clarke et al.[45] observed phylum *Proteobacteria* and

Verrucomicrobia and genus *Prevotella* (phylum *Bacteroidetes*) fecal counts to be higher, and those for genus *Bacteroides* (phylum *Bacteroidetes*) to be lower, in athletes than in sedentary controls (**Table 5** and **Figure 5**). Barton et al.[28] reported a positive association between physical activity level and fecal SCFA (butyrate, propionate, acetate and valerate) concentration (**Table 5**).

Bressa et al.[46] observed no association between objectively measured physical activity levels and bacterial alpha diversity in premenopausal women (**Table 5**). They did, however, report a positive association between physical activity and fecal counts for genus *Bifidobacterium* (phylum *Actinobacteria*) and *Akkermansia muciniphila* (phylum *Verrucomicrobia*) (**Table 5**). They also reported that within the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria*, some genera belonging to the same phylum were present in different amounts in active women compared to controls (**Table 5** and **Figure 5**). It should be noted that these authors adjusted their results for subject diet, as assessed via a food frequency questionnaire.

Estaki et al.[35] reported cardiorespiratory fitness to be positively associated with bacterial alpha diversity. Moreover, they reported, a positive association between cardiorespiratory fitness and fecal counts for order *Clostridiales* family *Lachnospiraceae*, family *Erysipelotrichaceae*, genus *Coprococcus*, genus *Roseburia* (all phylum *Firmicutes*) and genus *Adlercreutzia* (phylum *Actinobacteria*) (**Table 5**). In addition, they observed cardiorespiratory fitness to be positively associated with fecal butyrate, and to be negatively associated with other SCFAs such as propionate and acetate (**Table 5**). Yang et al.[36] observed premenopausal women with high cardiorespiratory fitness levels to have higher fecal counts of phylum *Bacteroidetes* after adjusting for nutritional intake. Finally, Durk et al.[33] reported a positive association between cardiorespiratory fitness and the *Firmicutes/Bacteroidetes* fecal count ratio in healthy young adults (**Table 5**).

Very short-term exercise intervention studies - detailed examination

Over two independent days, Lundgren-Kownacki et al.[38] assessed how water-rehydration affected the gut microbiota after a single load of physical work (a combination of different exercises). They only collected one fecal sample after each of two exercise days, but reported no change in the composition of the gut microbiota between these two days (the diet having been standardized before assessment) (**Table 6**). Zhao et al.[39] investigated the composition of the gut microbiota in 20 runners before and after a half-marathon (21.1 km), and fond the bacterial alpha diversity not to have changed after the race. However, they did observe an increase in fecal counts of class *Coriobacteria*, order *Coriobacteriales*, family *Coriobacteriaceae*, genus

Collinsella and *Collinsella aerofaciens* (all phylum *Actinobacteria*) after the race. Different families, genera and species belonging to the phyla *Proteobacteria* and *Firmicutes* also showed increased counts (**Table 6**). It should be noted that the study subjects received the same meal before each fecal collection. Shukla et al.[37], who performed a cycling test and collected fecal samples at baseline, 48 h and 72 h post-exercise, observed higher fecal counts for *Bacteroidetes* and other phyla (data not reported) at 72 h compared to baseline (**Table 6**). Finally, Scheiman et al.[32] evaluated how a marathon affected the gut microbiota after a marathon in 15 subjects. They observed that the fecal counts of genus *Veillonella* significantly increased after the marathon. Later, they confirmed the findings in a separate cohort of 11 ultramarathoners and Olympic rowers after a single bout of exercise. No study analyzed the effect of very short-term exercise on fecal SCFA concentrations (**Figure 3**).

Medium/long-term exercise intervention studies - detailed examination

Cronin et al.[40] ran an 8-week concurrent training intervention (three days per week), and observed the bacterial beta diversity, but not the alpha diversity, to have changed at the end of the study period. Similarly, Munukka et al.[29] ran a 6-week endurance intervention (cycling; three days per week) study involving women only, and observed bacterial beta diversity to have increased by the end of the study, whereas the alpha diversity remained unchanged (**Table 7**). However, after the intervention, fecal counts for phylum *Verrucomicrobia*, specifically family *Verrucomicrobiaceae* and genus *Akkermansia*, increased after adjusting for nutritional intake. In contrast, counts for phylum *Proteobacteria* had fallen (**Table 7**). Morita et al.[30] ran two exercise interventions of 12 weeks duration, endurance training (1 h of walking every day) or resistance training (1 h training per week). Fecal counts for the genus *Bacteroides* increased after endurance training while those for *Clostridium subcluster XIVa* (phylum *Firmicutes*) decreased. In contrast, *Clostridium cluster IX* (phylum *Firmicutes*) increased after the resistance intervention (**Table 7** and **Figure 5**). Keohane et al.[31] studied the effect of a long boat race (4 weeks) on the gut microbiota of three men. They collected four fecal samples (at baseline, mid-race, just before the race finished, and 3 months post-race), and showed bacterial alpha diversity to have changed from the middle of the race until the end. All participants returned increased fecal counts for the genus *Subdoligranulum UC*, *Dorea longicatena* and *Roseburia hominis*, and reduced counts for *Bacteroides finegoldii*. In one athlete, counts for *Prevotella copri* remained increased from the middle of the race until three months post-race. It should be noted that all athletes had the same diet during the race (**Table 7**). Allen et al.[47] conducted a 6-week endurance intervention (three days of endurance training per week) and collected

fecal samples at baseline, just after the intervention, and again 6 weeks later. The bacterial alpha and beta diversities did not change, either after the intervention or after the 6-week washout period. However, fecal counts for the order *Clostridiales*, genus *Roseburia*, genus *Lachnospira*, genus *Faeacalibacterium*, and genus *f_Lachnospiraceae unclass* (all phylum *Firmicutes*) increased after the intervention. The endurance training also increased some fecal SCFA concentrations (such as acetate and butyrate), but only in normal weight individuals. It should be noted that all the subjects in the latter study also followed an energy intake restriction diet during the intervention period (**Table 7**).

DISCUSSION

The present review indicates both physical activity level and cardiorespiratory fitness to be positively associated with bacterial alpha diversity in healthy humans, whereas the exercise interventions (either very short-term or medium/long-term) had little or no effect on bacterial alpha and beta diversities. Contrasting findings were detected regarding the effect of exercise interventions on fecal counts for the phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. In addition, some studies reported a positive association between physical activity/cardiorespiratory fitness and fecal SCFA concentration,[28,35] this agrees with the findings of one medium/long-term exercise intervention study.[47] Physical activity or exercise appears to have different effects on different species belonging to the same phylum. It is unclear whether diet has any bearing on these findings. Taking the examined results together, it would appear that exercise could influence the composition of the gut microbiota in healthy adults, but the heterogeneity of the available studies precludes any firm conclusions from being drawn.

How might exercise exert an effect on the gut microbiota?

Exercise affects several physiological systems,[14] including the skeletal muscle system.[23].Several studies have suggested bidirectional crosstalk to exist between the skeletal muscles and the gut - the so called muscle-gut[20] or gut-muscle[48] axis (**Figure 6**). This existence of this axis is based on the fact that the contraction of skeletal muscle during exercise has an anti-inflammatory effect due to the release of myokines.[49] Recently Hamasaki et al.[50] reported in a review that some myokines seem to play a role in mediating the secretion of glucagon-like peptide-1 (GLP-1, a key incretin involved in whole-body metabolism) in the gut during exercise.[11] Certainly, interleukin-6 (IL-6) is involved in the secretion of GLP-1 by the L-cells in the ileum.[51] Further evidence for the existence of the gut-muscle axis lies in the gut microbiota producing SCFAs - key mediators of energy metabolism in the mitochondria of skeletal muscles[52] that in turn help regulate

whole-body glucose metabolism.[53] Moreover, SCFAs interact with specific G-protein-coupled receptors (GPR41 and GPR43) on the intestinal L-cells,[54] stimulating the secretion of GLP-1.[55] Several bacteria are SCFA producers; *Bifidobacterium* (phylum *Actinobacteria*) produces acetate that can be transformed into butyrate, [56] whereas *Akkermansia* (phylum *Verrucomicrobia*) produces propionate and acetate.[57] Acetate and butyrate both enhance muscle fat oxidation, changing the oxidative status of muscle fibers.[58] Thus, acetate and butyrate enhance metabolic flexibility by improving the capacity to utilize and switch between lipid and carbohydrate fuels.[58] Butyrate also inhibits histone deacetylase, protecting against muscle protein catabolism and therefore preventing age-related muscle mass loss.[59] A recent study showed that the daily treatment of *Akkermansia munichipila* (*Verrucomicrobia* phylum), during 3 months, in obese individuals with metabolic syndrome is able to improve glucose and lipid metabolism, as well as body weight.[60] Curiously, work discussed in the present review suggests that fecal counts of the phyla *Actinobateria* and *Verrucomicrobia* are increased after very short-term[39] and medium/long-term[29] exercise interventions respectively, and that their numbers are related to physical activity[45,46] and cardiorespiratory fitness.[35] Recently, Scheiman et al.[32] demonstrated in mice that *Veillonella atypica* is able to metabolize lactate into acetate and propionate improving exercise performance, although this assumption remains to be demonstrated in humans. Moreover, Ehrenpreis et al. [61] showed exercise could make that SCFAs were more biologically available to colonic bacteria. We observed that SCFAs appear to increase after medium/long-term exercise interventions,[47] and are positively associated with physical activity level[28] and

cardiorespiratory fitness,[35] suggesting exercise to play a key role in the secretion of SCFAs, that stimulate the muscle-gut axis.

Figure 6: Main characteristics of the gut microbiota of a long-term-sedentary-behavior subject: dysbiosis, low bacterial alpha diversity, and low concentrations of short-chain fatty acids (SFCAs). It may be that after an exercise intervention, the gut microbiota becomes more eubiotic with a greater alpha diversity; SCFAs concentrations may also increase. The gold lines represent the possible cross-talk between the gut and the skeletal muscle (gut-muscle axis). The blue lines represent the possible cross-talk between the skeletal muscle and the gut (muscle-gut axis). However, most of the physiological mechanisms that explain these pathways remain unknown, or at least are not well understood; this is highlighted through the use of a question mark.

The diversity of microbes within the human body can be described in terms of their richness and evenness, i.e., by the number of species with respect to species abundance (alpha diversity). Several studies have shown that the greater the species diversity, the healthier the phenotype.[6,62] The reviewed results suggest that greater physical activity and cardiorespiratory fitness are associated with higher bacterial alpha diversity in healthy adults. [28,34,35,44,45] However, very short-term and medium/long-term exercise interventions (<8 weeks of endurance exercise) appear to have little effect on alpha diversity[29,38–40,47] (although after just 4 weeks of endurance exercise an effect was recorded in three athletes[31]). This suggests that human bacterial alpha diversity is affected only by longer exercise interventions. However, whether long term exercise really does increase bacterial alpha diversity, and the prevalence of species able to produce SCFAs (and therefore improve metabolic flexibility) remains a mystery. Some studies[63,64] suggest that long-term exercise interventions can indeed improve metabolic flexibility in humans, but the mechanisms that

might explain why are unknown. Further studies are needed to address these questions.

General limitations of the studies included in the present review

The cohorts included in the reviewed studies were quite heterogeneous. No comparisons between sexes or BMI categories could be made since the studies did not report the same kinds of data.

Most of the studies used self-reported questionnaires to determine physical activity,[27,28,34,44,45] with three[28,34,45] using validated questionnaires. However, it is well known that the estimation of physical activity by questionnaire (which is easy and inexpensive) is less accurate than more objective methods. [65] Bressa et al.[46] and Morita et al.[30] used an accelerometry-based method, which offers a potential solution to the problems associated with self-reported data.[65] Thus, the lack of observational studies that have used objective measurements of physical activity hinders drawing any reliable conclusions on the effect of physical activity on the gut microbiota.

Currently, all human and mouse studies that have addressed the effect of exercise on the gut microbiota have involved endurance interventions (running or cycling); less attention has been paid to resistance training,[30,40] which has a different physiological effect in humans.[66–68] Cronin et al.[40] made use of concurrent training (endurance + resistance exercises), although with this type of training it is impossible to know whether the endurance or resistance components have different effects. Morita et al.[30] ran endurance and resistance interventions in elderly adults, but these were dissimilar in terms of training time and intensity, making it impossible to know whether the effects on the gut microbiota were driven by the different types of exercise or the different total training times.

It should be remembered that gut microbiota is affected by the diet.[45,69–74] Four studies[31,38,39,47] used a standardized diet with their subjects before and during the intervention period, but this makes it impossible to know whether the changes seen in the gut microbiota were caused by the exercise intervention or the change in diet. New studies including a control group could solve this problem. Moreover, eight (44·4%) of the studies reviewed[28,33–35,37,40,44,45] did not take diet into account at all; the seven that did[27,29,30,32,36,46,47] monitored intake via food frequency questionnaires or 24 h diary records before collecting the fecal samples.

Eleven studies (61·1%)[27–29,31,32,35,39,40,44,46,47] used the advanced Illumina platform to sequence the gut microbiota, while seven used older techniques.[30,33,34,36–38,45] For assigning a taxonomic identity to the sequences, the most accurate platform is that of the Ribosomal Database Project (RDP; annotation error rate ~10%). This was used in only four studies,[27,37,39,47] while the SILVA and Greengenes databases

(error rate ~17%)[75] were used in two[29,45] and three[35,44,46] respectively. The remaining studies (n=9)[28,30-34,36,38,40] used other databases. Moreover, the studies examined in the present review reported their data at different taxonomic levels, making comparisons difficult.

Future research needs

Currently, we are not close to understanding the effect of exercise on the human gut microbiota. Future studies should bear in mind the shortcomings highlighted by the present review:

Data homogeneity. Future studies should report information with respect to age, BMI and gender. It needs to be clear whether the effect of exercise on the gut microbiota is different in these respects.

Physical activity. Most of the observational studies (55%) discussed in the present review used self-reported questionnaires to determine physical activity levels. Objectively measured physical activity (e.g., via accelerometry), would improve the reliability of any associations seen with changes in the gut microbiota.

Type of exercise. When designing future studies, the importance of the type of exercise (endurance vs. resistance), intensity (moderate vs. vigorous), and subjects status (untrained vs. trained individuals) should be understood.

Diet: The gut microbiota is easily affected by changes in food intake.[45,69-74] Future studies should control at least what their participants eat during the exercise interventions, and before stool collection. Reporting these data as descriptive values will help reveal the general effects of exercise.

Quantification of the gut microbiota. Interest in the human gut microbiota is growing fast, and the technology needed to examine it advancing rapidly. Future studies should use state-of-the-art technologies, such as the *Illumina* platform and RDP annotation. They should also focus on the effect of exercise interventions at all taxonomical levels. Some of the studies discussed in the present work[36,37] reported information at the phylum level, but species within the same phylum were seen to be affected differently by exercise. Reporting at the species level would be a great step forward.

Mechanistic studies. Mouse and human experiments should focus on elucidating the possible mechanisms via which exercise might influence the gut microbiota or *vice versa*. Basically, the unknown mechanistic pathways behind the gut-muscle and muscle gut axes need to be investigated (Figure 6).

Publication bias. Negative results in science are just as important as positive results.[76] In the present review, 11 (61·1%) studies reported only positive results; they did not report whether they

observed any negative or null effects. This type of information is crucial.

CONCLUSIONS

Based on the 18 studies included in the present review, physical activity and cardiorespiratory fitness seem to be positively associated with bacterial alpha diversity, fecal counts for certain bacterial phyla, and fecal SCFA concentrations in healthy adults. Exercise interventions seem to influence fecal counts for certain phyla. However, the heterogeneity of the examined studies precludes any stronger conclusions from being drawn. Thus, although the current evidence points towards exercise having an effect on the human gut microbiota, more and better designed studies are needed if this is to be confirmed, and the mechanism involved are to be understood.

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RESULTS AND DISCUSION

SECTION II

STUDY IV: A 24-week exercise intervention slightly modifies fecal microbiota composition in young sedentary adults: a randomized controlled trial

(In preparation)

ABSTRACT

Background

The human intestine is composed of different microorganisms, commonly known as gut microbiota, which influences the host's health. Exercise training induces a myriad of physiological adaptations in the human metabolism but it is not completely understood whether exercise training alters gut microbiota composition in humans. Based on that we investigated the effect of a 24-week supervised exercise intervention, at different intensities, on fecal microbiota diversity and composition in young sedentary adults.

Methods

61 young sedentary adults aged between 18-25 years participated in this study. The participants were randomized into control (CON: no exercise), (ii) moderate-intensity exercise (MOD-EX), and (iii) vigorous-intensity exercise (VIG-EX) groups. Fecal samples were collected before and 24 weeks after the exercise intervention, and fecal microbiota diversity and composition were analyzed with 16S rRNA sequencing.

Results

None of the exercise intensity programs modified beta or alpha diversities. However, VIG-EX group reduced the relative abundance of *Erysipelotrichia* class and *Erysipelotrichales* order (*Firmicutes* phylum) compared to the CON group (-0.4±1.2%), whereas both MOD-EX (-0.3±1.2%) and VIG-EX (-0.1±0.9%) groups reduced the relative abundance of *Erysipelotrichaceae* family compared to the CON group. Curiosly, MOD-EX group increased the relative abundance of *Acidaminoccocus* genus (*Firmicutes* phylum) (0.5±1.0%), likewise lactate dehydrogenase operon pathway in the feces (0.60±1.66%), compared to the CON and VIG-EX groups. Interestingly, the changes observed after exercise training in the fecal microbiota composition were not related to the cardiometabolic benefits induced by exercise.

Conclusions

A 24-week supervised exercise intervention, mainly at moderate intensity, slightly modifies gut microbiota composition in young sedentary adults.

INTRODUCTION

Microorganisms that colonize the gastrointestinal tract are known as gut microbiota, including bacteria, archaea, and eukarya[1]. All these microorganisms compose a gut ecosystem, which has multiple effects and metabolic functions on the organism[2]. The gut microbiota is composed of five dominant phyla (i.e., *Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia,* and *Actinobacteria*[3]), where *Firmicutes* and *Bacteroidetes* represent more than 70% of the total microorganisms within the gut in humans[4]. The equilibrium in the gut microbiota community is described as eubiosis, which is related to a healthier status[5]. However, when this homeostasis is disturbed is commonly known as dysbiosis that is related to an unhealthier status (e.g., obesity)[5].

Exercise is considered a non-pharmaceutical therapy to combat many chronic diseases such as obesity and type 2 diabetes[6,7], because it increases the overall metabolic activity [8]. Although exercise is a great tool to prevent and combat obesity, the molecular mechanisms mediating this protective effect are not well understood. In preclinical studies, exercise seems to restore eubiosis in the gut of metabolically compromised mice[9-11], whereas the evidence available in humans is scarce[12]. Observational studies have shown that higher levels of physical activity are related to higher fecal microbiota diversity and higher relative abundance of *Firmicutes* and *Bacteroidetes* [17-25] in humans. Similarly, findings from intervention studies indicate that exercising for less than three months modifies fecal microbiota composition in lean and overweight/obese men and women [26-28]. However, they observed that the exercise-induced changes in gut microbiota composition were not related to the exercise-induced benefits in cardiometabolic outcomes [26,27]. Altogether, these studies suggest that short-term exercise training might modify gut microbiota composition, the effects of longer exercise interventions being however unknown.

We investigated the effect of a 24-week supervised exercise intervention, at moderate and vigorous intensities, on fecal microbiota diversity and composition in young sedentary adults, and whether those changes are related to cardiometabolic benefits induced by exercise.

MATERIAL AND METHODS

Participants

A total of 145 young adults aged between 18-25 years old participated in the ACTIBATE study[29] (**Fig. 1**), an exercise-based randomized

controlled trial (ClinicalTrials.gov ID: NCT02365129) that aimed to investigate whether a 24-week exercise intervention was able to activate and recruit human brown adipose tissue. The inclusion criteria were: (i) being sedentary (less than 20min physical activity on less than 3 days per week), and (ii) having a stable body weight over the last 3 months. The exclusion criteria were: (i) smoker, (ii) suffering an illness that would limit the capacity to participate in the study, (iii) taking antibiotics or medication that affects the cardiovascular system, (iv) being pregnant. The study protocol and the written informed consent were performed in accordance with the last revised Declaration of Helsinki, and were approved by the Ethics Committee on Human Research of the University of Granada (n°.924), and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada). All participants signed informed consent.

Study design

The subjects were recruited from the province of Granada (Spain) through social networks, local media, and posters. After baseline examination, the participants were randomly assigned into three different groups using a computer-generated simple randomization[30]: (i) control group (CON), (ii) moderate-intensity exercise group (MOD-EX), and (iii) vigorous-intensity exercise group (VIG-EX). All participants recruited were instructed to not modify their daily routine, physical activity, and dietary patterns. All measurements were conducted before and 24-week after the intervention.

Supervised exercise training programs

A detailed description of the supervised exercise training program can be found elsewhere[29]. Briefly, the duration of the exercise training intervention was for 24-week, participants attended between 3-4 times per week accumulating a total of 80 minutes of resistance exercise and 150 minutes of endurance exercise training weekly. Both resistance and endurance training were individualized to the individual capacity of the participants. MOD-EX and VIG-EX groups performed the resistance training at 50% of the repetition maximum (RM) and 70% RM respectively. The load for resistance exercises was adjusted monthly. The endurance training was performed at 60% of the heart rate reserve (HRres) in MOD-EX, whereas VIG-EX performed 75 min/week at 60% HRres and 75min/week at 80% HRres. The exercise program was organized in 5 phases of the same extension, except for the first phase (familiarization phase).

Each group had 10-12 participants. They went at the same time of the day during the 24-week program: 8.30 a.m. to 10.30 a.m., 4 p.m. to 6 p.m., 6 p.m. to 8 p.m., and 8 p.m. to 10 p.m. We used an electronic

attendance sheet to register attendance, whereas we used heart rate monitors (Polar Electro Öy, Kempele, Finland) to quantify the adherence to the intensity that was prescribed. To maintain participant's attendance and adherence to the training program, which both were strictly monitored, participants could do the day workout outside of the research center when they could not attend to research training.

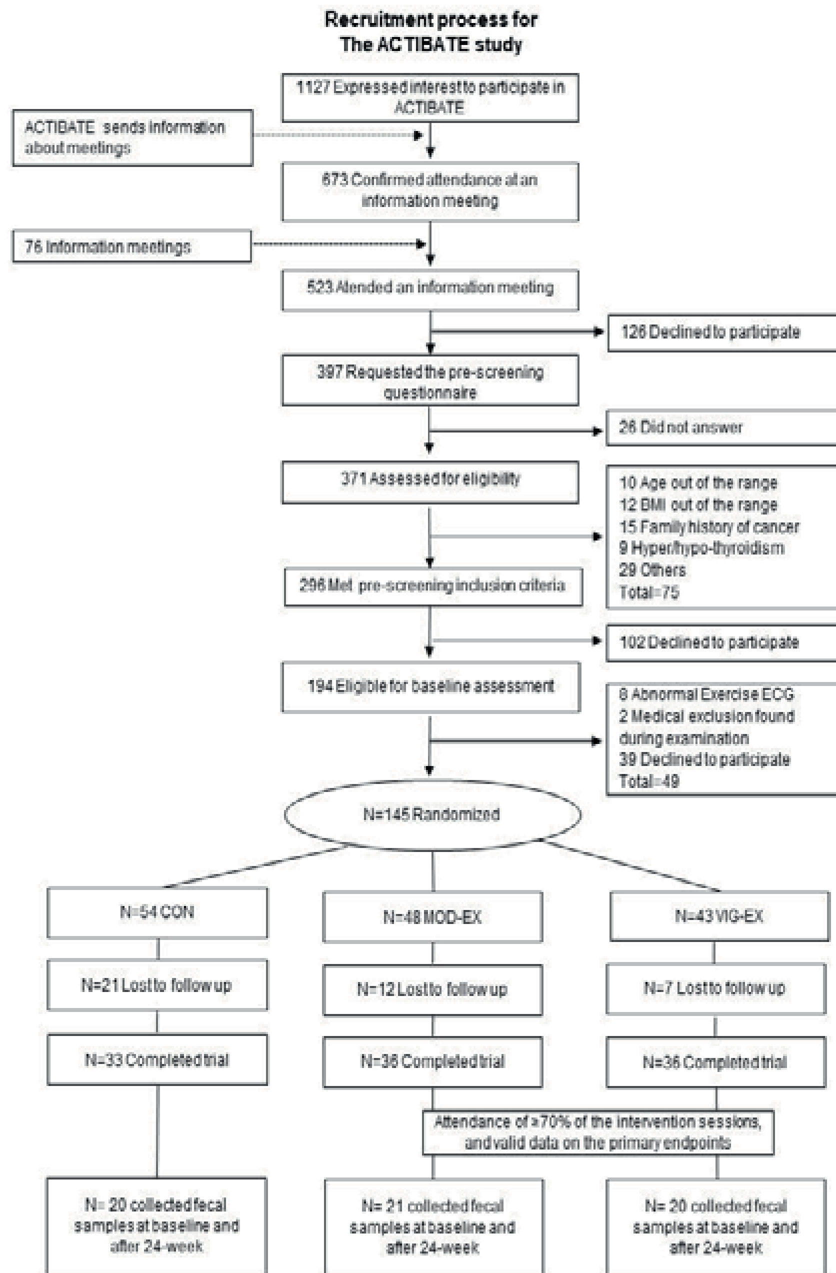

Figure 1: Flow-chart diagram of the study participants. *Abbreviation*: CON: control group; MOD-EX: moderate-intensity exercise group; VIG-EX: vigorous-intensity exercise group.

Primary endpoints: Fecal microbiota analysis

Fecal DNA extraction

Fecal samples (50-60g) were obtained before and just after the intervention (48-72h after the last exercise session) in a subsample of 61 individuals. Fecal samples were collected and introduced in a 60mL plastic sterile containers and were stored in the home freezer before being transported to our laboratory in a portable cooler with an ice plate. Upon arrival, the fecal samples were stored at -80°C until DNA extraction. We used a Stomacher® 400 blender (A. J. Seward and Co. Ltd., London, UK) to homogenize the fecal samples and QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain) to extract and purification DNA, according to the manufacturer's instructions. Finally, we used the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) to concentrate and purify DNA.

Sequencing

Extracted DNA was amplified by PCR targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene using primer pairs: Forward Primer (CCTACGGGNGGCWGCAG) and Reverse Primer (GACTACHVGGGTATCTAATCC). All PCRs were carried out in 25 μL final reaction volume, including: 12.5 μL of the 2X KAPA HiFi Hotstart prepared mixture (KAPA Biosystems, Woburn, MA, USA), 5 μL of each forward and reverse primers (1 μM), and 2.5 μL of extracted DNA (10ng). All PCRs following the next program: (i) denaturation at 95°C for 3 min, (ii) 8 denaturation cycles at 95°C for 30 s, (iii) annealing at 55°C for 30 s, (iv) elongation at 72°C for 30 s, (v) a final extension at 72°C for 5 min. Next, we used AMPure XP microspheres (Beckman Coulter, Indianapolis, IN, USA) to purify the 16S V3 and V4 amplicon away from free primers and primer-dimer species. Then, we used the Nextera XT index kit (Illumina, San Diego, CA, USA) to tag DNA with sequencing adapters. The pooled PCR products were purified using AMPure XP balls (Beckman Coulter, Indianapolis, IN, USA) before quantification. Lastly, we use Illumina MiSeq paired-end sequencing system (2x300nt) (Illumina, San Diego, CA, USA) to sequence amplicons.

Bioinformatics: Determination of fecal microbiota diversity, composition, and function

Dada 2[31] package version 1.10.1 in R software[32] was used for analyzing the raw sequences (FastQ files). All samples reached a cut-off of 10,000 reads were considered valid for subsequent analyses. Samples were standardized to equal size of 30,982 reads using the Phyloseq[33] package in R software returning 19,373 phylotypes. Phylotypes were

assigned to their specific taxonomic affiliation (phylum to genus) based on the naive Bayesian classification with a pseudo-bootstrap threshold of 80%[33] using the Classifier function in Ribosomal Data Project (RDP)[34]. In order to determine species taxonomies, we used Seqmatch function in RDP according to criteria published previously[35].

Diversities: Beta diversity is a measurement of the number of species shared among the bacterial community from the participants[36]. Alpha diversity is a measurement of the number of different phylotypes and the relative abundance of these phylotypes within the same participant[36]. Alpha diversity was calculated following 4 different proxies: i) species richness is the count of different phylotypes observed in the bacterial community[37]; ii) evenness index refers to the equitability of the phylotypes frequencies in a bacterial community[36]; iii) Shannon index is the number and equitability of the phylotypes in the bacterial community[37], and iv) inverse Simpson index is calculated by classical Simpson index, it is referred richness in a community with uniform evenness[38].

Composition: We obtained 16 different phyla and 240 genera. For the main analysis, we used relative abundances as the reads percent of each phylotype relative to the total number of reads. We performed the analyses when the average of the relative sequence abundance was higher than 1%.

Function: We annotated microbial genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KOs). The metabolic and functional contribution was inferred from 16rRNA data using the Tax4fun[39] program built-in Galaxy[40] private instance of the DengLab[41] (http://mem.rcees.ac.cn:8080). For the present study, we included estimations of the amino acid, carbohydrate, energy, lipid, and nucleotide metabolisms (54 pathways) and lactate functional pathways (11 pathways).

Secondary endpoints:

Body composition

We measured the participants´ weight and height barefoot, without shoes and wearing light clothing, using a SECA scale and stadiometer (model 799; Electronic Column Scale, Hamburg, Germany). Body composition (fat mass, lean body mass, and visceral adipose tissue mass) was measured by dual energy X-ray absorptiometry scan (Hologic Discovery Wi, Marlborough, MA). The body mass index (BMI) was calculated as weight/height^2 (kg/m^2).

Cardiometabolic profile

Blood samples were collected after 10-h overnight fasting, between 08:00 and 09:00 AM. Serum (obtained with Vacutainer® SST™ II Advance tubes) and plasma (obtained with Vacutainer® Hemogard™ tubes) were centrifuged directly in the laboratory (10000 rpm, 10 minutes, 4°C temperature). Then, they were stored at -80°C freezer for future analyses. Serum glucose, total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), and triacylglycerols concentrations were assessed following standard methods using an AU5832 biochemical analyzer (Beckman Coulter Inc., Brea CA, USA). Low-density lipoprotein-cholesterol (LDL-C) was estimated with the Friedewald formula [42]: [total cholesterol – HDL-C – (triglycerides/5)], in mg/dL[42]. Insulin was measured using the Access Ultrasensitive Insulin Chemiluminescent Immunoassay Kit (Beckman Coulter Inc., Brea CA, USA). The homeostatic model assessment (HOMA) index was calculated as [insulin (μU/mL) x glucose (mmol/L)/22.5][43]. C-reactive protein (CRP) was measured by immunoturbidimetric assay, employing the same AU5832 automated analyzer as above. Finally, plasma lactate concentration was determined by colorimetric methods (kit 1001330, Spinreact, Girona, Spain).

Dietary intake

The EvalFINUT® software was used to assess the dietary intake (energy and nutrient intake) from three 24-h dietary recalls, as we extensively described elsewhere[44]. Qualified and trained dietitians undertook the 24-h dietary recalls face-to-face on 3 separate days (2 weekdays and one weekend day). Two dieticians introduced all data from interviews independently in the software. Dietary energy density was calculated by dividing the energy contained in foods and beverages (excluding water) between the total weight of daily foods and beverages (expressed as kcal/g)[45].

Statistical analysis

The descriptive parameters are reported as mean and standard deviation unless otherwise stated. All analyses were conducted for men and women together since there was no sex*exercise interaction on the primary endpoints of this study (all $P \geq 0.05$; data not shown). Due to non-normal distribution (tested by D'Agostino & Pearson test) outcomes, the analyses were determined by non-parametric tests. Moreover, since a small value was obtained in microbial metabolic functional pathway outcomes, these were log2 transformed.

To study baseline phenotypical characteristics differences between groups, we used a one-way analysis of variance (ANOVA). We used permutational multivariate analysis of variance (PERMANOVA) with 9,999 random permutations based on Bray-Curtis dissimilarity, to

investigate differences in beta diversity between groups in Paleontological Statistics Software Package 3.0 (Past3)[46]. With the *Vegan* package in R software, we visualized beta diversity relationships at phylum and genus levels at Principal coordinate analysis (PCoA) in two-dimensional scatter plots. Differences in alpha diversity and composition between groups were performed with the Kruskal Wallis test.

A delta (Δ; post *minus* baselines values) was created for every single outcome. To study the effect of exercise on fecal microbiota diversity, composition and, microbial metabolic functional pathway analyses of covariance (ANCOVA) were performed adjusting by baseline values. These analyses were represented as heatmap plots using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Then, we used Spearman correlation to study the relationship between Δ fecal microbiota outcomes and Δ secondary outcomes. Bonferroni post hoc adjustments for multiple comparisons were used to examine differences between the three groups. Finally, Spearman correlations were performed to the investigated relationship between plasma levels of lactate and certain bacteria-consumer lactate (*Acidaminococcus* genus and *Acidaminococcus intestine* species) at baseline.

We performed sensitivity analyses adjusting by false discovery rate (FDR), using Benjamini, Krieger, and Yetutieli (desired false discovery rate = 5%). The level of significance was assumed at $P < 0.05$. We used the Statistical Package for Social Sciences (SPSS, v.22.0, IBM SPSS Statistics, IBM Corporation) to perform the statistical analysis. Graphical plots were generated using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and *gplot* package in *R* software.

RESULTS

Phenotypical characteristics were similar across groups at baseline

Figure 1 shows the flowchart and enrollment of the 61 participants included in the main analyses. At baseline, beta diversity was similar between groups at phylum and genus taxonomic levels (all $P \geq 0.205$; **Fig.2A**). Similarly, there were no differences in alpha diversity parameters (i.e., *species richness*, *evenness index*, *Shannon index*, or *inverse Simpson index*; all $P \geq 0.095$; **Fig.2B**), or fecal microbiota composition (all $P > 0.05$; **Fig.2C**). Moreover, no significant differences were observed at baseline in secondary endpoints (i.e., body composition, cardiometabolic profile, and energy and macronutrient intake) between groups (**Table 1**; All $P \geq 0.292$).

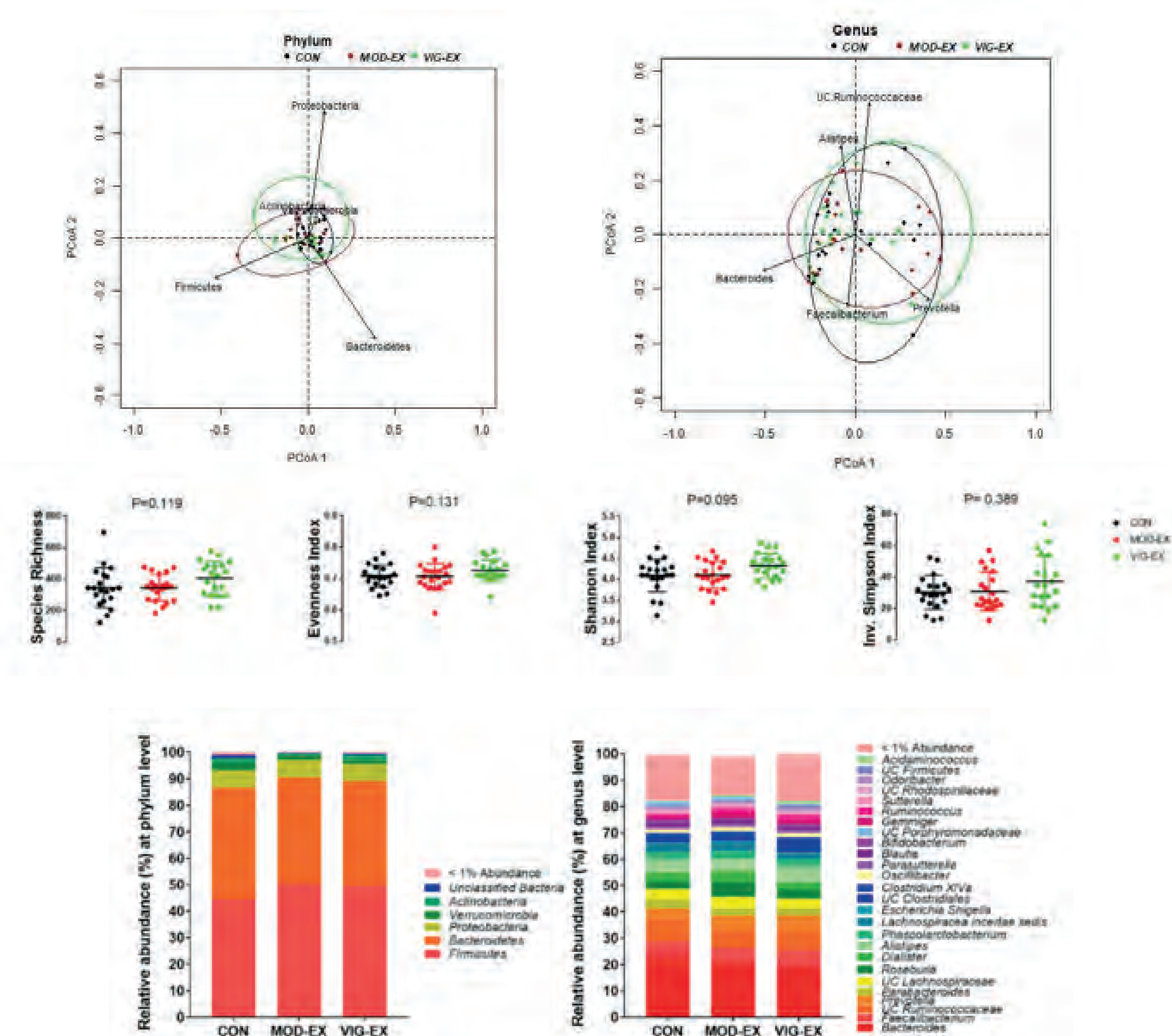

Figure 2: Descriptive fecal microbiota diversity and composition parameters at baseline. Panel A shows differences in the fecal microbiota community structure at phylum and genus taxonomic levels visualized by principal coordinate ordination analyses (PCoA) using Bray-Curtis dissimilarity; ellipses represent the 95% confident interval for every single group. Genus PCoA only shows the five genera of higher abundance. Panel B shows different indexes of fecal microbiota alpha diversity (species richness, evenness index, Shannon index and inverse Simpson index) Panel C indicates the relative abundance of the fecal microbiota composition at phylum and genus taxonomic level. Stacked bar represented percentage of relative abundance. All the results are depicted by CON (n=20), MOD-EX (n=21) and VIG-EX (n=20). P values were obtained from Kruskal-Wallis tests. *Abbreviation*: CON: control group; MOD-EX: moderate-intensity exercise group; UC: unclassified; VIG-EX: vigorous-intensity exercise group.

Table 1. Baseline characteristics of the study participants

	CON (n=20)			MOD-EX (n=21)			VIG-EX (n=20)			
	Mean	±	SD	Mean	±	SD	Mean	±	SD	P
Demographics										
Age (years old)	22.2	±	2.2	21.9	±	2.3	21.5	±	2.4	
Male (n/%)	7		35	5		24	6		30	
Female (n/%)	13		65	16		76	14		70	
Body composition										
BMI (kg/m²)	24.1	±	4.3	26.0	±	5.3	24.8	±	4.6	0.441
Lean mass (kg)	41.5	±	10.1	42.4	±	8.7	41.7	±	8.2	0.944
Fat mass (kg)	23.7	±	8.4	27.9	±	10.8	25.8	±	8.1	0.348
FM (%)	34.9	±	8.5	38.0	±	7.7	36.5	±	7.1	0.566
VAT mass (g)	321.8	±	178.4	380.4	±	220.2	344.1	±	178.7	0.623
Cardiometabolic profile										
Glucose (mg/dL)	88.4	±	7.3	88.7	±	6.6	86.4	±	5.2	0.461
Insulin (µUI/mL)	8.7	±	6.8	8.6	±	3.7	8.7	±	4.3	0.999
HOMA index	2.0	±	1.8	1.9	±	0.9	1.9	±	1.0	0.977
TC (mg/dL)	156.6	±	35.9	165.3	±	32.3	173.6	±	33.5	0.292
HDL-C (mg/dL)	51.6	±	10.1	51.7	±	14.0	52.4	±	14.4	0.981
LDL-C (mg/dL)	89.3	±	31.2	98.0	±	28.2	100.3	±	21.3	0.403
TG (mg/dL)	78.6	±	53.1	87.9	±	70.8	102.1	±	49.3	0.447
CRP (mg/L)	2.1	±	2.1	3.5	±	4.4	2.6	±	2.9	0.396
Energy and macronutrient intake										
Energy (kcal/day)	1905.2	±	381.2	1911.8	±	470.7	1919.2	±	465.7	0.995
Fat (g/day)	84.6	±	21.1	85.4	±	24.9	87.4	±	21.9	0.925
Protein (g/day)	76.7	±	21.0	77.0	±	22.8	80.7	±	18.4	0.794
Carbohydrate (g/day)	241.3	±	117.1	281.2	±	101.4	276.3	±	113.3	0.462
Fiber (g/day)	17.2	±	6.8	16.8	±	5.6	15.4	±	4.1	0.548

Data are presented as mean and standard deviation. P values were obtained from one-way analyses of variance. *Abbreviations*: BMI: Body mass index, CON: Control group, CRP: C-reactive protein, FM: Fat mass, HDL-C: high-density lipoprotein cholesterol, HOMA index: homeostatic model assessment–insulin resistance index, LDL-C: low density lipoprotein cholesterol, MOD-EX: Exercise moderate intensity group, TC: Total cholesterol, TG: triglycerides, VAT: Visceral adipose tissue, VIG-EX: Exercise vigorous intensity group.

24-week of exercise training does not modify fecal microbiota diversity, whereas slightly modifies fecal microbiota composition

The exercise intervention did not modify the fecal microbiota beta (data not shown) and alpha diversities (all P≥0.062, **Fig.3A**). However, VIG-EX decreased the relative abundance of *Erysipelotrichia* class and *Erysipelotrichales* order (*Firmicutes* phylum) compared to the CON, but not compared to the MOD-EX groups [Δ mean and standard deviation: Δ CON=0.4±1.3%; Δ MOD-EX=0.0±0.4%; Δ VIG-EX=-0.4±1.2%; P=0.015, **Fig.3B** and **C**; **Table 2**]. Moreover, MOD-EX and VIG-EX reduced the relative abundance of the *Erysipelotrichaceae* family (*Firmicutes* phylum) compared to the CON group [Δ CON=0.2±1.3%; Δ

MOD-EX=-0.3±1.2%; Δ VIG-EX=-0.1±0.9%; P=0.015, **Fig.3C** and **Table 2**]. However, MOD-EX increased the relative abundance of *Acidaminoccocus* genus (*Firmicutes* phylum), compared to CON and VIG-EX groups [Δ CON=-0.2±0.6%; Δ MOD-EX=0.5±1.0%; Δ VIG-EX=-0.5±1.3%; P=0.015, **Fig.3D** and **Table 2**]. However, MOD-EX was not able to modify the relative abundance of the bacterial species belonging to the *Acidaminococcus* genus (i.e., *Acidaminococcus intestini* and *Acidaminococcus fermentans;* all P≥0.135, **Fig.4**). All the significant differences remained after FDR corrections (data not shown).

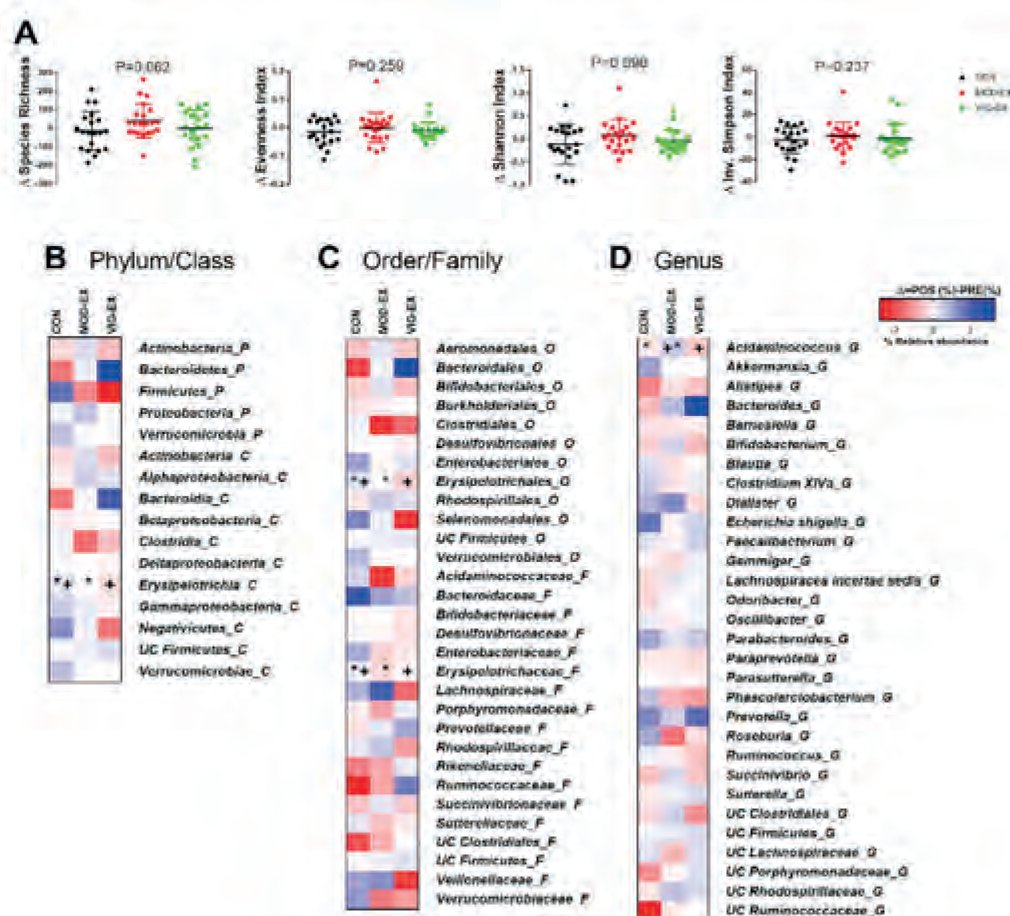

Figure 3: Effect of 24-week of exercise training on fecal microbiota diversity and composition. Panel A shows different indexes of fecal microbiota alpha diversity (species richness, evenness index, Shannon index and inverse Simpson index). Panel B shows differences in fecal microbiota composition at phylum (P) and class (C) taxonomic levels. Panel C shows differences in fecal microbiota composition at order (O) and family (F) taxonomic levels. Panel D shows differences in fecal microbiota composition at genus (G) taxonomic level. P values were obtained from one-way analyses of variance adjusted by baseline value. Symbols * and + represent significant differences between groups. *Abbreviation*: CON: control group; MOD-EX: moderate-intensity exercise group; UC: unclassified; VIG-EX: vigorous-intensity exercise group.

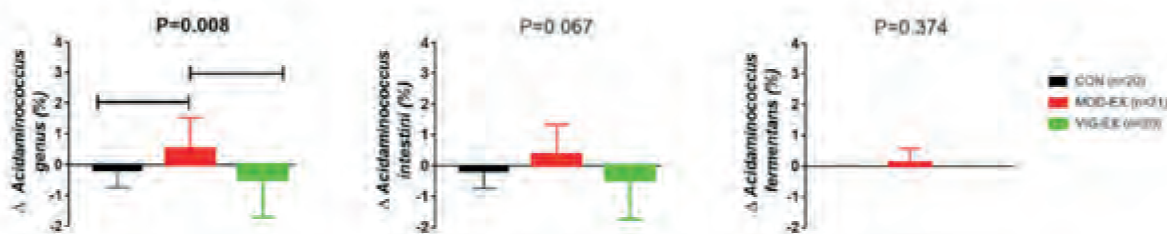

Figure 4: Effect of 24-week of exercise training on the relative abundance of *Acidaminococcus* genus, *Acidaminococcus intestini* and *Acidaminococcus fermentans* species. Data are presented as mean and standard deviation. Parallel horizontal bars represent significant differences between groups. P values were obtained from one-way analyses of variance adjusted by baseline values. *Abbreviation*: CON: control group; MOD-EX: moderate-intensity exercise group; VIG-EX: vigorous-intensity exercise group.

Table 2. Effect of a 24 weeks exercise intervention on fecal microbiota diversity and composition

	CON (n=20)			MOD-EX (n=21)			VIG-EX (n=20)			
	Mean	±	SD	Mean	±	SD	Mean	±	SD	P
Phylum (relative abundance %)										
Actinobacteria	-0.5	±	2.1	0.5	±	1.6	-0.9	±	1.5	0.341
Bacteroidetes	-2.1	±	12.3	0.4	±	13.1	4.0	±	10.6	0.407
Firmicutes	2.7	±	16.2	-1.9	±	14.0	-3.0	±	12.1	0.898
Proteobacteria	0.2	±	6.2	1.0	±	7.0	-0.1	±	4.5	0.720
Verrucomicrobia	1.0	±	6.0	0.1	±	3.3	0.0	±	2.5	0.349
Class (relative abundance %)										
Actinobacteria	-0.5	±	2.1	0.5	±	1.6	-0.9	±	1.5	0.341
Alphaproteobacteria	-0.2	±	2.6	0.8	±	2.9	0.4	±	2.8	0.577
Bacteroidia	-2.1	±	12.3	0.2	±	13.1	3.5	±	10.7	0.526
Betaproteobacteria	-0.2	±	3.2	-0.1	±	2.2	0.0	±	1.9	0.917
Clostridia	0.2	±	11	-2.5	±	13.5	-0.9	±	8.8	0.925
Deltaproteobacteria	0.05	±	1.10	0.00	±	0.70	-0.20	±	0.70	0.805
Erysipelotrichia	0.4*†	±	1.3	0.0*	±	0.4	-0.4†	±	1.2	0.015
Gammaproteobacteria	0.8	±	5.3	0.3	±	5.4	-0.2	±	2.9	0.716
Negativicutes	2.0	±	7.2	0.2	±	4.9	-2.1	±	4.6	0.080
UC Firmicutes	0.1	±	0.9	0.2	±	0.5	0.6	±	1.6	0.723
Verrucomicrobiae	1.1	±	5.8	0.1	±	3.3	0.0	±	2.5	0.345
Order (relative abundance %)										
Aeromonadales	-0.6	±	2.5	0.4	±	4.4	-0.5	±	2.2	0.476
Bacteroidales	-2.1	±	12.3	0.2	±	13.1	3.5	±	10.7	0.526
Bifidobacteriales	-0.4	±	1.9	0.4	±	1.5	-0.6	±	1.0	0.466
Burkholderiales	-0.2	±	3.2	-0.1	±	2.2	0.0	±	1.9	0.915
Clostridiales	0.2	±	11.5	-2.4	±	13.6	-0.9	±	8.9	0.925
Desulfovibrionales	0.1	±	1.1	0.0	±	0.7	-0.2	±	0.7	0.805
Enterobacteriales	1.5	±	4.5	0.0	±	3.0	0.4	±	2.0	0.301

Erysipelotrichales	0.4*†	±	1.4	0.0*	±	0.4	-0.4†	±	1.2	0.015
Rhodospirillales	-0.2	±	2.7	0.8	±	2.9	0.4	±	2.8	0.577
Selenomonadales	2.0	±	7.2	0.2	±	4.9	-2.1	±	4.6	0.093
UC Firmicutes	0.1	±	0.9	0.2	±	0.5	0.6	±	1.6	0.723
Verrucomicrobiales	1.1	±	5.9	0.1	±	3.3	0.0	±	2.5	0.345
Family (relative abundance %)										
Acidaminococcaceae	0.7	±	7.2	-2.5	±	10.0	-0.4	±	4.5	0.987
Bacteroidaceae	3.5	±	11.8	1.5	±	12.7	0.2	±	14.0	0.942
Bifidobacteriaceae	-0.1	±	2.0	0.0	±	1.8	-0.2	±	1.6	0.654
Desulfovibrionaceae	0.0	±	1.3	-0.1	±	1.1	-0.3	±	1.3	0.920
Enterobacteriaceae	1.3	±	5.7	-0.4	±	4.4	-0.2	±	3.3	0.323
Erysipelotrichaceae	0.2*†	±	1.3	-0.3*	±	1.2	-0.1†	±	0.9	0.015
Lachnospiraceae	1.2	±	11.9	2.9	±	9.6	-1.4	±	7.4	0.386
Porphyromonadaceae	-0.2	±	8.5	-0.9	±	4.8	0.0	±	5.4	0.855
Prevotellaceae	-0.3	±	13.6	0.4	±	9.5	1.3	±	15.5	0.910
Rhodospirillaceae	0.3	±	1.4	0.9	±	4.3	-0.9	±	5.2	0.760
Rikenellaceae	-1.4	±	4.5	-0.9	±	3.4	0.2	±	5.2	0.236
Ruminococcaceae	-2.8	±	8.7	-0.9	±	7.7	2.4	±	7.9	0.258
Succinivibrionaceae	-0.6	±	2.5	0.4	±	4.4	-0.5	±	2.2	0.476
Sutterellaceae	0.4	±	3.2	-0.6	±	4.4	-0.4	±	3.1	0.866
UC Clostridiales	-2.1	±	7.1	-0.8	±	5.8	0.5	±	4.6	0.240
UC Firmicutes	0.1	±	0.9	0.2	±	0.5	0.6	±	1.6	0.723
Veillonellaceae	1.5	±	6.9	1.8	±	8.7	-2.1	±	6.2	0.108
Verrucomicrobiaceae	1.7	±	8.1	-1.4	±	6.6	-1.0	±	4.6	0.187
Genus (relative abundance %)										
Acidaminococcus	-0.2†	±	0.6	0.5*†	±	1.0	-0.5*	±	1.3	0.008
Akkermansia	1.1	±	5.9	0.1	±	3.3	0.0	±	2.5	0.345
Alistipes	-1.4	±	2.7	-0.2	±	2.7	-0.6	±	2.6	0.360
Bacteroides	-0.6	±	11.5	0.8	±	10.4	2.6	±	10.0	0.865
Barnesiella	-0.3	±	1.2	-0.2	±	1.3	-0.4	±	1.4	0.985
Bifidobacterium	-0.4	±	1.9	0.4	±	1.5	-0.6	±	1.0	0.465
Blautia	0.3	±	0.9	-0.3	±	1.8	0.0	±	1.0	0.173
Clostridium XlVa	0.6	±	1.4	-0.3	±	1.2	0.2	±	1.0	0.169
Dialister	0.8	±	3.9	1.3	±	4.4	-0.2	±	2.2	0.167
Echerichia Shigella	1.6	±	4.9	-0.1	±	3.0	0.4	±	1.9	0.277
Faecalibacterium	0.3	±	5.5	-0.1	±	3.5	0.6	±	5.7	0.657
Gemmiger	-0.1	±	1.3	-0.3	±	2.0	0.2	±	1.5	0.547
Lachnospiracea incertae sedis	-0.4	±	2.3	0.2	±	2.8	0.0	±	2.3	0.503
Odoribacter	-0.3	±	0.6	0.0	±	0.7	0.1	±	0.7	0.075
Oscillibacter	0.2	±	1.2	-0.2	±	0.9	0.1	±	1.8	0.863
Parabacteroides	1.0	±	2.5	0.3	±	1.6	0.4	±	2.1	0.681
Paraprevotella	-0.2	±	0.7	-0.2	±	1.3	-0.3	±	1.0	0.261
Parasutterella	0.0	±	3.2	-0.1	±	1.7	-0.2	±	1.3	0.838
Phascolarctobacterium	0.7	±	4.7	-0.7	±	1.6	-0.9	±	3.7	0.334
Prevotella	1.4	±	8.0	0.5	±	5.4	1.7	±	6.0	0.846
Roseburia	1.0	±	4.2	-1.5	±	6.5	-0.2	±	2.1	0.794
Ruminococcus	0.2	±	1.5	-0.2	±	1.6	-0.6	±	2.1	0.368
Succinivibrio	-0.6	±	2.5	0.4	±	4.4	-0.5	±	2.2	0.476
Sutterella	-0.2	±	1.7	0.1	±	1.6	0.3	±	1.1	0.658

UC Clostridiales	-0.3 ± 1.7	0.5 ± 2.4	-1.0 ± 4.4	0.534
UC Firmicutes	0.1 ± 0.9	0.2 ± 0.5	0.6 ± 1.6	0.723
UC Lachnospiraceae	0.3 ± 3.2	-0.7 ± 3.2	0.3 ± 2.0	0.713
UC Porphyromonadaceae	-1.1 ± 3.0	-0.1 ± 1.9	0.3 ± 1.0	0.109
UC Rhodospirillaceae	-0.2 ± 2.7	0.6 ± 2.9	0.3 ± 2.7	0.659
UC Ruminococcaceae	-2.8* ± 6.4	-0.2 ± 3.5	0.2* ± 3.1	0.039

Data are presented as mean and standard deviation. P values were obtained from one-way analyses of variance adjusted by baseline values. Symbols * and † mean significant differences after Bonferroni corrections. Abbreviations: CON: Control group, MOD-EX: Moderate-intensity exercise group, UC: unclassified, VIG-EX: Vigorous-intensity exercise group.

24-week of exercise training upregulated lactate functional pathways in the feces

Overall, exercise did not modify microbial metabolic functional pathways in the feces (all P>0.05, **Fig.5A**). However, VIG-EX downregulated the glycerol lipid metabolism pathway (KO00561) compared to the CON group [Δ CON=0.04±0.09%; Δ MOD-EX=0.00±0.09%; Δ VIG-EX=-0.3±0.07%; P=0.007, **Fig.5A**]. Moreover, we found that MOD-EX upregulated lactate dehydrogenase operon pathway (K14348) compared to the CON and VIG-EX groups [Δ CON=0.01±1.93%; Δ MOD-EX=0.60±1.66%; Δ VIG-EX=-0.68±1.85%; P=0.007, **Fig.5B**]. Bacteria belonging to the *Acidaminococcus* genus consume lactate[47], and we observed that the relative abundance of *Acidaminococcus intestine* was positively correlated with the plasma levels of lactate at baseline (n=17, rho=0.62, P=0.008, **Fig.6**).

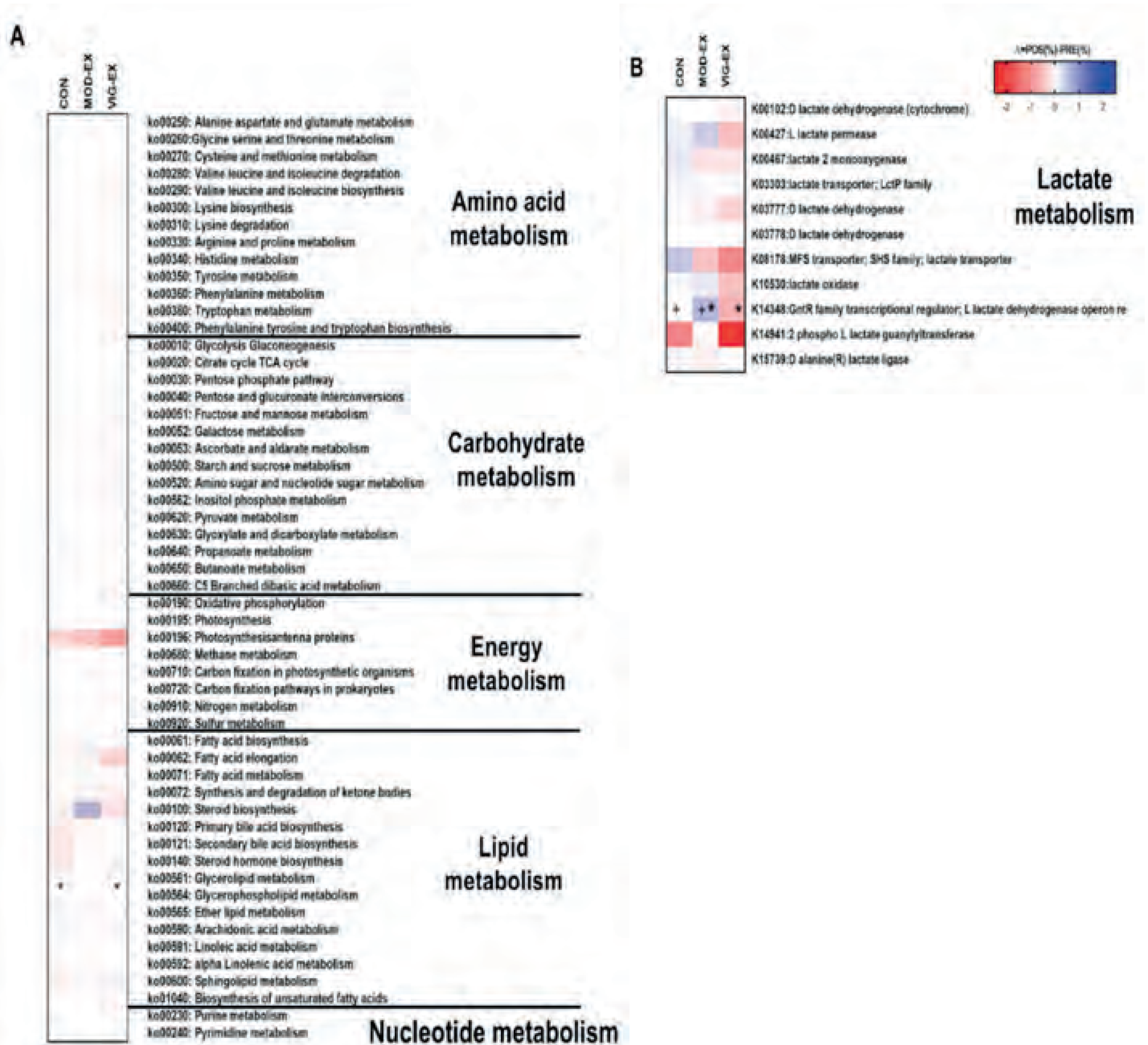

Figure 5: Effect of 24-week of exercise training on microbial metabolic functional pathways. Differences between microbial metabolic and functional pathways are represented as $Log_2POST-Log_2PRE$ values. The function was calculated considering the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Panel A shows metabolic pathways and panel B shows functional lactate pathways. Symbols * and + mean significant differences after Bonferroni corrections. P values were obtained from one-way analyses of variance adjusted by baseline values. *Abbreviation*: CON: control group; MOD-EX: moderate-intensity exercise group; VIG-EX: vigorous-intensity exercise group.

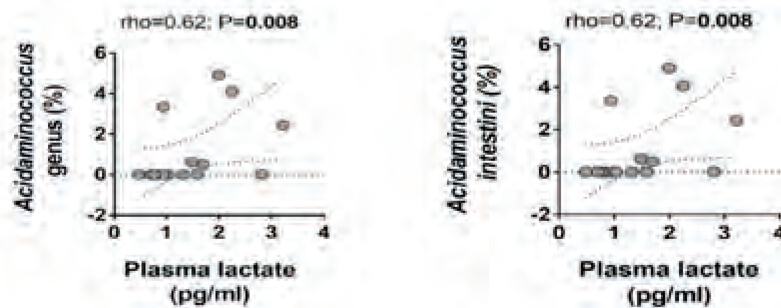

Figure 6: Spearman correlation between plasma levels of lactate and relative abundance of *Acidaminococcus* genus and *Acidaminococcus intestini* species at baseline (n=17). Rho=Spearman's correlations coefficient. P=p-value from univariate Spearman correlation.

Table 3. Effect of 24 weeks of exercise intervention training on body composition, cardiometabolic profile and energy and macronutrient intakes

	CON (n=20)			MOD-EX (n=21)			VIG-EX (n=20)			
	Mean	±	SD	Mean	±	SD	Mean	±	SD	P
Demographics										
Age (years)	22.2	±	2.2	21.9	±	2.3	21.5	±	2.4	
Sex (% female/n)	65 (n=13)			76.2 (n=16)			70 (n=14)			
Body composition										
BMI (kg/m^2)	0.2	±	1.3	-0.1	±	1.0	-0.6	±	1.7	0.130
Lean mass (kg)	1.0	±	2.4	1.7	±	1.2	1.8	±	1.8	0.358
Fat mass (kg)	0.0†	±	3.9	-1.6	±	2.3	-3.3†	±	4.0	**0.019**
FM (%)	0.5†	±	5.0	-2.7	±	3.0	-3.8†	±	3.4	**0.003**
VAT mass (g)	-6.7	±	97.4	-40.4	±	53.1	-60.8	±	86.1	0.126
Cardiometabolic profile										
Glucose (mg/dL)	2.1	±	5.7	1.8	±	6.6	0.9	±	5.2	0.163
Insulin (µUI/mL)	-0.7	±	4.5	-1.4	±	3.4	-0.9	±	4.6	0.860
HOMA index	-0.1	±	1.1	-0.3	±	0.8	-0.2	±	1.1	0.889
TC (mg/dL)	15.5	±	21.5	1.9	±	17.4	5.9	±	22.6	0.157
HDL-C (mg/dL)	4.7	±	8.9	4.8	±	9.9	6.2	±	9.0	0.829
LDL-C (mg/dL)	11.0*	±	14.0	-4.6*	±	18.5	1.4	±	19.3	**0.045**
TG (mg/dL)	0.1	±	17.5	0.0	±	23.9	-3.1	±	37.4	0.702
CRP (mg/L)	0.2	±	3.0	-0.9	±	6.8	0.2	±	5.3	0.861
Energy and macronutrient intakes										
Energy intake (kcal/day)	-94.6	±	546.5	295.5	±	571.4	218.7	±	564.5	**0.041**
Fat (g/day)	-4.1	±	29.7	13.5*	±	23.1	17.1*	±	36.1	**0.039**
Protein (g/day)	-2.3	±	22.9	10.6	±	22.8	3.8	±	21.9	0.181
Carbohydrate (g/day)	-16.0	±	99.2	14.1	±	144.9	30.9	±	109.2	0.155
Fiber (g/day)	-1.7*	±	8.5	3.9*	±	7.0	1.4	±	7.2	**0.007**

Data are presented as mean and standard deviation. P values were obtained from one-way analyses of variance adjusted by baseline values. Symbols * and † mean significant differences after Bonferroni corrections. *Abbreviations*: BMI: Body mass index, CON: Control group, CRP: C-reactive protein, FM: Fat mass, HDL-C: high-density lipoprotein cholesterol, HOMA index: homeostatic model assessment–insulin resistance index, LDL-C: low density lipoprotein cholesterol, MOD-EX: Exercise moderate intensity group, TC: Total cholesterol, TG: triglycerides, VAT: Visceral adipose tissue, VIG-EX: Exercise vigorous intensity group.

Table 4. Spearman correlation between changes in fecal microbiota composition parameters at different taxonomic levels with changes in body composition, cardiometabolic profile, and energy and macronutrient intakes.

	Δ Class *Erysipelotrichia* (%)			Δ Order *Erysipelotrichales* (%)			Δ Family *Erysipelotrichaceae* (%)			Δ Genus *Acidaminococcus* (%)		
	CON	MOD-EX	VIG-EX	CON	MOD-EX	VIG-EX	CON	MOD-EX	VIG-EX	CON	MOD-EX	VIG-EX
Δ Body composition												
BMI (kg/m2)	-0.04	-0.03	-0.02	-0.04	-0.03	-0.02	0.26	0.38	-0.06	-0.48	0.15	0.18
Lean mass (kg)	0.01	0.04	-0.05	0.01	0.04	-0.05	-0.14	0.29	0.25	-0.02	-0.03	-0.05
Fat mass (kg)	0.01	-0.06	<0.01	0.01	-0.06	-0.01	0.24	0.38	-0.13	-0.12	0.21	0.39
FM (%)	0.03	0.05	0.06	0.03	0.05	0.05	0.29	0.30	-0.17	-0.23	0.28	0.35
VAT mass (g)	0.03	-0.27	0.10	0.03	-0.27	0.09	0.22	0.30	0.01	0.13	0.52	0.39
Δ Cardiometabolic profile												
Glucose (mg/dL)	-0.26	-0.14	-0.25	-0.26	-0.14	-0.26	-0.05	0.29	-0.01	-0.01	0.24	0.35
Insulin (μUI/mL)	-0.23	0.09	0.00	-0.23	0.09	0.00	-0.02	0.22	0.06	-0.31	-0.28	0.14
HOMA index	-0.23	0.10	-0.06	-0.23	0.10	-0.06	-0.02	0.21	0.04	-0.20	-0.24	0.12
TC (mg/dL)	-0.12	0.11	0.10	-0.12	0.11	0.10	-0.02	0.13	0.17	0.12	-0.21	0.21
HDL-C (mg/dL)	-0.04	-0.15	0.16	-0.04	-0.15	0.16	-0.13	-0.07	-0.31	0.26	-0.17	0.30
LDL-C (mg/dL)	-0.21	-0.10	0.02	-0.21	-0.10	0.02	0.03	0.15	0.25	0.08	-0.27	0.00
TG (mg/dL)	0.15	0.19	-0.16	0.15	0.19	-0.16	-0.03	0.23	-0.18	-0.37	-0.13	0.04
CRP (mg/L)	-0.33	-0.39	-0.36	-0.33	-0.39	-0.37	-0.02	-0.28	-0.28	0.16	0.37	-0.14
Δ Energy and macronutrient intakes												
Energy intake (kcal/day)	0.23	-0.01	-0.06	0.23	-0.01	-0.05	-0.09	0.19	-0.01	0.03	-0.32	-0.20
Fat (g/day)	0.08	0.23	-0.04	0.08	0.23	-0.03	-0.14	0.31	0.00	0.04	-0.32	-0.12
Protein (g/day)	0.20	0.05	-0.16	0.20	0.05	-0.15	-0.22	0.08	0.12	0.07	-0.34	-0.24
Carbohydrate (g/day)	-0.19	0.06	-0.14	-0.19	0.06	-0.14	-0.21	-0.21	-0.05	0.15	0.17	-0.01
Fiber (g/day)	0.16	-0.15	-0.06	0.16	-0.15	-0.06	0.18	0.01	-0.24	0.17	-0.21	-0.09

Every value shows the correlation coefficient (rho). Bold values represent significant correlation (P<0.05). Abbreviations: Δ: post minus baselines values, BMI: Body mass index, CON: Control group, CRP: C-reactive protein, FM: Fat mass, HDL-C: high-density lipoprotein cholesterol, HOMA index: homeostatic model assessment—insulin resistance index, LDL-C: low density lipoprotein cholesterol, MOD-EX: Exercise moderate intensity group, TC: Total cholesterol, TG: triglycerides, VAT: Visceral adipose tissue, VIG-EX: Exercise vigorous intensity group.

The exercise-induced changes in fecal microbiota composition do not correlate with the exercise-induced changes in fatness, cardiometabolic profile and energy intake

Both exercise intensities reduced fat mass percentage [Δ CON=0.5±5 %; Δ MOD-EX=-2.7±3.0 %; Δ VIG-EX=-3.8±3.4 %; P=0.003, **Table 3**], and serum levels of LDL-C levels [Δ CON=11.0±14 mg/dl; Δ MOD-EX=-4.6±18.5 mg/dl; Δ VIG-EX=1.4±19.3 mg/dl; P=0.045, **Table 3**], whereas increased energy intake [Δ CON=-95.6±546.5 kcal/day; Δ MOD-EX=295.5±571.4 kcal/day; Δ VIG-EX=218.7±564.5 kcal/day; P=0.041, **Table 3**], mainly fiber

intake [Δ CON=-1.7±8.5 g/day; Δ MOD-EX=3.9±7.0 g/day; Δ VIG-EX=1.4±7.2 g/day; P=0.007, **Table 3**]. We did not find significant correlations between the Δ relative abundance of the aforementioned bacteria (*Erysipelotrichia* class, *Erysipelotrichales* order, *Erysipelotrichaceae* family and *Acidaminococcus* genus) and the Δ in the fatness, cardiometabolic profile and energy and macronutrient intakes (All P>0.05, **Table 4**).

DISCUSSION

This study shows that 24 weeks of exercise training was not able to modify beta or alpha diversities but induced subtle modifications in the relative abundance of bacteria belonging to *Firmicutes* phylum in the MOD-EX in young sedentary adults. MOD-EX increased the functional pathways related to lactate metabolism. The changes observed in fecal microbiota composition were not correlated with the cardiometabolic benefits induced by the exercise. These findings suggest that long-term exercise intervention, at moderate intensity, may play a role in the modification of gut microbiota composition in young adults.

Our results show that exercise did not modify beta and alpha diversities, which concur with previous studies[26,48-50]. More specifically, it has been shown that 8-week of endurance and resistance exercise does not affect alpha diversity[48] in middle-aged adults (18-40 years). Additionally, it seems that a similar exercise program neither affected alpha diversity in lean[28] nor in patients with overweight/obesity[26]. However, another study showed that 24-week VIG-EX intervention increased fecal alpha diversity in middle-aged overweight and obese participants[49]. Altogether, it shows that aging and the degree of obesity might be crucial parameters in the reduction of alpha diversity in the feces in humans[49]. Thus, it might be possible that exercise training can only increase alpha diversity when the baseline levels are already low[50]. This hypothesis concurs with the findings of our study since all participants were young and their fecal microbiota diversity indexes were similar between them. However, further studies are needed to fully understand how exercise training is able to modify fecal microbiota diversity.

We also observed that the VIG-EX group decreased the relative abundance of *Erysipelotrichia* class and also at lower taxonomical level, whereas MOD-EX group decreased the relative abundance of *Erysipelotrichaceae* family (*Firmicutes* phylum). Accordingly, a recent study demonstrated that mice that run in a voluntary wheel for 5-7 weeks decreased the relative abundance of *Erysipelotrichaceae* family, as well as body weight[9,51]. It has been also reported that higher physical activity levels were negatively related to the relative abundance of *Erysipelotrichaceae* family in older men (≥65 years old)[52].

Curiously, the relative abundance of *Erysipelotrichaceae* family is higher in patients with obesity and in obese mice, and it is related to higher prevalence of metabolic disorders[53]. In our data, we found a similar decreased in fat mass in MOD-EX and VIG-EX groups, and we observed that the decreased found in the relative abundance of *Erysipelotrichia* class and at lower taxonomical level was not related to the decreased observed in fat mass. These findings support the fact that MOD-EX and VIG-EX might reduce the relative abundance of the bacteria belonging to this phylum, but this decrease seem to be completely independent to the decreased observed in fat mass.

On the other hand, we found that MOD-EX increased the relative abundance of *Acidaminococcus* genus, in a similar way as it has been reported after a military training[54]. *Acidaminococcus* genus, and specifically *Acidaminococcus intestini* species, were reported to consume lactate[55] and produce short-chain fatty acids (SCFAs)[56]. Recently, it has been proved that *Veillonella atypica*, which is a bacterial specie able to metabolize lactate into SCFAs, improves exercise performance in mice[57], via the clearance of circulating lactate. It is well known that during exercise the vasoconstriction is essential to maintain a stable blood pressure, and it depends on the intensity and duration of exercise[58]. This very well described physiological process may explain why we observed that after MOD-EX training and not after VIG-EX training had an increase of the lactate dehydrogenase operon pathway in the bacteria, which is an enzyme responsible for utilizing lactate. Since, a partial vasoconstriction is happening during MOD-EX, circulating lactate can reach the gastrointestinal tract, through monocarboxylate transporter 4[61], and bacteria that consume lactate as a main metabolite, such as the *Accidaminococcus* genus, might increase their presence in the intestine. Unfortunately, the relative abundance of *Veillonella atypica* remained the same after exercise training (data not shown), which proves that further studies are needed to the fully understand the role of exercise training in the clearance of circulating lactate by bacteria colonizing the gut in humans.

CONCLUSIONS

A 24-week of exercise training, mainly at moderate intensity, slightly modified fecal microbiota composition and increased the lactate functional pathway in the feces of young adults. The exercise intervention reduced fatness and improved cardiometabolic profile; however, the observed change in fecal microbiota composition was not related to the cardiometabolic benefits induced by exercise training in young adults.

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RESULTS AND DISCUSION

SECTION II

STUDY V: Impact of an intermittent and localized cooling intervention on skin temperature, sleep quality and energy expenditure in free-living, young, healthy adults.

(J Therm Biol)

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ABSTRACT

Background

Where people live and work together it is not always possible to modify the ambient temperature; ways must therefore be found that allow individuals to feel thermally comfortable in such settings. The Embr Wave® is a wrist-worn device marketed as a 'personal thermostat' that can apply a local cooling stimulus to the skin. The aim of the present study was to determine the effect of an intermittent mild cold stimulus of 25°C for 15-20 s every 5 min over 3.5 days under free-living conditions on 1) skin temperature, 2) perception of skin temperature, 3) sleep quality and 4) resting energy expenditure (REE) in young, healthy adults.

Results

Ten subjects wore the device for 3.5 consecutive days. This intervention reduced distal skin temperature after correcting for personal ambient temperature ($P<0.05$), but did not affect the subjects' the perception of skin temperature, sleep quality or REE (all $P\geq0.051$).

Conclusion

Thus, the Embr Wave® device can reduce distal skin temperature, but wearing it under free-living conditions for 3.5 days does not seem to modify the perception of skin temperature, sleep quality or REE.

INTRODUCTION

The perception of the ambient temperature [1] is different in men and women [2] and a link may exist between thermal comfort and health [3-5]. Some studies report that the perception of an uncomfortable ambient temperature may lead to sleep disturbances [6], and may even be connected to sick building syndrome [7]. However, where people live, study and work together, the ambient temperature cannot often be 'individualized', which, given the above, might lead to health problems in some persons [8].

Embr Labs Inc. markets a device designed to help one achieve personal thermal comfort under different ambient temperatures. This device, the Embr Wave® (https://embrlabs.com/), which has the form of an adjustable bracelet no bigger than a smart watch, has an aluminum plate that can be warmed/cooled to 25-42°C at rates between 0.1-1°C/sec as customizable intensities, frequencies, and durations. The consumer product was developed to allow individuals to leverage localized thermal stimulation on the writs to change the wearer's perception of the ambient temperature and thus achieve greater thermal comfort [9]. The consumer product has been shown to improve perceived temperature by over 3°C [10] and has been found to offer sleep benefits to women experiencing disruptive night time hot flashes [11].

Over the last decade, it has been reported that short-term cold interventions (~10 days) might enhance the immune system and improve insulin sensitivity, among other factors [12] in overweight individuals via the activation of different thermogenic tissues [13,14]. Based on these findings, it has been suggested that the ambient temperature of buildings might be reduced to afford similar beneficial effects [15,16]. This however, is not really feasible; not everyone in a building feels comfortable at the same temperature. It has also been reported that, since sleep usually starts as core body temperature falls due to an increase in peripheral skin temperature in healthy subjects, manipulation of the skin temperature might modify sleep-onset latency [17] and sleep quality [18]. Knowing whether a local mild cold stimulus intervention under free-living conditions provides a feasible and effective means of improving human health and sleep quality is not without clinical and public health interest. The overarching goal of the study was to evaluate the effects of intermittent and localized cold exposure (15-20 seconds every 5 minutes, applied only to 6.25 cm^2 of one wrist), on human physiology. This cold exposure is significantly more intermittent than the thermal stimulation used in previous studies with Embr Wave® (and is not currently available in the consumer product).

The aim of the present study was to determine the effect of an intermittent mild cold stimulus of 25°C for 15-20 s every 5 min over 3.5 days under free-living conditions on 1) skin temperature, 2) the perception of skin temperature, 3) sleep quality, 4) resting energy expenditure (REE), and 5) the nutrient oxidation rate, in young healthy adults. This study represents a first investigation at the potential benefits of exposure to intermittent, localized cold sensations over prolonged periods of time.

MATERIAL & METHODS

Study subjects and ethics statement

The study subjects were 10 adults (5 women, 5 men; 25.8±3.4 years; **Table 1**); all were healthy, non-smokers, who took no medication that might affect their thermoregulatory response to cold exposure and included from March to April 2019. The study protocol was designed in accordance with the latest version of the Declaration of Helsinki and approved by the Ethics Committee on Human Research of the University of Granada (no. 793/CEIH/2019). Informed consent was obtained from all subjects.

Table 1. Characteristics of study subjects.

	All (n=10)			Women (n=5)			Men (n=5)		
Age (years)	25.8	±	3.2	27.4	±	2.9	24.2	±	2.8
Body mass index (kg/m2)	23.0	±	3.2	21.3	±	2.1	24.7	±	3.4
Fat Mass (kg)	21.0	±	8.2	24.2	±	9.6	17.8	±	5.8
Fat Mass (%)	32.2	±	9.5	33.2	±	10.7	31.1	±	9.3
Fat Mass Index (kg/m2)	7.3	±	2.5	8.1	±	3.01	6.5	±	1.9
Total Lean Mass (kg)	41.9	±	11.3	45.6	±	11.2	38.3	±	11.2
Lean Mass Index (kg/m2)	14.5	±	2.5	15.2	±	2.2	13.8	±	2.8
Fat Free Mass (kg)	44.1	±	11.7	48.1	±	11.7	40.2	±	11.5

Data are presented as means and standard deviations.

Embr Wave® device

Commercially available Embr Wave® devices were programmed to provide an intermittent cold stimulus, a ramp down to 25°C for 15-20 s every 5 min (Fig. 1). This functionality was developed to deliberately test the potential benefits of intermittent mild cold stimulation beyond commercially available operating modes). Subjects were instructed to remove the device, which was worn on the right wrist, only for hand-washing and bathing. Testing was performed on two subjects (one male, one female) per week.

Figure 1: **EMBR wave ®** device placed on a wrist of a participant.

Experimental procedure

The subjects worn the Embr Wave® device over a period of 3.5 days under free-living conditions.

Skin temperature measurements using iButtons

Skin temperature measurements were taken every 10 min using DS-1922 L Thermochron iButtons (resolution: 0.0625°C) (Maxim, Dallas, USA) [19], the validity and reliability of which have been established for the assessment of skin temperature in humans [20,21]. These were placed on the back of the hand, the inner part of the wrist, the forearm, in the supraclavicular area, and on the instep of both the right and left sides of the body. To measure the personal ambient temperature to which each subject was exposed (personal-AT), the subjects carried an iButton attached to a plastic fob on their person, though never in direct contact with their body or under clothing [16] (e.g., attached to a backpack or bag). Subjects were told to remove the iButtons only when bathing or washing their hands, and once finished, to put the iButtons on again by themselves; non-wear periods were recorded in a diary. All iButtons were programmed to start recording data every 10 minutes for 3.5 days, starting at 06:00 h on day 1. 24-hour means were determined, and overall 3.5-day means then determined using the Temperatus® software (http://profith.ugr.es/temperatus?lang=en)[22]. The control group was composed of the same subjects who received no intermittent mild cold stimulation for 3.5 days before the activation of the device.

Outdoor ambient temperature

To adjust for the effect of the mean outdoor ambient temperature (outdoor-AT), temperatures for the city of Granada, Spain (where this

work was performed) were downloaded every day of the study period from the Spanish National Meteorological Agency (www.aemet.es/es/portada). The test and control period outdoor-AT and the personal-AT for each subject were then calculated.

Perception of skin temperature

The perception of skin temperature was assessed using a 100-long mm visual analogue scale (VAS), where 0 mm represented "not cold at all" and 100 mm the "maximum tolerable cold". Subjects reported the perception of skin temperature over the different body sites (body, hands and feet) every day before they went to sleep [23].

Sleep quality

Sleep quality variables were determined as previously described [24,25] (a number of studies have shown that the wrist skin temperature provides a reliable proxy of sleep quality [26]). The interdaily stability (IS) of the wrist skin temperature (i.e., the constancy of the 24 h rhythmic pattern over the days of data collection), the intradaily variability (IV; i.e., the fragmentation of the rhythm), and the relative amplitude (RA) were determined as described elsewhere [25,27]. The RA was determined as the difference between the mean wrist skin temperature for the 5 consecutive hours with the maximum wrist skin temperature values (M5), and the mean wrist skin temperature for the 10 consecutive hours with the minimum values (L10), divided by their sum [28]. Finally, the times at which L10 and M5 occurred (TL10 and TM5, respectively) were calculated as previously described [28]. The mean daily pattern for wrist skin temperature was calculated per individual, and then the mean determined for all subjects. The mean daily determined for the test and control groups.

The subjects also wore an ActiGraph GT3X+ accelerometer (ActiGraph, Pensacola, FL, US) on their left wrist for the entire experimental period (except for water-based activities). The following sleep-related variables were determined using this device: (1) night onset (time at which the subject fell asleep); (2) wake-up time; (3) in-bed time (time between going to bed and waking up); (4) sleep duration (time between falling asleep and waking up); (5) sleep efficiency (ratio of sleep duration to in-bed time); (6) number and duration of periods spent awake after sleep onset (WASO). Daytime naps were not taken into account, and participants used a diary log for selecting sleeping periods. Before analysis, atypical data were eliminated and all non-wear time periods excluded. At least four valid days of data (i.e., each with >75% of the 100% possible data for a 24 h period) were required for a subject's results to be included in analyses.

Resting energy expenditure and nutrient oxidation

REE and nutrient oxidation rates were measured on three occasions following procedures described elsewhere, and the latest recommendations 29–31. Briefly, REE was measured at 08.30 h every day (i.e., after the overnight fast) in a quiet room with dim lighting under controlled environmental conditions (22-24°C; humidity 35-45%) [32]. The REE was assessed over 30 min using the Omnical metabolic cart equipped with a ventilated plastic-canopy for subject gases collection (Maastricht Instruments, Maastricht, Netherlands) previous a resting period (20 min). This cart has been previously validated for REE and nutrient oxidation rate determinations [33,34]. The calibration of the flow and gases analyzers was performed automatically before each measurement. The gas data returned were averaged for every minute using an Excel spreadsheet (the first 5-min of measurement were discarded) 32. The coefficients of variance (CV) of VO2, VCO2 and RQ were then calculated. A 5-min period that met the steady state criteria of CV<10% for VO2 and VCO2, and CV<5% for RQ was then selected for data analysis [29,35,36]. Lastly, using the same selected data period, and assuming zero urinary nitrogen excretion, the REE was estimated using Weir's equation [37], carbohydrate and fat oxidation rates were estimated using Frayn's equation [38], and the respiratory quotient (RQ; VCO_2/VO_2) calculated.

Body composition

Subject weight and height (barefoot and wearing standardized light clothes) were determined using a model 799 SECA scale and stadiometer (SECA, Hamburg, Germany). Body mass index (BMI) was calculated as weight/height squared (kg/m^2). Body composition was measured by dual energy X-ray absorptiometry using a Wi Discovery device (Hologic, Inc., Bedford, MA, USA). Lean and fat mass indices were calculated as lean mass/height squared and fat mass/height squared (kg/m^2).

Statistical analysis

Results are presented as means ± standard deviation, unless otherwise stated. General mixed model ANOVA was used to examine whether skin temperature and the perception of skin temperature differed under the test and control conditions. 'Time' (days 1, 2 and 3) was deemed to be a 'within-subjects' factors, whereas control and experimental conditions were regarded as 'between-subject' factors. Means were calculated for all iButtons daily temperatures, VAS perceptions of skin temperature, sleep quality variables, REE and the nutrient oxidation rate for the 3.5 days, and one-way ANOVA performed to examine the differences between the test and control

conditions. To take into account possible confounders, REE/lean body mass ratio, and skin temperature outcomes/personal-AT ratios were determined and compared between the control and test conditions using one-way ANOVA with *post hoc* Bonferroni correction. All calculations were made using the Statistical Package for the Social Sciences v.21.0 (IBM Corporation, Chicago IL, USA). Significance was set at P<0.05. All figures were created using GraphPad Prism v.7.00 software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Effect of EMBR wave® on skin temperature

Figure 2 shows that outdoor-AT varied slightly over the study period, whereas the personal-AT was constant. Thus, when outdoor-AT decreased, personal-AT remained stable. Moreover, the outdoor-AT data show the temperature of the first day of the intervention to be different compared to the remaining days (**Fig. 2**). The main analyses were performed performing a ratio with personal-AT only (**Fig. 3**).

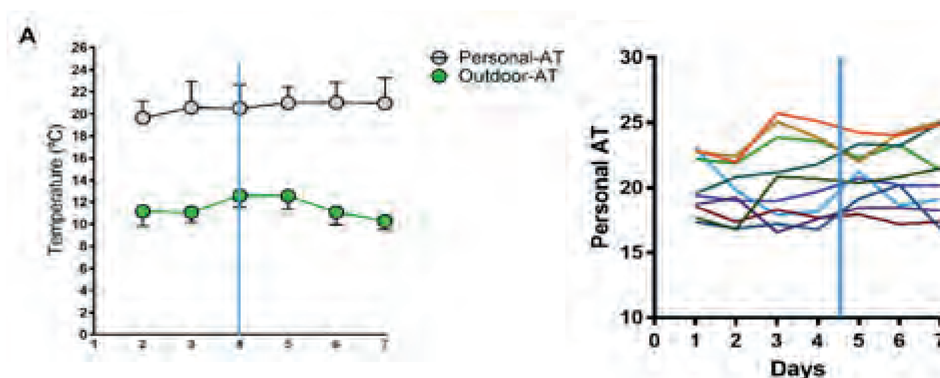

Figure 2: Personal ambient temperature (Personal-AT; grey dots) and outdoor ambient temperature (Outdoor-AT; green dots) for the 7 days of the experimental period (A). Individual variation of Personal-AT between participants (B). Blue vertical line represents the beginning of the 3.5 days mild-cold stimulus.

During the 3.5-day test condition period, the distal skin temperatures tended to be lower during the first 10 h, although not significantly so (data not shown), nor was it maintained. The 3.5-day means for the skin temperatures of the *right* hand, wrist and instep were lower than under control condition (**Fig. 3B**, **D** and **J**; P=0.027, P=0.031 and P=0.043 respectively). The skin temperature of the *left* instep was also lower (**Fig. 3I**; P=0.040). No significant differences were seen for the *left* supraclavicular area (**Fig. 3A, C, E, F, G** and **H**; all P≥0.059). When analyses were not adjusted for personal-AT, none of the above effects were apparent (see **Fig. 4**; all P≥0.112).

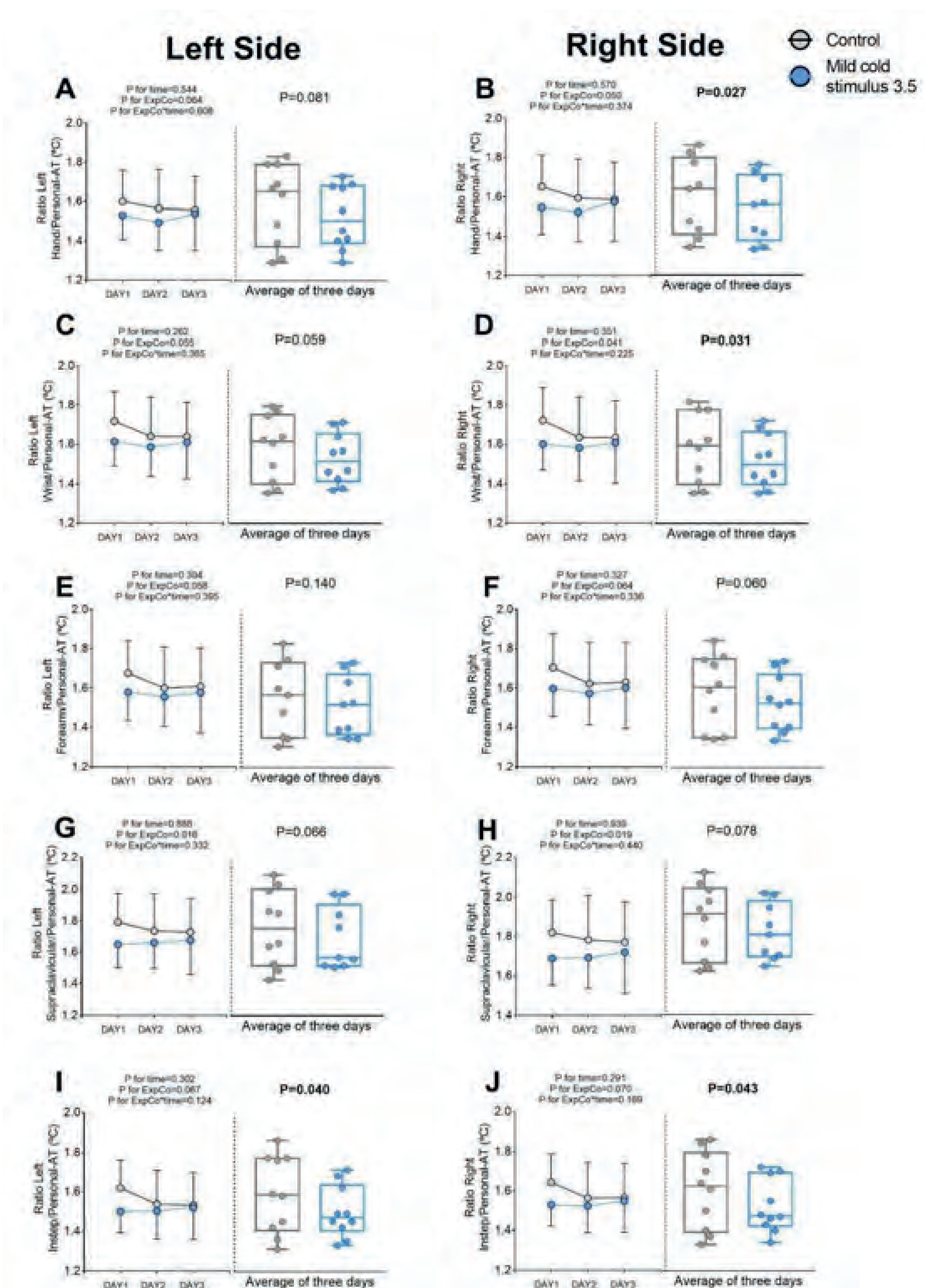

Figure 3: Effect of the 3.5-day intermittent mild cold stimulus on skin temperature (as measured by iButtons). Two-way ANOVA was used to detect differences between the experimental (blue lines) and control (grey lines) conditions. One-way ANOVA was used to determine whether the mean skin temperature in the experimental conditions (blue boxes) and control conditions (grey boxes) differed. Data used in two-way ANOVA are presented as means and 95% confidence intervals, whereas those for one-way ANOVA are presented as means and standard deviation. All data are adjusted for personal ambient temperature (personal-AT). Skin temperature was quantified on the left and right hands (A and B), left and right wrists (C and D), left and right forearms (E and F), left and right supraclavicular (G and H), and left and right insteps (I and J). ExpCo = experimental condition.

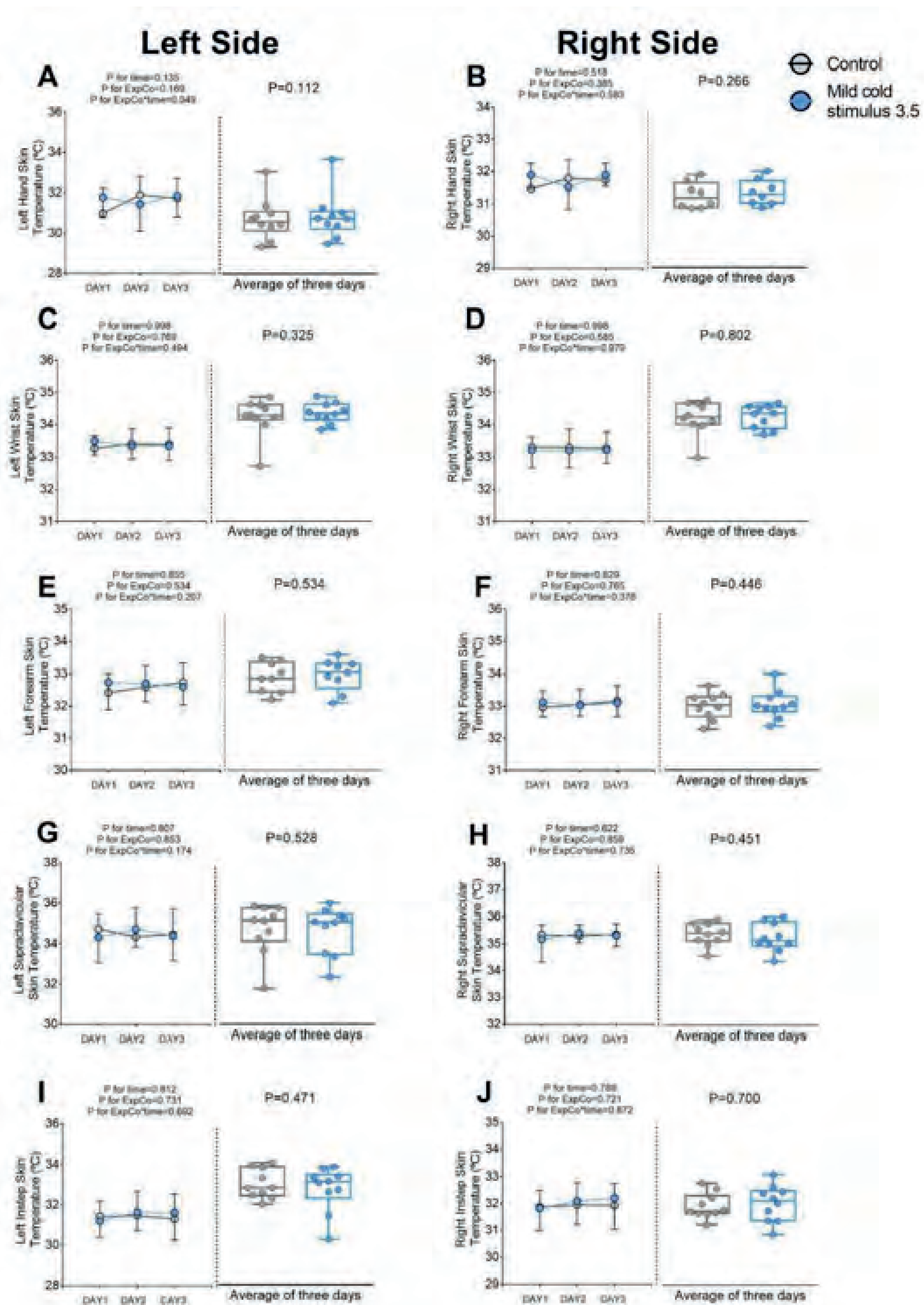

Figure 4. Effect of the 3.5-day intermittent mild cold stimulus on skin temperature (as measured by iButtons). Two-way ANOVA was used to detect differences between the experimental (blue lines) and control (grey lines) conditions. One-way ANOVA was used to determine whether the mean skin temperature in the experimental conditions (blue boxes) and control conditions (grey boxes) differed. Data used in two-way ANOVA are presented as means and 95% confidence intervals, whereas those for one-way ANOVA are presented as means and standard deviation. Skin temperature was quantified on the left and right hands (A and B), left and right wrists (C and D), left and right forearms (E and F), left and right supraclavicular area (G and H), and left and right insteps (I and J). ExpCo = Experimental condition.

Effect of EMBR wave® on perception of skin temperature

Figure 5 shows that the 3.5-day intermittent mild cold stimulus did not modify the perception of skin temperature anywhere before sleeping (**Fig. 5A, B** and **C**; all P≥0.297).

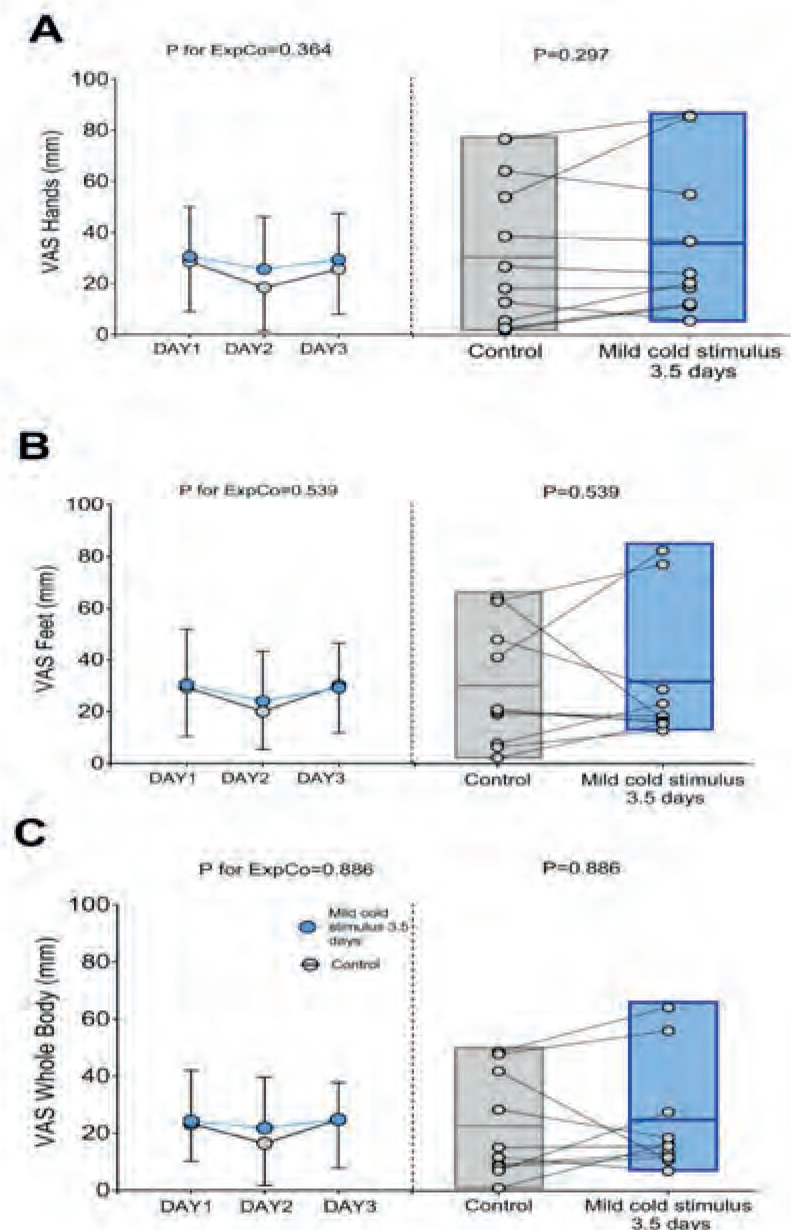

Figure 5. Effect of the 3.5-day intermittent mild cold stimulus on the perception of skin temperature, measured using a visual analogue scale (VAS), where 0 mm represented "not cold at all" and 100 mm the "maximum tolerable cold". Two-way ANOVA was used to detect differences between the experimental (blue lines) and control (grey lines) conditions. One-way ANOVA was used to determine whether the mean skin temperature in the experimental conditions (blue boxes) and control conditions (grey boxes) differed. Data used in two-way ANOVA are presented as means and 95% confidence intervals, whereas those for one-way ANOVA are presented as means and standard deviations. ExpCo = experimental condition.

Effect of EMBR wave® on sleep quality

The M5 for the left and right wrists was significantly higher during the time of the intermittent mild cold stimulus regimen (**Fig. 6E** and **F**; both P≤0.005), although these differences disappeared after adjusting for personal-AT (**Fig. 7E** and **F**; both P≥0.124). This suggests that ambient temperature is an important confounder of any quantification of the change in sleep quality that might be thought due to the stimulus provided by the device. The results returned by the accelerometers also suggested the stimulus had no significant effect on total sleep, sleep-onset times (**Table 2**; both P≥0.483) or sleep efficiency (**Table 2**; P=0.61).

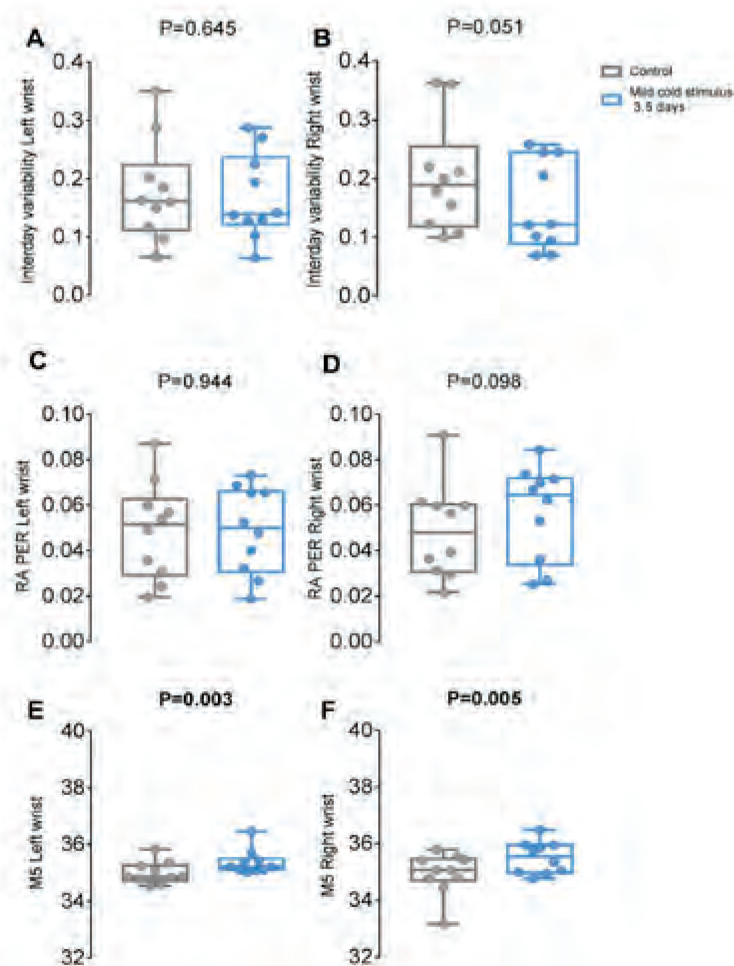

Figure 6. Effect of the 3.5-day intermittent mild cold stimulus on sleep quality. One-way ANOVA was used to identify differences in sleep quality variables between the control (grey boxes) and experimental conditions (blue boxes). Data used in one-way ANOVA are presented as means and standard deviations. Intradaily variability was quantified for the left and right wrist (A and B), as was relative amplitude (RA) (C and D), and M5 (E and F). Two-way ANOVA was used to detect differences between the experimental (blue lines) and control (grey lines) conditions.

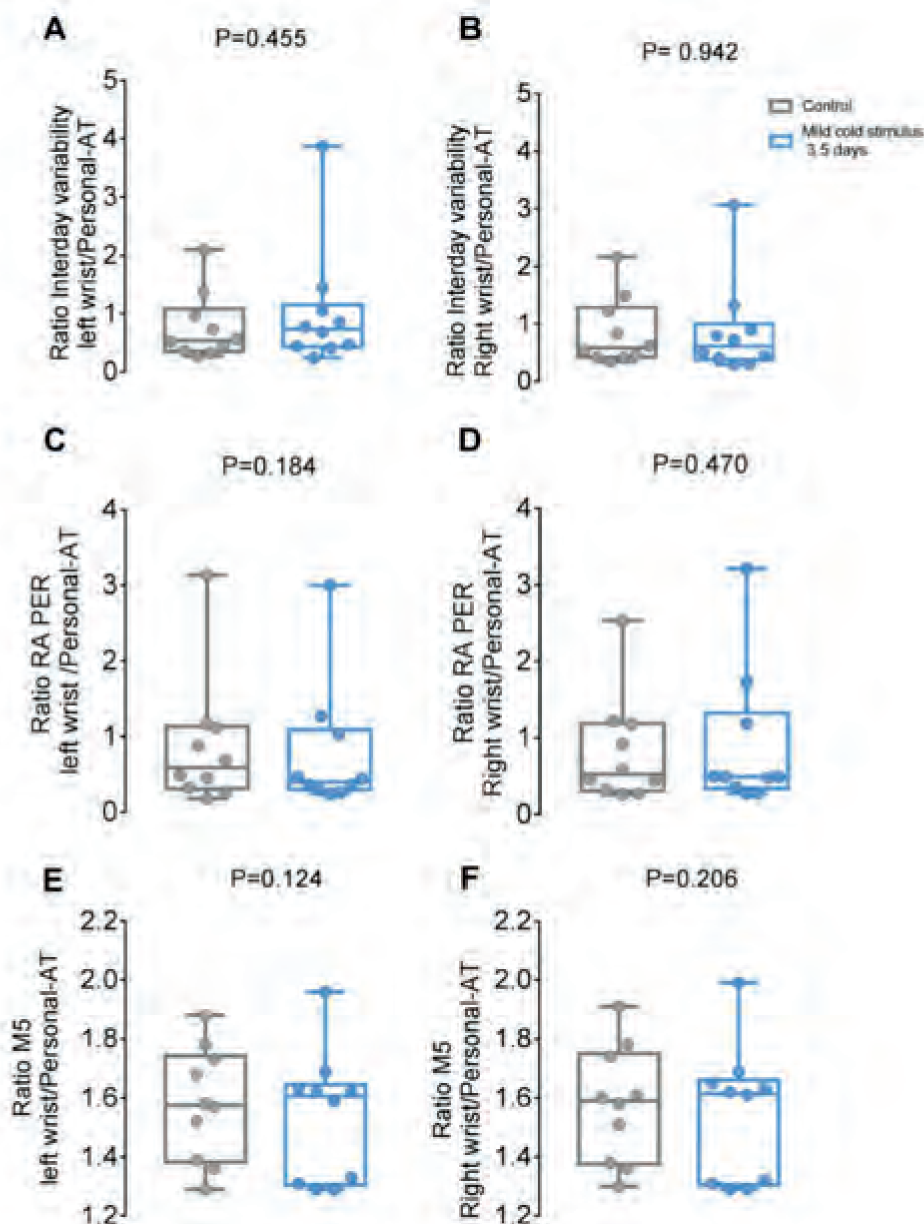

Figure 7. Effect of the 3.5-day intermittent mild cold stimulus on sleep quality. One-way ANOVA was used to study identify differences in sleep quality variables between the control (grey boxes) and experimental conditions (blue boxes). Data used in one-way ANOVA are presented as means and standard deviations. All data are adjusted for personal ambient temperature (Personal-AT). Intradaily variability was quantified for the left and right wrist (A and B), as was relative amplitude (RA) (C and D), and M5 (E and F). Two-way ANOVA was used to detect differences between the experimental (blue lines) and control (grey lines) conditions.

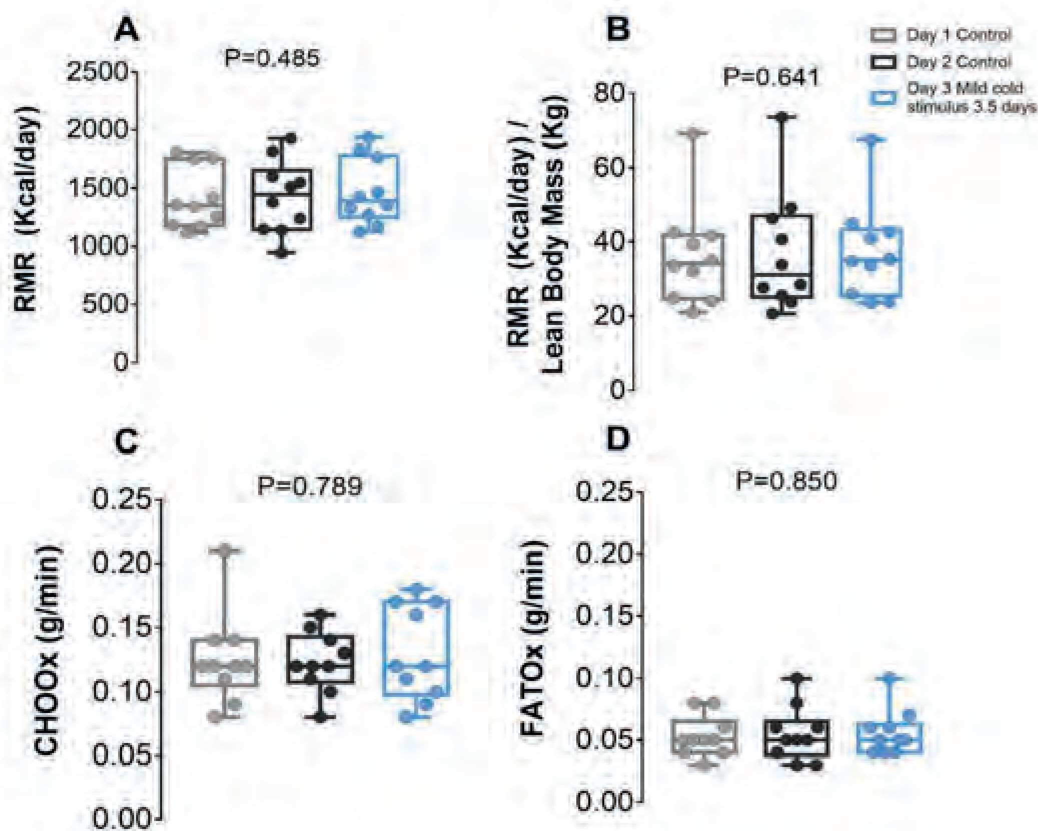

Figure 8. Effect of the 3.5-day intermittent mild cold stimulus on resting energy expenditure (REE) and nutrient oxidation as measured by indirect calorimetry. Two-way ANOVA was used to detect differences between the experimental (1 day, blue boxes blue lines) and control (2 days, grey and black boxes) conditions. Data used in two-way ANOVA are presented as means and 95% confidence intervals. REE was expressed as kilocalories per day (A) and as a ratio (REE/lean body mass; B). Nutrient

Table 2. Effect of the 3.5-day intermittent mild cold stimulus on accelerometer-measured sleep quality variables.

	InBedTime	OutBedTime	OnsetTime	Efficiency	TotalMinBed	TotalSleep Time	WakeAfter SleepOnset
ExpCo	0.155	0.109	0.384	0.379	0.378	0.582	0.378
Time	0.733	0.003	0.591	0.575	0.272	0.222	0.272
ExpCo*Time	0.228	<0.001	0.483	0.61	0.614	0.976	0.614

Two-way ANOVA was used to examine the differences between the values for sleep quality variables under control and experimental conditions. ExpCo = experimental condition.

Effect of EMBR wave® on REE and nutrient oxidation rate

The stimulus had no effect on REE, REE/lean body mass ratio, or the nutrient oxidation (carbohydrate and fat oxidation) rate (**Fig. 8A, B, C** and **D**; all P≥0.485). This lack of effect was observed even when using gas exchange data selection criteria different (i.e., Time Interval instead of steady state method) to those explained in Methods (data not shown).

DISCUSSION

The present results show that an intermittent mild cold stimulus regimen - 25°C for 15-20 s every 5 min over 3.5 days reduces the distal skin temperature, but it does not modify the perception of skin temperature, sleep quality, REE, or the nutrient oxidation rate.

The reduction in distal skin temperature - on both the left and right sides - caused by the 3.5 days stimulus regimen suggests the device might stimulate TRPM8 (transient receptor potential cation channel subfamily melastatin member 8) in the wrist area. Information would then be sent to the hypothalamus that the hand was being cooled down, leading to peripheral vasoconstriction [39,40] in an attempt to preserve the core body temperature. Different studies suggest that thermoregulatory responses occurring simultaneously on both sides of the body when only one side has been stimulated, might be a reflection of better cardiovascular health [41-43].

Additionally, the fall seen in the distal skin temperature over the initial 10 h of monitoring was not maintained, suggesting a physiological or behavioral adaptation to the intermittent mild-cold stimulus took place. This might also explain the lack of any change in the perception of skin temperature, REE or the nutrient oxidation rate. Future work might investigate whether different cold stimulus regimens are also induce such adaptation [44]. Moreover, skin temperature values were shown as a ratio to the Personal-AT due to the huge intraindividual variability observed (Fig. S1B). Surprisingly, we found that *right* distal skin temperatures (where the device was placed), were lower in comparison to *left* distal skin temperatures. This finding suggests that the effect of the device on distal skin temperature was independent of the Personal-AT, however, further and better studies are needed to confirm this hypothesis.

It was initially thought that the 3.5-day intermittent mild cold stimulus might impair sleep quality. However, no such effect was seen, perhaps again due the above-suggested physiological adaptation to the stimulus. Moreover, none of the present subjects had sleep or thermoregulatory problems; it remains to be seen whether the Embr

Wave® device modifies sleep patterns in those with poorer sleep and thermoregulatory health [45].

Brown adipose tissue (BAT) is one of the thermogenic tissues activated by cold [46]. In the present study, the supraclavicular skin temperature was measured by thermal imaging as a proxy of BAT activity, which validity has not been proven yet [47]. However, current evidence suggests that the supraclavicular skin temperature should be interpreted as the outcome of the combined responses of the blood vessels, skeletal muscles and BAT [47]. In any event, the supraclavicular skin temperature remained unaltered, suggesting that 3.5 days intermittent mild cold regimen to be insufficient to activate thermogenic responses. Further research to determine whether the intermittent mild cold exposure activates human BAT when applied to different locations on the body or with different frequencies, intensities or durations is warranted.

CONCLUSION

This study shows that an intermittent and localized mild cold stimulus provided over 3.5 days reduces distal skin temperature but does not induce a measurable modification of the perception of skin temperature, sleep quality, REE, or the nutrient oxidation rate in this cohort.

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GENERAL DISCUSSION

INTEGRATIVE DISCUSSION

Cardiometabolic diseases are the number-one cause of death, and current strategies used to combat it are not enough. Maybe, in the gut microbiota exist the solution to this problem. Consequently, numerous studies have been focused on elucidating the role of the gut microbiota on cardiometabolic diseases[1-5].

To gain more insights into the topic, we investigate novel markers of cardiometabolic health. Our results showed that genera belonging to *Firmicutes* phylum are always involved in all functions related to gut microbiota. In **study I**, we show that plasma levels of oxylipins are related to *Firmicutes* phylum, and this phylum might be responsible for the transformation of primary bile acids to secondary bile acids (**study II**). *Firmicutes* phylum is the most abundant bacterial community in a healthy gut, is composed of 3 classes, 4 orders, 17 families, and 54 genera[6], is more diverse than *Bacteroidetes* and *Actinobacteria* phyla. Furthermore, our systematic review (**study III)** shows that exercising less than 3 months influences the fecal microbiota composition, mainly in *Firmicutes* phylum. Therefore, in **study IV** we demonstrated that 24 weeks of exercise intervention training could slightly modify *Firmicutes* phylum composition of young adults. Additionally, in **study V**, we demonstrated that cold exposure is a therapeutic tool that can be applied at daily basis and future interventions understanding the role of cold exposure in the gut microbiota composition can be now developed. All this information should be taken into account in future studies because a modification of the *Firmicutes* phylum could trigger direct beneficial effects on host's metabolism (**Figure 1**).

From this International Doctoral Thesis, various new insights on the relationship between fecal microbiota and markers of cardiometabolic, and exercise as a potential tool to modify fecal microbiota composition have arisen which will be discussed and interpreted in this chapter.

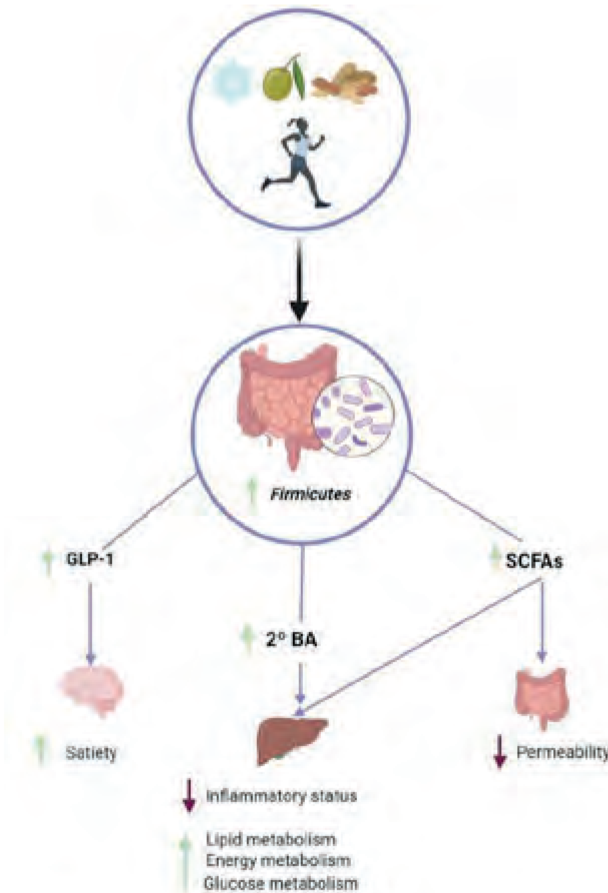

Figure 19: Integrative figure of this international thesis. *Abbreviation*: BA: Bile acids; GLP-1: glucagon-like peptide-1; SCFAs: Short-chain fatty acids.

Novel insights: Cardiometabolic markers

The second primary source of dietary energy for humans is fatty acids (FAs), 20-35% of total calories intake in adults[7]. The FAs are obtained from the diet, and >95% is available to the bloodstream through efficient processes of digestion and absorption in the intestine[8]. FAs furnish energy (9 kcal per gram), carry fat-soluble vitamins (A, D, E and K)[9], constitute the cell membrane phospholipids and act on its fluidity and signaling[8], and cholesterol metabolism[10]. The polyunsaturated fatty acids (PUFAs) are essential FAs, but they cannot be synthesized by humans and are required from dietary intake[11]. The dietary fatty acid (omega-3 and omega-6 PUFAs) can increase intestinal alkaline phosphatase (IAP) expression, which is known to maintain normal gut microbial homeostasis, and is also able to dephosphorylate LPS and ATP, therefore can reduce the survival and suppress some bacteria growth (*i.e., E.coli*). For all the above mentioned, different types of FAs dietary intake have a crucial role in modifying the composition of mice gut microbiota composition and influences the health improvement or disease (*i.e.,* obesity) of the host[12].

In **study I**, we showed that plasma levels of omega-3 PUFAs derived oxylipins positively correlated with *Clostridium IV* genus (*Firmicutes* phylum). Curiously, we also show that the relative abundance of *Clostridium leptum* species (*Clostridium IV* genus) is negatively correlated with plasma levels of primary BA (**study II**). *Clostridium* species are commensal bacteria and live in harmony with the intestinal environment, therefore exerting many salutary effects on our intestinal homeostasis[13]. Thus far, *Clostridium* metabolites, like butyrate and secondary BA, play a primary role in strengthening the intestinal barrier and interacting with host metabolism[13]. They can ferment a variety of nutrients, like carbohydrates to produce SCFAs. In addition, acetate and butyrate increase colonic glucagon-like peptide-1 (GLP-1) secretion with increased intracellular cAMP concentrations[14]. On the other hand, species belonging to *Clostridium IV* genus are involved in the production of primary to secondary BA through 7-α-dehydroxylases enzymes. Afterward, accumulating evidence has shown that secondary BA restrained the growth of *Clostridium difficile*[15], an opportunistic species that release toxins damaging the digestive system. The relative abundance of *Clostridium IV* genus could play an important role in the lipid metabolism and inflammatory host status. However, these assumptions should be addressed in the near future.

Current evidence indicates a bidirectional relationship between gut microbiota composition and the host. Thanks to technological advances, microbiota sequencing analysis have provided a great piece of information. In **study I**, plasma levels of oxylipins might drive gut microbiota composition, and gut microbiota composition and metabolites conversely may affect plasma levels of oxylipins and therefore host inflammatory status. Moreover, in **study II**, the relative abundances of gut microbiota affect the conversion to secondary BA through enzymes that express by bacteria, but the plasma levels of primary bile acids can also might affect the gut microbiota composition. Therefore, further intervention studies are needed to demonstrate what is the causal direction of these associations.

Potential interventions

There is a large amount evidence supporting the healthy effects of exercise on cardiometabolic health[16], including gut microbiota composition and gut permeability. In **study III**, we observed that exercise, acutely and chronically (>3 months of training) is able to modify fecal microbiota composition, especially the relative abundance of *Firmicutes* phylum. We did not find studies investigating the effect of longer and at different intensities exercise bouts on gut microbiota composition. This is the main reason why we conducted **study IV**, and found that after 24-week supervised exercise

intervention, at different intensities, can slightly modify the relative abundance of *Firmicutes* phylum.

During the exercise, the skeletal muscle receives 85% of blood flow[17]. However the blood flow in the mesenteric artery is reduced[18] and it depends on the intensity of exercise: in moderate exercise is decreased 50%, and in vigorous exercise is decreased ≈95%[19]. During the exercise, blood lactate concentration[20] and lactate clearance[21] are higher in vigorous exercise than moderate exercise. During exercise, the store of ATP in the muscle needs to be constantly replenished. If the oxygen supply is sufficient, this energy comes from pyruvate, however, if not sufficient, the energy comes from lactate. The lactate released by muscle during the exercise is moved to the liver and is converted to glucose, which then returns to the muscles and is metabolized back to lactate, and obtained 2 ATPs[22]. The reduction of mesenteric artery flow during moderate exercise, could permit blood lactate to cross the intestinal barrier[23] through monocarboxylate transporter 4[24]. In the gut, there are bacteria that express the lactate dehydrogenase enzyme, which is responsible for the breakdown of lactate, and can transform this lactate into SCFAs.

As we mentioned above, Scheiman et al.[25] showed that the relative abundance of *Veillonella atypica* species increased after a marathon. Then, they isolated this species from the fecal sample and inoculated it into mice. Surprisingly, mice increased treadmill run performance. *Veillonella atypica* species utilize lactate as their sole carbon source; moreover, it has the enzymes necessary to convert lactate into acetate and propionate. For this reason, using $^{13}C3$ –labeled lactate in mice, they found that lactate can cross the epithelial barrier into the lumen of the gut, and this lactate might be metabolized by *Veillonella atypica*. In our **study IV**, the relative abundance of *Acidaminococcus* genus increased in MOD-EX group. *Acidaminococcus* present lactate-dehydrogenase enzymes, which can transform lactate to SCFAs, and are used as a carbon source. Interestingly, *Acidaminococcus* belong to the same *Veillonella* taxonomy classification: *Firmicutes* phylum, *Negativicutes* class, *Selenomonadales* order. For this reason, we think *Acidaminococcus* and *Veillonella* share the same function during exercise.

On the other hand, Chevalier et al.[26] showed that the relative abundance of *Firmicutes* phylum increased from 18.6% to 60.5% under 30-days cold exposed mice. Then, they transplanted gut microbiota from thermoneutral and cold exposed mice into germ-free mice. Mice treated with the microbiota from cold-exposed mice showed a higher glucose and triglyceride uptake in the intestine compared to mice treated with the microbiota from thermoneutral mice. This study shows, that cold exposure modifies gut microbiota composition, and these bacteria improve intestinal absorption. In humans, the role of cold exposure in gut microbiota composition is still unknown. One of the main reasons is because cold exposure

cannot be applied at daily basis without side effects, such as overall discomfort or inability to be connected to a water-perfused suit. Thanks to the investigation performed in **study V**, we demonstrated that cold exposure can be applied at daily basis without side effects. Thus, future studies can be now developed for understanding the role of long-term cold exposure in gut microbiota composition and cardiometabolic health.

General limitations

The results presented in this International Doctoral Thesis should be considered with caution since there are some limitations that should be acknowledged:

- Cross-sectional design of **studies I** and **II** do not allow establishing any cause-effect direction. Therefore, due to bidirectional crosstalk between cardiometabolic markers and fecal microbiota composition, further studies are needed.

- All studies (except **study III**) were carried out in young healthy adults. Therefore, the results do not apply to other populations (i.e., old adults). Therefore, it is necessary to replicate the findings of the present International Doctoral Thesis on other populations.

- Fecal microbiota composition shows a high variability between individuals; therefore, in future studies, the sample sizes should be bigger than the sample size used in the **study IV** to collaborate the effect of exercise on fecal microbiota composition.

- The duration of the intervention was a continuous 3.5 days of stimulation every 5 minutes - apparently long enough for physiological or behavioral adaptation to occur, although longer, colder, or less continuous interventions might return different results (**study V**).

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CONCLUSIONS

GENERAL CONCLUSION

The present International Doctoral Thesis shows that there is an association between cardiometabolic health and the fecal microbiota composition in young adults. Moreover, exercise is able to modify fecal microbiota composition, whereas mild cold exposure is a promising therapy to improve energy metabolism, and finally all these effects seem to be mediated by bacteria species belonging to the *Firmicutes* phylum.

SPECIFIC CONCLUSIONS

SECTION 1: Novel insights: Cardiometabolic markers and fecal microbiota composition

- Specific conclusion 1.1: Plasma levels of omega-3 and omega-6 derived oxylipins are related to the relative abundance of *Clostridium IV* and *Sutterella* genera in young adults.
- Specific conclusion 1.2: The relative abundance of *Firmicutes* and *Bacteroides* phyla are related to the plasma levels of primary and secondary bile acids in young adults.

SECTION 2: Potential interventions to modify the fecal microbiota composition

- Specific conclusion 2.1.: Physical activity and cardiorespiratory fitness are positively associated with bacterial alpha diversity, fecal counts for certain bacterial phyla, and fecal Short-chain fatty acids concentrations in healthy adults. Exercise interventions seem to influence fecal counts for certain phyla in humans.
- Specific conclusion 2.2.: A 24-week of supervised exercise intervention, at moderate intensity, modifies the relative abundance of bacteria belonging to *Firmicutes* phylum. Furthermore, change in fecal microbiota composition is not related to the cardiometabolic benefits induced by exercise training in young adults.
- Specific conclusion 2.3.: An intermittent and localized mild cold stimulus over 3.5 days reduces distal skin temperature and wearing it under free-living conditions for 3.5 days does not seem to impair the perception of skin temperature and sleep quality or modify resting energy expenditure.

FUTURE PERSPECTIVES

FUTURE PERSPECTIVES

- **Study I**: It is known that the supplementation of omega-3 PUFAs improves cardiometabolic health. However, it is not clear the underlying mechanisms. Here we postulate that omega-3 PUFAs, via omega-3 oxylipins, may improve cardiometabolic health through a modification in the gut microbiota composition. Thus, studies investigating the effect of PUFAs supplementation on gut microbiota composition in mice and humans are needed it.
- **Study II**: It is crucial to confirm whether the bacterial species identified in this study can express enzymes involved in the transformation of primary bile acids to secondary bile acids. If this is confirmed, future research can be developed in this interesting field.
- It is known that patients that shows high levels of *Clostridium difficile* show low levels of secondary bile acids. Therefore, the use of specific bacteria able to transform primary BA to secondary BA might be a promising therapy to reduce the relative abundance of *Clostridium difficile*.
- **Study III**: It is crucial to investigate the effect of different types of exercise (strength vs. resistance) on gut microbiota composition in humans.
- **Study IV**: Additionally, it is of great scientific interest to perform fecal microbiota transplantation from humans that have done an exercise training program to mice. This study will prove whether the cardiometabolic benefits of exercise training are mediated by bacteria living within the gut.
- More specifically, it is needed it to investigate whether *Acidaminococcus Intestini* expresses all the enzymes needed it for the breakdown of lactate and the production of SCFAs.
- **Study V**: We have now identified a commercial tool able to cold exposed humans at daily basis without secondary side effects. Thanks to this finding, it can be developed a randomized controlled trial investigating whether long-term cold exposed and long-term warm exposed humans lead to different fecal microbiota composition. This future study will unveil the role of ambient temperature on fecal microbiota composition.

ANNEXES

MANUSCRIPTS DERIVED FROM THE THESIS

1. **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, Jonatan R. Ruiz, Patrick C.N. Rensen, Borja Martinez-Tellez. Plasma levels of omega-3 and omega-6 oxylipins are related to the relative abundance of *Proteobacteria* phylum in the feces of young adults. *In preparation*

2. **Huiwen Xu**, Francisco J. Osuna-Prieto, Lourdes Ortiz-Alvarez, Xinyu Di, Isabelle Kohler, Lucas Jurado-Fasoli, Jose Rubio-Lopez, Julio Plaza-Díaz, Ramiro Vilchez-Vargas, Alexander Link, Angel Gil, Jonatan R. Ruiz, Patrick C.N. Rensen, Borja Martinez-Tellez. Relative abundance of *Firmicutes* and *Bacteroidetes* bacteria is related to plasma levels of bile acids in young adults. *Submitted*

3. **Huiwen Xu**, Lourdes Ortiz-Alvarez, Borja Martinez-Tellez. Influence of Exercise on the Human Gut Microbiota of Healthy Adults. ***Clinical and Translational Gastroenterology.*** 2020 Feb;11(2):e00126. PMID: 32463624

4. **Huiwen Xu**, Lourdes Ortiz-Alvarez, Guillermo Sanchez-Delgado, Francisco M Acosta, Juan MA Alcantara, Francisco J. Amaro-Gahete, Wendy D. Martinez-Avila, Elisa Merchan-Ramirez, Victoria Muñoz-Hernandez , Francisco J. Osuna-Prieto, Lucas Jurado-Fasoli, Idoia Labayen, Ramiro Vilchez-Vargas, Alexander Link, Julio Plaza-Díaz, Angel Gil, Jonatan R. Ruiz, Borja Martinez-Tellez. A 24-week exercise intervention slightly modifies fecal microbiota composition in young sedentary adults: A randomized controlled trial. *In preparation*

5. **Huiwen Xu**; Antonio Martinez-Nicolas; Wendy D Martinez-Avila; Juan MA Alcantara; Juan Corral-Perez; David Jimenez-Pavon; Francisco M Acosta; Jonatan R Ruiz; Borja Martinez-Tellez. Impact of an intermittent and localized cooling intervention on skin temperature, sleep quality and energy expenditure in free-living, young, healthy adults. ***J Therm Biol.*** 2021 Apr;97:102875. PMID: 33863439

SHORT INDUSTRY CURRICULUM VITAE

Personal information

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Summary

- Forward-thinking **research application scientist** with a **strong technical background in human gut microbiota and data analysis** proved by successfully contributing to a *Danone* **40,000€** grant funding.
- **Quick learner** and **flexible team player** with **solid market and product knowledge** establishing collaborations with the BioPharma Company resulting in 5,000€+ cost savings.
- **Top communication and customer-facing skills** acquired by working with international teams leading to as evidenced by 8 peer-reviewed scientific publications in Q1 journals.

Key Industry Skills

Technical Application Scientist

Gained as PhD researcher at University of Granada

- Skilled scientist experienced in **customer training and effective troubleshooting** and proven ability to drive projects forward leading to the successful delivery of 5+ standard oparting procedures and 10+ databases.
- Knowledge of **current industry trends** with a strong background in human gut microbiota demonstrated by a short stay grant from the Spanish Ministry of Health valued in 5,000€.
- **Outstanding interpersonal skills** with experience managing 5 national and international students leading to 2 of them stay in our team with Spanish felloship.

Teamwork and Collaboration skills

Gained as MSc associate scientist at University of Granada

- Great conflict resolution skills and ability to **maintain positive relationships** with experience working with >50 scientists in >15 different projects.
- **Positive and intereactive personality** proven the succesful coordination of a randomized controlled trial study with 150 human participants, resulting a new grant funding of >500,000€.
- Excellent **ability to understand customer needs** demonstrated by contributing to a multidisciplinary research project which led to long-term partnerships with 2 national and 2 international research institutions.

Communication skills

Gained as visiting PhD researcher at University Hospital in Magdeburg, Germany

- Highly committed scientist with deep technical expertise and proven **capacity to support scientists** with experimental, software and instrument-related issues across multidisciplinary scientific projects.
- Detail-oriented and strategic planner with experience **developing impactful presentations** proved by 6 abstracts at international conferences.
- Extensive **experience communicating complex topics** evidenced by effectively delivering Biochemistry lectures in the University of Granada for 3+ years.

Professional skills

Key soft skills

- Client-facing skills
- Love travel
- Teamwork
- Communication

Technical transferable

- Troubleshooting
- Developing methods
- Experimental design
- Technical writing

Technical specific

- Faecal DNA extraction
- PCR
- Biostatistics analysis with SPSS and R
- Assistant testing software

Education

- **PhD in Biomedicine** 2017-Current
  University of Granada, Spain
- **MSc in Genetic, Nutritional and Environmental Conditions of Development and Growth** 2016-2017
  University of Granada, Spain
- **BSc in Nutrition and Dietetic Sciences** 2012-2016
  University of Granada, Spain

Top 3 professional achievements

- **Achievement 1**: Managing and coordinating 150 participants for 7 different experimental tests in a 2-year randomised trial study and achieving succesful data colletion allowing the project to publish >50 articles.

- **Achievement 2**: Getting a BSc Extraordinary Award (2nd best out of 100+ students) which granted my PhD fundings (FPU16/05159) proving great time- and resource-management skills.

- **Achievement 3**: Having the great opportunity to travel abroad to other scientific centers (e.g. Germany, and The Netherlands) improving my empathy and teamworking skills .

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4. LinkedIn:

   https://www.linkedin.com/in/hw-xu/

Education

2017-2022	PhD student in Biomedicine, University of Granada, Spain
2021-2021	Course "The transition from academy career to industry", on-line, "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 PhD Student.*

Participation in Research Project

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

2019-2021 **Skin teEMBRature project:** Impact of an intermittent and localized cooling intervention on skin temperature, sleep quality and energy expenditure in free-living, young, healthy adults. Financed by EMBR lab.

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€.

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

1. 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.

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5. Borja Martinez-Tellez; Yolanda Garcia-Rivero; Guillermo Sánchez-Delgado; **Huiwen Xu;** Francisco Amaro-Gahete; Francisco M Acosta; Patrick CN Rensen; Mariëta R. Boon; Jose M Llamas-Elvira; Jonatan R Ruiz. Supraclavicular skin temperature measured by iButtons and 18F-fluorodeoxyglucose uptake by brown adipose tissue in adults. **J Therm Biol.** 2019 May;82:178-185.

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6. **Huiwen Xu;** Borja Martinez-Tellez; Guillermo Sánchez-Delgado; Acosta FM; Patrick CN Rensen; Jose M Llamas-Elvira; Jonatan R Ruiz. Association of wrist and ambient temperature with cold-induced brown adipose tissue and skeletal muscle 18F-FDG uptake in young adults. **Am J Physiol Regul Integr Comp Physiol.** 2018 Dec 1;315(6):R1281-R1288.

   [IF JCR 2018: 3.17. Rank 26/81. Q2. PHYSIOLOGY]

7. Borja Martinez-Tellez; Lourdes Ortiz-Alvarez; 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]

8. 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]

Teaching experience

2020-2021 Physiology and clinical biochemistry. Degree in Pharmacy (1.5 ECTS Credits) University of Granada, Spain.

Structural biochemistry. Degree in Pharmacy (3 ECTS Credits) University of Granada, Spain.

Biotechnology. Degree in Pharmacy (1.5 ECTS Credits) University of Granada, Spain.

2019-2020 Structural biochemistry. Degree in Pharmacy (1.5 ECTS Credits) University of Granada, Spain.

Structural biochemistry. Degree in Food Science and Technology (3 ECTS Credits) University of Granada, Spain.

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

2018-2019 Structural biochemistry. Degree in Food Science and Technology (4.5 ECTS Credits) University of Granada, Spain.

Structural biochemistry. Degree in Food Science and Technology (1.5 ECTS Credits) University of Granada, Spain

Congress communications as first author

2019

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

Congress: 13th European Nutrition Conference, Dublin, Ireland

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

2018

Title: Meal-induced thermogenesis in metabolically healthy but obese adults. *Oral presentation*

Congress: XVII Congreso de la Sociedad Española de Nutrición (SEÑ) and X Jornada de l'Associació Catalana de Ciències de l'Alimentació (ACCA), Bacelona , Spain.

Author: Huiwen Xu; Guillermo Sanchez-Delgado; Borja Martínez Téllez; Victoria Muñoz-Hernandez; Wendy D. Martinez-Avila; Francisco J. Amaro-Gahete; Jonatan Ruiz Ruiz.

Title: Meal-induced thermogenesis in metabolically healthy but obese adults. *Poster*

Congress: XVII Congreso de la Sociedad Española de Nutrición (SEÑ) and X Jornada de l'Associació Catalana de Ciències de l'Alimentació (ACCA), Bacelona , Spain.

Author: Huiwen Xu; Guillermo Sanchez-Delgado; Borja Martínez Téllez; Victoria Muñoz-Hernandez; Wendy D. Martinez-Avila; Francisco J. Amaro-Gahete; Jonatan Ruiz Ruiz.

Title: Meal-induced thermogenesis in metabolically healthy but obese adults. *Oral Participation*

Congress: V Reunión de Jóvenes Investigadores, Barcelona, Spain.

Author: Huiwen Xu; Guillermo Sanchez-Delgado; Borja Martínez Téllez; Victoria Muñoz-Hernandez; Wendy D. Martinez-Avila; Francisco J. Amaro-Gahete; Jonatan Ruiz Ruiz.

Title: Asociación entre la temperatura de la muñeca y las temperaturas ambientales con la captación de glucosa por el tejido adiposo pardo y el músculo esquelético en adultos jóvenes. *Oral Participation*

Congress: III Jornadas / I Congreso Nacional de Investigadores en Formación Fomentando la Interdisciplinaridad (JIFFI), Granada, Spain.

Author: Huiwen Xu; Borja Martínez Téllez; Guillermo Sanchez-Delgado; Francisco Miguel Acosta Manzano; Yolanda García Rivero; Jonatan Ruiz Ruiz.

Grant and awards

2019- Internship grant: "Estancia breve en el extranjero financiada por el programa estatal de promoción del talento y su empleabilidad subprogram estatal de movilidad: estancias breves y traslados temporales FPU". ≈4100€

2018- 3 top oral presentation award in "V Reunión de Jóvenes Investigadores, Barcelona, Spain."

2018- Award to assist to 13th European Nutrition Conference, Dublin, Ireland.

Dissemination news

2020- News about our revision "Influence of Exercise on the Human Gut Microbiota of Healthy Adults: A Systematic Review" in CanalUGR.

2018- News about our article "Skin temperature response to a liquid meal intake is different in men than in women" in Ideal newspaper.

Courses

2021 Title: "Charla Moticadxs 2" – 1 hour
Organizer: University of Granada

Title: "Incorporación de datos ómicos a proyectos de investigación" – 20 hours
Organizer: University of Granada

Title: "Machine learning y big data para bioinformatica" -100 hours
Organizer: University of Granada

Title: "Cómo construir el marco teórico" -1 hour
Organizer: University of Granada

Title: "Formación de un document de Word para la tesis" -1 hour
Organizer: University of Granada

2020 Title: "Atajos bibliográficos" -1 hour
Organizer: University of Granada

Title: "Preguntas fundamentales sobre el acceso abierto a la ciencia-open Access" -1 hour
Organizer: University of Granada

2019 Title: "Bioinformática y bioestadística en microbiología ambiental. Nivel avanzado ambiental y speech training" -30 hours

Organizer: University of Granada

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: "Herramientas para el desarrollo de la investigación" -9 hours
Organizer: University of Granada

Title: "Taller sobre procesos y procedimientos administrativos" - 2 hours
Organizer: University of Granada

Title: "Técnicas estadísticas aplicadas en el ámbito de la nutrición y de la salud" -10 hours
Organizer: University of Granada

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