Patrícia Costa Morais
Bachelor's in Biomedical Sciences

Study of human gut microbiome dynamics in general population

As part of the Citizen Science project
Microbioma Comunidade Portugal

MASTER'S IN MEDICAL MICROBIOLOGY
NOVA University Lisbon
September, 2025

DEPARTMENT OF
LIFE SCIENCES

Study of human gut microbiome dynamics in general population

As part of the Citizen Science project *Microbioma Comunidade Portugal*

Patrícia Costa Morais

Bachelor's in Biomedical Sciences

Supervisor: Isabel Gordo, Principal Investigator,
Gulbenkian Institute for Molecular Medicine

Co-supervisor: Karina Xavier, Principal Investigator,
Gulbenkian Institute for Molecular Medicine
Jaime Mota, Associate Professor,
NOVA School of Science and Technology,
NOVA University Lisbon

Examination Committee:

President: José Paulo Sampaio,
Full Professor, NOVA School of Science and Technology, NOVA University Lisbon

Arguer: Mariana Machado,
Adjunct Assistant Professor, Faculty of Medicine, University of Lisbon

Supervisor: Isabel Gordo,
Principal Investigator, Gulbenkian Institute for Molecular Medicine

MASTER'S IN MEDICAL MICROBIOLOGY

NOVA University Lisbon
September, 2025

Study of human gut microbiome dynamics in general population

As part of the Citizen Science project *Microbioma Comunidade Portugal*

In the present work, generative artificial intelligence tools were used for grammatical review, text reformulation, and code optimization. These tools were employed under the supervision of the author, and all generated content was verified for accuracy. The authorship and validation of the content remain entirely the responsibility of the author, and the use of artificial intelligence is in accordance with institutional standards of academic integrity.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the support of the many people and institutions who were part of this journey over the past year.

First, I would like to thank my supervisor, Isabel Gordo, for accepting me into this exciting project on the Portuguese human microbiome. Thank you for always being available to meet, helping with what was needed, for solving every problem along the way, and for ensuring I would have the results I needed for this thesis. Your guidance, support, and shared knowledge about evolution and science made all the difference. To my co-supervisor, Karina Xavier, thank you for your support, insights, shared knowledge in metabolomics, and for always ensuring everything was going well. Both of you made sure I had everything I needed for this project to succeed, and I'm truly grateful for that.

To my internal co-supervisor, Jaime Mota, thank you for your support, for always being available, and for replying so quickly to my questions and doubts.

A special thanks to Maria João for the support with participant communication, for always being present during challenges, and for staying positive and motivated. Thank you also for taking me to the SciComPT conference, an amazing opportunity to learn about science communication.

To all my colleagues and former colleagues from the Evolutionary Biology Lab and Bacterial Signaling Lab, thank you. You made this journey lighter, funnier, and full of learning. Special thanks to Elsa and Marta for answering all my endless questions, helping me at every stage, and for always bringing calm and laughter to my day. To Raphael for your help and scientific feedback on the thesis and presentations. To Joana, Monica, Patrícia, and Raquel, thank you for your kind words and continuous support. And to everyone else who was part of my journey, even if not mentioned here, please know that I truly appreciate each one of you.

To André, from Advanced Data Analysis, thank you for all the support and shared knowledge on the 16S analysis. And to Helena, from CERMAX, and Miguel, thank you for your help with metabolomics. To all the partners and institutions who collaborated on this project, thank you for your support and valuable feedback on documents and important decisions.

To my family, my parents and brother, thank you for always being there, even from afar. Your words and support through calls and messages meant the world to me. To my grandparents, uncles, and cousins, thank you for making Lisbon feel like home and for helping me in so many ways. To my friends from Valpaços, Aveiro, and Lisbon, thank you for always being there, no matter the distance or the time.

And finally, to all the participants who contribute to this study with their time, samples, and suggestions.

Without all of you, none of this would have been possible!

ABSTRACT

The human gut microbiome is highly diverse and plays a crucial role in health, influenced by factors such as diet, environment, and genetics. Although its composition has been extensively studied in several countries, the Portuguese microbiome remains poorly characterized. Moreover, only recently have a few longitudinal studies begun to emerge.

This gap is addressed in this thesis through *Microbioma Comunidade Portugal*, a citizen science project involving 32 families, mainly from Oeiras municipality. Participants collected three fecal samples over time, and the family-based design allowed the inclusion of individuals across different ages and contributed to sustained motivation.

Results revealed that these Portuguese cohort's gut microbiota was dominated by Bacillota and Bacteroidota, consistent with other studies, and remained stable over time at the phylum level. The Bacillota/Bacteroidota ratio was consistent across time, age, and body mass index.

Interestingly, alpha diversity increased overtime, possibly reflecting seasonal and/or behavior changes of the participants. Surprisingly, alpha diversity was higher in the elderly. Similar to other studies, intraindividual diversity was lower than interindividual diversity.

Fecal metabolomic profiling did not show notable changes over time, and no correlations were found between key short-chain fatty acid-producing genera and the concentrations of these metabolites.

Participants actively contributed feedback and engaged in laboratory visits and events, enhancing scientific literacy and trust in science. This study constitutes the first longitudinal analysis of the Portuguese gut microbiome, with an adherence rate of 94%, highlighting the valuable role of citizen science in microbiome research. Future studies involving larger and more geographically diverse cohorts will be essential to clarify the observed alpha diversity variations and further explore the functional dynamics of the gut microbiome.

Keywords: Portuguese microbiota, Longitudinal study, Citizen science, Short-chain fatty acids, Microbiome diversity, 16S rRNA sequencing

RESUMO

O microbioma intestinal humano é altamente diverso e desempenha um papel crucial na saúde, sendo influenciada por fatores como dieta, ambiente e genética. Embora a sua composição tenha sido amplamente estudada em vários países, o microbioma português permanece pouco caracterizado. Além disso, apenas recentemente alguns estudos longitudinais começaram a surgir.

Estas lacunas são colmatadas nesta tese através do *Microbioma Comunidade Portugal*, um projeto de ciência cidadã que envolveu 32 famílias, maioritariamente do concelho de Oeiras. Os participantes recolheram três amostras fecais ao longo do tempo e o desenho familiar permitiu incluir indivíduos de diferentes idades, contribuindo para uma elevada adesão e motivação contínua.

Os resultados mostraram que a microbiota intestinal desta coorte portuguesa foi dominada pelos filos Bacillota e Bacteroidota, em concordância com outros estudos, mantendo-se estável ao longo do tempo ao nível do filo. A razão Bacillota/Bacteroidota manteve-se consistente ao longo do tempo, idade e índice de massa corporal.

Interessantemente, a diversidade alfa aumentou ao longo do tempo, possivelmente refletindo alterações sazonais e/ou comportamentais dos participantes. Surpreendentemente, a diversidade alfa foi maior nos idosos. Assim como em outros estudos, a diversidade intraindividual foi menor que a diversidade interindividual.

A análise metabolómica das fezes não revelou alterações significativas ao longo do tempo, nem correlações entre os géneros produtores de ácidos gordos de cadeia curta e as concentrações destes metabolitos.

Os participantes contribuíram com feedback e envolveram-se em visitas laboratoriais e eventos, promovendo a literacia científica e a confiança na ciência. Este estudo representa a primeira análise longitudinal do microbioma intestinal português, com uma taxa de adesão de 94%, destacando o valor da ciência cidadã na investigação do microbioma. Estudos futuros com coortes maiores e geograficamente mais diversas serão essenciais para clarificar as variações observadas na diversidade alfa e aprofundar a compreensão da dinâmica do microbioma intestinal.

Palavras chave: Microbiota portuguesa, Estudo longitudinal, Ciência cidadã, Ácidos gordos de cadeia curta, Diversidade do microbioma, Sequenciação 16S rRNA

CONTENTS

FIGURES INDEX

TABLES INDEX

ABBREVIATIONS AND ACRONYMS

^{1}H-NMR	Proton Nuclear Magnetic Resonance
ADHD	Attention-Deficit/Hyperactivity Disorder
AMPs	Antimicrobial Peptides
ASVs	Amplicon Sequence Variants
BMI	Body Mass Index
BSC	Bristol Stool Chart
BSL-2	Biosafety Level 2
C+C Program	*Programa Ciência + Cidadã*
CSI	Chemical Shift Imaging
CBR	Católica Biomedical Research Centre
D_2O	Deuterated Water
DNA	Deoxyribonucleic Acid
ECSA	European Citizen Science Association
GALT	Gut-Associated Lymphoid Tissue
GIMM	Gulbenkian Institute for Molecular Medicine
GIT	Gastrointestinal Tract
GLP-1	Glucagon-Like Peptide-1
HMP	Human Microbiome Project
IBD	Inflammatory Bowel Disease
IQR	Interquartile Range
ITQB NOVA	Instituto de Tecnologia Química e Biológica António Xavier
ITS	Internal Transcribed Spacers
KH_2PO_4	Potassium Dihydrogen Phosphate
K_2HPO_4	Dipotassium Hydrogen Phosphate
MetaHIT	METAgenomics of the Human Intestinal Tract
NaN_3	Sodium Azide
NGS	Next-Generation Sequencing
NH_3	Ammonia

PBS	Phosphate-Buffered Saline
PCR	Ploymerase Chain Reaction
PYY	Peptide Tyrosine Tyrosine
Q1	First Quartile
Q3	Third Quartile
rRNA	Ribosomal Ribonucleic Acid
SCFAs	Short-Chain Fatty Acids
sIgA	Secretory Immunoglobulin A
TP	Time Point
TSP-d4	3-(trimethylsilyl) propionic acid-2,2,3,3-d4 sodium salt
Tregs	Regulatory T Cells
UK	United Kingdom
USA	United States of America

INTRODUCTION

A. The Human Gut Microbiota

Our bodies host trillions of microorganisms, including bacteria, viruses, eukaryotes, and archaea, forming the human microbiota. Together, they colonize distinct ecological niches, such as the skin, oral cavity, respiratory system, vaginal canal, and gastrointestinal tract (GIT)[1]. Each site represents a unique microenvironment, influencing the composition and activity of its microbial community[2].

Among all these microbial ecosystems, the GIT is the most complex and densely populated. It is home to a highly dynamic and structured community of microbes, where both the number and diversity of microorganisms increase along the gut[3]. In adults, this ecosystem harbors more than 10^{13} bacteria, representing hundreds of different species[4,5].

This complex ecosystem, rich in commensal bacteria and viruses, plays a crucial role in maintaining our health through mutual interactions. While our body provides nutrients and helps to maintain ecological homeostasis, these microbes help us in return. They contribute to processes such as nutrient metabolism, immune modulation, and protection against pathogens[1]. These symbiotic relationships reflect a coevolutionary history, which has shaped both humans and microbial communities[6,7].

Previously, it was believed that gut colonization began at birth. However, recent studies raise the possibility that microorganisms might already be present in the womb. Nonetheless, it is during birth that the newborn is exposed to diverse microbial ecosystems, from the mother and the surrounding environment[8]. This early colonization evolves rapidly through infancy and becomes relatively stable in adulthood[9]. The structure and functions of the gut microbiota are shaped by complex bidirectional interactions with the host and are influenced by diet, geography, lifestyle, and medication, making your microbiota almost as unique as a fingerprint[10].

This ecosystem is not only taxonomically diverse but also genetically rich. Each resident species contains several thousand genes, and in total, the microbiome is estimated to contain about 2 million genes, which is over 100 times the number of human genes[5]. This genetic richness allows rapid adaptations to environmental changes, such as shifts in diet, infections, or antibiotic use[11]. Although such perturbations can temporarily alter microbial composition, the adult gut microbiota usually shows ecological resilience, meaning that it can return to a stable and functional state as it was previously[12].

Given the central role of the intestinal microbiota in our health, disturbances in its composition, known as dysbiosis, have been linked to health problems, including gastrointestinal, metabolic, and neurological disorders[3]. These associations highlight the need to understand the constitution and roles of the gut microbiota, as well as its variation across individuals and over time. Large-scale projects like the METAgenomics of the Human Intestinal Tract (MetaHIT) and the Human Microbiome Project (HMP) have played a crucial role in the understanding of how the gut

microbiome is related to human health and diseases. This knowledge is essential for advancing biomedical research with the development of new strategies to promote and restore health[13,14].

B. 16S rRNA Gene Sequencing: Principles, Applications, and Limitations in Gut Microbiome Profiling

Traditionally, scientists studied complex microbial ecosystems by cultivating and isolating each individual strain on culture plates. However, this method has major limitations because many microorganisms are either slow-growing or simply cannot be cultured under standard laboratory conditions[15]. Estimates suggest that over 80% of microbes present in our gut and in the environment remain unculturable with traditional methods, leading to an underestimation of the microbial diversity[16].

Because of this, researchers develop new culture-independent molecular techniques capable of providing a more complete and accurate representation of microbial ecosystems. Progress in sequencing technology, molecular biology tools, and the increase of microbial genome databases throughout the 20th century have made possible the discovery of a wide range of new species and expanded our understanding of microbial diversity in the GIT and other ecosystems[16].

One major advance in microbial ecology was the use of the 16S ribosomal ribonucleic acid (rRNA) gene as a phylogenetic marker to identify and classify prokaryotes. This gene functions as a “molecular clock” and is present in all bacteria and archaea. It encodes a conserved segment of the 30S small subunit of the 70S ribosome complex, a key component required for protein synthesis[17].

The 16S rRNA gene has about 1500 base pairs and contains nine hypervariable regions (V1–V9), separated by ten conserved regions. The conserved segments make it possible to design universal primers for polymerase chain reaction (PCR) amplification, while the hypervariable regions, which evolve more rapidly due to higher mutation rates, reflect their role as an effective “molecular clock”, enabling estimation of evolutionary differences between microbial groups. This combination of conserved and variable regions makes this gene a powerful phylogenetic marker for profiling microbial communities[18].

Different hypervariable regions provide different levels of taxonomic resolution and amplification efficiency. Among them, the V4 region, highlighted in Figure 1.1, is often chosen to identify bacteria because of its high phylogenetic resolution at the genus level and has been shown to be accurate in simulated microbial communities[18].

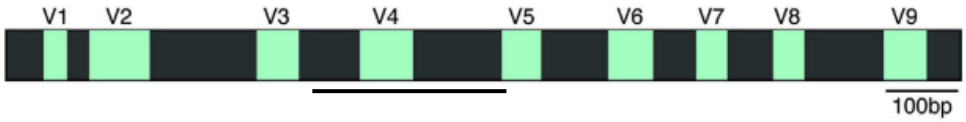


Figure 1.1. Structure of the 16S rRNA gene, highlighting the nine hypervariable regions (V1–V9, in blue-green) flanked by ten conserved regions (in dark gray). This structure enables the design of universal primers and allows taxonomic discrimination among prokaryotes. The V4 region highlighted is commonly used in microbial community profiling. Adapted from Jay-Hyun Jo et al.[17]

Another advantage of 16S profiling is that primers are designed to target only prokaryotes. Although they can sometimes amplify non-target sequences from eukaryotes, such as mitochondrial deoxyribonucleic acid (DNA), which also contains the 16S rRNA gene, the use of optimized primers allows for a more effective profiling of the microbial community in complex samples. This contrasts with untargeted next-generation sequencing (NGS) approaches, which sequence all the DNA present in the sample, including human DNA, reducing the sensitivity of prokaryotic analysis[19].

While the 16S rRNA sequencing remains the standard for profiling bacteria and archaea, other genetic markers are needed for different organisms. For example, the 18S rRNA gene of the 40S ribosomal small subunit is used to study eukaryotes, while fungi are typically profiled using internal transcribed spacers (ITS) regions together with parts of the 18S, 5.8S, and 28S genes. NGS based on rRNA enabled the characterization of complex microbial ecosystems in environments such as soil, water, and the human body, aiding in the detection of ecological disturbances, such as contamination and disease-associated shifts[20].

1. 16S rRNA Amplicon Sequencing Workflow

A typical 16S rRNA amplicon sequencing workflow involves several key steps, beginning with sample collection, storage, and transport (1). These initial steps must follow protocols to minimize biases introduced by sample handling and preservation methods[21].

Next, DNA extraction (2) is performed using optimized and reproducible methods to minimize biases caused by different lysis efficiencies among bacteria, since gram-positive bacteria have a thicker peptidoglycan layer, and to remove potential inhibitory substances that could interfere with the PCR amplification process (3). In the PCR amplification step, barcoded primers that anneal to conserved regions flanking the selected hypervariable region of the 16S gene are used. After DNA amplification, the samples are sequenced (4), typically on Illumina platforms, which use sequencing-by-synthesis technology, where fluorescently labeled nucleotides are incorporated into DNA strands, imaged, and the fluorescent label is cleaved before the next base is added, allowing the DNA sequence to be read[20].

The downstream computational analysis involves several steps, the demultiplexing of pooled samples (1), removal of adapters and primers (2), quality control (3), filtering and trimming (4) to ensure high-quality data, dereplication, and denoising to correct sequencing errors (5), merging of paired-end reads (6), and removal of chimeric sequences (7)[22]. Additional contaminant filtering is applied to improve data accuracy[23]. Taxonomic classification of the resulting amplicon sequence variants (ASVs) is performed using reference databases, allowing phylogenetic inference and quantitative comparison across samples[17,18]. This approach estimates the relative abundance of microbial taxa and differences between samples using ASVs, which enables single-nucleotide resolution in community profiling[15].

2. Limitations of 16S rRNA Sequencing

The 16S rRNA gene-based approach is widely used for microbial community profiling because it is fast, relatively cheap, and does not require bacterial culturing. However, this method presents several limitations[21].

A major limitation is the limited taxonomic resolution of short amplicons. The 16S gene shows a high level of conservation among closely related species, and short-read sequencing of hypervariable regions often fails to distinguish taxa at the species or strain level. Consequently, taxonomic assignments are frequently limited to the genus level, limiting ecological and clinical interpretations. In addition, 16S rRNA sequencing does not provide information about the functional activity of the sequenced microorganism[21]. To bypass some of these limitations, several studies use full-length 16S sequencing or shotgun metagenomic sequencing, which can provide species and strain level resolution and insights into the functional capacity[18].

Another limitation is that 16S rRNA sequencing does not provide information about microbial viability. Since it targets DNA, it cannot distinguish between live and dead microbes[24], nor identify whether detected DNA originates from active community members or transient sources such as diet or environmental contaminants[25].

Additionally, gene copy number variation presents a challenge. The number of 16S copies varies among bacteria, some species have a single copy, while others possess multiple copies, leading to biased abundance estimates[24].

Contamination is an additional concern throughout the workflow, from sample collection and DNA extraction to library preparation. Sampling devices, DNA extraction kits, and reagents can contain microbial DNA, confounding the results[26,27]. Biological samples may contain inhibitors that interfere with the amplification process, such as bile salts, humic acid, and polysaccharides[24,27]. Furthermore, differences in primer affinity, such as the GC content or the specificity to the target DNA, can lead to amplification bias, favoring certain taxa over others[17]. PCR can also introduce sequence errors, such as base substitutions, insertions, deletions, and chimera reads due to incomplete extension of the gene, which may artificially inflate diversity estimates[18].

Technical variability, including batch effects from DNA extraction or sequencing runs, can introduce systematic bias into downstream analyses[27]. Therefore, it is critical to apply rigorous quality control. This includes positive and negative sequencing controls, as well as blank controls to detect contamination, in which the same extraction protocol is performed without adding any sample. Also, normalization procedures across batches minimize technical artifacts[18].

Despite these challenges, 16S rRNA sequencing has revolutionized microbiome research. It has enabled the identification of hundreds of previously uncharacterized taxa and enhanced our understanding of microbial diversity and host-microbe interactions, particularly in the human gut, where important associations between microbial composition and host health or disease have been demonstrated[13].

C. Overview of Gut Microbiota Composition at the Phylum Level

The human gut microbiota harbors a highly diverse microbial community, with bacteria being the most extensively studied group. Taxonomically, these bacteria are classified into hierarchical levels including phyla, classes, orders, families, genera, and species. Although the gut microbiota is composed of hundreds of species, they belong to a small number of phyla, whose relative abundances vary significantly among individuals and populations. The two dominant phyla are Bacillota and Bacteroidota (formerly called Firmicutes and Bacteroidetes, respectively), which together account for approximately 90% of the gut's microbiota population[3]. Other less abundant phyla found in healthy populations include Pseudomonadota (Proteobacteria), Actinomycetota (Actinobacteria), and Verrucomicrobiota (Verrucomicrobia)[28].

Bacillota is the most diverse and abundant bacterial phylum in the gut, making up about 50 to 80% of the bacteria present. They are mainly gram-positive and include classes like Clostridia and Bacilli. The class Clostridia includes the families Lachnospiraceae and Ruminococcaceae, the most abundant in gut microbes. The class Bacilli includes genera such as *Lactobacillus*, *Enterococcus*, and *Streptococcus*, which are predominant in the upper GIT. These bacteria can degrade different types of dietary fibers and produce butyrate and lactate, which help maintain gut pH balance and support key functions, including barrier integrity, anti-inflammatory responses, glucose regulation, and fat metabolism[29,30].

Bacteroidota is the most abundant phylum of gram-negative bacteria in the gut, with approximately 7000 species and including classes such as Bacteroidia, Sphingobacteria, Flavobacteria, and Cytophagia. Dominant genera within this phylum include *Bacteroides*, *Prevotella*, *Alistipes*, and *Parabacteroides*, which specialize in the degradation and fermentation of complex polysaccharides into short-chain fatty acids (SCFAs), carbon dioxide, hydrogen, and other metabolites important for our health. These bacteria help prevent pathogen colonization and modulate the immune system[30,31].

The phylum Actinomycetota, composed of gram-positive bacteria, includes the families Bifidobacteriaceae, Propionibacteriaceae, Corynebacteriaceae, and Streptomycetaceae. The genus *Bifidobacterium* is the most abundant in the GIT in vaginally delivered newborns, and its abundance decreases with age. This phylum comprises around 8% of the adult microbiota[32]. These microorganisms ferment carbohydrates into lactate and produce antimicrobial and anti-inflammatory compounds that inhibit pathogen growth, contributing to gut barrier development and immune homeostasis[30,32].

Pseudomonadota is a metabolically and morphologically diverse phylum. The main class in the gut is Gammaproteobacteria, which includes the Enterobacteriaceae family. Although generally minor components of the gut microbiota, their overrepresentation is often associated with dysbiosis[33]. For example, overgrowth of genera such as *Escherichia* and *Desulfovibrio* has been associated with metabolic syndrome[34].

Verrucomicrobiota is typically present at low abundance but includes *Akkermansia muciniphila*, a species capable of degrading mucin present in the intestinal mucus layer. This bacterium has been associated with better metabolic health, enhanced glucose metabolism regulation, intestinal barrier function by reducing levels of inflammatory cytokines, and protection against obesity[30].

D. Functional Role of the Gut Microbiota in Host Health

The role of the human gut microbiota extends beyond taxonomy, contributing significantly to host physiology, acting like a metabolic "organ" that contributes to a wide range of essential biological processes. It is vital for maintaining gut homeostasis and promoting host health[35].

This microbial community complements the host's genetic capacities through its vast number of genes. It helps protect against pathogen colonization, digestion of dietary components, produces key metabolites, and contributes to immune system development, gastrointestinal development, fat storage, behavior, epithelial barrier integrity, and efficacy of certain treatments. These functional roles are summarized in Figure 1.2 and will be discussed in more detail below[36].

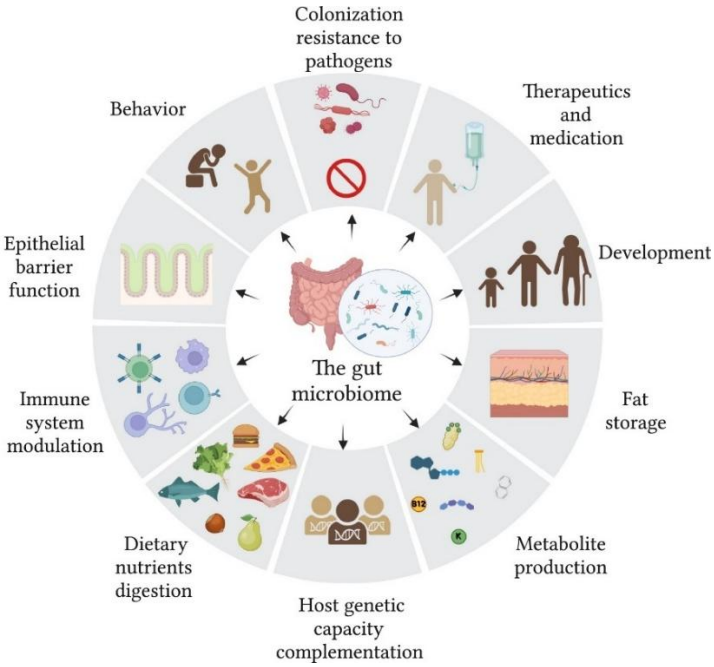


Figure 1.2. Schematic summary of gut microbiota–host interactions. The gut microbiota expands the host's genetic capacity, contributes to the digestion and production of essential metabolites, protects against pathogens, and influences immune development, gut barrier function, behavior, and drug metabolism. Adapted from Oliveira et al.[36]

1. Digestion

One of the main functions of gut microbiota is fermenting complex dietary polysaccharides that are indigestible by human enzymes. This fermentation converts complex carbohydrates into SCFAs, which serve as energy sources for colon cells and influence host metabolism, fat storage, and immune modulation. SCFAs will be discussed in more detail in the next chapter[37,38].

The microbiota plays an important role in the availability of vitamins. It contribute to the biosynthesis of vitamin K and several B vitamins, which are essential for numerous biological processes in our body[39].

Both SCFAs and vitamins are essential for maintaining gut health, energy production, and modulating intestinal inflammation[8].

Additionally, gut microbes help protein metabolism, transforming amino acids into compounds like indoles, phenols, and bacteriocins, which are antimicrobial peptides that can shape the microbiome and inhibit pathogenic microbes[9,40].

Regarding bile acids, the liver produces primary bile acids that aid in fat digestion[35]. Once in the gut, microbes deconjugate and dehydroxylate them into secondary bile acids, which affect fat storage, metabolism, and absorption, and energy homeostasis[9,40].

In addition, gut microbes can modulate the efficacy of xenobiotics, such as antibiotics, bioactive dietary compounds, and drugs. The microbiota metabolizes these compounds, which can lead to reduced treatment effectiveness or altered drug availability, influencing the therapeutic outcomes[36,41].

2. Protection

The gut microbiota acts as a first line of defense against the colonization and dissemination of pathogens, also known as colonization resistance[42]. Commensal microbes inhibit pathogens' proliferation by consuming available nutrients and competing for adhesion sites along the mucus layer[3].

They also lower the lumen pH through SCFAs production, and the production of secondary bile salts also influences the growth of certain species, such as *Clostridioides difficile*[43]. Moreover, they secrete antimicrobial peptides and modulate host cell-signaling pathways that reinforce tight-junction integrity[8]. Collectively, these mechanisms limit pathogen growth and help preserve epithelial barrier function[44].

3. Immune System

The gut microbiota is essential for the development, maturation, and regulation of both the innate and adaptive immune systems, especially in early life[9,11]. After birth, exposure to microbial antigens helps the formation of gut-associated lymphoid tissue (GALT)[11], which "educates" the immune system to distinguish between commensal and pathogens[45], promoting immune tolerance while maintaining vigilance[8].

As the immune system matures, commensal bacteria contribute to the differentiation and expansion of immune cell populations, such as T and B lymphocytes[11], supporting the balance between pro- and anti-inflammatory responses. For example, certain bacteria promote the differentiation of regulatory T cells (Tregs), which are essential for immune tolerance and prevent exaggerated inflammatory responses to harmless antigens[45].

A critical part of this defense is the intestinal mucus barrier, particularly in the colon, where two distinct mucus layers protect the epithelial surface. The inner layer is dense and sterile, while the outer layer is more dynamic and rich in glycans secreted by goblet cells. These host-derived carbohydrates can be utilized as nutrients by commensal microbes, promoting their colonization and protection of the GIT[9]. Other defense mechanisms include the secretion of antimicrobial peptides (AMPs) and secretory immunoglobulin A (sIgA). sIgA, produced by B cells, binds microbial antigens and forms immune complexes that are presented to dendritic cells from GALT, facilitating immune tolerance and mucosal immunity[1]. Together, AMPs, mucin glycoproteins, and sIgA limit microbial overgrowth, reinforce the epithelial barrier, and modulate immune responses[7].

A stable and diverse microbiota is important for maintaining long-term immune balance by preventing overreactions to food antigens and to commensals, while enhancing defense against pathogens, and reducing the risk of allergic, inflammatory, and autoimmune diseases through immune cell signaling modulation[8].

4. Gut-Brain Axis

The dynamic interplay between the gut microbiota and the central nervous system, known as the gut-brain axis, has gained significant attention in recent studies. This complex interaction occurs through neural, endocrine, immunological, and metabolic pathways[46], involving microbial metabolites such as SCFAs and amino acid derivatives[47].

The gut microbiota can influence host behavior, mood, stress responses[46], and cognitive functions. Alterations in the microbial community have been linked to various neuropsychiatric conditions, including anxiety, depression, and autism spectrum disorders[47]. Understanding this intricate relationship offers promising avenues for novel therapeutic strategies, including psychobiotics, which are live organisms that, when consumed in adequate quantities, may benefit mental health by acting through the gut-brain axis[48].

E. Factors That Shape the Human Gut Microbiota

Although the microbiota shows resilience and functional redundancy, it remains a dynamic ecosystem that is continuously modulated throughout life by a complex interplay of intrinsic (host-related) and extrinsic (environmental) factors, resulting in significant inter-individual variability[49].

External environmental exposures, such as dietary habits, living environment, cohabitation, neonatal feeding practices, medication use, and smoking, play a significant role in shaping the gut microbial community. In addition, host-related factors like genetic background, mode of delivery, physical activity, immune status, and age also influence its composition and functional capacity. These factors are summarized in Figure 1.3 and will be discussed in more detail below[36].

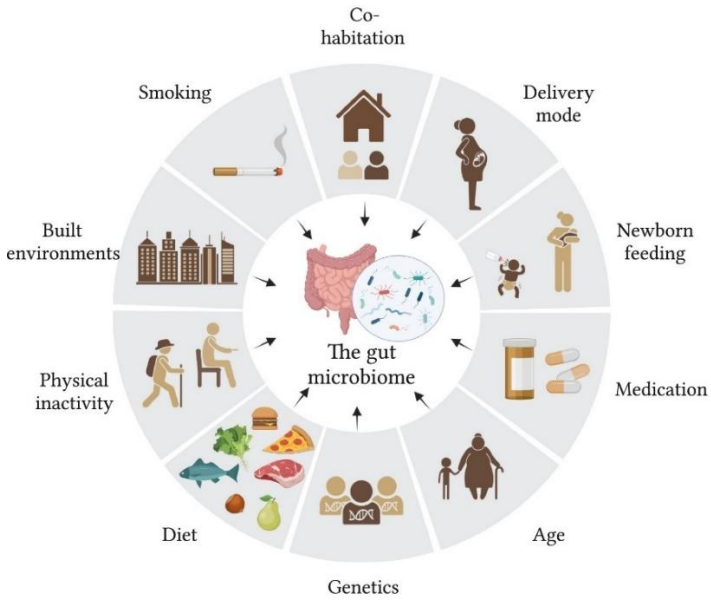


Figure 1.3. Factors influencing the composition of the human gut microbiota. Both extrinsic environmental factors, including the built environment (the environment where we live), cohabitation, diet, infant feeding, medication, and smoking, and intrinsic host factors such as genetics, mode of delivery, physical activity, and age contribute to shaping gut microbial communities. Adapted from Oliveira et al.[36]

1. Intrinsic Factors

The fetus is thought to be colonized by the placenta microbiome, which may shape the fetal immune system and microbiota[8]. However, this hypothesis remains controversial, and current data are limited, requiring further investigation[50]. From the moment of birth, this dynamic ecosystem starts developing and progresses rapidly during infancy[49].

The mode of delivery influences the initial microbial composition of the newborn. Infants born vaginally acquire microbes resembling the mother's vaginal microbiota, including the genera *Lactobacillus* and *Prevotella*, while those born by cesarean section are colonized by skin and environment microbes, such as *Staphylococcus*, *Corynebacterium*, and *Cutibacterium* (known as *Propionibacterium* in the old nomenclature)[8,9]. Cesarean-delivered infants typically exhibit lower microbial diversity and a higher risk of immune-related diseases[3].

Infant feeding also shapes the early colonization of microbes. Breastfed infants usually harbor diverse and complex microbiota, dominated by the genera *Bifidobacterium* and *Lactobacillus*[9], while formula-fed infants tend to have higher abundances of the genera *Escherichia*, *Bacteroides*, and *Clostridium*[3].

The gut microbiota gradually increases in diversity and complexity during the first years of life, reaching an adult-like composition by approximately three years of age[51]. Throughout adulthood, the microbiota remains relatively stable but undergoes notable shifts during aging, often characterized by decreased diversity, reduction in beneficial Bacillota, and increased proportions of Bacteroides and Clostridiota, which may contribute to increased susceptibility to dysbiosis and age-associated diseases[45].

Host genetics also plays a role in shaping the microbiota composition[52], however, new evidence suggests that this influence has a minor role. This is supported by findings of microbiota similarities in genetically unrelated individuals sharing the same house[53] and by a twin study reporting an average genetic contribution of only 8.8% of influence[25]. Additionally, sex-related differences in microbiota composition have been reported, potentially driven by the interaction between sex hormones and the gut microbiota[54,55].

2. Extrinsic Factors

The environment where we live can influence our microbiota composition. Factors like exposure to pollutants, chemicals, and socioeconomic conditions vary with geography and cultural practices. These factors can shape our diet, medication, lifestyle, hygiene practices, and rural or urban living[56]. They are also reflected in the built environments we inhabit, such as transport systems and buildings, which harbor and transmit microbes across different areas and individuals[57].

Gut bacteria proliferation depends on the nutrients from the host's diet as their primary energy source, making diet the most influential and modifiable factor in shaping microbiota composition. Diets rich in fiber promote the growth of SCFA-producing bacteria, which support a more beneficial effect on the immune system, weight, glucose homeostasis, insulin sensitivity, and food intake[58]. In contrast, Western diets high in saturated fat, refined carbohydrates, and animal protein are associated with reduced microbial diversity and with inflammatory diseases and allergies[10]. Since the diet is a modifiable factor, it is a potential therapeutic strategy to improve the diversity and stability of microbiota composition[25].

Medication use, especially antibiotics, can profoundly disrupt microbiota, reduce their diversity, and even lead to the loss of some species. It can also lead to dysbiosis, which can facilitate pathogen overgrowth in cases of resilience failure[3]. Other drugs, including proton-pump inhibitors, nonsteroidal anti-inflammatory drugs, metformin, and atypical antipsychotics, also alter microbial community structure and function[59].

Physical activity has been associated with increased diversity, improved metabolic functions of the gut microbiota, and less intestinal inflammation[60,61]. Other lifestyle factors, including stress, sleep quality, alcohol, and smoking, have been shown to affect both the composition and function of the gut microbiota[3,5,56].

F. Microbial Metabolites and Short-Chain Fatty Acids

Beyond their taxonomic composition, gut microbial communities are functionally dynamic and produce a variety of metabolites that significantly influence host physiology[38].

Dietary plant fibers and polysaccharides are complex molecules that the human host cannot digest because of the absence of enzymes to decompose them[29]. These compounds reach the cecum and large intestine, where they are degraded by the gut microbiota. This process is done by anaerobic saccharolytic bacteria that ferment polysaccharides into various metabolites[58], and

gases such as carbon dioxide, hydrogen, and methane[62]. Among these microbial products, SCFAs are the most studied due to their significant impact on host physiology[38].

SCFAs are saturated, aliphatic organic acids containing between one and six carbon atoms. For example, acetate (C2), propionate (C3), butyrate (C4), isobutyrate (C4), isovalerate (C5), and valerate (C5)[62]. The most abundant SCFAs in the gut are acetate, propionate, and butyrate, accounting for more than 95% of SCFAs production, and in a molar ratio of 60:20:20 in the colon and stool. Their production is influenced by the type and quantity of ingested dietary fibers, especially plant-based polysaccharides, resistant starches, and oligosaccharides. While the Western diet generally contains 20 to 25 grams of fiber per day, plant-rich diets provide 60 grams, affecting microbiota composition and type and quantity of metabolites produced[58].

Fecal SCFAs concentration can be between 10 and 30 millimoles per day in rich-fiber diets, and in normal diets, they can reach 5 to 15 millimoles per day. However, these concentrations in feces do not reflect the rates of SCFAs production in the GIT, because 95% of them are rapidly absorbed by the colonocytes and utilized by the host, and only a small fraction is secreted in feces[58]. SCFAs provide between 5 to 15% of human daily energy requirements and provide 60% to 70% of energy for colonic epithelial cells[63].

In the cecum and proximal colon, genera such as *Roseburia* and *Faecalibacterium*, from the Bacillota phylum, can constitute up to 20% of the gut microbiota in some humans. These bacteria help maintain an acidic environment of 5.5 through butyrate production. In contrast, in the distal colon, where fiber availability is reduced, the pH increases to around 6.5, and bacteria from the Bacteroidota phylum predominate, producing acetate and propionate. This pH variation helps prevent the overgrowth of pH-sensitive bacteria, like the families Enterobacteriaceae and Clostridiaceae[58].

SCFAs are also involved in the modulation of the enteric and central nervous system, being key in gut-brain communication, by modulating inflammation and serving as an energy source. SCFAs act locally via the enteric nervous system and influence the central nervous system, regulate intestinal inflammation, act as metabolic regulators, and improve the gut barrier. Changes in mood, behavior, and cognitive function have been linked to SCFAs, but the mechanism is poorly understood[63].

Butyrate is produced by different bacteria, such as *Roseburia*, *Faecalibacterium*, and *Eubacterium*[58]. It is formed from the condensation of two molecules of acetyl-CoA. After that, it can be converted by phosphotransbutyrylase and butyrate kinase to form butyryl-CoA, which is known as the classical pathway. Alternatively, the acetyl-CoA molecules can be transformed via the butyryl-CoA: acetate CoA-transferase pathway[38]. There are also other ways to produce butyrate, using pathways that involve glutamate or lysine, known as butyrogenic pathways[62]. Butyrate has been associated with the inhibition of histone deacetylases, leading to changes in gene expression through epigenetic regulation. This will influence cell differentiation, proliferation, and apoptosis in cancer cells, inhibiting tumor growth, suppressing cancer cell motility, and inducing their death[63]. It can also regulate genes associated with the reduction of pro-

inflammatory cytokines, and induce macrophages to produce antimicrobial proteins, modulating the immune system[62]. Butyrate helps regulate gut permeability as it is the preferred energy source for colonocytes[58] , by promoting activation of genes to form the tight junctions, and also modulates mucus production by stimulating goblet cells. Additionally, it can cross the blood-brain barrier and has been associated with neurotransmitter production and energy metabolism, improving insulin sensitivity and reducing inflammation in adipose tissue[63]. Most butyrate is consumed locally in the colon by colonocytes, and a smaller portion is absorbed into the bloodstream and oxidized in the liver[58].

Acetate is produced by bacteria such as *Clostridium*, *Bifidobacterium*, and *Akkermansia*[37], and is synthesized from pyruvate. This production can occur through two main pathways, the classic pathway, where acetate is produced via acetyl-CoA hydrolysis, and the Wood-Ljungdahl pathway, which has two branches. The first branch involves the reduction of the CO_2 to formate, while the second branch involves the reduction of CO_2 to CO, which then combines with a methyl group[38,62]. Around 70% of acetate is used by the liver for energy production and the synthesis of cholesterol, fatty acids, glutamine, and glutamate, as well as gluconeogenesis. The remaining acetate is distributed to peripheral tissues, like the heart, adipose tissue, kidneys, and muscles[58]. Acetate can also cross the blood-brain barrier and has been shown to help reduce appetite in rodents[63]. In addition, it has anti-inflammatory effects and protects the epithelial barrier by increasing tight junction expression and reducing intestinal permeability[63].

Propionate is produced by bacteria such as *Bacteroides* and *Clostridium* genera[58] and is synthesized from succinate, via succinate pathway, or from acrylate with lactate, via acrylate pathway, and also from fucose and rhamnose via propanediol pathways, found in *Lachnospiraceae* and *Ruminococcaceae* species[38]. About 30 to 50% of propionate in the peripheral circulation is taken up by the liver from the portal circulation, where it is used for gluconeogenesis and to lower cholesterol synthesis[58]. Propionate also regulates gut hormones, such as peptide tyrosine tyrosine (PYY) and glucagon-like peptide-1 (GLP-1), which help control appetite, energy balance, and reduce weight gain. Additionally, it reduces inflammation, regulates tight junction proteins, and supports gut barrier function by promoting mucin production[63].

Nitrogen is essential for bacterial growth, and approximately 50% of the host's urea is hydrolyzed to ammonia (NH_3) in the large intestine. Proteolytic fermentation of bacterial proteins and amino acids produces potentially toxic metabolites, including amines, phenolic compounds, and volatile sulfur compounds. This process also generates branched-chain fatty acids such as isovalerate, isobutyrate, and 2-methylbutyrate, and branched-chain amino acids valine, isoleucine, and leucine. These amino acids contribute to the production of SCFAs[38,58].

The balance and availability of SCFAs are closely linked to gut health. Low levels are associated with a negative impact on human health and are observed in individuals with inflammatory bowel disease (IBD), type 2 diabetes, autoimmune disorders, obesity, irritable bowel syndrome, and colorectal cancer. Conversely, high levels are associated with a decrease in their absorption,

some studies show high levels of propionate linked to type 2 diabetes and obesity, and increased acetate levels promote lipogenesis and obesity[37].

Metabolomics is used to uncover and characterize the metabolites in a biological sample. With this, it is possible to understand how food is digested, absorbed, and fermented in the GIT and how these processes impact human metabolism. It provides information about bioaccessibility, absorption, food residues in the colon, and microbiota activity. Fecal metabolites may reflect gut and host health and could serve as potential diagnostic biomarkers, however, some studies report individual variability[64]. Current research suggests that functional capabilities may be more indicative of health than microbiota composition[49].

One technique used to detect SCFAs in human samples is proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. This method can detect more than 70 metabolites in feces and is based on the magnetic properties of the hydrogen nuclei[37]. With high reproducibility, low sample preparation requirements, moderate sensitivity, and rapid data acquisition, ^{1}H-NMR is a reliable method for metabolite analysis[37]. Also, the peaks can be quantified using internal standards[64]. However, it can be expensive due to the use of deuterated solvents, which are used to minimize interference from water protons and allow spectra visualization[37].

G. Current Knowledge Lacks Longitudinal Studies

Despite significant progress in gut microbiome research over the past two decades, defining a healthy gut microbiota is a challenge in humans, since there is a substantial individual variation[49]. Most microbiome studies are based on cross-sectional data, which provide a single snapshot of the microbial community at one point in time[10].

To understand the temporal dynamics of the microbiome, how it changes over time, and the relation with various factors and their implications for health and diseases, longitudinal studies are essential. In these studies, the same individuals are sampled across multiple time points (TPs)[49]. These studies provide a unique opportunity to understand intra-individual variation over time, explore the impact of early microbiome signatures through life, the effects of social and lifestyle factors on microbial composition, and observe microbial responses to perturbations, such as antibiotic use or dietary changes. They can also help identify early microbial signatures that predict future disease or response to treatment[10,65].

Sampling the same individual several times can provide information about host genetic effects, identify microbial taxa that are heritable, minimize cohort effects, and reveal age-related changes in the microbiome. This approach can reveal host-associated microbes. If they remain stable over time, it may suggest better adaptation to the environment. On the other hand, if variance occurs, it could indicate a higher capacity for adaptation to environmental changes or other perturbation. Such studies can help distinguish which microbial communities are more stable and which ones fluctuate more over time[10,65].

However, longitudinal microbiome studies remain limited due to several scientific, logistical, and financial challenges[18]. Maintaining participant engagement over extended periods is difficult, and dropout rates can be high. In addition, sampling intervals may be inconsistent, which can complicate data interpretation. Despite these challenges, longitudinal studies remain a crucial tool for uncovering how the gut microbiome interacts with the host over time and for distinguishing between expected variations or fluctuations and changes that may reflect environmental shifts or imbalances potentially associated with diseases[65].

H. Citizen Science Approach: Engaging the General Portuguese Population (Focus on Lisbon)

To address the lack of gut microbiome data in Portugal, particularly in the context of longitudinal studies, this work adopted a citizen science framework. Citizen science is defined as the active participation of the public in scientific research, either as collaborators or contributors, with the aim of advancing and generating scientific knowledge. Participants may contribute in various stages of the research process and receive feedback on their participation. They can help with data and sample collection, help formulate research questions, and even contribute to data analysis and interpretation[66].

Effective citizen science projects must be accessible, transparent, and collaborative. These projects should provide mutual benefit, citizens can share their knowledge, improving research quality, while scientists promote scientific literacy, contributing to the truths in science[67]. This approach aligns with the ten principles of the European Citizen Science Association (ECSA). The data generated must be available in open access, in accordance with ethical guidelines and the principles of open and democratic science[68].

Several large-scale microbiome studies have successfully implemented this approach. Notable examples of citizen science projects include the *American Gut Project*[54], the *British Gut Project*[69], and the *Flemish Gut Flora Project*[70], where citizens contributed fecal samples to advance our understanding of gut microbiome diversity. Other initiatives include *Isala*, focused on the vaginal microbiome in Belgium[71], and *Saca La Lengua*, which explores the oral microbiome in Spain[72].

Citizen Science strategies have enabled large-scale sample collection over time, with public involvement in microbiome research and promoting scientific and health literacy[66]. Moreover, it aligns with the priorities of the European Commission, which encourages open science, data democratization, and public engagement in research[73].

The citizen science approach of *Microbioma Comunidade Portugal* was launched in 2024 under the *Programa Ciência + Cidadã* (C+C Program), as part of the Open Science strategy in Oeiras. The program currently supports nine dynamic citizen science projects that address critical challenges in sustainable agriculture, microbiology, health, environment, and climate change. Through the active involvement of citizens, these projects have already produced peer-reviewed

publications, gained international recognition, and secured European Union funding and awards, thereby demonstrating the transformative potential of collaborative science[74, 75, 76].

In the present study, the composition of the intestinal microbiota in the portuguese population was analysed, with a focus on families living around Oeiras, and the variations over time at the phylum level were examined. Microbial diversity at the first time point was evaluated, along with metabolomic profiling on some individuals, particularly the temporal variation of SCFA levels. Overall, this project gives a general picture of how the microbiota and its metabolic activity change over time in a local portuguese group.

MATERIALS AND METHODS

A. Experimental Design and Data Collection Procedures

1. Study Design

The *Microbioma Comunidade Portugal* project was a longitudinal observational study, designed to analyse the microbiome composition over time of participants from different families. The study involved non-invasive sample collection and data gathering from a diverse population living in the Lisbon metropolitan area, especially in the Municipality of Oeiras.

This study was conducted at the Gulbenkian Institute for Molecular Medicine (GIMM) in Oeiras, where the scientific coordinators Isabel Gordo and Karina Xavier are based. The project was carried out in collaboration with several institutions, such as Católica Biomedical Research Centre (CBR, Luis Teixeira), GIMM CARE (Ana Margarida Almeida), Everything is New (Patrícia Morais), Instituto de Tecnologia Química e Biológica António Xavier (ITQB NOVA, Sarela Santamarina and Maria João Leão), C+C Program (Maria João Leão), and Oeiras Municipality.

2. Ethical Approval

The study was approved by the Ethics Committee of IHMT – ITQB – NSL – IGC on March 13, 2024, following ethical guidelines.

3. Inclusion Criteria

- Participants must be between 5 and 99 years old and reside in the metropolitan area of Lisbon, particularly in the Municipality of Oeiras.
- Samples from at least two family members (individuals with a kinship relationship or living in the same household) are required.
- Participants must provide some personal data and report whether they have any diagnosed diseases, medication, including antibiotics.
- People with chronic diseases such as diabetes, obesity, hypertension, and thyroid disorders were not considered exclusion factors due to their high prevalence in our community.

4. Exclusion Criteria

- Individuals with diagnosed intestinal diseases or undergoing treatment for oncological diseases were excluded.
- Pregnant individuals were not eligible to participate.

5. Recruitment and Selection of Participants

The recruitment of participants was carried out under the coordination of the C+C Program. The project was disseminated through the social networks of partner institutions, by email, flyers,

newsletters, and video. It was also promoted during the Open Day events of GIMM and ITQB NOVA, in schools, universities, and senior academies. During these events, the objectives of the project were presented, and flyers containing a QR code linking to a quiz were distributed, offering participants the option to provide an email address for further contact.

With this study, the goal was to recruit about 30 families, with a total of between 120 and 150 participants. Each person was asked to collect three fecal samples during a period of six months, one sample every two months, and the collection could be different between participants, even within the same family.

For the selection, the inclusion and exclusion criteria were considered, as well as the order of inscription, with preference for those residing in Oeiras. This study was designed to last 1 to 2 years.

6. Kit Composition

The kit *Microbioma Comunidade* was developed based on the Kit EasySampler® (Ref. 107000) with the addition of a tube with DNA stabilizer from Invitek (Ref. 1038111200). These kits were funded by the C+C Program and assembled at GIMM Oeiras with the help of citizen scientists and contains:

- The sampling base;
- Two spoons to assist with sample collection;
- One pair of gloves to prevent contamination;
- The protocol *Microbioma Comunidade Portugal* for sample collection;
- An informative leaflet about the importance of the microbiome;
- Three stickers, two for labelling the tubes and one for the envelope;
- Three documents:
    - Informed consent of the project;
    - Informed consent for photos, as part of the Citizen Science project;
    - A questionnaire to be filled out during each sample collection.
- Two tubes:
    - Tube from the original kit, without any liquid;
    - Tube with DNA stabilizer from Invitek. To prevent leakage, this tube was placed inside a larger tube, with absorbent paper to ensure safety in case of any spillage.

      Note: To avoid mistakes during sample collection, a one-pea icon was placed on the tube with the DNA stabilizer and a three-pea icon on the empty tube for easy identification.
- A bag and an envelope for storing the samples after collection.

All items were packed inside the plastic bag, and each box included the number of kits needed according to the number of participants per family. If they did not cohabit, a new box was delivered. The box and its composition are present in Figure 2.1.

Figure 2.1. (A) Box and (B) contents of the Kit *Microbioma Comunidade*. It includes all materials required for sample collection, including sampling base, gloves, spoons, two tubes, three stickers, the sample collection protocol, informed consent documents, a questionnaire, an informative leaflet, and storage materials.

7. Sample Collection and Documentation

The sample collection was done before the day scheduled for delivery, since they needed to be delivered to GIMM Oeiras within 24 hours. The participants collected the samples at their homes using the kit and following the protocol *Microbioma Comunidade Portugal*. After sample collection, the tubes were placed in the plastic bag, then in the envelope, to be stored in the refrigerator at 4ºC.

When the first sample was delivered, the informed consents and questionnaires were also received. Informed consent for children and adolescents was obtained from their parents or legal guardians. Then the second box of the kit for the second collection was provided, and the following collection was done in the same manner. The schedule for the collections was arranged by phone call, with approximately two months apart, and based on the availability of the participants.

8. Participant Data

Personal data were collected through the questionnaire. All this information was transferred to an Excel file for the metadata of the samples. This included information on health, probiotics, diseases, medication, including antibiotics, and pregnancy. It also covered lifestyle factors such as diet, alcohol consumption, fruit and vegetable intake, dairy intake, and smoking. For bowel health, participants provided data using the bristol stool chart (BSC), stool colour, bowel

movements, and frequency. Additionally, birth-related information was gathered, including birth method, place of birth, and country, breastfeeding history, as well as personal details like year of birth, weight, height, sex, residence, blood type, family, and profession. These responses were filled out with the participant's code to maintain anonymity.

All participant information was encrypted with a family code and a personal code to ensure privacy. The code was delivered to the responsible of the family, following the European Union General Data Protection Regulation (2016). Only the scientific coordinator and the project logistics manager know the password and have access to these files. The information will be stored in the office of the scientific coordinator for a period of three years, after which it will be destroyed.

9. Fecal Sample Processing

Fecal samples were delivered to GIMM Oeiras by a member of the family or collected at participants' homes. They were stored at 4°C on the laboratory refrigerator until further processing, with a maximum of 48 hours. To ensure biosafety conditions and prevent contamination, all procedures were carried out in a controlled environment in Biosafety Level 2 (BSL-2). Before sample processing, five eppendorf tubes of 2 mL were pre-labelled and weighed. Each fecal sample present in the tube without any liquid was aliquoted into those sterile eppendorf tubes, with approximately 100 mg per tube. Tubes were then cleaned with virkon and ethanol to minimize contamination and weighed.

For metabolomic analysis, the aliquoted samples were immediately frozen on dry ice. It is recommended that samples be frozen as soon as possible after collection to preserve their integrity. For cultivation purposes, 800 µL of phosphate-buffered saline (PBS) was added to each tube with the sample, and the mixture was homogenized using pestles. Subsequently, 800 µL of a 40% glycerol / 0.2% cysteine hydrochloride monohydrate solution was added and homogenized by vortexing. The tubes were also placed on dry ice. After processing all the tubes, they were cleaned with virkon and ethanol and placed in a plastic bag to leave BSL-2. One of the tubes for cultivation was prepared and delivered to Sarela Santamarina at the ITQB for studies beyond this project. The other four tubes were stored at -80ºC freezers in GIMM Oeiras.

B. Microbiota of the Human Fecal Samples

1. Aliquots and DNA Extraction

The tubes with the DNA stabilizer were maintained in the refrigerator until the delivery to GIMM CARE in Lisbon, at a maximum of two-month intervals. When the tube arrived, the samples were aliquoted into five tubes and frozen at -20ºC. DNA extraction was performed in two aliquots, following the ZymoBIOMICS DNA Kits protocol. The DNA concentration was measured using Qubit and then frozen at -20ºC. One aliquot was sent to Novogene for sequencing, and the other remained at the Biobank under the responsibility of Ana Margarida Almeida.

2. Library Construction and Sequencing

Novogene performed the sample quality control by first assessing DNA quantification using a NanoDrop spectrophotometer, followed by evaluation of DNA degradation and contamination using agarose gel electrophoresis. After passing this first quality check, PCR amplification was performed targeting the V4 region of the 16s rRNA gene using the forward primer GTGCCAGCMGCCGCGGTAA and reverse primer GGACTACHVGGGTWTCTAAT, both containing sample-specific barcodes. The PCR products were then purified to remove primers and enzymes through a 2% agarose gel electrophoresis, and size-selected for the library preparation. The same amount of the PCR products from each sample was used for library construction, which included end repair, A-tailing, and ligation of Illumina adapters. The integrity and size of the library were confirmed using the Agilent 5400 system and quantification by qPCR. Sequencing was then performed in one run on the Illumina NovaSeq platform. The original data generated by the platform were demultiplexed by Novogene based on sample-specific barcodes and transformed to Sequenced Reads, also known as Raw Data, and recorded in a FASTQ file, with the sequenced reads and their corresponding quality.

3. Processing and Analysis of 16S rRNA Sequencing Data

i. Barcodes and Primers Removal

The initial step involved the removal of barcodes and primers, using Cutadapt (v2.3), to obtain the sequenced reads.

ii. Quality Assessment of Reads

Then the quality of raw reads was assessed with FastQC (v0.11.9). Individual reports for each sample were then summarized using MultiQC (v1.19), which provided an easy visualization of the quality results across all samples.

iii. Import Data into R

The FASTQ files were imported into R (v4.4.2) through RStudio (v2024.12.1.563).

iv. DADA2 Pipeline

The data was processed with the DADA2 Pipeline Tutorial (v1.16) and using the DADA2 package (v1.34.0).

Quality Inspection and Trimming:
Quality profiles of forward and reverse reads were visually inspected and then truncated at 210 bp to remove low-quality ends.

Error Rate Model:
DADA2 sequencing error models are specific to each dataset, and based on them, it learns error rates. Since NovaSeq platforms use binned quality scores, which means that instead of a continuous quality score, they are discrete, the monotonic error function was used to adjust the model appropriately.

Dereplication and Sample Inference:
Reads were dereplicated automatically, which identifies and groups identical reads into a unique sequence with their respective abundance. Sample interference was then conducted with the monotonic error model, correcting substitutions, insertions, and deletions. This step produces ASVs, representing the biological DNA sequence present in each sample, with single-nucleotide resolution.

Merging Paired-End Reads:
Forward and reverse reads were assembled, requiring sufficient overlap and perfect matching in the overlapping region. Non-overlapping or mismatched reads were discarded.

Chimera Removal:
During the PCR, the DNA strand can not be fully extended, detach from the template, and anneal to a different template. This form of chimeric sequences needs to be removed to trust our data. This remotion was performed using the consensus method, which identifies ASVs that can be reconstructed from the combination of more abundant sequences.

Taxonomic Assignment
Taxonomy was assigned to the resulting ASVs using the SILVA reference database (v138.2). This classifier was recently updated and is highly used in microbiome studies, allowing classification at the species level. However, the V4 region has a limited resolution at that taxonomic level, since it is a small part of the 16s rRNA gene.

v. Phylogenetic Tree Construction

A phylogenetic tree was constructed using the neighbor-joining method implemented in the phangorn package (v2.12.1). This method offers a good balance between speed and accuracy for the dataset analysis.

vi. Construction of the Phyloseq Object

A complete microbiome object was created using the phyloseq package (v1.50.0), which integrates the taxonomic table, sample metadata, ASV abundance table, and the phylogenetic tree into a single data structure for downstream analysis.

vii. Contaminant Filtering

To identify potential contaminants, the decontam package (v1.26.0) was used, applying both the prevalence and frequency-based methods based on the AVSs of the negative controls.

viii. Filtering Steps

The dataset was refined by removing ASVs not classified as Bacteria at the kingdom level, ASVs unclassified at the phylum level, and low-abundance ASVs with fewer than 100 total reads.

ix. Data Visualization and Diversity Analysis

Microbiota composition at the phylum level was visualized using ggplot2 (v3.5.1). Microbial diversity was assessed using alpha and beta diversity. These metrics were calculated to

characterize the richness and variability of the gut microbiota across TPs and participants. Data dispersion was assessed using the interquartile range (IQR), represented by the first (Q1) and third (Q3) quartiles.

Alpha diversity is a metric that measures the diversity of a sample, and has different metrics, such as the observed or richness, that uses the number of ASVs in the sample, Chao 1 index estimate the richness based on the total species richness, Shannon index measure the richness and the equity based on the abundance distribution, and Simpson index is the probability of two random reads being from the same species.

Beta diversity is a metric that compares the composition between different samples. It uses different distance metrics such as the Bray Custis distance, that is based on the dissimilarity and the abundance, the Jaccard Index, that measures the dissimilarity based on the presence and absence of species, and the Unifrac distance is based on the phylogeny and can be related with the presence and absence, Unweighted Unifrac, and based on the relative frequence, Weighted Unifrac.

Note: These steps were also performed using rarefaction at 20,000 reads per sample, and similar results were obtained. However, since rarefaction can affect the results[77], all analyses presented in the following section were conducted without rarefaction.

4. Statistical Analysis

To the analysis at the phylum level and the ratio Bacillota/Bacteroidota, the Kolmogorov–Smirnov test was used to understand if the dispersion would be different between time points, and the Wilcoxon signed-rank test to compare the paired samples, also between time points. Also, the Fligner-Killeen test was used to test if the variances were similar at the phylum level between time points.

To correlate different variables, the Spearman correlation test was used to understand if one variable influences the other variable.

For the alpha analysis, a Wilcoxon rank-sum test for independent samples with only two factors and a Kruskal-Wallis test for variables with more than two factors. The beta analysis for intra- and inter-individual variation was conducted with the Wilcoxon rank-sum test.

The statistically significant data was considered when the p-value < 0.05.

C. Metabolomics of the Human Fecal Samples

1. Samples Chosen for Metabolomic Analysis

For the metabolomic analysis, to simplify and due to time constraints associated with analysing such a large dataset, one sample per family was selected. The criteria for sample selection included availability at both time points, adult individuals of approximately the same age, a balanced gender ratio, and a shorter time interval between sample collection and processing.

2. NMR Sample Preparation and Filtration of Fecal Metabolites

To determine the metabolic environment in the fecal samples of humans, we used ^{1}H-NMR analysis. Sample preparation and filtration steps were performed under BSL-2 conditions, while filter preparation was carried out in a standard laboratory environment.

Fecal samples were diluted in 1 ml of deuterated water (D_2O, 99,9% Sigma-Aldrich), and 0.3 g of 0.1 mm glass beads (Scientific Industries SI-BG01) were added. Tubes were sealed with parafilm, wiped with virkon and ethanol, and transferred out of BSL-2 for bead-beating using a QIAGEN Retsch Tissuelyser II for two 1-minute cycles with a pulse of 30 Hertz per second, and with plate inversion between cycles. Samples were transported to BSL-2, unsealed, and centrifuged at 14000 RPM for 30 minutes at 4°C to remove the glass beads and debris. Supernatant was collected and filtered through a 0.22 µm syringe filter (Avantor) and followed by 3 KDa filters (Vivaspin® 500) in a centrifuge at 15000 RCF and 4ºC for seven hours or left overnight to obtain at least 150 µl of filtrate. Samples were stored at -80°C freezer until spectrum acquisition.

3. NMR Spectrum Acquisition for Fecal Sample Metabolomics

For NMR spectrum acquisition, samples were defrosted at room temperature, and the preparation was carried out in a previously washed 5 mm glass NMR tube.

The phosphate buffer was prepared using KH_2PO_4 (potassium dihydrogen phosphate) and K_2HPO_4 (dipotassium hydrogen phosphate) at 350 mM and with 2% of NaN_3 (sodium azide) to obtain a pH close to 7.

To each NMR tube, 380 µL of D_2O, 60 µL of phosphate buffer, 10 µL of a 0.05% (w/v) 3-(trimethylsilyl) propionic acid-2,2,3,3-d_4 sodium salt (TSP-d4, Sigma-Aldrich, 0.04949 mM, as internal standard for quantification), and 150 µL of the fecal sample were added. The mixture was then homogenized by inversion, and the pH was measured.

The samples were run on a Bruker AVANCE II+ 500 MHz instrument equipped with a 5 mm TCI(F)-z H-C/N Prodigy cryo-probe. With the TopSpin program (v4.0.7), the system was locked on the deuterium signal from D_2O to maintain the stability of the magnetic field. Tuning and matching adjustments were performed to optimize the frequency of resonance of the probe and the signal transference between the probe and the spectrometer, minimizing energy loss. Followed by shimming to ensure homogeneity of the magnetic field. The receiver gain, or the amplification of the received signal by the probe, was adjusted to 101. The 90° pulse was calibrated to obtain a better signal, and water suppression was optimized to allow spectrum visualization. Spectrum acquisition was done with the 1D NOESY pulse sequence with pre-saturation (noesypr1d), enabling suppression of the solvent signal while detecting spatial interactions between protons. The acquired spectrum was 200 scans at 25ºC, and the phase correction was performed to obtain peaks symmetric and clear. Samples were processed sequentially following the same procedure.

4. Metabolites Identification in Chenomx

Using the Chenomx program (v8.11), the acquired spectrum was imported into the Processor tool. The processing needs the pH of the sample and the concentration of the standard used. Then, the removal of water peak, baseline correction using Autospline, and chemical shift imaging (CSI) calibration were performed to obtain a good spectrum enabling the correct quantification of the metabolites based on the internal standard peak (TSP-d4). The spectrum was saved as a Chenomx spectrum file and imported into the Compound Builder tool, where peaks were manually assigned to reference peaks from the compound library of the program, enabling the determination of the SCFAs concentration in the sample.

5. Quantification of Metabolite Concentration

The first step is adjusting the concentration of the sample to the dilution factor. With the NMR spectra, we obtain the concentrations in µM of the contents inside the NMR tube. Since 150 µl of the sample was diluted in the tube with phosphate buffer, TSP-d4, and D_2O, the concentration must be adjusted by multiplying by a factor of 4, since the total volume of the tube was 600 µl.

The next step is to convert the concentration in the 150 µl of sample filtrate back to the initial volume in which the fecal material was resuspended. The initial volume is considered as the weight of the fecal material (assuming 1 g = 1 mL) plus the 1 mL of D_2O added. To obtain the quantity of each fecal metabolite in µmol, the concentration was multiplied by the total sample volume in liters, assuming no loss of measurable entities during extraction.

Finally, mass normalization accounts for different fecal sample masses. To obtain the amount of metabolites (µmol) per mass of the sample (g), we divide by the total sample weight.

These steps allow the quantification of metabolites normalized to both volume and sample mass, ensuring consistency across different experimental conditions.

The next equation represents the normalization procedure.

$$\frac{Dilution\ factor * NMR\ concentration\ (\mu M) * \frac{Sample\ Volume}{1000}(l)}{Sample\ Weight\ (g)}\ \frac{\mu mol}{g}$$

6. Statistical Analysis

Statistical analyses of metabolomic data were performed in R, using the same packages applied in the microbiota analysis.

To compare metabolite concentrations between the two time points, the Wilcoxon signed-rank test for paired samples was used.

Correlations between microbiota composition and metabolite levels were assessed using Spearman correlation, with a Benjamini-Hochberg correction applied to account for multiple testing.

Statistical significance was defined as a p-value < 0.05.

RESULTS AND DISCUSSION

A. Logistics and Demographic Characterization of the Citizen Science Cohort

To obtain a longitudinal characterization of the microbiome population in the Lisbon area, mainly in Oeiras, we started the *Microbioma Comunidade Portugal* project, a pioneering citizen science microbiome project in Portugal.

This section describes the logistical organization of the project, how participants were recruited, the profile of participating families, the data collected, and the challenges encountered in a longitudinal study.

1. Project Launch and Participant Recruitment

The launch of the *Microbioma Comunidade Portugal* project took place on July 10, 2024, at the Municipal Library of Oeiras. This event was promoted through social media, flyers, and posters across partner institutions, attracting more than one hundred participants. During the session, the project goals, kits, and sample collection protocols were presented by Patrícia Morais and the other team members. Partners also discussed the importance of microbiome research with the community. Attendees interested in participating were invited to leave their email addresses for future contact.

Following this event, additional outreach activities were conducted, under C+C Program with Patrícia Morais collaboration, to engage more participants. These included participation in the International Microorganism Day event organized by ITQB NOVA at Santo Amaro Beach, the NOS Alive music festival in Algés, and the European Researchers' Night at Oeiras Marina. At these events, attendees could complete a microbiome quiz via QR code and leave their contact information.

In August and September, the participant information package and forms were sent to the collected emails. Eligible participants completed the forms to provide data such as name, phone number, email, number of family or household members, presence of gastrointestinal diseases, ages of family or household members, area of residence, pregnancy status, and an open field for any additional questions or concerns.

2. Participant Selection and Inclusion

In total, 56 registrations were received. Of these, 11 were excluded for not meeting the study inclusion criteria. From the remaining 45 families, 37 were contacted by phone to confirm their interest in participating. Four families did not respond to phone calls and were subsequently contacted by email, but only two confirmed their participation. Three of the contacted families declined to participate in the study.

For the 32 families who agreed to participate, an appointment was scheduled at GIMM Oeiras in September with a representative from each family.

During this visit, participants received a kit and a detailed explanation of its contents, along with instructions for sample collection and storage based on the *Microbioma Comunidade Portugal* protocol. Any questions or concerns about participation were addressed during this appointment. After the explanation, the first sample collection was scheduled, and participant codes were provided via email. For participants who requested home sample collection, appointments were arranged accordingly.

3. Longitudinal Sample Collection Workflow

Participants were instructed to collect the sample as close as possible to the delivery time, with a maximum of 24 hours allowed between collection and delivery, and a maximum of 48 hours until processing. The samples should be kept in the refrigerator, and for travel times exceeding 15 minutes to GIMM Oeiras, samples should be transported in a thermal bag with ice or a similar cooling method. Samples were required to be always refrigerated to prevent bacterial overgrowth and preserve integrity.

Sample deliveries were scheduled for the morning, with a daily limit of 12 samples, to ensure timely processing under controlled conditions. This limit was established to minimize the time between collection and processing, thereby preserving sample quality. Maintaining homogeneity in sample handling is essential to minimize possible confounding factors in microbiome studies[21].

However, since samples were not always delivered immediately after collection, some variation in the time from collection to processing occurred. On average, 5.4 samples were processed per day over 64 collection days, and the median time until processing from the sequenced samples was 18 hours and 30 minutes.

To support the continuity of longitudinal sampling, new kits for the subsequent collection were distributed after each delivery. Appointments for these collections were arranged by phone, depending on participant availability, and were scheduled approximately two months apart. If the family contact did not answer, a reminder message was sent. It was occasionally observed that one of the questionnaires was missing following sample delivery. When that occurred, the family responsible was contacted to provide the missing document.

The first time point (TP1) took place between August and October, the second time point (TP2) from October to December, and the third time point (TP3) from December to February. Although the intended interval between collections was approximately two months, scheduling conflicts sometimes occurred, leading to deviations from this timeline.

The main challenges during the collection process included difficulties in contacting participants, the need to reschedule appointments, and occasional delays in delivery. In some cases, families requested home collection, requiring the team to remain flexible and available on short notice. Good communication and adaptability were key to keeping participants engaged and ensuring

the quality of both biological and contextual data. Maintaining participant involvement is particularly important in longitudinal studies, where repeated engagement is needed over time[78].

The logistics of the sample collection across the three TPs are summarized in Table 3.1. The numbers reflect the family representative, who requested home collection, was contacted multiple times, needed to reschedule, or delivered family samples on different days.

Table 3.1. Logistics of sample collection across three time points based on one contacted person from the 32 families. Each value refers to the number of families represented by the family representative contact, who required logistical support during each phase of sample collection. Categories include requests for home collection, multiple contact attempts, rescheduled appointments, and deliveries on different days.

Collection Logistics	First sample collection	Second sample collection	Third sample collection
Home collection requested	5	8	6
Contacted more than once	17	21	20
Rescheduled appointments	4	9	9
Delivered on different days	14	15	15

As shown in Table 3.1, the number of families requesting home sample collection increased from five at TP1 to eight at TP2, then slightly decreased to six at TP3. This trend suggests that while most families initially managed in-person delivery, some required more assistance as the study progressed.

Similarly, the number of families contacted multiple times increased, from 17 at TP1 to 21 at TP2 and remained high at TP3. This reflects the increasing effort required to maintain engagement over time.

The number of rescheduled appointments also increased, from four families in TP1 to nine in both TP2 and TP3, likely due to difficulties balancing study commitments with personal and family responsibilities.

Additionally, nearly half of the families delivered their samples on different days within the same collection round. This often occurred due to difficulties coordinating collection among family members, especially in households with young children, complex work schedules, or non-cohabiting relatives.

These findings highlight the logistical complexity of longitudinal sample collection in community studies. They reinforce the need for flexible scheduling, ongoing communication, and responsive participant support to ensure high-quality, consistent data and maintain long-term participation[78].

4. Composition of the Study Cohort

In total, 119 individuals participated in the study, distributed across 32 families. Table 3.2 summarizes the age group and gender distribution of the participating families.

Table 3.2. Summary of the number of participants, their age group, and gender distribution across families. Each row represents a family, and the final row contains the total count across all families. Age groups are defined as children and adolescents (5–17 years), adults (18–65 years), and seniors (over 65 years). Gender is indicated by the letters M for male and F for female.

Families	Kids/Adolescents (Gender)	Adults (Gender)	Seniors (Gender)	TOTAL
1	3(M,M,M)	2(M,F)		**5**
2		2(M,F)		**2**
3			2(M,F)	**2**
4		3(M,M,F)		**3**
5	3(F,F,M)	2(M,F)		**5**
6		2(M,F)		**2**
7	2(M,F)	1(F)		**3**
8	2(F,F)	2(M,F)		**4**
9	2(M,F)	2(M,F)		**4**
10	2(M,M)	2(M,F)		**4**
11	2(M,M)	2(M,F)		**4**
12	1(M)	2(M,F)		**3**
13	1(M)	2(M,F)		**3**
14	1(F)	2(M,F)		**3**
15		2(M,F)		**2**
16		4(M,M,M,F)		**4**
17	4(F,F,F,M)	6(M,M,M,F,F,F)	1(F)	**11**
18			2(M,F)	**2**
19	2(F,F)	5(M,M,M,F,F)	1(F)	**8**
20		1(M)	1(F)	**2**
21	2(F,F)	2(M,F)		**4**
22	2(F,F)	3(M,M,F)	3(M,F,F)	**8**
23		2(M,F)		**2**
24	2(F,F)	2(M,F)		**4**
25		3(F,F,F)	2(M,F)	**5**
26		3(M,M,F)		**3**
27		2(M,F)		**2**
28	3(M,M,F)	2(M,F)		**5**
29		2(M,F)		**2**
30		4(M,M,F,F)		**4**
31			2(M,M)	**2**
32		2(F,F)		**2**
Total	**34(15M,19F)**	**71(36M,35F)**	**14(6M,8F)**	**119**

Regarding age groups, 34 participants were classified as children or adolescents (28.6%), 71 as adults (59.7%), and 14 as seniors (11.8%), allowing for a broad representation across different stages of life. The predominance of adults is related to the wider age range in this category and to the fact that adults are typically the primary contact and legal guardians responsible for providing consent. The inclusion of children, adolescents, and older adults supports microbiome analysis across life stages[79].

Participation was based on either biological or legal family ties, or on shared household residence, meaning that not all participants from the same family necessarily lived in the same household.

This inclusive approach allowed the recruitment of diverse family structures, including cohabiting relatives, non-cohabiting family members, and individuals connected through household dynamics.

Based on family size, 17 families were composed of two to three members and were classified as small, 12 families had four to five members and were considered medium-sized, and only three families had more than five members, they were classified as large families. This variation in family size was relevant for microbiome research, as both familial relationships and cohabitation are known to influence microbial composition through genetic background, environment, shared meals, hygiene habits, and interpersonal contact[80,81].

Human genetics is known to influence the gut microbiome, and even in cases where family members do not reside together, shared genetic traits may still contribute to microbial similarities[81]. One example is the host genetic predisposition to diseases[11].

Larger families, particularly those with both cohabiting and non-cohabiting members, offer a unique opportunity to investigate the dynamics of microbial transmission[82].

This diversity in family contributed to a rich and diverse dataset for the study. However, it also presented logistical challenges for organizing sample collection, especially in cases where family members lived in different households.

5. Characterization of the Study Population

In addition to biological samples, participants were asked to complete a questionnaire at each sample collection. This provided contextual information on personal, health, and lifestyle factors that may influence microbiome composition.

The questionnaire included questions about year and place of birth, gender, height, weight, residence, blood type, dietary habits, alcohol consumption, smoking status, consumption frequency of fruits, vegetables and dairy, medication use, health conditions, antibiotic and probiotic use, birth-related information, and bowel health indicators such as stool type, color, and evacuation frequency. The data collected enabled a comprehensive characterization of the study population and offered relevant context for interpreting microbiome results.

Although the questionnaire also included questions about pregnancy, bowel movement type, and profession, these variables were not included in the tables. No participants were pregnant or became pregnant during the study period. Additionally, the average evacuation frequency over five days overlapped with data already shown in Table 3.4, making this variable redundant. Finally, information on professional occupations was too heterogeneous to be categorized or interpreted. Interestingly, 20 families had at least one participant working in a research institution, possibly reflecting how the project was disseminated within scientific networks.

A summary of the main data regarding age, weight, height, and BMI is shown in Table 3.3.

Table 3.3. Participant demographics and anthropometric data. The numbers show the median values with interquartile ranges, sample size, and missing data per category. Age is expressed in years, weight in kilograms (kg), height in meters (m), and body mass index in kg/m². Groups are divided by age groups as described earlier. All data refer to responses in the first questionnaire, since some changes were observed over time.

Variable	Group	Median (IQR)	Sample Size (n)	Missing Data (n)
Age (years)				
	Kids/Adolescents	12.50 (10.00-14.00)	34	-
	Adults	46.00 (35.00-52.00)	71	-
	Seniors	76.50 (67.75-82.00)	14	-
Weight (kg)				
	Kids/Adolescents	41.50 (31.05-52.25)	32	2
	Adults	73.00 (62.00-85.00)	71	-
	Seniors	67.00 (58.00-77.25)	14	-
Height (m)				
	Kids/Adolescents	1.48 (1.39-1.63)	32	2
	Adults	1.72 (1.63-1.76)	71	-
	Seniors	1.67 (1.57-1.76)	14	-
Body Mass Index (kg/m^2)				
	Kids/Adolescents	18.43 (16.04-21.41)	32	2
	Adults	24.68 (22.06-28.08)	71	-
	Seniors	24.47 (23.27-27.52)	14	-

Table 3.3 summarizes the study population across the three age groups: Kids/Adolescents, Adults, and Seniors. Median ages were consistent with these categories, with 12.50 years for kids and adolescents, 46.00 years for adults, and 76.50 years for seniors. As expected, anthropometric values varied across groups.

Children and adolescents had the lowest median weight (41.50 kg) and height (1.48 m). Adults were generally heavier and taller, with a median weight of 73.00 kg and height of 1.72 m. Seniors showed a slight decrease in both weight (67.00 kg) and height (1.67 m), likely influenced by the higher proportion of female participants in this group.

Body mass index (BMI) values were mostly within the normal (values between 18.5 and 24.9) or slightly elevated range. Adults and seniors had median BMIs of 24.68 and 24.47 kg/m^2, respectively, indicating a generally normal population. Among children and adolescents, the slightly lower median BMI of 18.4 kg/m^2 reflects their growth stage. Two missing entries for height and weight in the children's group limited BMI calculations for those participants, though this is unlikely to affect the overall trend.

Table 3.4 provides additional details on participants' residence, lifestyle habits, digestive health, and overall health status.

Table 3.4. Overview of the study participant background, lifestyle, and health-related characteristics. Data is presented as counts of participants and percentages for each category. Variables include gender, residence, country of birth, blood type, dietary habits, alcohol intake, smoking status, daily consumption of fruits, vegetables and dairy, antibiotic and probiotic use, presence of diseases and medication use, mode and place of birth, breastfeeding history, stool characteristics (bristol stool scale, color, and evacuation frequency), and menstruation regularity. All responses refer to the first questionnaire, except for antibiotic use [a], which was updated after the first collection.

Variable	Category	Number (n)	Percentage (%)
Gender			
	Masculine	57	47.90%
	Feminine	62	52.10%
Residence			
	Oeiras	79	66.39%
	Lisbon	27	22.69%
	Cascais	11	9.24%
	Sintra	1	0.84%
	Seixal	1	0.84%
Born in Portugal			
	Yes	107	89.92%
	No	12	10.08%
Blood Type			
	A	48	40.34%
	B	2	1.68%
	AB	1	0.84%
	O	41	34.45%
	Did not know	27	22.69%
Type of Diet			
	Non-specific diet	105	88.24%
	Plant-based diet	9	7.56%
	Gluten-free diet	2	1.68%
	Intermittent fasting	1	0.84%
	Paleolithic diet	1	0.84%
	Plant-based and gluten-free diet	1	0.84%
Alcohol Intake			
	Never	34	28.57%
	No	15	12.61%
	Yes, sometimes	54	45.38%
	Yes, frequently	12	10.08%
	Did not answer	4	3.36%
Smoke			
	Never	80	67.23%
	No	31	26.05%
	Yes	8	6.72%

Portions of Fruits and Vegetables Per Day		
One	13	10.92%
Two to three	80	67.23%
Four or more	26	21.85%
Portions of Dairy Per Day		
One	42	35.29%
Two to three	67	56.30%
Three to four	9	7.56%
Five or more	1	0.84%
Antibiotic Use[a]		
After the first collection	4	3.36%
Probiotics Consume		
No	112	94.12%
Yes	7	5.88%
Diseases		
No	50	42.02%
Yes	69	57.98%
Medication Use		
No	47	39.50%
Yes	71	59.66%
Did not answer	1	0.84%
Mode of Delivery		
Vaginally	89	74.79%
C-section	30	25.21%
Local of Birth		
Hospital	110	92.44%
Home	8	6.72%
Did not know	1	0.84%
Breastfeeding		
No	8	6.72%
Yes	104	87.39%
Did not know	7	5.88%
Bristol Stool Chart		
Type 1	3	2.52%
Type 2	6	5.04%
Type 3	45	37.82%
Type 4	43	36.13%
Type 5	10	8.40%
Type 6	8	6.72%
Did not answer	4	3.36%

Color			
	Light brown	78	65.55%
	Brown	2	1.68%
	Dark	24	20.17%
	Green	1	0.84%
	Brown and green	1	0.84%
	Brown and red	1	0.84%
	Did not answer	12	10.08%
Evacuation Frequency			
	Less than one time per day	30	25.21%
	One time per day	60	50.42%
	More than one time per day	27	22.69%
	Did not answer	2	1.68%
Regularity of Menstruation			
	No	38	61.29%
	Yes	24	38.71%

Table 3.4 presents a detailed overview of the study population's lifestyle and health status. All data refers to the first questionnaire since changes were observed over time, except for antibiotic use.

The gender distribution was balanced, with 57 males (47.90%) and 62 females (52.10%). Most participants resided in Oeiras, reflecting the main recruitment area, and 89.92% were born in Portugal.

Most participants reported not following a specific diet (88.24%), though small numbers followed plant-based, gluten-free, intermittent fasting, or other diets.

Portugal traditionally follows the Mediterranean diet, rich in fruits, vegetables, legumes, olive oil, fish, dairy products, and small amounts of meat. A similar pattern is the Atlantic diet, which emphasizes seasonal, local foods but includes more tubers, cabbage, chestnuts, temperate fruits like apples and pears, and cereals such as rye and corn. The adherence to these traditional diets may influence the population's gut microbiota and reduce disease risk[83].

Regarding blood type, most participants were type A (40.34%) or type O (34.45%), with smaller percentages of type B (1.68%) and AB (0.84%). These numbers align with known blood type distributions in Portugal, where type O and A are the most prevalent, followed by type B and AB[84].

Alcohol consumption was reported by 55.46% of participants, while only 6.72% reported smoking. Most participants had never smoked (67.23%).

Fruit and vegetable intake was moderate to high, with 67.23% consuming two to three portions per day. Dairy consumption followed a similar pattern, with 56.30% consuming two to three portions daily.

In terms of health, 57.98% of participants reported at least one chronic condition, and 59.66% reported taking medication regularly. These conditions, such as diabetes, hypercholesterolemia, and hypertension, are common in the general population and were included in the study inclusion criteria. Other reported conditions included asthma, allergies, and neurological or psychological disorders such as anxiety, depression, and Attention-Deficit/Hyperactivity Disorder (ADHD).

Antibiotic use after the first sample collection was low (3.36%), and probiotic use was also rare (5.88%).

Concerning birth history, most participants were born in hospitals (92.44%) and delivered vaginally (74.79%). Breastfeeding was common, with 87.39% reporting having been breastfed.

Stool-related data, including BSC, stool color, and evacuation frequency, indicated overall healthy bowel patterns. Most participants reported stool types 3 and 4 (37.82% and 36.13%, respectively), which are considered normal. The most frequent stool color was light brown (65.55%). Half of the participants reported a bowel movement once per day. These indicators are normal for the gut function in healthy individuals[70].

Among menstruating participants, 61.29% reported irregular cycles, possibly due to continuous use of birth control pills, menopause, or being premenarcheal.

Together, these data provide a robust and detailed profile of the study population, supporting the interpretation of microbiome results despite some missing values.

6. Sample Processing

Sample collection was performed at the participants' homes, where each stool sample was divided into two tubes. A schematic representation of the sample processing steps is presented in Figure 3.1.

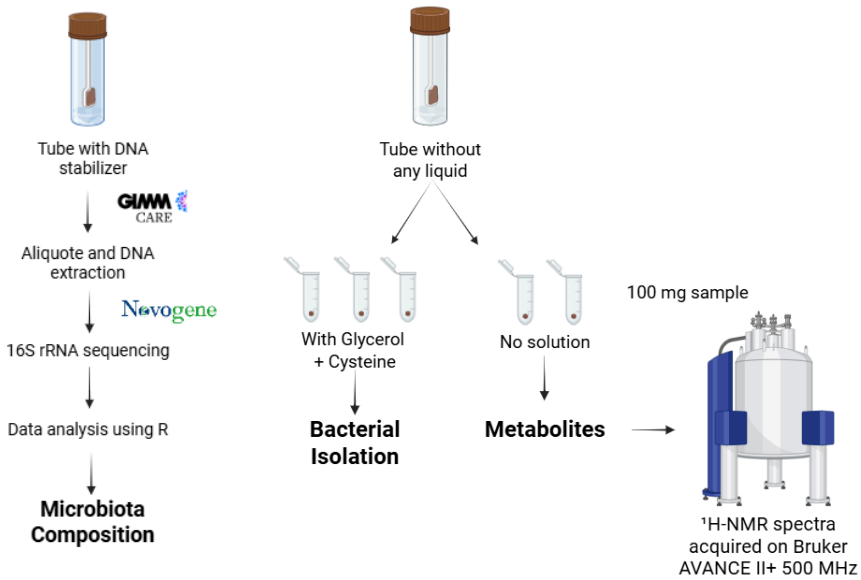


Figure 3.1. Tubes used for sample collection and experimental workflow for analysing microbiota composition, bacterial isolation, and metabolite profiling from stool samples. Stool samples were collected in two types of tubes: one containing a DNA stabilizer for the microbiota composition analysis, and one without any liquid, used for both bacterial isolation and metabolites profiling.

One tube contained a DNA stabilizer, which was selected based on previous optimization by one of the GIMM CARE projects. This method was shown to be the most effective for DNA preservation and extraction in two months apart, particularly in individuals with intestinal diseases, ensuring high-quality data for microbiota composition analysis.

The second tube did not contain any liquid, as it was used for two distinct purposes, with approximately 100 mg of sample in each eppendorf tube: (1) bacterial isolation, which required the addition of glycerol and cysteine to preserve viable bacteria, and (2) metabolomic profiling of the SCFAs, which requires the absence of solutions in the sample to avoid interference with ^{1}H-NMR spectroscopy analysis.

7. DNA Sequencing

The tube containing the DNA stabilizer was delivered to GIMM Care in Lisbon for subsequent DNA extraction. Samples were transported to the laboratory in five separate batches, with each stored in refrigeration for no longer than two months before delivery. Table 3.5 summarizes the delivery dates, number of samples, and the corresponding time points (TP1, TP2, TP3) included in each batch.

Table 3.5. Delivery dates and number of fecal samples per batch, preserved in DNA stabilizer, and sent to GIMM Care (Lisbon) for DNA extraction. "TP" refers to the time point of sample collection (TP1, TP2, or TP3). The table details the number of samples from each time point per delivery, the delivery date, and the total number of samples in each batch.

Batch Number	Delivery Date	Samples Included	Total Samples
1	17/09/2024	70 TP1	**70**
2	10/10/2024	47 TP1 + 4 TP2	**51**
3	21/11/2024	2 TP1 + 93 TP2	**95**
4	17/01/2025	18 TP2 + 89 TP3	**107**
5	26/02/2025	23 TP3	**23**

As shown in the table, the first batch included only TP1 samples. Subsequent batches included an increasing number of TP2 and TP3 samples, reflecting the progressive nature of sample collection across participants. The largest batch was the fourth, which corresponds to the peak of collection activity during TP3. Since participants delivered samples at different times, some batches included a mix of time points.

To ensure DNA stability, samples were refrigerated at 4ºC and processed within a maximum of two months after collection. This is essential to minimize microbial profile alterations due to prolonged storage time and ensure DNA stability for accurate downstream extraction and analysis[85]. Batch processing also allowed consistent sample handling and minimized technical variability, known as batch effects, which are known confounding factors in microbiome studies[18]. Despite the logistical challenges of longitudinal community studies, the protocols implemented helped ensure sample integrity and quality throughout the study.

However, as expected in long-term studies, some participants dropped out. One family left the study after TP1, and three individuals from other families were unable to provide a third sample for personal reasons. Consequently, the total number of samples collected decreased slightly

over time: 119 samples were collected at TP1, 115 at TP2, and 112 at TP3, resulting in a dropout rate of 5.88%.

After collection and DNA extraction, samples were stored at -20ºC. Samples from batches 1 to 3 were sent to Novogene for sequencing. However, not all samples met the minimum DNA concentration threshold of 10 ng/µL and had to be excluded. In these three batches, seven DNA extractions were performed in total.

A visual overview of the entire workflow, from sample collection to sequencing, is presented in Figure 3.2, highlighting the number of samples processed at each step and those excluded due to low DNA concentration.

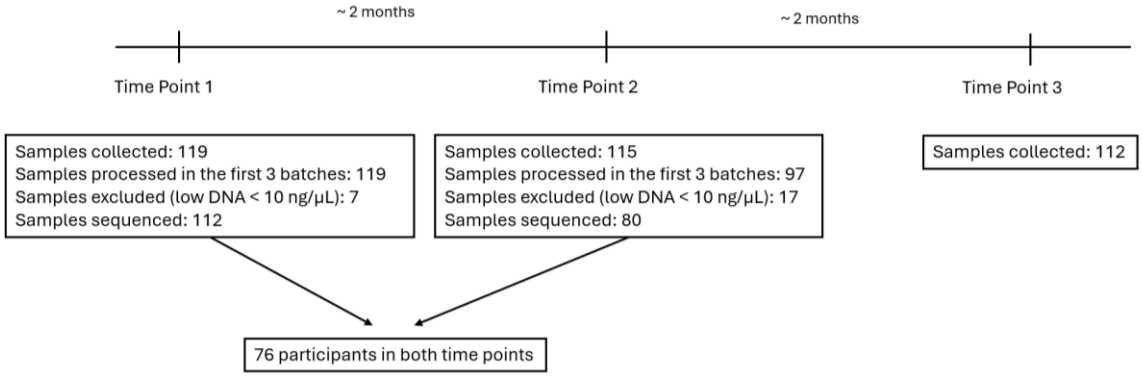


Figure 3.2. Workflow overview of sample collection, processing, and sequencing across the three time points of the study (TP1, TP2, and TP3), with emphasis on the first three batches sent to GIMM CARE for DNA extraction. Each time point was approximately two months apart. Samples with low DNA concentration (<10 ng/µL) were excluded from sequencing. A total of 76 participants had samples sequenced at both time points.

From the 119 samples collected at TP1, 112 passed the quality check and were sequenced. At TP2, from the 115 samples collected, 97 were included in the sequencing batches, but only 80 had sufficient DNA concentration. TP3 samples had 112 samples collected and processed, but these had not yet been sequenced by the time of this analysis.

Notably, 76 participants had usable, sequenced samples for both TP1 and TP2, enabling longitudinal analysis. However, three individuals lacked sequenced samples at both TPs due to either quality control failure or insufficient fecal material collected into the DNA stabilizer tube.

In total, 11% of sequenced samples were excluded due to low DNA concentration, which is a common limitation in microbiome studies involving self-collection. This finding emphasizes the importance of proper sample collection to avoid compromising the results[20].

B. Microbiota Composition Analysis

1. In the Portuguese Community

We began by analysing the median relative frequency of the gut microbiota composition at the phylum level using 112 samples from TP1 and 80 samples from TP2. Figure 3.3 shows the median relative abundance of the most prevalent phyla, along with the interquartile range (IQR).

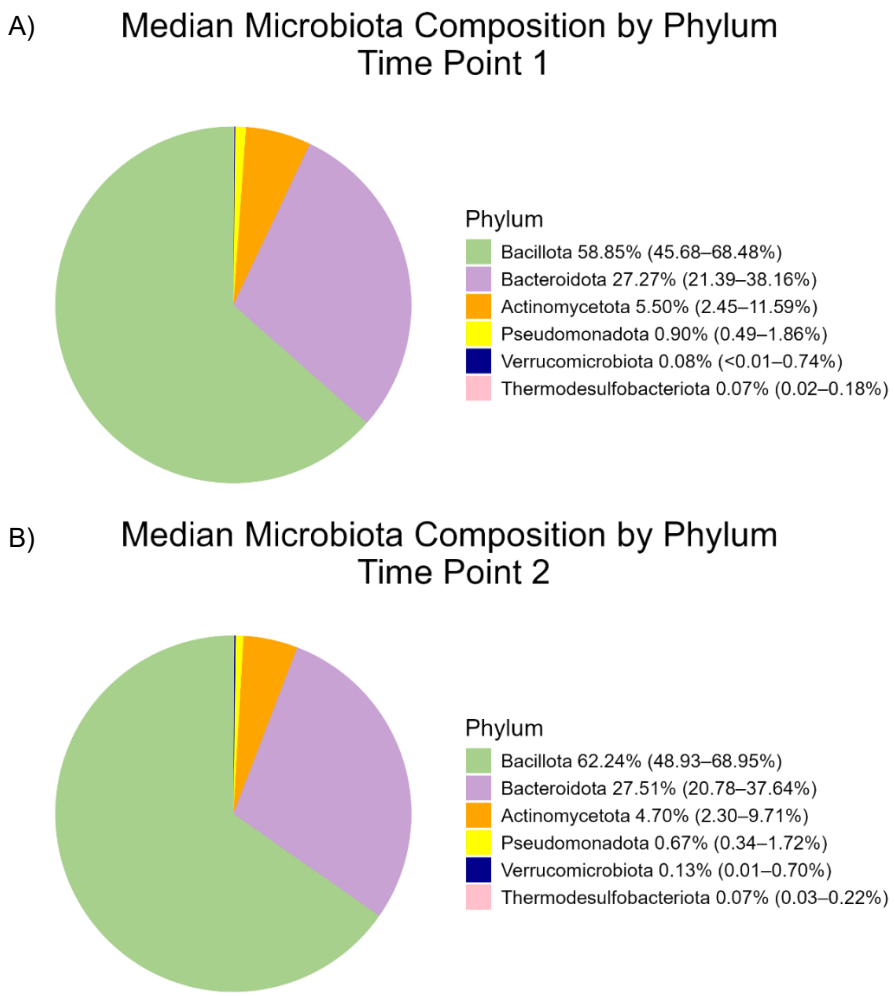


Figure 3.3. Median gut microbiota composition at the phylum level in (A) time point 1 and (B) time point 2. Each sector represents the median relative abundance, with the interquartile range, calculated across 112 samples at time point 1 and 80 samples at time point 2.

At TP1, the dominant phylum was Bacillota, with a median relative abundance of 58.85%, followed by Bacteroidota at 27.27%. Other phyla present at lower abundances included Actinomycetota (5.50%), Pseudomonadota (0.90%), and Verrucomicrobiota (0.08%).

At TP2, the overall profile remained similar. Bacillota increased slightly to 62.24%, while Bacteroidota remained stable at 27.51%. Actinomycetota and Pseudomonadota showed slight decreases (4.70% and 0.67%, respectively), and Verrucomicrobiota slightly increased to 0.13%.

The observed microbiota composition is consistent with what has been reported in healthy adults, where Bacillota and Bacteroidota typically dominate the gut community[13,14]. Although exact relative abundances may vary between studies due to population differences and methodological approaches.

The presence of Actinomycetota, Pseudomonadota, and Verrucomicrobiota at very low levels aligns with previous findings that these phyla are less prevalent in the human gut microbiome[86].

Another notable phylum, Thermodesulfobacteriota, was formerly classified within the Deltaproteobacteria class of Proteobacteria. This phylum includes sulphate-reducing bacteria

such as *Bilophila wadsworthia* and *Desulfovibrio piger*, which are commonly found in low abundance in healthy individuals[87,88].

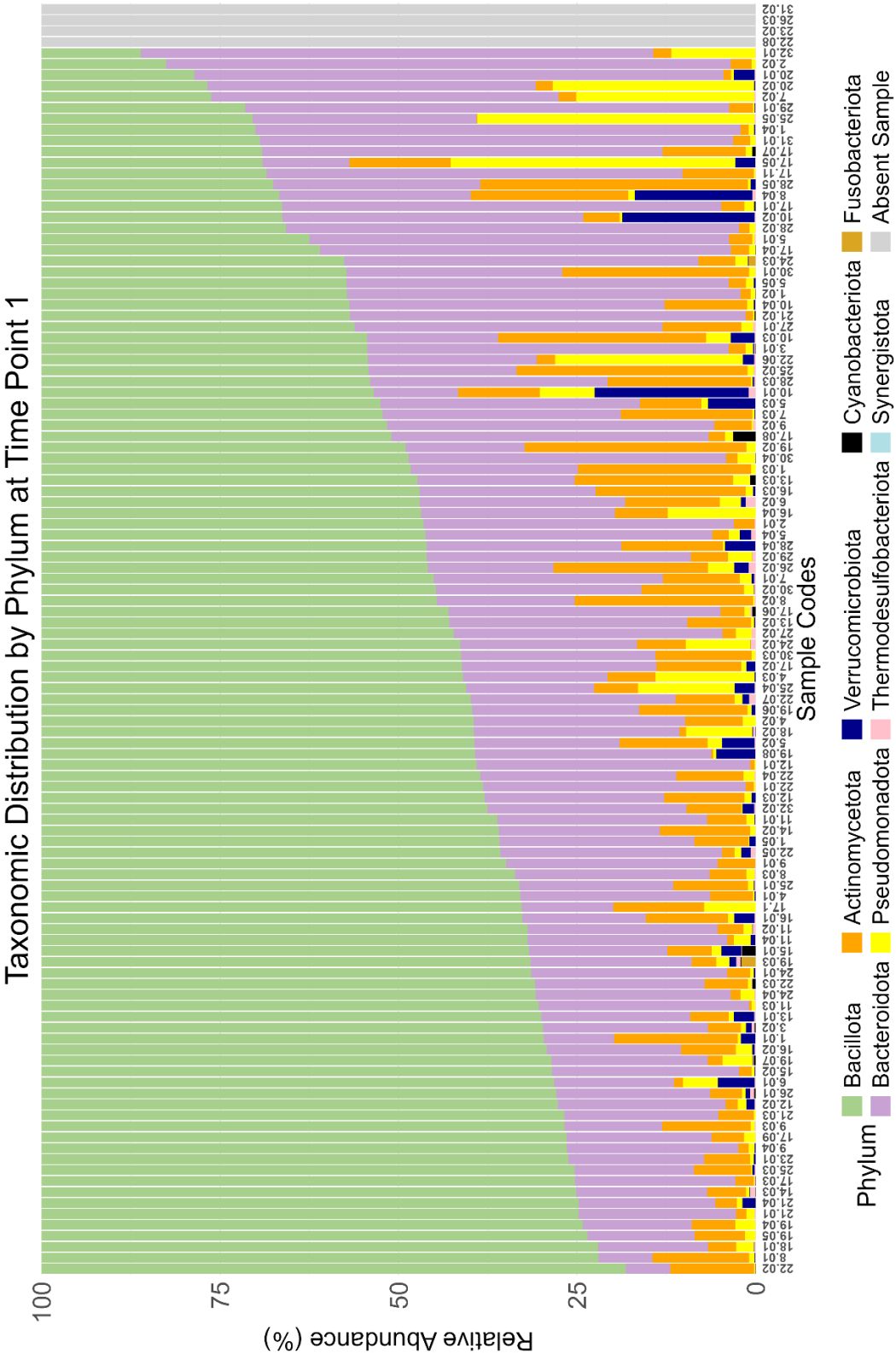
The presence of less common phyla in low or variable abundances is a typical finding in microbiome studies and reflects natural inter-individual variability[14].

2. Gut Microbiota Composition Dynamics Over Time

i. Changes at the Phylum Level

To understand how gut microbiota composition changes over time, we analysed the relative abundance of the bacterial phyla for each sample at both time points (TP1 and TP2). Results are shown in Figure 3.4. In panel A (TP1), samples are sorted in ascending order by the relative abundance of the phylum Bacillota, the most dominant phylum. The same sample order was kept in panel B (TP2) to allow easy intra-individual comparison. Gray lines indicate samples from individuals who had insufficient DNA concentration at one of the TPs or were not sequenced but were sequenced in the other.

A)


Taxonomic Distribution by Phylum at Time Point 1
Relative Abundance (%)
0
25
50
75
100
Sample Codes
Phylum
Bacillota
Bacteroidota
Actinomycetota
Pseudomonadota
Verrucomicrobiota
Thermodesulfobacteriota
Cyanobacteriota
Synergistota
Fusobacteriota
Absent Sample

B)

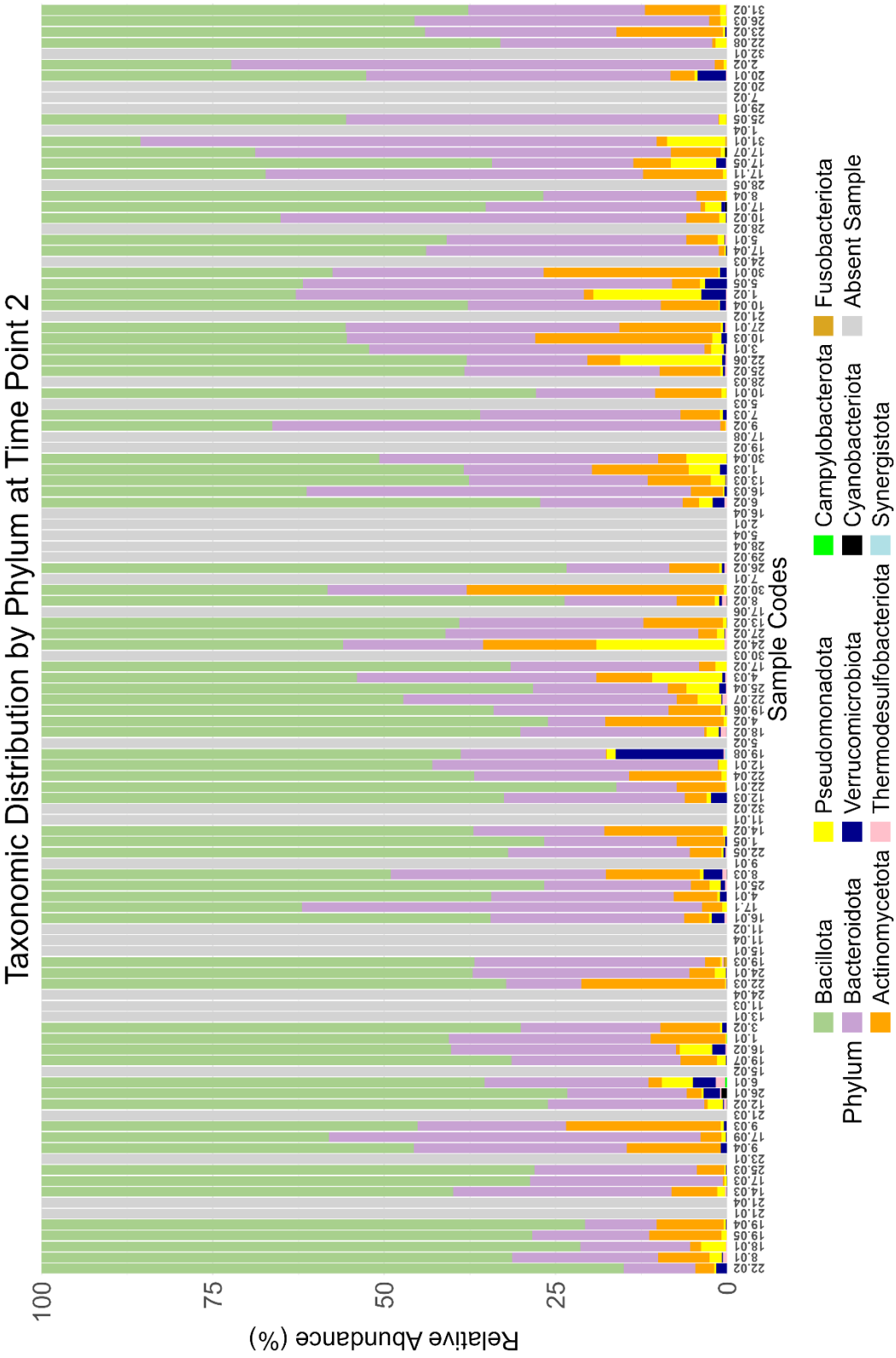


Figure 3.4. Relative abundance of microbiota composition at the phylum level for each sample at (A) time point 1 and (B) time point 2. Samples in (A) are organized in ascending order based on the abundance of the Bacillota phylum, and the order in (B) follows the same code sequence as in (A). Gray lines represent missing samples due to insufficient DNA concentration or not sequenced at the respective time point.

Figure 3.4. shows considerable inter-individual variation in the relative abundance of the five main phyla. This variation is well-documented in other studies[14]. Also, variation between TP1 and TP2 within the same individual, known as intra-individual variation over time, was also observed.

Bacillota and Bacteroidota were detected in all individuals at consistently high levels, confirming their dominant role in the gut microbiome[30].

In contrast, Actinomycetota, Pseudomonadota, and Verrucomicrobiota were present in lower amounts, and data visualization indicates greater variability across and within individuals.

Actinomycetota was detected in all individuals, indicating a stable prevalence, although fluctuations reflect the normal microbial dynamics or host-related factors[89].

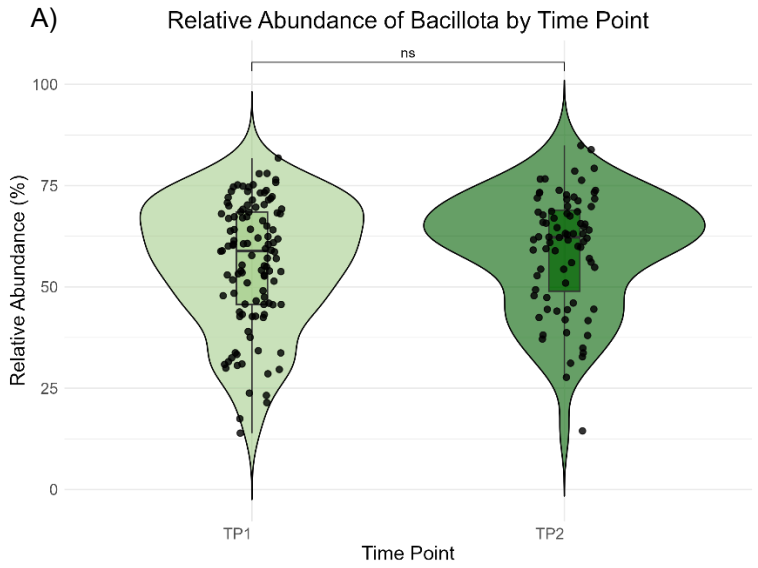
Pseudomonadota showed unusually high levels in a few samples. Such increases have been associated with dysbiosis and an imbalanced microbiome, suggesting it may serve as a potential marker of disease risk[90]. Additionally, this phylum's overrepresentation might be linked to high-fat diets[91].

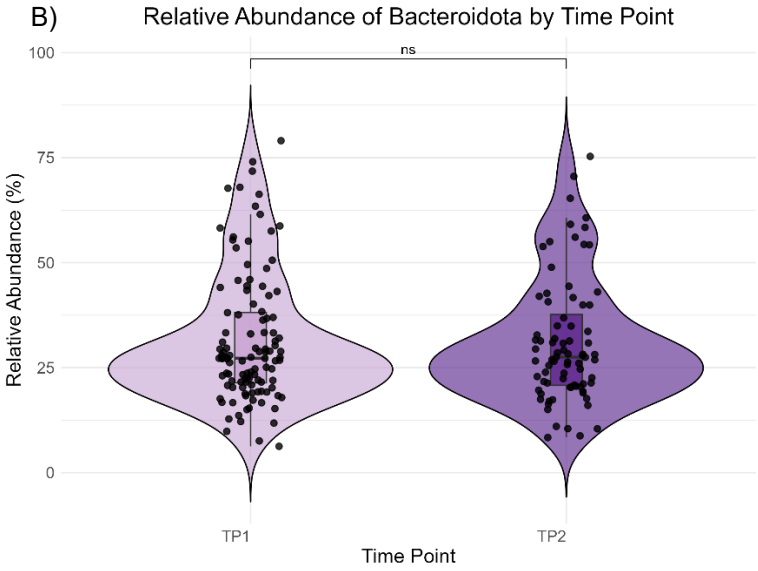
Verrucomicrobiota is mainly represented by *Akkermansia muciniphila* in the human gut. This bacterium is frequently associated with metabolic health and is being investigated as a potential probiotic, although further studies are needed to confirm its efficacy[92]. Since this bacterium is found in higher abundances in the mucus layer[93], variations in mucus content in stool samples could influence the detected levels of this phylum.

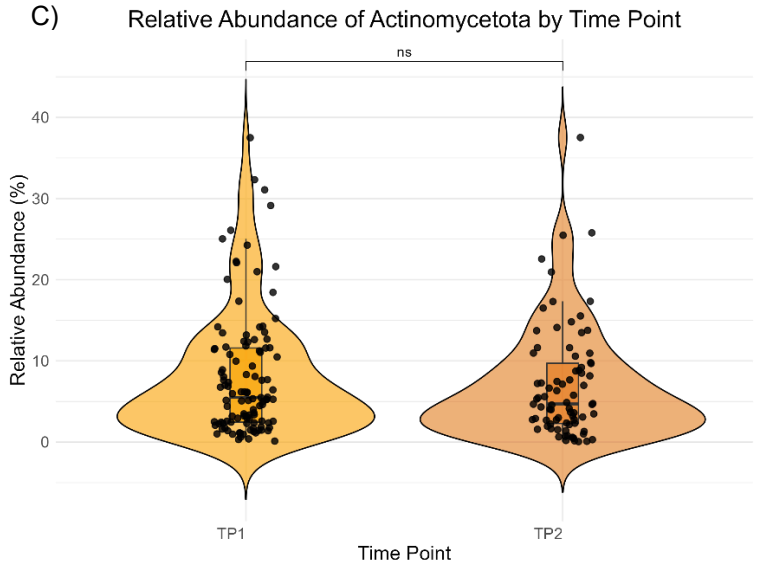
Other phyla, such as Cyanobacteriota, Fusobacteriota, and Synergiatota, were detected in low abundances and not consistently across individuals, which may result in very low or even undetectable median values.

Interestingly, Campylobacteriota (formerly Epsilonproteobacteria) was found only at TP2 in two individuals from different families. This suggests it may represent transient or opportunistic colonization[94].

To better visualize temporal variation in each of the five dominant phyla, we analysed their relative abundances at two TPs. This comparison enabled the assessment of community variability and highlighted phylum shifts over time (Figure 3.5).


A)
Relative Abundance of Bacillota by Time Point
ns
100
75
50
25
0
Relative Abundance (%)
TP1
TP2
Time Point


B)
Relative Abundance of Bacteroidota by Time Point
ns
100
75
50
25
0
Relative Abundance (%)
TP1
TP2
Time Point


C)
Relative Abundance of Actinomycetota by Time Point
ns
40
30
20
10
0
Relative Abundance (%)
TP1
TP2
Time Point

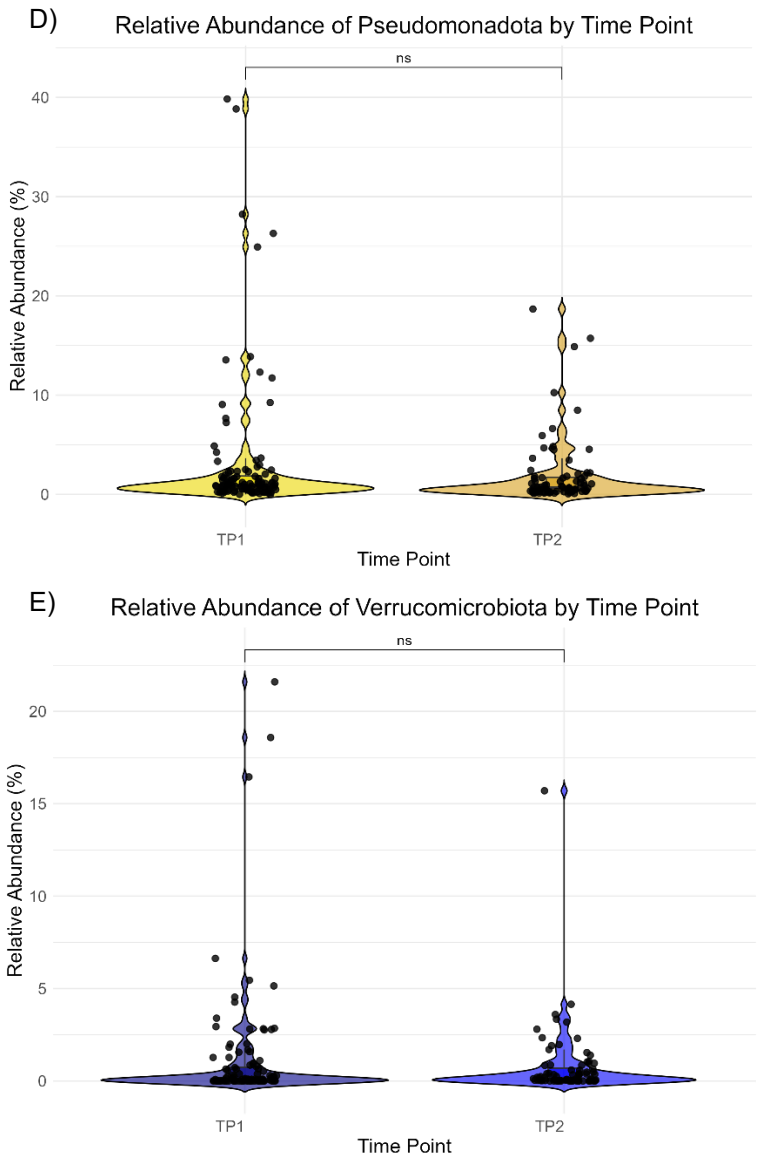


Figure 3.5. Violin plots representing the distribution of relative abundance for the five major bacterial phyla at two time points. Each panel shows the relative abundance for (A) Bacillota, (B) Bacteroidota, (C) Actinomycetota, (D) Pseudomonadota, and (E) Verrucomicrobiota. Black dots represent individual samples, with 112 samples at time point 1 (left) and 80 at time point 2 (right). For the comparison between time points, a Wilcoxon signed rank test for the 76 individuals at both time points revealed p-values of: Bacillota 0.20, Bacteroidota 0.54, Actinomycetota 0.05, Pseudomonadota 0.08, and Verrucomicrobiota 0.73. A Kolmogorov-Smirnov test comparing overall distributions between time points resulted in the following p-values: Bacillota 0.14, Bacteroidota 0.99, Actinomycetota 0.64, Pseudomonadota 0.12, and Verrucomicrobiota 0.53.

Bacillota remained the most abundant phylum at both TPs. At TP1, its mean relative abundance was 55.73% ± 15.57%, ranging from 13.91% to 81.81%, increasing slightly to 59.10% ± 14.28% at TP2 and ranging from 14.50% to 84.91%. A visual inspection shows greater spread and dispersion at TP1, and at TP2, a more concentrated distribution around the 65% in relative abundance.

Bacteroidota was the second most abundant phylum, with a mean of 31.56% ± 15.59% at TP1, and a range from 6.28% to 79.04%. At TP2, the mean was 30.93% ± 14.69% and ranged between 8.37% to 75.30%. The distributions at both TPs were very similar, with no indication of significant shifts.

Actinomycetota had a mean relative abundance of 8.17% ± 7.72% at TP1, ranging from 0.12% to 37.49% and slightly decreased to 7.05% ± 6.95% at TP2, and a similar range of 0.06% to 37.52%. Although violin plots suggest slightly greater dispersion at TP1, overall distributions remained similar. A few individuals had higher abundances at TP1, whereas at TP2, only one exceeded 28% in relative abundance.

Pseudomonadota and Verrucomicrobiota were generally present at low relative abundances across the cohort. Pseudomonadota had a mean of 3.12% ± 6.88% at TP1, with five individuals exceeding 20%. At TP2, the mean decreased to 1.96% ± 3.45%, with a maximum of 18.67%. Although the average decreased, the distribution shape remained consistent between TPs. Figure 3.5. D) also shows individuals with higher relative abundances at TP1, which decreased to approximately half at TP2. Finally, Verrucomicrobiota showed low overall abundance, averaging 1.16% ± 3.24% at TP1, with a maximum of 21.60% and 0.76% ± 1.93% at TP2, with a maximum of 15.71%. At TP1, three individuals exhibited high values, while only one individual remained with high abundance at TP2.

To further investigate temporal variability, statistical analyses were performed to detect significant shifts in overall microbiota composition between TPs. A Kolmogorov-Smirnov test compared the distributions of relative abundances across 112 samples from TP1 and 80 samples from TP2 to identify population-level shifts. Additionally, a Wilcoxon signed-rank test was conducted on 76 individuals with samples at both TPs to assess intra-individual changes over time, providing insight into microbiota stability within subjects. No statistically significant differences in relative abundances were detected between TPs for any phylum.

To further assess temporal variability, the variance in relative abundances for each phylum was calculated, and the results are presented in Table 3.6.

Table 3.6. Variance of the relative abundance of five major bacterial phyla at time points 1 and 2. This table presents the variance for Bacillota, Bacteroidota, Actinomycetota, Pseudomonadota, and Verrucomicrobiota. A total of 112 samples were analysed at time point 1 and 80 samples at time point 2. The Fligner-Killeen test was used to assess differences in the variances between time points.

Phylum	Variance TP1	Variance TP2	Fligner-Killeen test (p-value)
Bacillota	242.57	203.99	0.26
Bacteroidota	243.08	215.81	0.81
Actinomycetota	59.56	48.37	0.43
Pseudomonadota	47.39	11.91	0.32
Verrucomicrobiota	10.47	3.73	0.11

Table 3.6 summarizes the variance in relative abundance across five microbial phyla at two TPs. The Fligner-Killeen test was used to assess differences in the variances between TPs. No statistically significant differences in variance were observed, supporting the results of compositional stability at the phylum level.

Despite some minor variations, the overall gut microbial composition remained stable over the study period at the phylum level. No significant differences were found in the relative abundance distributions of the five major phyla between TPs, nor were there significant intra-individual changes or differences in variance detected. Although small changes across individuals may

reflect natural temporal dynamics or external influences such as diet variation, without no major shifts at the phylum level in the studied community.

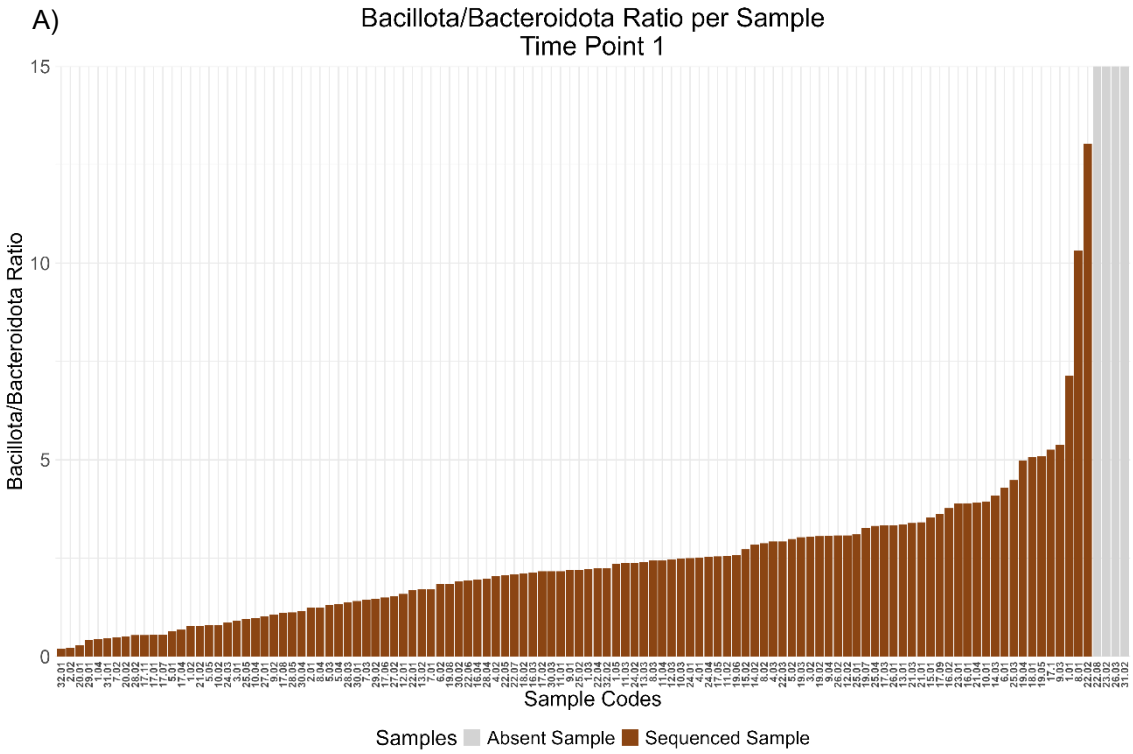
It is also important to note that these results are based on relative abundances, not absolute bacterial counts. Small shifts in relative abundance do not necessarily indicate real changes in total bacterial load, as they may simply reflect changes in the proportion of other taxes[95].

ii. Bacillota to Bacteroidota Ratio Analysis

The ratio between Bacillota and Bacteroidota (often called the Firmicutes/Bacteroidetes) is one of the most used measures in microbiome research to explore gut microbial balance. While it is commonly studied in the context of metabolic diseases, such as obesity[96], it can also provide important insights into the general structure and natural variation of gut bacteria in the community.

In our study, since all participants were free from intestinal disease, we looked at this ratio to explore how stable it remains over time and whether intra-individual or age-related differences exist.

To do this, we calculated the Bacillota/Bacteroidota ratio for each participant at both TPs to explore how stable this ratio is over time, and how much it varies between individuals. These results can provide information about the natural fluctuation of this ratio and may serve as a reference for future longitudinal studies investigating gut imbalances or diseases. Individual results are shown in Figure 3.6.



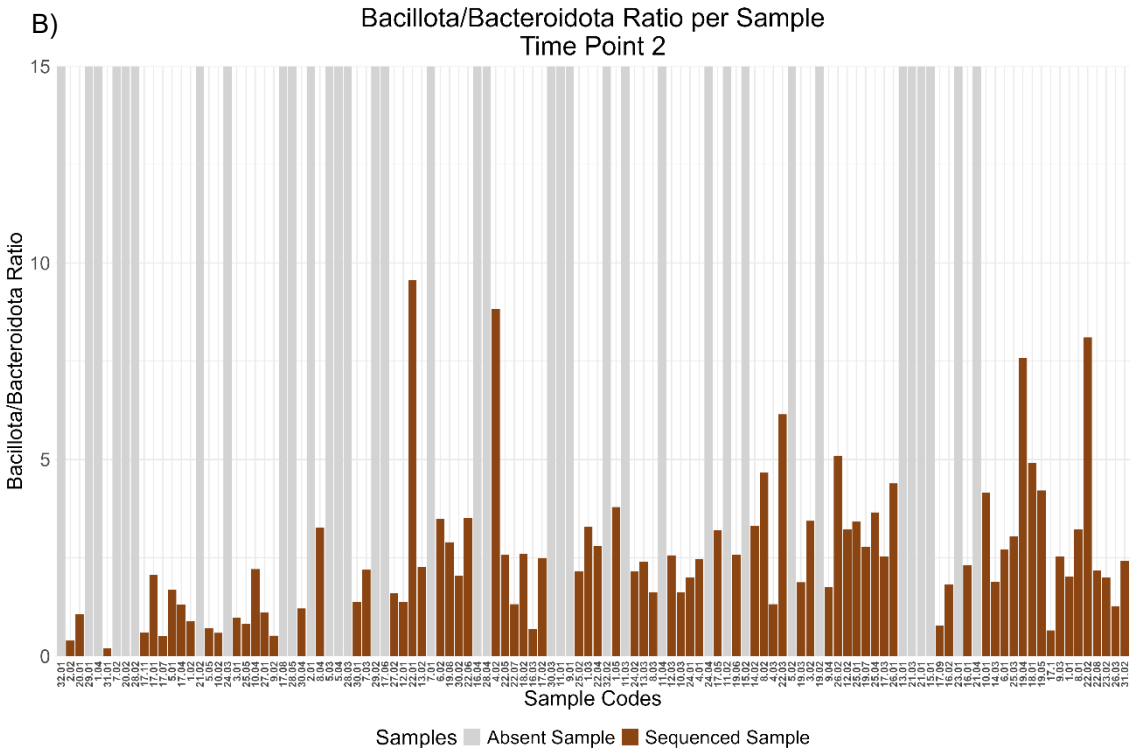


Figure 3.6. Bacillota to Bacteroidota ratio for each individual at (A) time point 1 and (B) time point 2. In panel (A), samples are ordered by an increasing ratio to illustrate their distribution at time point 1. In panel (B), samples are aligned according to individual codes to preserve correspondence with panel (A). Gray lines represent missing samples due to insufficient DNA concentration or not sequenced at the respective time point.

At TP1, three individuals showed high values above 6, showing a greater dominance of Bacillota in those cases. The median Bacillota/Bacteroidota ratio at TP1 was 2.20 (IQR: 1.24-3.07), reflecting a general predominance of Bacillota over Bacteroidota in most participants.

At TP2, although individual values changed, some increased and others decreased, the median and interquartile range remained similar, 2.24 (IQR: 1.36-3.24), suggesting no major shift between TPs.

To evaluate these changes statistically, we used a Kolmogorov–Smirnov test to compare variations in the distribution between TP1 (n = 112) and TP2 (n = 80). The results showed no significant difference (p-value = 1.00). To understand if this ratio changed significantly within individuals over time, we performed a Wilcoxon signed-rank test for the 76 individuals with samples at both TPs. The test also revealed no significant differences (p = 0.77).

These results suggest that the balance between these two dominant phyla remained relatively stable throughout the study period, supporting the concept of gut microbiota resilience and stability at the individual level[89]. However, it is important to consider that individual-level changes still occur, reflecting the dynamic and personalized nature of these microbial ecosystems[14].

Previous research has shown that the Bacillota/Bacteroidota ratio may vary with age. Namely, some studies observed an increase in the ratio among adults[97], while others noted an increase from children to seniors[98].

To explore this in our cohort, participants were divided into the three previously defined age groups. Figure 3.7 presents the Bacillota/Bacteroidota ratio across these age groups at TP1.

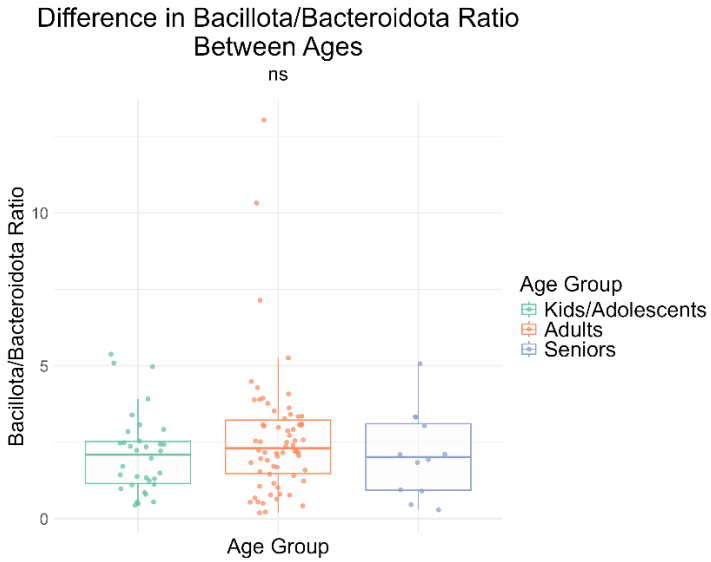


Figure 3.7. Boxplots of the Bacillota/Bacteroidota ratio at time point 1, stratified by age group. The data represents 112 individuals sampled at time point 1. Each boxplot illustrates the distribution of Bacillota/Bacteroidota ratios within each age group, with each dot corresponding to an individual. A Kruskal-Wallis test was performed to compare the groups (p-value = 0.36).

The Bacillota/Bacteroidota ratio at TP1 was approximately similar across age groups. Median values were 2.10 for kids and adolescents, 2.30 for adults, and 2.01 for seniors. A Kruskal-Wallis test was used to assess differences between groups (p-value = 0.36), showing that, in our study, age did not influence the Bacillota/Bacteroidota ratio at TP1.

Interestingly, the three individuals with the highest ratios belonged to the adult group. These higher ratios have sometimes been linked to greater energy extraction from the diet and a higher risk of obesity and weight gain[99], and obese individuals show higher levels of Bacillota[100].

Given previous studies linking the Bacillota/Bacteroidota ratio to obesity, we also examined whether changes in this ratio were associated with changes in BMI, an index associated with obesity. We compared the Bacillota/Bacteroidota ratio with BMI at TP1 (n = 112) and assessed whether changes in the ratio between T1 and T2 were associated with changes in BMI among participants with data at both TPs (n = 76). Results are shown in Figure 3.8.

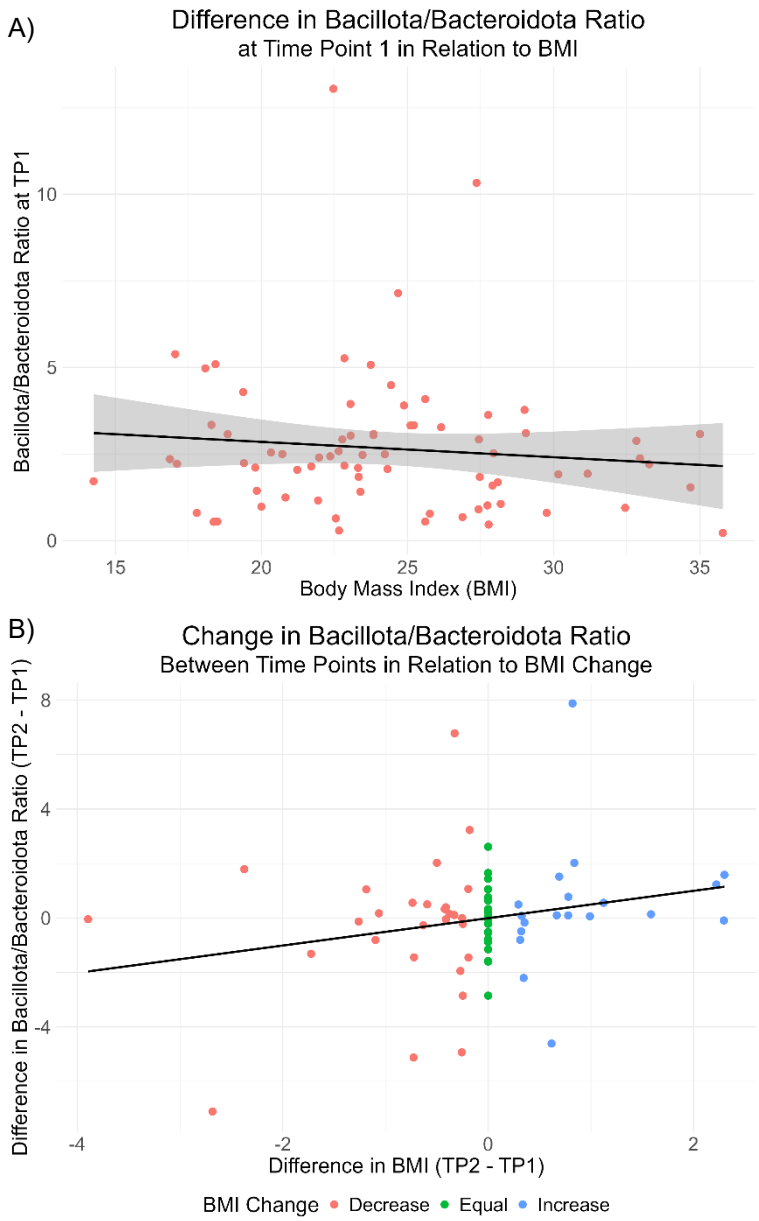


Figure 3.8. Scatter plot of the relationship between changes in the Bacillota/Bacteroidota ratio and body mass index (BMI) from time point 1 (A) and between time points (B). Each point represents one individual, with n = 112 for TP1 and n = 76 for between time points. The line indicates the tendency of the dots. In B, the color indicates the BMI change category: decrease (red), no change (green), and increase (blue). A Spearman correlation test was performed (p-value = 0.38 and 0.17, respectively).

Figure 3.8 shows some dispersion in the data, with no visible relation between BMI and ratio. In A, the tendency line seems to be negatively correlating BMI with the ratio, and in B, individuals with increased, decreased, or unchanged BMI all showed a wide range of Bacillota/Bacteroidota ratio changes, and no clear trend was observed, the tendency line is almost flat. To test for an association between changes in the Bacillota/Bacteroidota ratio and BMI, we used a Spearman correlation test, which indicated no statistically significant relationship between the two variables.

Although some studies hypothesized that an increase in the Bacillota/Bacteroidota ratio may accompany weight gain[101], our data suggest that, at least over the study period and within this cohort, changes in this ratio do not directly correlate with BMI changes. This may be because most participants had a normal or healthy BMI and were not obese. It also highlights the

complexity of microbiota-host interactions, where factors such as metabolic health, diet quality, or genetics may play a larger role than BMI alone.

In summary, the Bacillota/Bacteroidota ratio remained stable over time in each person and did not show significant differences between age groups or with changes in BMI. These results suggest that this ratio is a relatively stable characteristic of the gut microbiota and may not respond quickly to short-term changes in body weight or age.

3. Western VS Non-Western Microbiota Composition

Studies have reported that certain bacterial genera are more prevalent in Western versus non-Western populations, with *Bacteroides* typically dominating in Western and *Prevotella* in non-Western populations. These findings are thought to be related to the dietary habits and lifestyle differences between industrialized and non-industrialized countries[102]. *Prevotella* is associated with high fiber intake and lower meat consumption, whereas *Bacteroides* is linked to low-fiber diets rich in fat, refined sugars, and red meat[62]. To explore this pattern in our cohort, we examined the relative abundance of these two genera at TP1. Figure 3.9 shows the results.

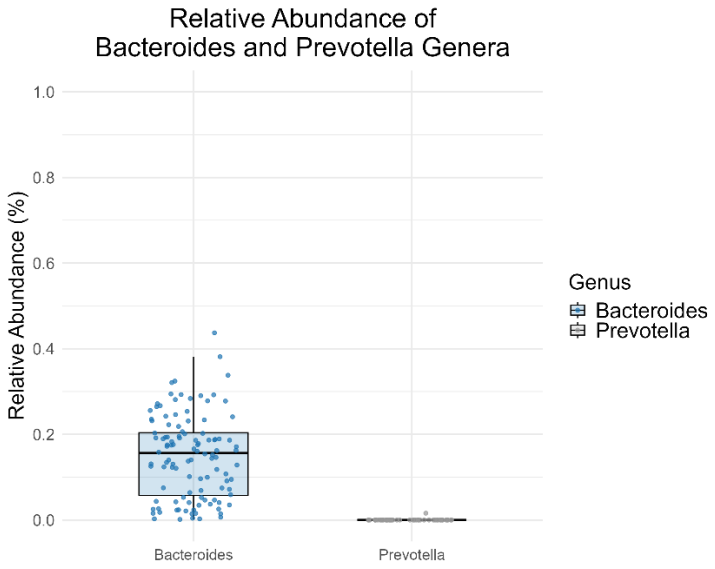


Figure 3.9. Relative abundances of *Bacteroides* and *Prevotella* genera at time point 1. Each point represents an individual participant (n = 112). The abundance of *Bacteroides* is shown in blue and *Prevotella* in gray.

Figure 3.9 shows a higher relative abundance of *Bacteroides* and minimal presence of *Prevotella* among the individuals analysed. Only one participant exhibited detectable levels of Prevotella, and even then, at low abundance.

These results suggest that the gut microbiota of this Portuguese cohort aligns with a Western microbial profile, supporting the idea that this population follows dietary habits and lifestyle patterns common to industrialized countries.

4. Microbial Diversity Analysis

Following the microbiota composition analysis at the phylum level, we next explored how the gut microbiota varied in terms of diversity. Understanding microbial diversity is essential in microbiome research, as it helps us learn how many different types of microbes are present (richness) and how distributed and balanced the community is (evenness). In this section, we looked at two types of diversity, alpha diversity, which assesses the microbial diversity within a single sample, and beta diversity, which looks at the differences between samples. With this, we can better understand the microbial community structure and its variations over time.

i. Alpha Diversity

Alpha Diversity Over Time

To evaluate the microbial diversity within individual samples, known as alpha diversity, we compared several metrics between TP1 and TP2, including Observed richness, Chao1, Shannon, and Simpson indices. The results are shown in Figure 3.10.

This analysis provides insight into how the complexity and composition of each individual's gut microbiota changes over time.

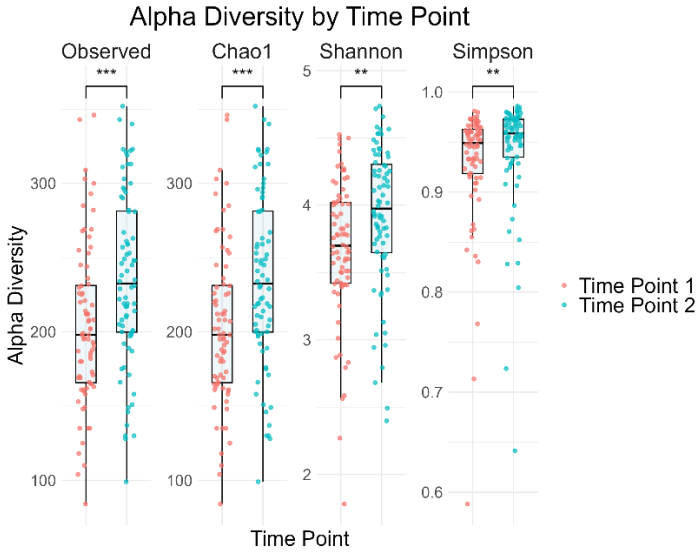


Figure 3.10. Alpha diversity metrics of the gut microbiota at time points 1 and 2. Alpha diversity was assessed using four indices: Observed richness, Chao1, Shannon, and Simpson. Data from individuals present at both time points (n = 76) are shown, with red representing time point 1 (TP1) and blue representing time point 2 (TP2). Paired comparisons were performed using the Wilcoxon signed-rank test. The resulting p-values were: Observed richness = 4.22×10^{-7}, Chao1 = 4.22×10^{-7}, Shannon = 4.10×10^{-4}, Simpson = 5.93×10^{-3}.

Figure 3.10 shows an increase in median alpha diversity from TP1 to TP2 across all metrics. The Wilcoxon signed-rank test confirmed that these increases were statistically significant. This suggests that the gut microbiota became more diverse over time within this population.

Since the participants reported no major changes in antibiotic use or intrinsic health factors during the study period, these diversity changes are likely explained by external influences such as diet, stress, medication, or other environmental factors.

Diet can rapidly and reproducibly alter the gut microbiota within just one day, especially with shifts between plant-based and animal-based diets[3]. In our study, the two sample collections were two months apart, so dietary influence could have contributed to the observed variation in microbial diversity. However, based on dietary data collected, the reported intake of fruits and vegetables showed no major changes between TP1 and TP2. At TP1, 10 participants consumed 1 portion daily, 51 consumed 2 to 3 portions, and 14 consumed 4 or more portions. At TP2, these numbers were 14 for 1 portion, 6 for 1 to 2 portions, 43 for 2 to 3 portions, and 13 for 4 or more portions. This suggests that fruit and vegetable intake alone is unlikely to explain the observed increase in microbial diversity.

Although fruit and vegetable intake remained relatively stable, small or unreported dietary changes, such as seasonal changes in food, can influence the gut microbiota composition[103]. Since sample collection occurred in summer and autumn, this seasonal variation in the availability of fruits and vegetables could explain the observed changes, also shown in other studies[104].

This seasonal variation in alpha diversity may also be influenced by fluctuations in insulin sensitivity across the year. During late summer, people often become less sensitive to insulin, possibly due to hotter weather, which can affect sleep and reduce physical activity. These changes in lifestyle and metabolism can influence the gut microbiome[105].

Another possible explanation is a change in participant behavior and awareness. Being part of a longitudinal citizen science study may have influenced participants' attitudes toward diet and lifestyle, potentially leading to subtle but meaningful changes in dietary fat intake or other lifestyle factors not explicitly captured by our questionnaires. A recent study found that increased awareness of the microbiome's role in health can influence dietary and lifestyle choices[106]. Such behavioral changes could help explain the observed increase in microbial diversity over time.

Alpha Diversity Across Families

Families have a unique microbial ecosystem, as they share a related genetic background, common dietary patterns, and household environments, creating a distinct microbiome profile. Several studies have identified higher microbiome similarity among family members compared to unrelated individuals, with the household environment emerging as one of the strongest predictors of gut microbial composition[52,82]. These observations suggest that families develop their microbiome through years of shared microbial exposures, cohabitation, and similar lifestyle factors.

To investigate how microbial diversity varies across different families, we analysed alpha diversity metrics in families with five or more individuals at TP1. This threshold was chosen because statistical analyses below five may yield unreliable results. These criteria resulted in three families with five members, two with eight, and one with eleven members. However, in the two families with eight members, one participant did not have the necessary DNA amount for analysis, so the final dataset included 40 individuals across six families. The findings are presented in Figure 3.11.

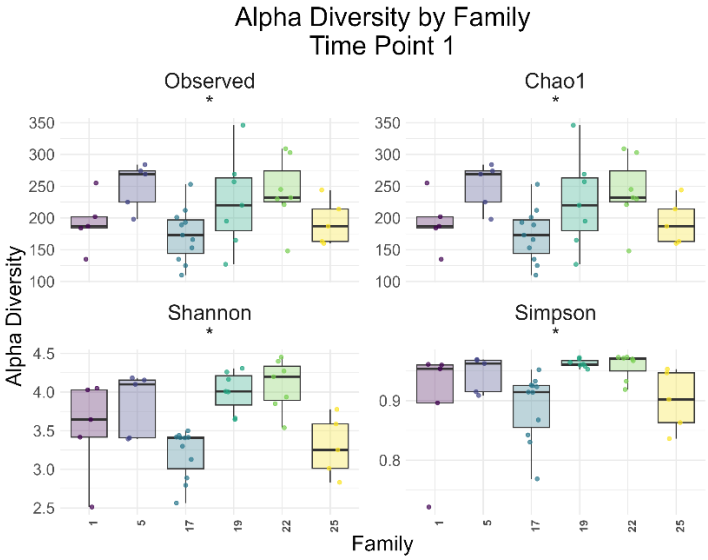


Figure 3.11. Alpha diversity metrics of the gut microbiota in families with five or more members at time point 1. Each boxplot represents the distribution of alpha diversity values within a family (n = 40 individuals present in the six families). The x-axis shows family numbers as defined in Table 3.2. Four diversity indices were assessed: Observed Richness, Chao1, Shannon, and Simpson. Differences across families were evaluated using the Kruskal–Wallis test, with the following p-values: Observed Richness = 3.41×10^{-2}, Chao1 = 3.41×10^{-2}, Shannon = 7.16×10^{-4}, Simpson = 1.89×10^{-3}.

Figure 3.11 shows that some families had higher or lower alpha diversity values, and some exhibited greater variability in microbial diversity among their members. These differences may be influenced by different dietary habits, environmental exposures, such as the workplace, genetic factors in related individuals, cohabitation, or medication use.

To further explore this variation, a Kruskal-Wallis test was conducted to determine whether there were statistically significant differences in alpha diversity between these families. The analysis revealed significant differences in family diversity, indicating that gut microbiota composition significantly varies across families. This suggests that some families harbor a more diverse bacterial community, while others present lower diversity. These findings reinforce the idea that the household environment and shared lifestyle are important contributors to individual microbiome profiles.

Alpha Diversity Across Age Groups

Age has also been reported as one factor that influences the microbiome composition, with an increase until a stable adult microbiome composition is reached around the age of three[3], followed by a gradual decline in diversity and richness in older adults[45].

To assess this, we compared alpha diversity metrics across age groups for the 112 individuals present in the TP1. The results are presented in Figure 3.12.

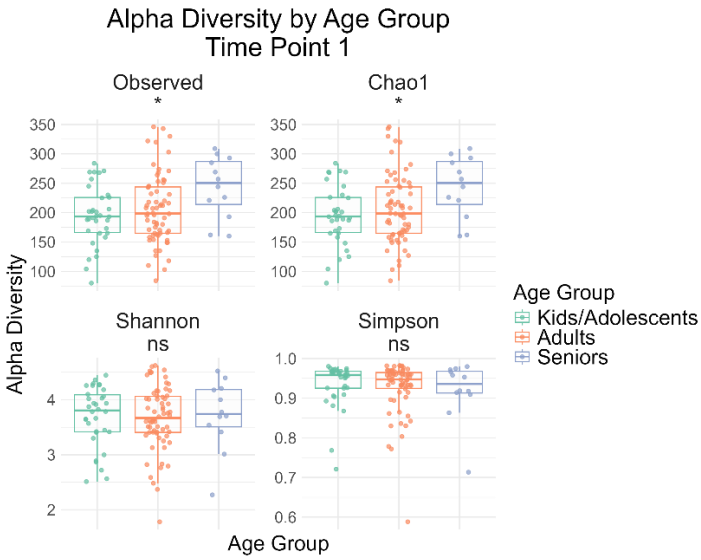


Figure 3.12. Alpha diversity metrics of the gut microbiota across age groups at time point 1. Each boxplot represents the distribution of alpha diversity values within each age group (n = 112 individuals present at time point 1). Four diversity indices were assessed: Observed richness, Chao1, Shannon, and Simpson. Differences across age groups were evaluated using the Kruskal-Wallis test, with the following p-values: Observed richness = 4.90×10^{-2}, Chao1 = 4.90×10^{-2}, Shannon = 8.20×10^{-1}, Simpson = 7.27×10^{-1}.

Figure 3.12 illustrates an unexpected increase in microbial diversity within the senior group, measured by the Observed richness and Chao1 index. To confirm this statistically, a Kruskal-Wallis test was conducted, revealing statistically significant differences across age groups, probably driven by the higher richness observed in the senior group. This suggests that older individuals may have a more diverse microbiome based on the number of unique sequence variants detected.

However, the Shannon and Simpson indices did not show significant differences between age groups. These indices also consider how evenly the microbes are distributed, and not just how many ASVs are present. This suggests that while seniors may host more bacterial types (richness), the balance or dominance among those bacteria (evenness) might be similar across age groups.

Interestingly, the older population in our study appears to have a more favorable microbial composition, similar to what has been observed in centenarians, who are known to exhibit higher alpha diversity[107,108]. However, it is important to note that the observed increase in diversity may be influenced by the smaller sample size of this group, which may not fully represent the whole population. Therefore, these findings should be interpreted with caution and highlight the need for further investigation in larger and more representative cohorts.

Other Variables in Alpha Diversity Analysis

To explore additional factors potentially influencing gut microbial diversity within individuals, we examined several variables selected from the participant questionnaires, based on associations previously reported in the literature.

Physiological and hormonal factors, including sex and menstrual cycle regularity, were analysed due to evidence that sex hormones can modulate microbial composition[109,110]. Anthropometric data, such as BMI, were also considered, as higher body weight has been associated with reduced gut diversity[79]. Blood type was evaluated based on findings that secreted blood group antigens may influence microbial composition[111].

Lifestyle and dietary factors, including fiber intake, alcohol consumption, smoking, and dairy consumption, were assessed due to their known links to microbiota structure[25,112,113]. Medication use and chronic treatments were also included because of their potential impact on microbial diversity[1].

Finally, bowel function and early-life exposures, such as the delivery mode and breastfeeding, were evaluated, as both stool consistency and developmental factors have been shown to shape the gut microbiome[8,114].

For all these variables, appropriate non-parametric tests (Wilcoxon rank-sum or Kruskal-Wallis) were used to assess associations with alpha diversity metrics (Observed species, Chao1, Shannon, and Simpson). No statistically significant associations were found for any of the tested variables.

These non-significant findings may reflect limited statistical power, especially in groups with small sample sizes, such as specific blood types or smoking status. Additionally, certain variables, such as dietary intake, were collected in broad categories without detailed food-type differentiation, which may have reduced sensitivity to detect microbiota-related effects. Overall, these findings emphasize the complexity of the gut microbiota ecology and highlight the need for larger, more detailed studies to clarify the role of individual factors.

ii. Beta Diversity

Beta Diversity Over Time

To further explore the variation in microbial communities between individuals over time, we calculate the beta diversity, which provides information about how similar or different these communities are between the two TPs. We used several distance metrics, including Bray-Curtis, Jaccard, Weighted Unifrac, and Unweighted Unifrac. The results are shown in Figure 3.13.

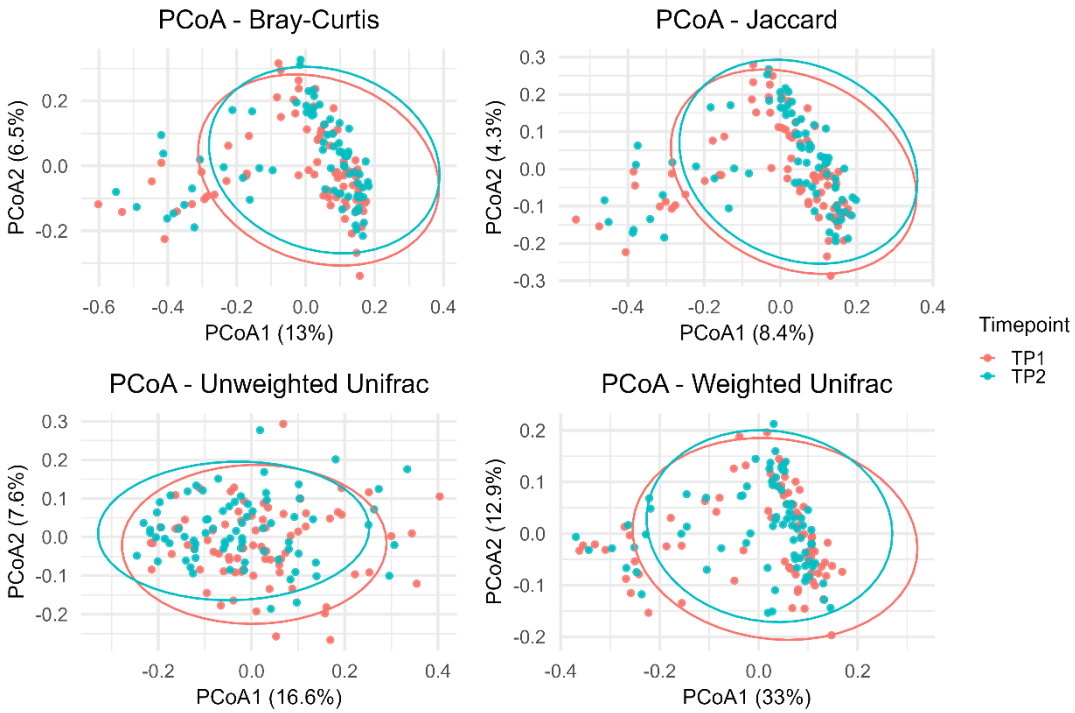


Figure 3.13. Beta diversity metrics of the gut microbiota at time points 1 and 2. Principal Coordinates Analysis plots based on four beta diversity metrics: Bray-Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac. Each dot represents an individual (n = 76 individuals present at both time points), with red indicating time point 1 (TP1) and blue indicating time point 2 (TP2). Ellipses were added to highlight clustering patterns and potential differences in microbiota composition between time points.

The principal coordinates analysis (PCoA) plots in Figure 3.13 show substantial overlap between TP1 and TP2, with no clear separation of the samples across all metrics. This suggests that the overall gut microbiota composition remained relatively stable over the two-month period. This finding contrasts with the significant increase observed in alpha diversity, suggesting that while individual microbial richness and evenness increased, the overall community composition remained stable.

These results support existing literature showing that the gut microbiota is generally resilient and with limited changes in the short term under normal conditions[89]. The absence of major external factors, as in experimental studies, has likely contributed to this compositional stability.

Beta Diversity Across Families

We next investigate how microbiota composition varied across the 32 different families at TP1, considering that family members often share diets, environments, genetics, and lifestyles, which can influence microbial transmission and similarity[82]. The results are presented in Figure 3.14.

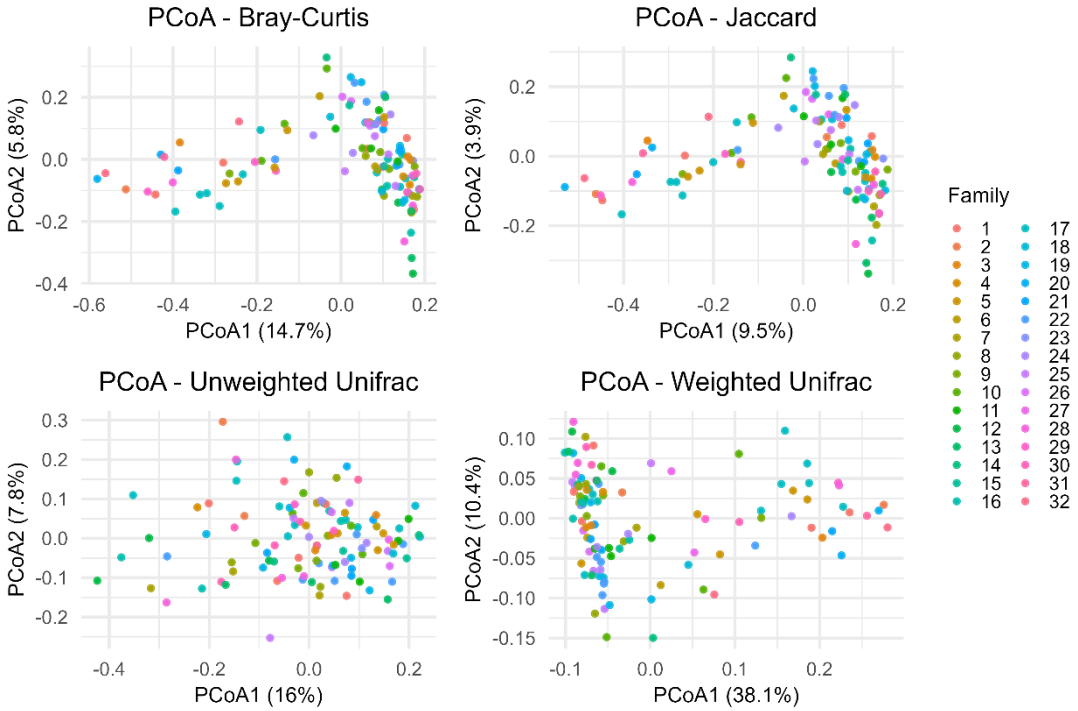


Figure 3.14. Beta diversity metrics of the gut microbiota across 32 families at time point 1. Principal Coordinates Analysis plots based on four beta diversity metrics: Bray-Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac. Each dot represents an individual (n = 112 individuals present at time point 1), and colors indicate family membership. The plots illustrate patterns of microbiota composition across different families at the same time point.

Figure 3.14 shows no distinct clustering by family, indicating that family membership did not significantly influence the overall gut microbiota composition in this cohort.

Despite known intra-family microbial similarities reported in the literature[52], our results show no strong clustering by family across any of the distance measures. This suggests that while families may differ in microbial diversity levels, the overall microbial composition and structure do not group by family in our cohort. Additionally, although families share environments, individual behaviors, diets, and exposures can still differ substantially, leading to a similar global structure of the microbiota composition. This lack of correlation may also be influenced by logistical factors such as the different days of sample collection and delivery, which could introduce variability unrelated to family membership.

Beta Diversity Across Age Groups

To assess whether age influenced the overall structure of the gut microbiota, we compared beta diversity across age groups at TP1. Age-related microbiome shifts have been reported, particularly during early development and in the elderly. Results are present in Figure 3.15.

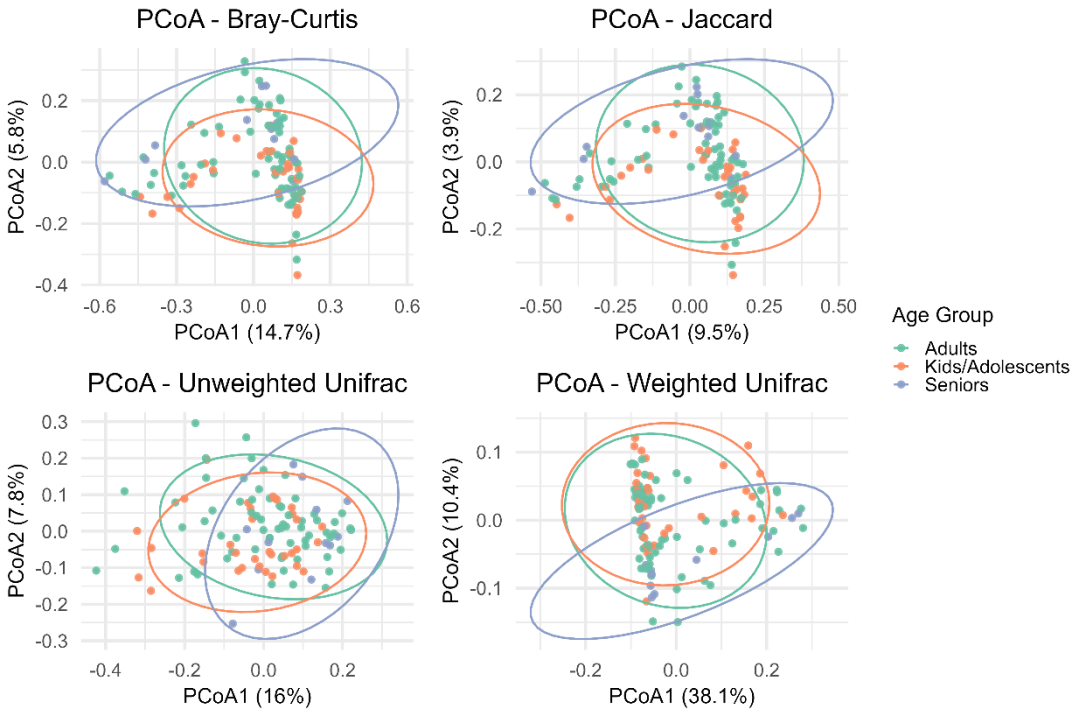


Figure 3.15. Beta diversity metrics of the gut microbiota across age groups at time point 1. Principal Coordinates Analysis plots based on four beta diversity metrics: Bray-Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac. Each dot represents an individual (n = 112 individuals present at time point 1), colored according to age group. Ellipses were added to highlight clustering patterns and potential differences in microbiota composition between age groups.

Similarly, no clear clustering or separation of samples by age group was observed in any of the four beta diversity PCoAs, as shown in Figure 3.15. This suggests that age was not a major driver of microbial community differences in this cohort, using this analysis.

Since differences were observed in older individuals with higher diversity, which may indicate a greater number of microbial ASVs (increased richness), the overall community structure, the types, and relative abundances of taxa did not differ from those of other individuals. Another possibility is that the older group in this study was small, limiting our ability to detect differences in the microbial community.

Together, these beta diversity analyses show that the overall structure of the gut microbiota remained relatively stable over time and was not significantly affected by family membership or age group in this cohort.

After exploring time, family, and age, we also analysed other variables from the questionnaire, such as diet, BMI, gender, stool consistency, medication use, and early-life factors, but none of them showed significant associations with beta diversity metrics in our cohort.

It is possible to observe two clusters in the beta diversity analyses, except for the Unweighted UniFrac metric. However, we were unable to identify, based on these variables, any factors that could explain the presence of these two clusters. This highlights the complexity of factors influencing the gut microbiota and suggests that larger studies might be needed to detect subtle effects.

This lack of association may be due to the cohort's relatively similar lifestyles or limited sample sizes in some categories. Still, the findings support the idea that the gut microbiota is quite stable and does not change much in the short term unless disrupted by stronger influences such as antibiotics, disease, or major lifestyle changes[89].

Intra and Inter Individual Microbiota Variability

To evaluate the stability of the gut microbiota over time within individuals, we compared intra-individual variability, differences between samples from the same individual at TP1 and TP2, with inter-individual variability, differences between samples from different individuals at the same TP. Beta diversity distances were calculated using four commonly used metrics: Bray-Curtis, Jaccard, Weighted UniFrac, and Unweighted UniFrac, based on the 76 participants who had samples at both TPs.

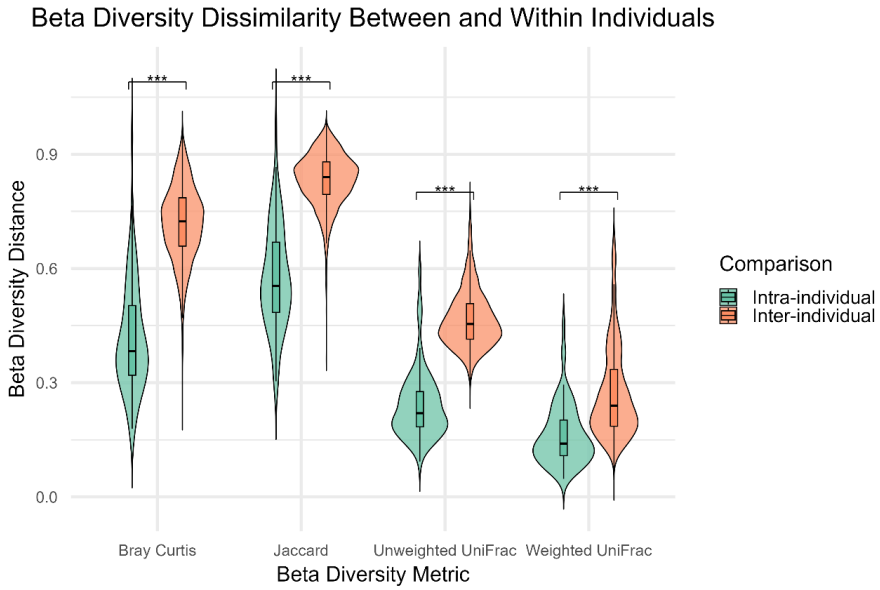


Figure 3.16. Comparison of intra-individual and inter-individual variability in gut microbiota composition. Boxplots represent beta diversity distances calculated using four metrics: Bray-Curtis, Jaccard, Unweighted UniFrac, and Weighted UniFrac, and violin plots the distribution of the data. Blue bars correspond to intra-individual temporal variability (paired samples from the same individuals across two time points; n = 76 pairs), while red bars represent inter-individual variability in the same time point (comparisons between different individuals, for time point 1 and time point 2). Statistical differences were assessed using the Wilcoxon rank-sum test, with the following p-values: Bray-Curtis = 2.20×10^{-16}, Jaccard = 4.90×10^{-2}, Unweighted UniFrac = 8.20×10^{-1}, Weighted UniFrac = 7.27×10^{-1}.

Figure 3.16 illustrates that temporal intra-individual variability is significantly lower than inter-individual variability. This difference was confirmed statistically using the Wilcoxon rank-sum test, indicating that the gut microbiota composition remains more consistent within individuals than between different individuals over the study period. These findings are consistent with previous studies that have also demonstrated the individuality of the gut microbiota[115].

Overall, this analysis reinforces the concept of a personalized and stable gut microbiota. Each individual's microbial community appears to follow its own unique trajectory over time, with more similarity to its own previous state than to that of others in the population, likely due to environmental factors such as diet, chemical exposure, and biological influences[105].

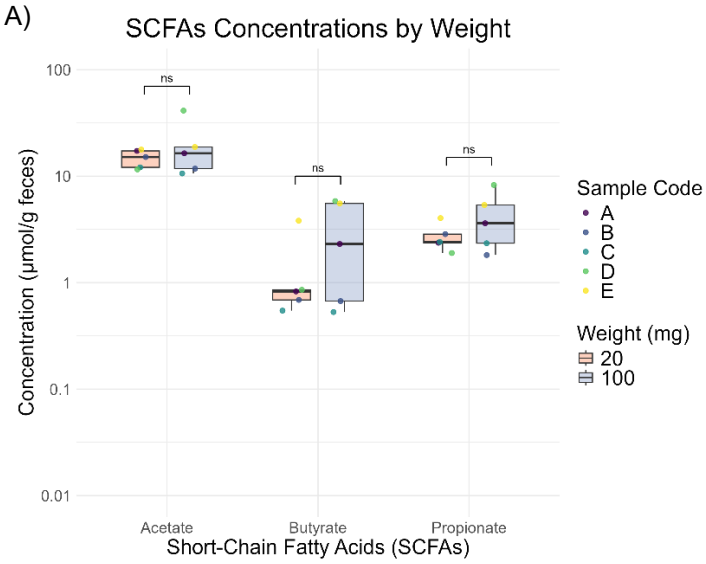
C. SCFA Production by Gut Microbes

To further explore the functions of the gut microbiota, we focused on the production of SCFAs, namely acetate, propionate, and butyrate. Based on our inclusion criteria, 20 individuals from different families were selected for this part of the study. This allowed us to investigate SCFAs production, its variation over time, and possible associations with the gut microbial community. One Eppendorf tube from the collection tube without buffer was used for metabolite quantification by ^{1}H-NMR.

Participants were selected to include an equal number of males and females, to avoid gender related differences in SCFA levels. Previous studies have reported that older males tend to have higher levels of butyrate and propionate, probably due to differences in nutrient intake. Age was also considered, and all participants were adults of a similar age, with a median age of 49 years. SCFA concentrations have been reported to vary with age, being higher in adults between 18 and 64 years and lower after 65 years[116].

1. Optimizing Sample Weight for NMR Quantification

The NMR protocol used in this study was originally developed for mouse fecal pellets. Therefore, before applying it to human samples, we tested different sample weights to determine the best amount for accurate SCFA measurement. We tested 20 mg, 100 mg, and 200 mg of fecal material, and the results are shown in Figure 3.17.



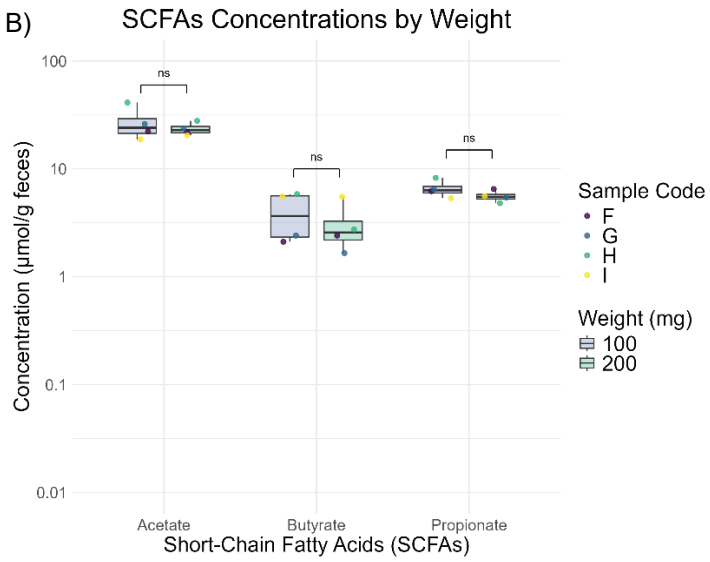


Figure 3.17. Short-chain fatty acid concentrations (acetate, propionate, and butyrate) were measured from samples with different weights of fecal material: 20 mg vs 100 mg (A) and 100 mg vs 200 mg (B). Each dot with the same color represents a sample from the same individual. Red bars correspond to the 20 mg, blue bars represent 100 mg, and green bars represent 200 mg. A Wilcoxon signed-rank test was applied to evaluate differences between weights. In A, the p-values were: Acetate = 1.00, Butyrate = 0.31, Propionate = 0.31, and in B: Acetate = 0.38, Butyrate = 0.38, Propionate = 0.63.

Figure 3.17.A) shows that acetate concentrations were similar between 20 mg and 100 mg. However, butyrate and propionate concentrations were lower in the 20 mg samples, probably because the amount of material was too small for accurate quantification. In B, the concentrations remained similar between 100 mg and 200 mg, suggesting that 100 mg is enough for reliable results. A Wilcoxon signed-rank test was performed to assess differences between weights in the concentrations of SCFAs, and no significant differences were found.

The higher variation observed in butyrate levels may be due to its chemical structure (a three-carbon chain), which results in three distinct peaks in the ^{1}H-NMR spectrum, making its measurement slightly more variable than acetate or propionate.

Although no statistically significant differences were found between the sample weights, we chose to use approximately 100 mg per sample. This decision was based on the lower signal intensity and increased baseline noise observed in the spectra from 20 mg samples, particularly for propionate and butyrate. Samples with weights slightly above 100 mg were accepted, since no impact on the SCFAs quantification was observed.

This optimization step contributed to improved reproducibility and accuracy in the quantification of metabolites present in one sample, making comparisons between samples more reliable.

2. Stability of SCFAs at 4 °C

To assess whether SCFA concentrations remain stable at refrigeration temperature, we evaluated their levels after storage at 4 °C for 24 hours. This consideration was based on previous reports indicating that bacterial metabolism can continue at low temperatures[117].

SCFA concentrations were measured at two TPs, immediately after processing (time 0) and after 24 hours of refrigeration, using ^{1}H-NMR spectroscopy, the results are presented in Figure 3.18.

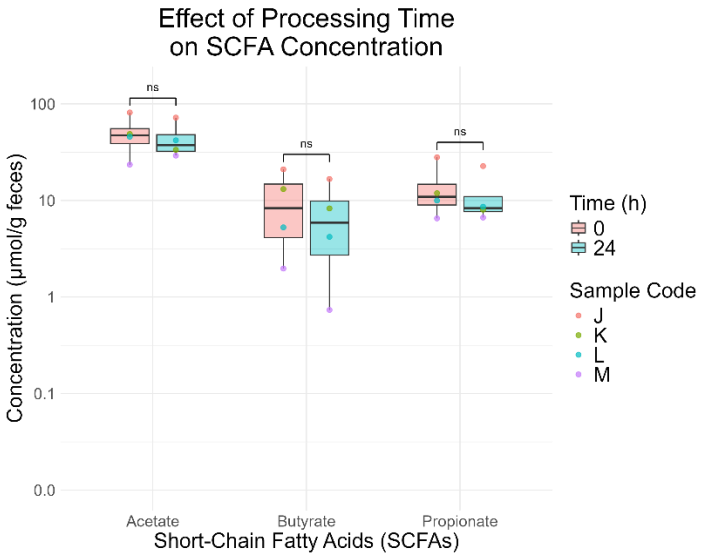


Figure 3.18. Short-chain fatty acid concentrations (acetate, propionate, and butyrate) were measured at time 0 and after 24 hours at 4 °C. Each dot with the same color represents a sample from the same individual. Red bars correspond to time 0, and blue bars represent 24 hours of storage. Statistical differences were assessed using the Wilcoxon signed-rank test, with the following p-values: Acetate = 0.38, Butyrate = 0.13, Propionate = 0.25.

Figure 3.18 shows no major differences in the concentrations of acetate, propionate, and butyrate after 24 hours of refrigeration. The median concentrations at time 0 were 47.26 µmol/g (IQR: 40.17 – 56.96) for acetate, 9.20 µmol/g (IQR: 4.44 – 15.10) for butyrate, and 10.94 µmol/g (IQR: 9.11 – 15.92) for propionate. After 24 h, the median concentrations were 37.70 µmol/g (IQR: 32.37 – 49.48) for acetate, 6.23 µmol/g (IQR: 3.33 – 10.37) for butyrate, and 8.32 µmol/g (IQR: 7.68 – 12.10) for propionate.

To assess whether these changes were statistically significant, a Wilcoxon signed-rank test was performed, revealing no significant differences in SCFA levels between the two TPs. Although a slight decrease was noted, particularly for butyrate and propionate, this change was not statistically significant.

Initially, it was hypothesized that SCFA concentrations might increase during refrigeration due to ongoing microbial fermentation. However, the observed decrease suggests that SCFAs may have been consumed by microbes or that some bacteria died due to oxygen exposure.

These results indicate that storing fecal samples at 4 °C for up to 24 hours does not significantly alter SCFA levels, although a decreasing trend is observed. This finding is important as it supports the use of refrigerated storage before analysis without compromising results, providing flexibility in sample handling. Nonetheless, minimizing the time between collection and processing remains preferable to ensure optimal data quality.

3. Dynamics of SCFA Concentrations Over Time

To understand how SCFA concentrations change over time within individuals, we analysed fecal samples collected from 20 participants from different families, between the two TPs. In total, 40 fecal samples were analysed. Samples were refrigerated at 4 °C for a median duration of 19 hours, and a maximum of 30 hours prior to processing.

This design allowed us to assess intraindividual variability in SCFA concentrations over time. Figure 3.19 shows the concentrations of acetate, propionate, and butyrate produced for each participant at TP1 and TP2.

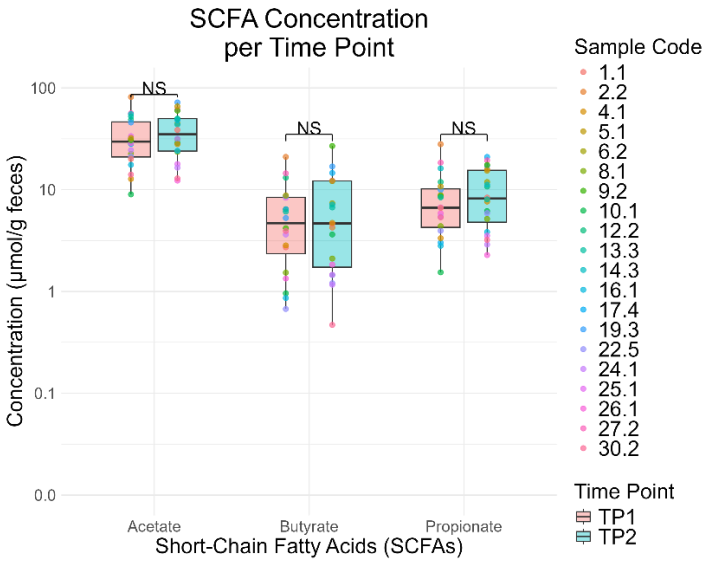


Figure 3.19. Short-chain fatty acid concentrations (acetate, propionate, and butyrate) from 20 individuals at time point 1 (red) and time point 2 (blue). Each dot with the same color represents a sample from the same individual. Red bars correspond to time point 1 (TP1), and blue bars represent time point 2 (TP2). Statistical differences across time points were assessed using the Wilcoxon signed-rank test, with the following p-values: Acetate = 0.18, Butyrate = 0.55, Propionate = 0.39.

Figure 3.19 shows some interindividual variation in SCFA levels between individuals at both TPs, which is expected due to differences in gut microbiota composition and diet, particularly the types and amounts of fiber consumed. This interindividual variability has also been reported in previous studies[118]. Moreover, fecal SCFA levels are influenced not only by microbial production but also by intestinal transit time, as these metabolites can be used as substrates for the production of others over time in the GIT[119].

Within individuals, however, SCFA profiles appeared relatively stable over the two-month period, suggesting a resilient metabolic output of the gut microbiota despite potential fluctuations in microbial composition and external factors. Median concentrations at TP1 were 29.70 µmol/g (IQR: 20.94 – 46.49) for acetate, 4.72 µmol/g (IQR: 2.42 – 8.42) for butyrate, and 6.66 µmol/g (IQR: 4.27 – 10.17) for propionate. At TP2, the median concentrations were 35.15 µmol/g (IQR: 23.95 – 49.86) for acetate, 4.66 µmol/g (IQR: 1.74 – 12.15) for butyrate, and 8.19 µmol/g (IQR: 4.83 – 15.53) for propionate. We observed a slight increase in acetate and propionate levels between TP1 and TP2.

To statistically assess these differences, Wilcoxon signed-rank tests were performed for each SCFA, and no significant differences were found for any of the three metabolites evaluated between the two TPs.

This stability suggests that, in most healthy adults, fecal SCFA levels remain relatively constant over a two-month period despite normal fluctuations in diet and lifestyle. These findings align with previous research showing stability in fecal metabolites over time and after diet intervention[120] and its stability over a period of one moth[121].

Although fecal SCFA concentrations represent only a small fraction of total SCFA production, since approximately 95% of SCFAs are absorbed by colonocytes before excretion, they remain useful, non-invasive markers of gut microbial activity. They reflect the balance between microbial production, host absorption, and metabolic usage, serving as an indirect measure of microbial function. This makes them valuable biomarkers in both clinical and research settings, particularly for longitudinal sampling. For example, changes in fecal SCFA levels have been linked to metabolic diseases, suggesting that monitoring SCFAs over time could be a helpful tool in disease surveillance[64].

However, it is important to note the limitations. Our sample size was small (n = 20), and larger cohorts would be needed to confirm these findings. Additionally, including the third time point (TP3) could strengthen the evidence for SCFA stability over time.

4. Correlation Between SCFAs and Gut Microbial Genera at TP1

Since SCFAs are metabolic products of microbial fermentation, we explored the relationship between gut microbiota and their levels. Specifically, we assessed the correlation between the SCFA concentrations and the relative abundance of bacterial genera associated with the production of these metabolites at TP1. Figure 3.20 presents a heatmap showing these correlations.

The median relative abundance of these genera at TP1 were 12.63% (IQR: 5.03 – 18.82) for *Bacteroides*, 12.09% (IQR: 7.17 – 17.05) for *Faecalibacterium*, 1.28% (IQR: 0.53 – 19.34) for *Eubacterium*, 1.05% (IQR: 0.24 – 6.95) for *Bifidobacterium*, 0.73% (IQR: 0.35 – 2.18) for *Roseburia*, 0.20% (IQR: 0.39 – 0.58) for *Clostridium*, and 0.05% (IQR: 0.00 – 1.02) for *Akkermansia*. Faecalibacterium and Bacteroides were the most abundant genera, consistent with previous studies[122].

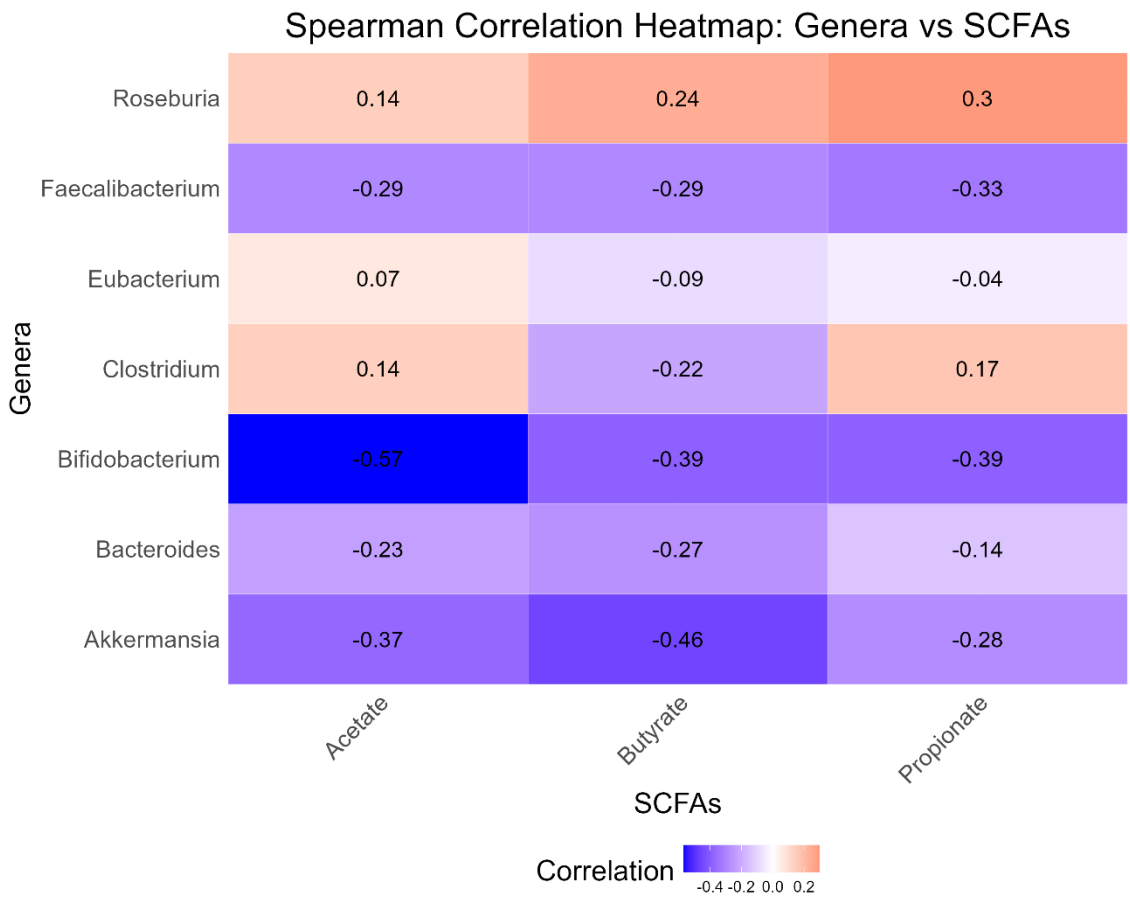


Figure 3.20. Heatmap of Spearman correlation coefficients between short-chain fatty acid concentrations (acetate, butyrate, and propionate) and the relative abundance of selected bacterial genera in time point 1 (n = 20). Red indicates positive correlation, blue indicates negative. The Benjamini-Hochberg correction was applied to control the false discovery rate in multiple comparisons. No correlations were statistically significant.

Figure 3.20 shows moderate negative correlations between *Bifidobacterium* and acetate, and between *Akkermansia* and butyrate, which was unexpected. This may be explained by their low relative abundance in the samples, suggesting that acetate levels may be related to other genera and not only these. Additionally, *Roseburia*, typically associated with butyrate production, showed a stronger correlation with the levels of propionate. This might be due to these bacteria's location in the proximal colon, where the butyrate-producing bacteria are present in higher abundance[62].

To confirm that none of the six bacterial genera were associated with SCFA production at TP1, a Benjamini-Hochberg correction for multiple comparisons was performed, and no significant correlation was found. One factor for this lack of correlation is the limited sample size, reducing statistical power. Additionally, the use of relative rather than absolute abundances may also have influenced the results, potentially masking true associations.

These results highlight the complexity of microbial ecosystem functions. Functional redundancy, strain-level differences, and microbial interactions such as cross-feeding complicate the relationship between taxonomic abundance and metabolite concentrations. Furthermore, microbial metabolism depends on substrate availability, such as fiber intake, which was not fully controlled in this study.

Overall, while some genera are known contributors to SCFA production, functional outcomes may not linearly correlate with taxonomic abundance, especially in small cohorts and when using 16S rRNA gene sequencing alone. Future studies integrating multi-omics approaches, including metagenomics and gene expression analysis, are needed to better understand SCFA dynamics and microbial function in the gut[123].

D. Citizen Science as an Engagement and Communication Strategy and Study Limitations

As previously mentioned, a distinctive feature of this study was its citizen science approach, implemented within the framework of the C+C Program. Participants helped with the data and sample collection, and shared ideas to improve the study, acting as contributors[124].

Self-sampling at home is practical but presents challenges that require pilot testing to optimize technical and logistical aspects. For example, lack of control over refrigeration and timing during collection may affect anaerobic bacteria viability and metabolite stability[67]. Although our results showed no significant impact of collection timing on SCFA levels, future studies could provide participants with kits to freeze samples immediately at home, ensuring better preservation and more flexible delivery scheduling. Such kits could include a plastic scraper and a blister-style container with breakable compartments, allowing aliquots to be thawed individually without compromising the rest of the sample. For larger studies, designated collection points with freezers, such as pharmacies, might be useful but would require additional funding.

1. Participant Suggestions and Proposed Improvements

Participants also gave valuable feedback on the questionnaires and the sample collection protocol. One suggestion was to use digital questionnaires to reduce handwriting errors, data loss, and forgotten responses. However, because of privacy and ethical rules, this wasn't possible. To ensure data security and anonymity, a secure paid platform would be required.

Since this was a pilot study, the same questionnaire was given at all three TPs. Several participants mentioned that this repetition felt redundant, especially when their answers didn't change. Some even wrote "same as previous" instead of repeating their responses, causing missing data. Changing the questionnaire over time while retaining key questions could improve data quality and participant engagement.

In addition, participants suggested specific changes to the questionnaire:

- In the first section, change "Mandatory Information" to "Essential Information" with a note like "This information is needed to characterize the population. Without it, participation cannot be considered."
- Add "Mediterranean diet" and "Lactose-free diet" as options in question 8.
- For questions 10 and 11, add "1 portion per day" as an option.
- In question 12, add "Yes, sporadically."

- Change questions 13 and 14 to ask: “Did you take any antibiotics/probiotics in the last month?” or, for later questionnaires, “Did you take any antibiotics/probiotics between sample collections? If yes, when and which?”
- In question 15, ask “Do you have some disease or condition?”, such as hyperactivity or ADHD.
- In question 16, add supplements and vitamins to the list, not just medications.

In the second section:

- Add a note to question 3: “Choose only one option,” since this wasn’t clear.
- For question 4, add “dark brown” and “reddish” instead of just “red.”
- Add new questions to question 7: “Do you take the contraceptive pill regularly?”, “Are you a child or adolescent?”, and “Are you in menopause or pre-menopause?”

Regarding the sample collection protocol, participants suggested:

- Stickers should be applied only at step 26 to avoid contamination.
- The image labeled as step 25 does not resemble a toilet and should be replaced for clarity.
- The “x 2-3” shown in the image of step 25 is not explained in the text and needs clarification.
- Adding a video tutorial to explain the sample collection process would make the instructions easier to follow and help clarify the images.

Question 6 in the second section, regarding average evacuation frequency, was often answered similarly to the previous question. To avoid redundancy and improve clarity, this question should be changed to a multiple-choice format.

Moreover, question 7 in the first section, about family and cohabitation, should be reformulated, since not all the family live in the same house, and some infants could be adopted and not genetically related, it should be divided into two. One about genetics and another about cohabitation.

A question about physical activity could also be asked, since it is documented that athletes have a higher diversity compared to sedentary ones[60]. Another important question could be whether the woman was recently pregnant or is currently breastfeeding, since pregnancy alters the microbiota composition[125].

Refining the questionnaire to include more specific diet questions will complement our metadata and improve subsequent analyses.

2. Outreach Activities with Participant Involvement

An important part of this project was the active involvement of citizen scientists, who not only contributed samples and feedback but also helped raise awareness about the intestinal microbiome within their communities. They took part in public engagement activities aimed at

raising awareness about the importance of gut bacteria, serving as co-ambassadors of the project. Examples include their participation in the ITQB NOVA International Microorganism Day at Santo Amaro Beach and in the European Researchers' Night at the Oeiras Marina (Figure 3.21).

Figure 3.21. Participation of the citizen scientists during public events to raise awareness about the intestinal microbiome. The (A) ITQB NOVA International Microorganism Day at Santo Amaro beach, and (B) the European Night of Investigators at Oeiras Marina.

These fun and interactive activities were particularly designed for children, to help them understand that the human gut hosts a diverse community of microorganisms. Activities included blowing ink drawings to mimic microbes and painting rocks to represent gut bacteria. By engaging participants directly, these public outreach initiatives played a key role in fostering community interest and understanding of the gut microbiome, helping to demystify microorganisms and highlight their relevance to human health.

In addition to public engagement activities, the project organized two visits to partner laboratories (Figure 3.22). The first visit, in December 2024, included the Católica Biomedical Research Center, GIMM Oeiras, and the BSL2 facility, guided by researchers of the team Luis Teixeira, Isabel Gordo, and Karina Xavier. The second visit, in June 2024, took place at ITQB NOVA, in collaboration with another citizen science project, guided by Sarela Santamarina and Mónica Serrano. These visits promoted transparency in sample processing and trust in the scientific process. During the events, the team provided project updates, answered questions about sample handling, and discussed the potential effects of probiotics on the gut microbiome. The second visit additionally allowed participants to explore advanced facilities such as the NMR unit and microscopy laboratories.

Figure 3.22. First visit to the partner laboratories. In (A), Católica Biomedical Research Center with Luis Teixeira, in (B), Gulbenkian Institute for Molecular Medicine, Oeiras pole, presented by Karina Xavier and Isabel Gordo, and (C) the presentation of the BSL2 facility.

Outreach activities and laboratory visits play an important role in citizen science projects. They build trust in science, enhance motivation to stay involved in the project, and promote knowledge about the microbiome and the ongoing scientific research. Similar initiatives have been shown to improve public awareness and even influence health-related behaviors in a positive way[69].

The project was presented at the SciComPT Conference in Madeira in April. During the event, the project kits were shown, and discussions were held about the importance of this project and the crucial role of communication in science for the public. Additionally, activities were conducted to show how we can involve different age groups and raise awareness in the community about the importance of the microbiome for our health.

At the conclusion of the project, personalized reports on the microbiota were developed in collaboration with the C+C Program for all families participating in the study. A report summarizing the results for the broader community involved in the study was also prepared and will be disseminated through the channels of the Municipality of Oeiras and the C+C Program, thus closing the loop and acknowledging participants' contributions to science. Annex H presents a provisional version to be delivered to participating families.

3. Study Limitations and Future Perspectives

One of the main limitations of this study is that only samples from TP1 and most of the samples from TP2 were sequenced, primarily due to time constraints and limited resources. As a result, the temporal resolution of the dataset is restricted, limiting our ability to capture longer-term microbial fluctuations or identify trends. Future analyses that include samples from TP3 will be crucial for enhancing the longitudinal aspect of the study. This would allow the application of more robust and complex statistical models to better characterize natural microbial variation and stability over time within this population. Re-analysis of the existing data using updated methodologies and at different taxonomic levels or integrating TP3 samples could provide a more comprehensive understanding of temporal dynamics in the gut microbiota.

Additionally, this study collected only three fecal samples over a six-month period, which is not enough to capture seasonal variations throughout the year. Longer studies are needed to detect changes related to environmental factors and seasonal diets, to better understand the normal variation in gut microbiome dynamics over time. Although some studies with two-year durations

and six-month intervals between collections did not report seasonal differences, due to the large time between sample collection[126]. Future studies should aim to collect samples specifically during different seasons of the year. However, longer follow-up periods may increase participant dropout, which should be carefully considered when designing such studies.

Also, quantifying fecal samples to obtain absolute abundances, rather than relying on relative abundances alone, gives a clearer and more accurate picture of the microbial community[127]. Relative abundances show the proportion of each microbe compared to the total, but this can be misleading because if one microbe increases, others appear to decrease even if their actual numbers stay the same. Measuring absolute abundances helps us see real changes in microbial populations, which is important to better understand their behavior, interactions, and possible effects on health. This can be done using flow cytometry, allowing the count of microbial cells in the fecal sample, which helps adjust the amount of DNA extracted based on the microbial load[95].

Additionally, sequencing was limited to genus-level resolution, restricting the identification of species differences, strain-level resolution, and detection of functional genes. This limitation affected our ability to detect functions such as SCFA production, pathogenic potential, or antibiotic resistance. Future studies using shotgun metagenomics, metatranscriptomics, or metabolomics could provide a deeper and more precise understanding of microbial functions, activity, and dynamics.

CONCLUSION

Microbioma Comunidade Portugal is a pilot project that demonstrates the potential of citizen science as an effective approach in gut microbiome research. It demonstrates how involving the community in the research process, through data and sample collection, feedback, public events, and laboratory visits, can help recruit participants, increase their engagement, raise awareness about the microbiome, and foster trust in science.

The time interval of two months between fecal sample collections was chosen to detect microbial variability and stability under natural conditions, without lifestyle or clinical interventions. With the collection of three fecal samples to understand the equilibrium abundances across time and estimate the temporal variation in the gut microbiota[127].The logistics of conducting a longitudinal study were complex, requiring flexibility, ongoing communication, and participant motivation to maintain high-quality data and long-term commitment. The high number of study registrations reflected strong interest in participating, with individuals with gastrointestinal conditions being more inclined to participate than those without, this also occurred in other studies[127]. The dropout rate was low (6%), likely due to the scientific interest and background of many participants.

At the taxonomic level, the phylum-level composition in this cohort was consistent with findings from other microbiome studies, with Bacillota and Bacteroidota being the most abundant phyla.

For instance, in Southwest China, Bacillota is the dominant phylum, averaging 42.29% with a wide inter-individual range (0.48%–97.46%), followed by Bacteroidota (0–94.60%), Proteobacteria (0–99.32%), Actinomycetota (0–92.69%), and Verrucomicrobiota (0–24.58%)[128]. Our cohort also showed inter-individual variability of the gut microbiota, although not as significant.

Comparatively, African populations exhibit a distinct microbiota profile, with Bacillota dominance at 78%, a higher Actinomycetota average of 16.5%, and a much lower average abundance of Bacteroidota, only 2.2%[129], emphasizing the strong influence of cultural, dietary, environmental, and genetic factors on microbiome composition across populations.

In the Spanish population, Bacillota also dominates, with an average of 53.9%, followed by Bacteroidota (37.2%), Proteobacteria (5%), Verrucomicrobiota (1.8%), and Actinomycetota (0.9%)[122], closely aligning with our Portuguese data. However, slight differences, particularly in Actinomycetota relative abundance, may be explained by methodological variations, participant characteristics (including age, health status, and medication use), or environmental exposures, despite expected dietary similarities between Spain and Portugal.

No significant changes were observed at the phylum level across the study TPs, suggesting that the gut microbiota remained stable and resilient at this taxonomic level[89]. The five main phyla maintained consistent relative abundance, distribution patterns, and variance. However, probably at the genus level, a substantial variation in intra- and inter-individual is likely to be seen, as shown

in other longitudinal studies with daily sampling[127] (see Appendix A for relative abundance by TP at the genus level).

Analysis of the Bacillota to Bacteroidota ratio, a frequently cited microbial marker, also showed no significant variation across TPs, age groups, or BMI categories. This supports the concept that this ratio is a stable feature in healthy individuals and may serve as a reliable metric in future studies aiming to detect deviations associated with disease or metabolic disturbances.

When comparing the relative abundances of *Bacteroides* and *Prevotella* in our cohort with data from previous studies, our results are consistent with the gut microbiota composition typically found in Western populations, such as those in the United Kingdom (UK) and the United States of America (USA), with the dominance of Bacteroides and low levels of *Prevotella*. This supports the classification of our Portuguese cohort as exhibiting a Western-type gut microbiota[102].

Regarding diversity, we observed a significant increase in alpha diversity over time, suggesting improved richness and evenness in microbial communities throughout the study period. This increase can be related to seasonal differences, such as lower levels of insulin sensitivity in late summer, or to changes in diet associated with participation in a longitudinal citizen science study. Further longitudinal studies are needed to better understand the underlying causes of this increase.

Previous studies have shown that individuals with gastrointestinal diseases often have reduced alpha diversity. However, our longitudinal study suggests that these differences may depend on the timing of sample collection. This highlights the importance of considering the intrinsic dynamics of the gut microbiota in clinical studies, as ignoring these fluctuations can bias the definition of health and disease states[115].

Relying on a single time point may lead to misclassification because differences between controls and patients could fall within the normal range of variation over time. Therefore, these findings support the use of longitudinal study designs in microbiome research, despite the increased financial and logistical challenges they present[127].

Family- and age-related differences were also observed, suggesting that shared environments and host factors may influence the composition of the gut microbiome. Older individuals tended to have greater ASVs richness, a pattern also observed in centenarians' studies. However, no significant associations were found with diet, lifestyle, or early life exposures, possibly due to limited variation in these factors or small subgroup sizes.

In contrast to alpha diversity, beta diversity remained stable over time, with no significant shifts across TPs, families, or age groups. Intra-individual comparisons confirmed that each participant's gut microbiota was like their own at different TPs than to others, highlighting the personalized and consistent nature of the adult gut microbiome. This reinforces the idea that, while richness and diversity may fluctuate, the core composition of an individual gut microbiota remains resilient in the absence of major perturbations[89].

Fecal SCFA concentrations, which are key metabolic products of microbial fermentation, also remained stable across TPs. This aligns with the stability observed in microbiota composition at the phylum level, suggesting functional maintenance of the gut ecosystem in normal individuals. These results support the potential of SCFAs as useful biomarkers, as they reflect the complexity of the gut microbiota, diet, and host physiology[121]. However, more longitudinal studies are needed to evaluate their dynamics over time and confirm their stability and associations with disease. Detecting changes beyond normal fluctuations could make SCFAs valuable tools for disease monitoring and early detection[64].

To contextualize our SCFA results, we compared them with published data from healthy cohorts.

In Italy, a study of 16 healthy individuals, with a median age of 46 years, reported median concentrations of 30.9 ± 14.4 µmol/g for acetate, 7.6 ± 4.3 µmol/g for propionate, and 7.1 ± 5.9 µmol/g for butyrate, using gas chromatography-mass spectrometry[130].

In the USA, a study of 21 healthy adults, between 18 and 65, reported median acetate levels of 107.5 mmol/kg (IQR: 57.9–141.1), propionate at 23.5 mmol/kg (IQR: 12.2–36.1), and butyrate at 20.4 mmol/kg (IQR: 10.7–35.1), using liquid chromatography[119].

In Thailand, a larger study with 157 healthy participants, between 18 and 60 years, reported median concentrations of 12.61 ± 6.45 µmol/g for acetate, 9.61 ± 5.09 µmol/g for propionate, and 7.27 ± 4.42 µmol/g for butyrate[131].

Despite methodological and demographic differences, all studies found acetate as the most abundant SCFA, followed by propionate and butyrate, a pattern that aligns with our findings. Our SCFA levels were more similar to those of Italy, a European country. While absolute concentrations varied, especially between regions, these differences likely reflect variation in ethnicity, dietary patterns, sample handling, and analytical methods[37]. This supports the idea that SCFA concentrations are relatively stable and reliable biomarkers of gut microbiota activity, even when microbial diversity shifts over time. In other words, variability in the gut microbiome does not necessarily imply functional changes within the microbial community[121].

However, we did not find significant correlations between SCFA levels and the relative abundance of known SCFA-producing genera. This suggests that metabolic functions can be carried out by multiple bacterial taxa, not limited to a specific set of genera. This idea is further supported by the lack of major phylum-level changes over time and the stability of SCFA profiles. Such functional redundancy is essential for ecosystem resilience and may explain the absence of genus-level correlations. To better understand the relationships between microbial composition and function, future studies should incorporate metagenomic, transcriptomic, and metabolomic approaches capable of capturing strain-level differences and functional activity more accurately.

In summary, this pilot project not only establishes a strong foundation for future microbiome studies in the Portuguese population but also contributes to the broader understanding of gut microbiota dynamics. We observed stability at the phylum level, including a consistent Bacillota to Bacteroidota ratio. Alpha diversity varied over time, likely influenced by seasonal factors, as

well as family and age-related differences, with older individuals showing greater richness. Beta diversity remained stable, with microbiomes being more similar within individuals over time than between different people, highlighting the personalized and resilient nature of the gut microbiome. Our results reinforce the role of SCFAs as reliable metabolic biomarkers and highlight the limitations of relying only on taxonomic profiling, especially in small cohorts. These findings emphasize the complexity of microbiome function, where ecological redundancy, metabolic interactions, and strain-level differences all help maintain stable functional outcomes. To confirm and expand these findings, more long-term and detailed longitudinal studies are needed.

REFERENCES

1. Hou, K. *et al.* Microbiota in health and diseases. *Signal Transduct. Target. Ther.* **7**, 1–28 (2022).
2. Lloyd-Price, J., Abu-Ali, G. & Huttenhower, C. The healthy human microbiome. *Genome Med.* **8**, 1–11 (2016).
3. Rinninella, E. *et al.* What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms* **7**, 14 (2019).
4. Sender, R., Fuchs, S. & Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol.* **14**, e1002533 (2016).
5. Gilbert, J. A. *et al.* Current understanding of the human microbiome. *Nat. Med.* **24**, 392 (2018).
6. Berg, G. *et al.* Microbiome definition re-visited: old concepts and new challenges. *Microbiome* **8**, 1–22 (2020).
7. Thursby, E. & Juge, N. Introduction to the human gut microbiota. *Biochem. J.* **474**, 1823–1836 (2017).
8. Catassi, G. *et al.* The importance of gut microbiome in the perinatal period. *Eur. J. Pediatr.* **183**, 5085 (2024).
9. Jandhyala, S. M. *et al.* Role of the normal gut microbiota. *World J. Gastroenterol.* **21**, 8787 (2015).
10. Govender, P. & Ghai, M. Population-specific differences in the human microbiome: Factors defining the diversity. *Gene* **933**, 148923 (2024).
11. Sommer, F. & Bäckhed, F. The gut microbiota — masters of host development and physiology. *Nat. Rev. Microbiol.* **11**, 227–238 (2013).
12. Cho, I. & Blaser, M. J. The human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* **13**, 260–270 (2012).
13. Qin, J. *et al.* A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**, 59–65 (2010).
14. Methé, B. A. *et al.* A framework for human microbiome research. *Nature* **486**, 215–221 (2012).
15. Grice, E. A. & Segre, J. A. The Human Microbiome: Our Second Genome. *Annu. Rev. Genomics Hum. Genet.* **13**, 151–70 (2012).
16. Lagier, J. C. *et al.* The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. *Clin. Microbiol. Rev.* **28**, 237–264 (2015).
17. Jo, J. H., Kennedy, E. A. & Kong, H. H. Research Techniques Made Simple: Bacterial 16S Ribosomal RNA Gene Sequencing in Cutaneous Research. *J. Invest. Dermatol.* **136**, e23–e27 (2016).
18. Combrink, L. *et al.* Best practice for wildlife gut microbiome research: A comprehensive review of methodology for 16S rRNA gene investigations. *Front. Microbiol.* **14**, 1092216 (2023).
19. Cena, J. A. de, Zhang, J., Deng, D., Damé-Teixeira, N. & Do, T. Low-Abundant Microorganisms: The Human Microbiome's Dark Matter, a Scoping Review. *Front. Cell. Infect. Microbiol.* **11**, 689197 (2021).
20. Bharti, R. & Grimm, D. G. Current challenges and best-practice protocols for microbiome analysis. *Brief. Bioinform.* **22**, 178–193 (2021).
21. Knight, R. *et al.* Best practices for analysing microbiomes. *Nat. Rev. Microbiol.* **16**, 410–422 (2018).
22. Callahan, B. J. *et al.* DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).
23. Pollock, J., Glendinning, L., Wisedchanwet, T. & Watson, M. The Madness of Microbiome: Attempting To Find Consensus "Best Practice" for 16S Microbiome Studies. *Appl. Environ. Microbiol.* **84**, e02627-17 (2018).
24. Weinroth, M. D. *et al.* Considerations and best practices in animal science 16S ribosomal RNA gene sequencing microbiome studies. *J. Anim. Sci.* **100**, 1–18 (2022).
25. Leeming, E. R., Johnson, A. J., Spector, T. D. & Roy, C. I. L. Effect of Diet on the Gut Microbiota: Rethinking Intervention Duration. *Nutrients* **11**, 2862 (2019).
26. Davis, N. M., Proctor, Di. M., Holmes, S. P., Relman, D. A. & Callahan, B. J. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome* **6**, 226 (2018).

27. Nearing, J. T., Comeau, A. M. & Langille, M. G. I. Identifying biases and their potential solutions in human microbiome studies. *Microbiome 2021* **9**, 1–22 (2021).
28. Zakerska-Banaszak, O. *et al.* The Role of Host Genetics and Intestinal Microbiota and Metabolome as a New Insight into IBD Pathogenesis. *Int. J. Mol. Sci.* **25**, 9589 (2024).
29. Sun, Y. *et al.* Gut firmicutes: Relationship with dietary fiber and role in host homeostasis. *Crit. Rev. Food Sci. Nutr.* **63**, 12073–12088 (2023).
30. Nalage, D. *et al.* Bacterial phyla: microbiota of kingdom animalia. *Acad. Biol.* **2**, 1–19 (2024).
31. Cheng, J., Hu, J., Geng, F. & Nie, S. Bacteroides utilization for dietary polysaccharides and their beneficial effects on gut health. *Food Sci. Hum. Wellness* **11**, 1101–1110 (2022).
32. Binda, C. *et al.* Actinobacteria: A relevant minority for the maintenance of gut homeostasis. *Dig. Liver Dis.* **50**, 421–428 (2018).
33. Sorbara, M. T. & Pamer, E. G. Microbiome-based therapeutics. *Nat. Rev. Microbiol.* **20**, 365–380 (2022).
34. Bradley, P. H. & Pollard, K. S. Proteobacteria explain significant functional variability in the human gut microbiome. *Microbiome* **5**, 1–23 (2017).
35. Bui, T. P. N. The Human Microbiome as a Therapeutic Target for Metabolic Diseases. *Nutrients* **16**, 2322 (2024).
36. Oliveira, R. A. & Pamer, E. G. Assembling symbiotic bacterial species into live therapeutic consortia that reconstitute microbiome functions. *Cell Host Microbe* **31**, 472–484 (2023).
37. Archana *et al.* Gut microbiota derived short-chain fatty acids in physiology and pathology: An update. *Cell Biochem. Funct.* **42**, e4108 (2024).
38. Koh, A., De Vadder, F., Kovatcheva-Datchary, P. & Bäckhed, F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. *Cell* **165**, 1332–1345 (2016).
39. LeBlanc, J. G. *et al.* Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Curr. Opin. Biotechnol.* **24**, 160–168 (2013).
40. Fan, Y. & Pedersen, O. Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* **19**, 55–71 (2020).
41. Ursell, L. K. *et al.* The Intestinal Metabolome—an Intersection Between Microbiota and Host. *Gastroenterology* **146**, 1470 (2014).
42. Buffie, C. G. & Pamer, E. G. Microbiota-mediated colonization resistance against intestinal pathogens. *Nat. Rev. Immunol.* **13**, 790 (2013).
43. Sorbara, M. T. & Pamer, E. G. Interbacterial mechanisms of colonization resistance and the strategies pathogens use to overcome them. *Mucosal Immunol.* **12**, 1 (2018).
44. Kamada, N., Seo, S. U., Chen, G. Y. & Núñez, G. Role of the gut microbiota in immunity and inflammatory disease. *Nat. Rev. Immunol.* **13**, 321–335 (2013).
45. Popkes, M. & Valenzano, D. R. Microbiota–host interactions shape ageing dynamics. *Philos. Trans. R. Soc. B Biol. Sci.* **375**, 20190596 (2020).
46. Collins, S. M., Surette, M. & Bercik, P. The interplay between the intestinal microbiota and the brain. *Nat. Rev. Microbiol.* **10**, 735–742 (2012).
47. Cryan, J. F. *et al.* The microbiota-gut-brain axis. *Physiol. Rev.* **99**, 1877–2013 (2019).
48. Sarkar, A. *et al.* Psychobiotics and the Manipulation of Bacteria–Gut–Brain Signals. *Trends Neurosci.* **39**, 763 (2016).
49. Van Hul, M. *et al.* What defines a healthy gut microbiome? *Gut* **73**, 1893–1908 (2024).
50. Gil, A. *et al.* Is there evidence for bacterial transfer via the placenta and any role in the colonization of the infant gut? – a systematic review. *Crit. Rev. Microbiol.* **46**, 493–507 (2020).
51. Yatsunenko, T. *et al.* Human gut microbiome viewed across age and geography. *Nature* **486**, 222 (2012).
52. Goodrich, J. K. *et al.* Human genetics shape the gut microbiome. *Cell* **159**, 789 (2014).
53. Rothschild, D. *et al.* Environment dominates over host genetics in shaping human gut microbiota. *Nature* **555**, 210–215 (2018).
54. de la Cuesta-Zuluaga, J. *et al.* Age- and Sex-Dependent Patterns of Gut Microbial Diversity in Human Adults. *mSystems* **4**, e00261-19 (2019).
55. Zommiti, M. & Feuilloley, M. G. J. Sex hormones–Gut microbiome axis: An update of what is known so far. *Curr. Opin. Endocr. Metab. Res.* **38**, 100571 (2025).
56. Ahn, J. & Hayes, R. B. Environmental Influences on the Human Microbiome and

Implications for Noncommunicable Disease. *Annu. Rev. Public Health* **42**, 277 (2021).
57. Browne, H. P., Neville, B. A., Forster, S. C. & Lawley, T. D. Transmission of the gut microbiota: spreading of health. *Nat. Rev. Microbiol.* **15**, 531–543 (2017).
58. Den Besten, G. *et al.* The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J. Lipid Res.* **54**, 2325 (2013).
59. Maier, L. *et al.* Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* **555**, 623–628 (2018).
60. Penney, N. *et al.* Investigating the Role of Diet and Exercise in Gut Microbe-Host Cometabolism. *mSystems* **5**, e00677-20 (2020).
61. Clarke, S. F. *et al.* Exercise and associated dietary extremes impact on gut microbial diversity. *Gut* **63**, 1913–1920 (2014).
62. Hays, K. E., Pfaffinger, J. M. & Ryznar, R. The interplay between gut microbiota, short-chain fatty acids, and implications for host health and disease. *Gut Microbes* **16**, 2393270 (2024).
63. Mansuy-Aubert, V. & Ravussin, Y. Short chain fatty acids: the messengers from down below. *Front. Neurosci.* **17**, 1197759 (2023).
64. He, W. & Bertram, H. C. NMR-Based Metabolomics to Decipher the Molecular Mechanisms in the Action of Gut-Modulating Foods. *Foods* **11**, 2707 (2022).
65. Grieneisen, L., Blekhman, R. & Archie, E. How longitudinal data can contribute to our understanding of host genetic effects on the gut microbiome. *Gut Microbes* **15**, 2178797 (2023).
66. Marks, L., Laird, Y., Trevena, H., Smith, B. J. & Rowbotham, S. A Scoping Review of Citizen Science Approaches in Chronic Disease Prevention. *Front. Public Heal.* **10**, 743348 (2022).
67. Ahannach, S., Van Hoyweghen, I., Verhoeven, V. & Lebeer, S. Citizen science as an instrument for women's health research. *Nat. Med.* **30**, 3445–3454 (2024).
68. ECSA (European Citizen Science Association). Ten Principles of Citizen Science. *ECSA* (2015).
69. Savio, L. Del, Prainsack, B. & Buyx, A. Motivations of participants in the citizen science of microbiomics: data from the British Gut Project. *Genet. Med.* **19**, 959–961 (2017).
70. Falony, G. *et al.* Population-level analysis of gut microbiome variation. *Science.* **352**, 560–564 (2016).
71. Lebeer, S. *et al.* A citizen-science-enabled catalogue of the vaginal microbiome and associated factors. *Nat. Microbiol.* **8**, 2183–2195 (2023).
72. Willis, J. R. *et al.* Citizen-science reveals changes in the oral microbiome in Spain through age and lifestyle factors. *NPJ biofilms microbiomes* **8**, 38 (2022).
73. Strasser, B. J., Baudry, J., Mahr, D., Sanchez, G. & Tancoigne, E. "Citizen Science"? Rethinking Science and Public Participation. *Sci. Technol. Stud.* **32**, 52–76 (2019).
74. Henriques, B. *et al.* MicroMundo@Oeiras: Citizen Science promoting antibiotic stewardship, discovery of new antimicrobials and monitoring of soil resistance. *FEMS Microbiol. Lett.* (2025).
75. Gil-Serna, J. *et al.* Citizen Science to Raise Antimicrobial Resistance Awareness in the Community: The MicroMundo Project in Spain and Portugal. *Microb. Biotechnol.* **18**, e70123 (2025).
76. Maria Leão *et al.* Oeiras Experimenta: climate-smart crops for sustainable food production - Impetus4cs - Impetus For Citizen Science Project. *December 18* https://impetus4cs.eu/oeiras-experimenta-climate-smart-crops-for-sustainable-food-production/ (2024).
77. McMurdie, P. J. & Holmes, S. Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. *PLOS Comput. Biol.* **10**, e1003531 (2014).
78. De Moor, T., Rijpma, A. & Prats López, M. Dynamics of Engagement in Citizen Science: Results from the "Yes, I do!"-Project. *Citiz. Sci. Theory Pract.* **4**, 1–17 (2019).
79. Komodromou, I. *et al.* Exploring the Dynamic Relationship between the Gut Microbiome and Body Composition across the Human Lifespan: A Systematic Review. *Nutrients* **16**, 660 (2024).
80. Vemuri, R., Shankar, E. M., Chieppa, M., Eri, R. & Kavanagh, K. Beyond Just Bacteria: Functional Biomes in the Gut Ecosystem Including Virome, Mycobiome, Archaeome and Helminths. *Microorganisms* **8**, 483 (2020).
81. Valles-Colomer, M. *et al.* The person-to-person transmission landscape of the gut and oral microbiomes. *Nature* **614**, 125–135 (2023).

82. Song, S. J. *et al.* Cohabiting family members share microbiota with one another and with their dogs. *Elife* **2**, e00458 (2013).
83. Guiné, R. P. F., Florença, S. G., Amaral, A. L. & Costa, C. A. Reference dietary patterns in Portugal: Mediterranean diet vs Atlantic diet. *Open Agric.* **10**, 20220406 (2025).
84. Duran, J. A., Chabert, T., Rodrigues, F. & Pestana, D. Distribuição dos Grupos Sanguíneos na População Portuguesa. *ABO* **29**, 5–17 (2007).
85. Gemmell, M. R. *et al.* Optimised human stool sample collection for multi-omic microbiota analysis. *Sci. Rep.* **14**, 1–14 (2024).
86. Eckburg, P. B. *et al.* Diversity of the Human Intestinal Microbial Flora. *Science* **308**, 1635 (2005).
87. Waite, D. W. *et al.* Proposal to reclassify the proteobacterial classes deltaproteobacteria and oligoflexia, and the phylum thermodesulfobacteria into four phyla reflecting major functional capabilities. *Int. J. Syst. Evol. Microbiol.* **70**, 5972–6016 (2020).
88. Huttenhower, C. *et al.* Structure, function and diversity of the healthy human microbiome. *Nature* **486**, 207–214 (2012).
89. Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K. & Knight, R. Diversity, stability and resilience of the human gut microbiota. *Nature* **489**, 220 (2012).
90. Shin, N. R., Whon, T. W. & Bae, J. W. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* **33**, 496–503 (2015).
91. Méndez-Salazar, E. O., Ortiz-López, M. G., Granados-Silvestre, M. D. L. Á., Palacios-González, B. & Menjivar, M. Altered gut microbiota and compositional changes in firmicutes and proteobacteria in mexican undernourished and obese children. *Front. Microbiol.* **9**, 308672 (2018).
92. Gao, F. *et al.* The role of Akkermansia muciniphila in maintaining health: a bibliometric study. *Front. Med.* **12**, 1484656 (2025).
93. Donaldson, G. P., Lee, S. M. & Mazmanian, S. K. Gut biogeography of the bacterial microbiota. *Nat. Rev. Microbiol.* **14**, 20–32 (2015).
94. Cornelius, A. J. *et al.* Epsilonproteobacteria in Humans, New Zealand. *Emerg. Infect. Dis.* **18**, 510 (2012).
95. Vandeputte, D. *et al.* Quantitative microbiome profiling links gut community variation to microbial load. *Nature* **551**, 507–511 (2017).
96. Lee, Y. & Lee, H. Y. Revisiting the Bacterial Phylum Composition in Metabolic Diseases Focused on Host Energy Metabolism. *Diabetes Metab. J.* **44**, 658 (2020).
97. Mariat, D. *et al.* The firmicutes/bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol.* **9**, 1–6 (2009).
98. Vaiserman, A. *et al.* Differences in the gut Firmicutes to Bacteroidetes ratio across age groups in healthy Ukrainian population. *BMC Microbiol.* **20**, 221 (2020).
99. Turnbaugh, P. J. *et al.* An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* **444**, 1027–1031 (2006).
100. Amabebe, E., Robert, F. O., Agbalalah, T. & Orubu, E. S. F. Microbial dysbiosis-induced obesity: role of gut microbiota in homoeostasis of energy metabolism. *Br. J. Nutr.* **123**, 1127–1137 (2020).
101. Magne, F. *et al.* The Firmicutes/Bacteroidetes Ratio: A Relevant Marker of Gut Dysbiosis in Obese Patients? *Nutrients* **12**, 1474 (2020).
102. Heaver, S. L., Johnson, E. L. & Ley, R. E. Sphingolipids in host–microbial interactions. *Curr. Opin. Microbiol.* **43**, 92–99 (2018).
103. Davenport, E. R. *et al.* Seasonal Variation in Human Gut Microbiome Composition. *PLoS One* **9**, e90731 (2014).
104. Smits, S. A. *et al.* Seasonal cycling in the gut microbiome of the Hadza hunter-gatherers of Tanzania. *Science (80-.).* **357**, 802–805 (2017).
105. Zhou, X. *et al.* Longitudinal profiling of the microbiome at four body sites reveals core stability and individualized dynamics during health and disease. *Cell Host Microbe* **32**, 506-526.e9 (2024).
106. Rook, O. & Zwart, H. Awareness of human microbiome may promote healthier lifestyle and more positive environmental attitudes. *Commun. Med.* **5**, 39 (2025).
107. Kong, F. *et al.* Gut microbiota signatures of longevity. *Curr. Biol.* **26**, R832–R833 (2016).
108. Sepp, E. *et al.* Comparative Analysis of Gut Microbiota in Centenarians and Young People: Impact of Eating Habits and Childhood Living Environment. *Front. Cell. Infect. Microbiol.* **12**, 851404 (2022).
109. Valeri, F. & Endres, K. How biological sex of the host shapes its gut microbiota. *Front.*

Neuroendocrinol. **61**, 100912 (2021).
110. Siddiqui, R., Makhlouf, Z., Alharbi, A. M., Alfahemi, H. & Khan, N. A. The Gut Microbiome and Female Health. *Biology (Basel).* **11**, 1683 (2022).
111. Ewald, D. R. & Sumner, S. C. J. Human microbiota, blood group antigens, and disease. *Wiley Interdiscip. Rev. Syst. Biol. Med.* **10**, e1413 (2018).
112. Chen, E. *et al.* Dairy Consumption and the Colonic Mucosa-Associated Gut Microbiota in Humans—A Preliminary Investigation. *Nutr.* **17**, 567 (2025).
113. Capurso, G. & Lahner, E. The interaction between smoking, alcohol and the gut microbiome. *Best Pract. Res. Clin. Gastroenterol.* **31**, 579–588 (2017).
114. Roager, H. M. *et al.* Colonic transit time is related to bacterial metabolism and mucosal turnover in the gut. *Nat. Microbiol.* **1**, 1–9 (2016).
115. Olsson, L. M. *et al.* Dynamics of the normal gut microbiota: A longitudinal one-year population study in Sweden. *Cell Host Microbe* **30**, 726-739.e3 (2022).
116. Cui, M. *et al.* Influence of Age, Sex, and Diet on the Human Fecal Metabolome Investigated by 1H NMR Spectroscopy. *J. Proteome Res.* **20**, 3642–3653 (2021).
117. Cunningha, J. L. *et al.* Impact of time and temperature on gut microbiota and SCFA composition in stool samples. *PLoS One* **15**, e0236944 (2020).
118. McOrist, A. L., Abell, G. C. J., Cooke, C. & Nyland, K. Bacterial population dynamics and faecal short-chain fatty acid (SCFA) concentrations in healthy humans. *Br. J. Nutr.* **100**, 138–146 (2008).
119. Waseem, M. R. *et al.* Associations of Fecal Short Chain Fatty Acids With Colonic Transit, Fecal Bile Acid, and Food Intake in Irritable Bowel Syndrome. *Clin. Transl. Gastroenterol.* **14**, e00541 (2022).
120. Ferreira, H. *et al.* Impact of a legumes diet on the human gut microbiome articulated with fecal and plasma metabolomes: A pilot study. *Clin. Nutr. ESPEN* **63**, 332–345 (2024).
121. Sangermani, M. *et al.* Stability in fecal metabolites amid a diverse gut microbiome composition: a one-month longitudinal study of variability in healthy individuals. *Gut Microbes* **16**, (2024).
122. Latorre-Pérez, A. *et al.* The Spanish gut microbiome reveals links between microorganisms and Mediterranean diet. *Sci. Rep.* **11**, 1–12 (2021).
123. Louis, P. & Flint, H. J. Formation of propionate and butyrate by the human colonic microbiota. *Environ. Microbiol.* **19**, 29–41 (2017).
124. Wiggins, A. & Wilbanks, J. The Rise of Citizen Science in Health and Biomedical Research. *Am. J. Bioeth.* **19**, 3–14 (2019).
125. Fasano, A. *et al.* Microbiota during pregnancy and early life: role in maternal-neonatal outcomes based on human evidence. *Gut Microbes* **16**, (2024).
126. Fu, B. C. *et al.* Temporal variability and stability of the fecal microbiome: the Multiethnic Cohort Study. *Cancer Epidemiol. Biomarkers Prev.* **28**, 154 (2018).
127. Vandeputte, D. *et al.* Temporal variability in quantitative human gut microbiome profiles and implications for clinical research. *Nat. Commun.* **12**, 1–13 (2021).
128. Qu, Q. *et al.* Population-level gut microbiome and its associations with environmental factors and metabolic disorders in Southwest China. *npj Biofilms Microbiomes* **11**, 1–15 (2025).
129. Doumatey, A. P. *et al.* Gut Microbiome Profiles Are Associated With Type 2 Diabetes in Urban Africans. *Front. Cell. Infect. Microbiol.* **10**, 497714 (2020).
130. Niccolai, E. *et al.* Evaluation and comparison of short chain fatty acids composition in gut diseases. *World J. Gastroenterol.* **25**, 5543 (2019).
131. Manokasemsan, W. *et al.* Quantifying fecal and plasma short-chain fatty acids in healthy Thai individuals. *Comput. Struct. Biotechnol. J.* **23**, 2163 (2024).

Appendix A – Supplementary Table 1

Supplementary Table 1. Median relative abundance and interquartile range (IQR) of gut microbiota genera at time point 1 (TP1) and time point 2 (TP2). This table presents the median values and interquartile ranges (IQR) of the relative abundance (%) for each genus detected across samples at TP1 (n=112) and TP2 (n=80). Genera with a median abundance of 0.00% at both time points were excluded to simplify the table.

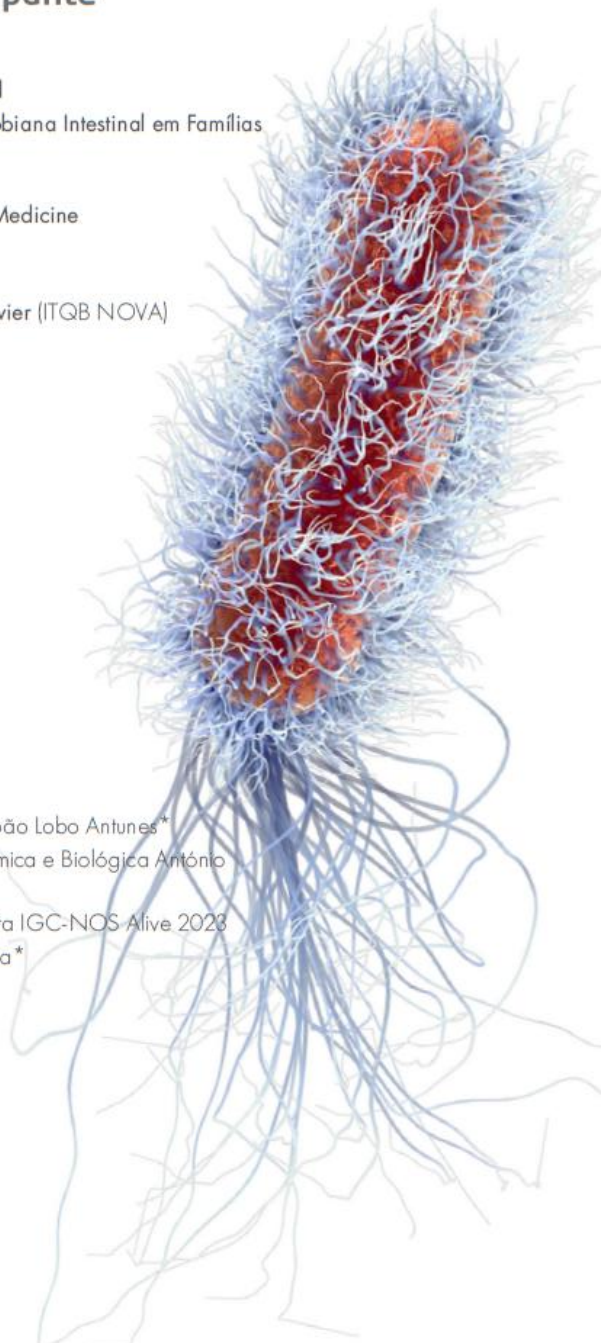
Genera	Median (IQR) TP1	Median (IQR) TP2
[Eubacterium] eligens group	0.23%(0.05–0.78%)	0.29%(0.12–0.71%)
[Eubacterium] siraeum group	0.03%(0–0.12%)	0.09%(0.01–0.29%)
[Eubacterium] ventriosum group	0.08%(0.03–0.16%)	0.12%(0.06–0.23%)
[Eubacterium] xylanophilum group	0.05%(0–0.16%)	0.11%(0.04–0.3%)
[Ruminococcus] gauvreauii group	0.23%(0.11–0.37%)	0.28%(0.15–0.55%)
Acutalibacter	0.07%(0.03–0.12%)	0.08%(0.04–0.2%)
Adlercreutzia	0.01%(0–0.04%)	0.01%(0–0.07%)
Agathobacter	2.46%(0.92–5.25%)	3.05%(1.31–5.7%)
Agathobaculum	0.09%(0.05–0.19%)	0.14%(0.09–0.29%)
Akkermansia	0.02%(0–0.74%)	0.06%(0–0.61%)
Alistipes	0.63%(0.23–1.3%)	0.66%(0.28–1.35%)
Anaerobutyricum	0.05%(0.25–1%)	0.90%(0.56–1.41%)
Anaerostipes	0.58%(0.23–1.09%)	0.84%(0.31–1.59%)
Angelakisella	0.00%(0–0.02%)	0.01%(0–0.02%)
Asaccharobacter	0.02%(0–0.06%)	0.02%(0.01–0.06%)
Bacteroides	14.47%(5.25–19.4%)	14.58%(7.99–22.11%)
Barnesiella	0.20%(0.04–0.58%)	0.18%(0.05–0.57%)
Bifidobacterium	1.78%(0.33–6.73%)	1.35%(0.07–6.98%)
Bilophila	0.04%(0.01–0.08%)	0.03%(0.01–0.08%)
Blautia	4.13%(2.68–5.74%)	5.11%(3.77–7.49%)
Butyribacter	0.10%(0.02–0.29%)	0.11%(0.02–0.42%)
Butyricimonas	0.06%(0–0.16%)	0.05%(0–0.13%)
CAG-56	0.26%(0.08–0.5%)	0.27%(0.1–0.42%)
Christensenellaceae R-7 group	0.41%(0.11–1.53%)	0.70%(0.18–1.99%)
Clostridium	0.24%(0.04–0.76%)	0.32%(0.1–0.83%)
Colidextribacter	0.02%(0.01–0.06%)	0.05%(0.02–0.08%)
Collinsella	1.50%(0.84–2.79%)	1.32%(0.5–2.51%)
Coprobacter	0.00%(0–0.02%)	0.02%(0–0.04%)
Coprococcus	0.96%(0.41–1.57%)	1.22%(0.84–1.79%)
Dialister	0.28%(0–1.17%)	0.23%(0–0.9%)
Dorea	0.95%(0.54–1.46%)	1.22%(0.91–1.63%)
Enterocloster	0.13%(0.07–0.21%)	0.16%(0.09–0.27%)
Erysipelotrichaceae UCG-003	0.21%(0.04–0.65%)	0.15%(0.03–0.35%)
Escherichia-Shigella	0.03%(0–0.15%)	0.01%(0–0.13%)
Faecalibacterium	11.43%(8.57–15.72%)	8.71%(6.32–12.49%)
Faecalimonas	0.02%(0.01–0.04%)	0.03%(0.02–0.06%)
Family XIII AD3011 group	0.03%(0.01–0.07%)	0.06%(0.02–0.13%)
Family XIII UCG-001	0.01%(0–0.02%)	0.02%(0.01–0.03%)
Flavonifractor	0.00%(0–0.01%)	0.01%(0–0.02%)
Fusicatenibacter	1.08%(0.55–1.95%)	1.39%(0.72–2.12%)

GCA-900066575	0.02%(0.01–0.03%)	0.03%(0.01–0.05%)
Gemmiger	2.29%(1.31–4.16%)	1.96%(1.29–4.16%)
Intestinibacter	0.11%(0.01–0.47%)	0.16%(0.01–0.6%)
Intestinimonas	0.02%(0–0.09%)	0.06%(0.02–0.17%)
Lachnoclostridium	0.24%(0.1–0.34%)	0.25%(0.19–0.37%)
Lachnospira	0.45%(0.13–0.89%)	0.47%(0.26–0.96%)
Lachnospiraceae FCS020 group	0.12%(0.06–0.18%)	0.15%(0.09–0.24%)
Lachnospiraceae ND3007 group	0.31%(0.16–0.63%)	0.47%(0.21–0.82%)
Lachnospiraceae NK4A136 group	0.70%(0.26–1.51%)	0.82%(0.41–1.5%)
Lachnospiraceae UCG-004	0.11%(0.06–0.2%)	0.17%(0.08–0.25%)
Lachnospiraceae UCG-010	0.03%(0.01–0.06%)	0.05%(0.02–0.12%)
Lachnotalea	0.00%(0–0.03%)	0.02%(0–0.05%)
Marvinbryantia	0.10%(0.05–0.16%)	0.16%(0.08–0.23%)
Mediterraneibacter	0.62%(0.27–1.16%)	0.70%(0.5–1.08%)
Monoglobus	0.06%(0.02–0.12%)	0.13%(0.04–0.18%)
Negativibacillus	0.01%(0–0.05%)	0.03%(0.01–0.12%)
NK4A214 group	0.12%(0.02–0.32%)	0.23%(0.08–0.62%)
Odoribacter	0.09%(0.03–0.15%)	0.12%(0.05–0.19%)
Oscillibacter	0.02%(0.01–0.06%)	0.03%(0.01–0.06%)
Oscillospira	0.01%(0–0.02%)	0.01%(0–0.03%)
Paludicola	0.00%(0–0.01%)	0.01%(0–0.02%)
Parabacteroides	0.42%(0.19–0.93%)	0.62%(0.29–0.97%)
Parasutterella	0.03%(0–0.09%)	0.02%(0–0.08%)
Phascolarctobacterium	0.12%(0–0.6%)	0.13%(0–0.51%)
Romboutsia	0.37%(0.1–0.98%)	0.52%(0.15–1.11%)
Roseburia	0.78%(0.35–1.68%)	0.97%(0.47–1.62%)
Ruminococcus	0.99%(0.37–2.39%)	2.05%(0.86–3.08%)
Ruthenibacterium	0.02%(0–0.04%)	0.02%(0–0.04%)
Schaalia	0.01%(0–0.02%)	0.01%(0–0.02%)
Senegalimassilia	0.06%(0–0.17%)	0.07%(0–0.19%)
Slackia	0.02%(0–0.1%)	0.02%(0–0.13%)
Streptococcus	0.06%(0.02–0.23%)	0.08%(0.02–0.29%)
Sutterella	0.22%(0–0.55%)	0.20%(0–0.33%)
Terrisporobacter	0.03%(0–0.29%)	0.05%(0–0.18%)
Turicibacter	0.01%(0–0.04%)	0.02%(0–0.09%)
UCG-002	1.14%(0.31–2.24%)	1.34%(0.61–2.45%)
UCG-003	0.10%(0.05–0.23%)	0.10%(0.05–0.22%)
UCG-005	0.30%(0.08–1.15%)	0.73%(0.25–1.42%)
UCG-009	0.00%(0–0.01%)	0.01%(0–0.02%)
Unknown	3.48%(1.31–6.71%)	3.72%(1.91–10.02%)

Appendix B - Participant Information Package

Microbioma Comunidade Portugal | município Oeiras

Pacote Informativo para o Participante



Título do Estudo
Microbioma Comunidade Portugal – Intestino Saudável
Ciência Cidadã em Estudo pioneiro da Dinâmica Microbiana Intestinal em Famílias

Instituição Promotora
Fundação GIMM - Gulbenkian Institute for Molecular Medicine

Parceiros
Instituto de Tecnologia Química e Biológica António Xavier (ITQB NOVA)
Católica Biomedical Research Centre (CBR)
Everything is New
Programa Ciência + Cidadã
Município de Oeiras

Coordenação da Equipa de Investigação
Isabel Gordo, Instituto Gulbenkian de Ciência*

Coordenação Ciência Cidadã
Maria João Leão, Programa Ciência + Cidadã

Equipa de Investigação
Isabel Gordo, Instituto Gulbenkian de Ciência*
Karina Xavier, Instituto Gulbenkian de Ciência*
Luís Teixeira, Católica Biomedical Research Centre
Ana Santos Almeida, Instituto de Medicina Molecular João Lobo Antunes*
Sarela Garcia-Santamarina, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa
Patrícia Morais, Instituto Gulbenkian de Ciência*/Bolseira IGC-NOS Alive 2023
António Gomes da Costa, Instituto Gulbenkian de Ciência*
Maria João Leão, Programa Ciência + Cidadã

Comissão de Ética Responsável
Comissão de Ética conjunta do IHMT-ITQB-NSL-IGC

Data de Aprovação pela Comissão de Ética
13 de março de 2024

---

* O Instituto Gulbenkian de Ciência (IGC) e o Instituto de Medicina Molecular João Lobo Antunes (IMM) uniram-se para criar a Fundação GIMM – Gulbenkian Institute for Molecular Medicine.

GIMM Gulbenkian Institute for Molecular Medicine | itqb nova | CATOLICA BIOMEDICAL RESEARCH CENTRE | Everything is New | CIÊNCIA + CIDADÃ | OEIRAS VALLEY | MUNICÍPIO OEIRAS

Pacote Informativo para o Participante

Introdução e Perguntas

Obrigado pelo seu interesse em participar no projeto Microbioma Comunidade Portugal - Intestino Saudável. Este projeto de Ciência Cidadã tem como objetivo principal um estudo de investigação pioneiro sobre a Dinâmica Microbiana Intestinal em Famílias. A investigação será realizada no pólo de Oeiras da Fundação GIMM, Rua da Quinta Grande, 6, 2780-157 Oeiras, Portugal, em parceria com o polo de Lisboa da Fundação GIMM, o Instituto de Tecnologia Química e Biológica (ITQB NOVA), o Católica Biomedical Research Centre (CBR), a Everything is New, o Programa Ciência + Cidadã e o Município de Oeiras.

Este pacote contém informações que o ajudarão a decidir se deseja participar e colaborar voluntariamente neste estudo de Ciência Cidadã. Se decidir participar como cientista cidadão/ã neste projeto, para além de contribuir para um importante e inovador estudo científico que visa caracterizar o microbioma intestinal da comunidade residente na região onde vive, ficará a conhecer os microrganismos que compõem o seu microbioma intestinal e o da sua família e irá adquirir mais informações sobre a importância do microbioma intestinal para a nossa saúde individual e global.

Por favor, leia calmamente este pacote de informações e faça todas as perguntas necessárias à equipa de investigação, através do e-mail: microbiomapt@gimm.pt.

Quem é o promotor do Microbioma Comunidade Portugal - Intestino Saudável, quem é responsável pela sua condução e quem o aprovou?

Este estudo de investigação é promovido pela Fundação GIMM - Gulbenkian Institute for Molecular Medicine e é realizado em parceria com o ITQB NOVA, o CBR, a Everything is New e o Município de Oeiras. Este projeto de investigação está a ser implementado no âmbito do Programa Ciência + Cidadã e é coordenado cientificamente pela Doutora Isabel Gordo, em estreita colaboração com a restante equipa. Este estudo foi analisado e aprovado pela Comissão de Ética conjunta do IHMT-ITQB-NSL-IGC e está em conformidade com a Lei n.º 21/2014, de 16 de abril que regulamenta a realização de investigação clínica.

Qual é o objetivo do Microbioma Comunidade Portugal - Intestino Saudável?

O Microbioma Comunidade Portugal é um projeto de Ciência Cidadã que pretende, com a sua ajuda, caracterizar os microrganismos (por exemplo as bactérias e os fungos) que vivem nos nossos intestinos e a sua diversidade genética (microbioma) ao longo do tempo que é influenciada por hábitos alimentares, estilos de vida, uso de antibióticos, entre outros fatores. O microbioma de cada indivíduo é único e a abundância das espécies que o constituem flutua ao longo do tempo. Conhecer a composição e a flutuação do microbioma de cada indivíduo, e compará-la com o da sua família e da comunidade onde vive é o objetivo do Microbioma Comunidade Portugal.

A informação que irá ser recolhida neste projeto é essencial para podermos definir o que é uma alteração normal da composição do microbioma. Com esse conhecimento poderemos no futuro detetar alterações significativas que possam ser preditivas de doença. Neste sentido, ao conhecermos melhor a dinâmica do microbioma intestinal estaremos em melhor condição para desenvolver novos tratamentos para doenças cuja patologia possa estar relacionada com alterações no microbioma intestinal.

Pacote Informativo para o Participante

Acreditamos que uma abordagem inovadora de Ciência Cidadã neste projeto enriquecerá a investigação científica proposta, ao aliar a participação e o envolvimento de cientistas cidadãos com o potencial de contribuir para a saúde e bem-estar de todos.

O que é o Microbioma Intestinal e porque é importante o seu estudo?

O microbioma intestinal é um ecossistema composto por biliões de microrganismos (i.e. bactérias, fungos e protozoários) que habitam o nosso intestino e pelos seus genes, que desempenham um papel crucial na nossa saúde. Estes microrganismos interagem de maneira complexa com o hospedeiro que colonizam, protegendo-o de invasores patogénicos e educando o seu sistema imunológico. A harmonia entre este dueto, hospedeiro e microrganismo, é essencial para o funcionamento adequado do nosso corpo.

Projetos de investigação realizados em vários países demonstraram que diversos fatores como a dieta, o uso de antibióticos e outros tratamentos influenciam a composição das espécies que compõem o microbioma intestinal humano. Estudos recentes conduzidos por membros deste consórcio revelaram também que a composição do microbioma é mais dinâmica do que se esperava, mesmo em ambientes controlados de laboratório. É precisamente essa dinâmica de variação temporal que é essencial ser estudada na área do microbioma intestinal humano.

Todas as pessoas estão expostas a uma série de variações ambientais ao longo das suas vidas, muitas das quais podem contribuir para a composição única do seu microbioma. Estas variações naturais manifestam-se provavelmente em flutuações temporais no microbioma, cuja amplitude não está devidamente estudada. É, portanto, extremamente importante incorporar a dimensão tempo na caracterização do microbioma intestinal humano para podermos estudar a sua evolução e identificar quais as espécies de microrganismos que são transitórias ou estáveis no intestino humano e quantificar o nível de flutuação natural de cada uma destas espécies que compõem o microbioma intestinal.

Neste sentido, o Microbioma Comunidade Portugal – Intestino Saudável tem como principais objetivos:
- Realizar um estudo de amostragem temporal para quantificar as flutuações no microbioma intestinal de pessoas saudáveis;
- Estabelecer relações entre as flutuações do microbioma intestinal em membros da mesma família e coabitantes, considerando a relação genética e estilo de vida. Isso ajudará a compreender como esses fatores contribuem para a composição única do microbioma intestinal em cada indivíduo e em grupos.

Eu e a minha família podemos participar no Microbioma Comunidade Portugal - Intestino Saudável?

O Microbioma Comunidade Portugal está aberto a 30 famílias da zona metropolitana de Lisboa, mais em particular nesta primeira fase do estudo no Município de Oeiras, que serão selecionadas pela equipa de investigação do projeto de acordo com os critérios de inclusão e com a ordem de inscrição. Este estudo pretende recrutar um total entre 120 e 150 participantes que terão de recolher 3 amostras fecais durante um período de 6 meses (3 amostras por participante - 1 amostra recolhida de 2 em 2 meses; a data de recolha da amostra pode ser diferente de participante para participante, mesmo dentro da mesma família). Este tamanho da amostra é suficiente para uma análise exploratória com poder estatístico.

Pacote Informativo para o Participante

Critérios de Inclusão:

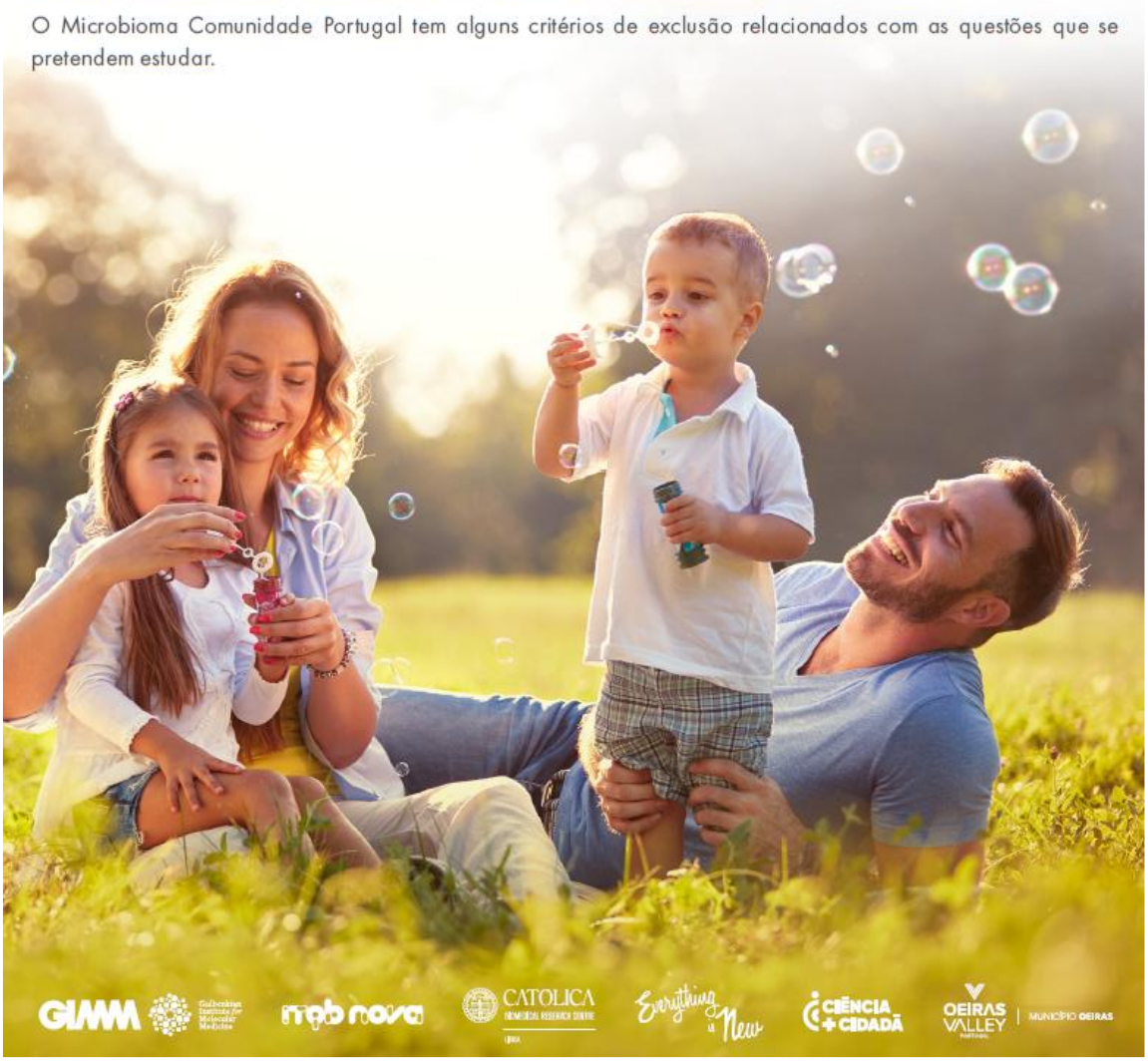
- Participantes com idades compreendidas entre os 5 e os 99 anos e residentes na área metropolitana de Lisboa (em particular no Município de Oeiras);

- São necessárias amostras de pelo menos 2 membros da família (pessoas com relação de parentesco ou que habitem a mesma casa);

- Facultar alguns dados pessoais e reportar se tem alguma doença diagnosticada ou se está a tomar algum antibiótico, conforme descrito mais à frente (Inquérito).

Existem quaisquer restrições ou impedimentos que me impeçam de participar no projeto Microbioma Comunidade Portugal - Intestino Saudável?

O Microbioma Comunidade Portugal tem alguns critérios de exclusão relacionados com as questões que se pretendem estudar.



Pacote Informativo para o Participante

Critérios de exclusão:

- Pessoas com doença intestinal diagnosticada ou em tratamento de doenças oncológica não serão consideradas neste estudo uma vez que o objetivo é caracterizar o microbioma de pessoas sem este tipo de doenças; no entanto, doenças crónicas como diabetes, obesidade, hipertensão arterial e doenças da tiroide não serão consideradas fatores de exclusão dado a sua elevada prevalência na nossa comunidade;

- Pessoas grávidas não poderão participar neste estudo.

Porque é importante a minha participação?

Este estudo de Ciência Cidadã pretende, com a sua ajuda e colaboração, conhecer melhor a composição do microbioma intestinal em famílias, com potenciais benefícios em diversas frentes, nomeadamente:

- Ao nível de Investigação fundamental: a contribuição para o conhecimento das diversas comunidades de bactérias que compõem o microbioma intestinal, e da sua estabilidade temporal. Ajudará também na caracterização de níveis de resistência a antibióticos, um problema de saúde global;

- Ao nível da Educação: na transferência de conhecimento e sensibilização sobre a importância do microbioma, bem como os métodos científicos que se usam para estudar este ecossistema. Este estudo proporcionará uma oportunidade única para promover a conscientização pública sobre a relevância do microbioma intestinal para a nossa saúde e bem-estar;

- No conhecimento sobre o seu próprio microbioma intestinal num contexto familiar e comunitário;

- Os participantes, tal como os cientistas, serão também embaixadores deste projeto e estarão a contribuir de forma significativa para o bem-estar da comunidade em geral.

Quais são as perguntas de investigação deste estudo?

Este projeto de Ciência Cidadã pretende, através de uma estreita colaboração entre cientistas e cidadãos, desenvolver um estudo pioneiro em Portugal sobre a dinâmica microbiana intestinal e responder às seguintes questões:

- **Quão dinâmico é o microbioma intestinal humano? Como evoluem os microrganismos que colonizam os nossos intestinos? Que fatores influenciam a dinâmica do microbioma intestinal?**

  Estas questões referem-se à variabilidade ecológica e evolutiva do microbioma intestinal humano, que pode ser influenciada por diversos fatores como a dieta, estilo de vida, uso de medicamentos, exposição a microrganismos externos, entre outros.

- **Quais as relações na dinâmica do microbioma intestinal entre as pessoas da mesma família e entre as várias famílias, em comunidade?**

Pacote Informativo para o Participante

A análise das relações na dinâmica do microbioma intestinal entre membros da mesma família é crucial para entendermos como os fatores genéticos e ambientais influenciam a composição e a função do microbioma intestinal. Através deste projeto, será possível recolher amostras de diferentes membros de uma família ao longo do tempo e investigar como as relações entre membros da mesma família se manifestam. Iremos também comparar os resultados entre as várias famílias que participarão neste estudo. O projeto proposto vai permitir identificar padrões de microbioma intestinal compartilhados entre familiares e compreender como a interação entre os membros de uma família influencia os seus microbiomas intestinais.

Acreditamos que uma abordagem inovadora de Ciência Cidadã enriquecerá a investigação científica proposta neste projeto, ao aliar cientistas a cidadãos neste estudo sobre um tema de importância para a saúde e bem-estar de todos.

O que é a Ciência Cidadã e como se enquadra neste projeto?

A Ciência Cidadã envolve a "participação pública em atividades de investigação científica, onde os cidadãos contribuem ativamente para a ciência, seja por meio de seus esforços intelectuais, conhecimento pessoal e local, ou recursos e ferramentas disponíveis" (Comissão Europeia, 2014). Desta forma, a Ciência Cidadã pode contribuir ativamente para a democratização da ciência e para a confiança da população no método científico.

De realçar que este projeto de investigação científica, inovador em Portugal, será desenvolvido no âmbito do Programa de Cidadania Ativa - Ciência + Cidadã que tem como missão desenvolver projetos de ciência cidadã, pensar a ciência com as pessoas e consultar a sociedade civil na tomada de decisões para a ciência.

Neste contexto, pretendemos uma participação ativa dos cidadãos neste projeto, envolvendo-os em várias fases da investigação a decorrer e incentivando-os a tornarem-se embaixadores do projeto, promovendo nas suas comunidades locais a importância do microbioma intestinal para a nossa saúde.

O que envolve participar com a minha família no projeto de Ciência Cidadã Microbioma Comunidade Portugal - Intestino Saudável?

Se aceitar participar neste projeto com membros da sua família serão necessários os seguintes procedimentos:

1 Dados Pessoais e assinatura do formulário de consentimento informado

- Ser-lhe-á solicitado, a si e aos membros da sua família ou coabitantes que vão participar neste projeto, que preencham um inquérito; a informação recolhida será pseudoanonimizada, apenas conhecida pela coordenadora científica do projeto e outro membro da equipa;
- Os dados recolhidos serão digitalizados num ficheiro eletrónico, que será armazenado num disco rígido externo protegido por senha, e os formulários escritos e o disco rígido externo serão mantidos num arquivo fechado no escritório da coordenadora científica do projeto. Os formulários de consentimento informado assinados e os formulários manuscritos com dados pessoais serão retidos por 3 anos após o encerramento do estudo num arquivo fechado no escritório da coordenadora científica do projeto. Este prazo é determinado pela necessidade de acesso a esta informação no caso de um problema imprevisto ou de uma reclamação. Após os 3 anos, os arquivos serão destruídos, mas a base de dados, que contém os meta-dados anonimizados, será conservada num repositório de dados;

Pacote Informativo para o Participante

- Cada participante terá de assinar o formulário de consentimento informado e, caso aplicável, dos filhos menores, em duplicado, para autorizar a participação neste estudo. Uma cópia do consentimento informado assinado será para seu registo e mantê-la no decorrer do projeto. A outra cópia será mantida pela equipa de investigação, até 3 anos após a duração do estudo. Será contactado por telefone, e-mail ou sms a confirmar a sua participação no presente estudo;

- Será também solicitado que forneça alguns dados pessoais, para contacto no âmbito do estudo, e que preencha um questionário de saúde e estilo de vida. Receberá um e-mail ou um sms a confirmar a participação no presente estudo.

2 Recolha das amostras através do Kit Microbioma Comunidade Portugal

- Será contactado via e-mail, sms e/ou chamada telefónica para agendar:
  a. a entrega dos Kits Microbioma Comunidade Portugal para auto-recolha de fezes;
  b. o levantamento das amostras na morada que indicar (caso residam na mesma morada a entrega dos kits e levantamento de amostras dos vários membros da sua família registados no estudo poderão ser marcadas na mesma altura).

- Aquando da receção do seu kit, ser-lhe-á enviado o seu código de participante por e-mail e/ou sms, que posteriormente deverá registar no autocolante que se encontra dentro do Kit Microbioma Comunidade Portugal;

- Ser-lhe-á solicitado, a si e a todos os participantes registados, a recolha de 3 de amostras de fezes por cada membro da família durante um período de 6 meses (1 amostra por pessoa de 2 em 2 meses) utilizando os Kits Microbioma Comunidade Portugal (no caso de crianças com a ajuda dos pais);

- Para proceder à auto-recolha de fezes na comodidade da sua casa, deve consultar as instruções do folheto informativo contido no interior da embalagem do Kit Microbioma Comunidade Portugal;

- Um representante da família ficará responsável por guardar as amostras no frigorífico (não congelar) até serem recolhidas por um representante do projeto (estafeta) na sua residência;

- As amostras terão de ser preservadas no frigorífico (não congelar) até serem recolhidas por um estafeta na residência;

- Em caso de alguma dúvida ou questão contacte-nos através do e-mail: microbiomapt@gimm.pt

3 Inquéritos: Dados pessoais, saúde e estilos de vida

No início do estudo, será conduzido um inquérito a cada membro participante para avaliar a relação entre o nível de flutuação do microbioma intestinal e diversos fatores como a dieta, o uso de antibióticos e outros medicamentos, doenças, alergias, idade, grau de parentesco e convivência, entre outros.

O mesmo inquérito será também realizado antes da segunda e terceira recolhas de amostras para atualização dos dados.

4 Apresentação e envolvimento dos cidadãos

Por se tratar de um estudo de Ciência Cidadã, os participantes serão incentivados a participarem em ações informativas e de sensibilização e a serem embaixadores do projeto nas suas comunidades locais.

Pacote Informativo para o Participante

Estão previstas 4 sessões de sensibilização com os participantes, de 2 em 2 meses, com duração prevista de 2-3 horas, na Fundação GIMM - Gulbenkian Institute for Molecular Medicine e/ou num local publico de Oeiras (Ex. Biblioteca Municipal de Oeiras).

4.1 Na primeira sessão, realizada antes da primeira auto-recolha de fezes, estão planeados os seguintes pontos:

a. Apresentação do projeto de Ciência Cidadã Microbioma Comunidade Portugal;

b. Apresentação dos investigadores e colaboradores do projeto;

c. Esclarecimento de dúvidas que os participantes possam ter sobre as várias etapas do projeto;

d. Explicação e entrega dos kits de auto-recolha de fezes para a primeira colheita.

Nota: os participantes podem ajudar o grupo de investigação a preparar os kits de auto-recolha.

4.2 Nas segunda e terceira sessões, antes da segunda e terceira auto-recolhas de fezes, estão programados os seguintes procedimentos:

a. Os investigadores farão um ponto de situação sobre os avanços do projeto de investigação até à data;

b. Os participantes vão receber um folheto informativo sobre os resultados obtidos relativos ao microbioma intestinal da comunidade analisada;

c. Serão entregues os kits para as recolhas de fezes seguintes;

d. Os participantes colaborarão na cocriação de uma publicação (em formato de mini-livro ou brochura) sobre o microbioma intestinal e a sua importância para a saúde humana.

Nota: os participantes podem ajudar o grupo de investigação a preparar os kits de auto-recolha.

4.3 Na quarta sessão temos previstos os seguintes pontos:

a. Apresentação dos resultados finais desta fase do projeto Microbioma Comunidade Portugal;

b. Apresentação de planos futuros relativos ao projeto de investigação;

c. Apresentação da publicação sobre o Microbioma Intestinal desenvolvida em cocriação com os participantes e que, posteriormente, será divulgada em parceria com o Município de Oeiras em escolas, hospitais, universidades séniores, entre outros.

Qual é o procedimento na recolha das amostras e de dados pessoais?

Os dados pessoais dos membros que participam no estudo serão preenchidos no inquérito sobre dados pessoais, saúde e estilo de vida. Ao participar neste estudo, concorda em fornecer as informações mínimas requeridas neste inquérito e fornecer a sua amostra fecal (ou dos filhos menores) e concorda em transferir a propriedade da mesma para a instituição coordenadora do projeto que a utilizará para investigação, nomeadamente para o estudo Microbioma Comunidade Portugal. A instituição coordenadora do projeto também poderá utilizar o material para outras investigações relacionadas ao objeto deste estudo, após ser revisto por um comité de ética competente. As amostras fecais serão armazenadas ultracongeladas indefinidamente na Instituição Promotora do projeto. Os micróbios isolados ou coleções de micróbios cultivados a partir das amostras fecais, serão armazenados na Instituição Promotora

Pacote Informativo para o Participante

por tempo indeterminado e serão usados para fins de investigação no estudo Microbioma Comunidade Portugal ou noutros estudos relacionados, após ser revisto por um comité de ética competente.

Quais são os potenciais benefícios da minha participação neste projeto de Ciência Cidadã?

Ao fazer parte do Microbioma Comunidade Portugal está a participar ativamente, como cientista cidadão/ã, num importante projeto de investigação científica e a contribuir para a produção de conhecimento e sua divulgação na sociedade civil.

Todos os participantes receberão informações sobre o seu microbioma intestinal e o de suas famílias. Receberão também materiais educativos em formato digital e em folhetos, abordando a importância do microbioma intestinal para a saúde, como mantê-lo saudável e preservá-lo e sobre os desafios científicos atuais relacionados com a investigação deste ecossistema. Este projeto tem unicamente fins de investigação e de educação no âmbito do microbioma intestinal humano. A informação que irá receber não tem fins de diagnóstico ou aconselhamento médico.

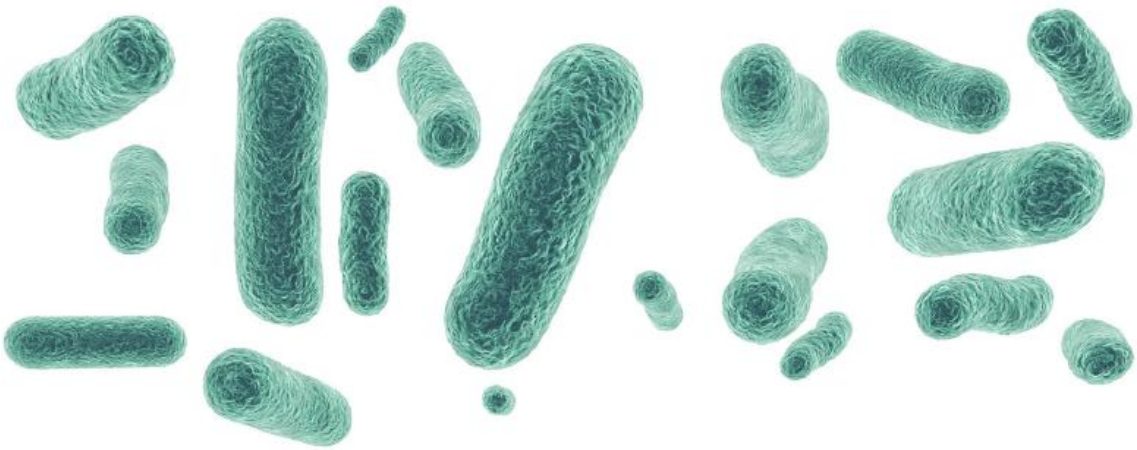
Divulgação do Estudo e Envolvimento dos Cidadãos

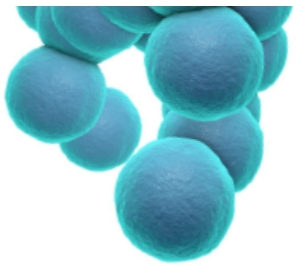
Os resultados da investigação no âmbito deste projeto serão divulgados em revistas científicas internacionais de acesso aberto e em congressos e outros encontros nacionais e internacionais.

Este projeto de Ciência Cidadã pretende envolver os participantes nas várias fases deste estudo. Além de recolha das amostras, as famílias selecionadas serão envolvidas através de sessões regulares de informação, visitas aos laboratórios, participação em reuniões com os investigadores, cocriação de materiais de sensibilização, entre outros.

As conclusões do estudo através dos resultados globais da comunidade serão também divulgadas online numa página destinada a este projeto nos websites da Instituição Promotora e do Programa Ciência + Cidadã.

A identidade dos participantes e das suas famílias será mantida anónima na divulgação dos resultados.





Pacote Informativo para o Participante

A minha participação é voluntária?

Sim, a sua participação neste estudo de Ciência Cidadã é voluntária e a equipa de investigação agradece desde já a sua participação e envolvimento.

Há algum custo associado à participação no Microbioma Comunidade Portugal?

Os participantes não terão quaisquer custos associados nem receberão pagamentos pela sua participação.

Durante quanto tempo vou colaborar e estar envolvido neste projeto de Ciência Cidadã?

Este estudo terá a duração de 1 a 2 anos. Os participantes estarão envolvidos durante um período de 6 meses, na fase da recolha de 3 amostras de fezes (1 amostra por participante de 2 em 2 meses). No decorrer e depois de terminadas as recolhas das amostras, os participantes serão também incentivados a continuarem a participar ativamente neste projeto.

Quais são os riscos potenciais da minha participação?

Este estudo apenas recolhe informações através de amostras de fezes e dos inquéritos, pelo que é improvável a existência de riscos relacionados com o mesmo. O seu estilo de vida não será afetado pela sua decisão de participar ou não no estudo. Deverá utilizar as luvas fornecidas e consultar a informação de procedimentos de higiene nas instruções do Kit Microbioma Comunidade Portugal para evitar contaminação com possíveis micróbios patogénicos nas fezes (do próprio e do meio ambiente).

O que será estudado na minha amostra fecal?

As amostras de fezes recolhidas serão utilizadas para estudar apenas a composição do microbioma intestinal ao longo do tempo e os produtos bioquímicos produzidos por estes micróbios. Para isso, as amostras serão manuseadas no laboratório e a análise será feita utilizando técnicas de genética, bioquímica e bioinformática específicas para este fim. As bactérias isoladas do intestino serão analisadas através da técnica de sequenciação do amplicon 16S rRNA que visa estudar especificamente o gene 16S rRNA. Este gene é encontrado em todas as bactérias e utilizado para identificar e classificar bactérias com base nas suas sequências genéticas.

Onde serão armazenadas as minhas amostras fecais?

Todas as amostras de fezes e micróbios isolados das amostras serão conservados indefinidamente na Instituição Promotora sob a responsabilidade da equipa de investigação associada ao projeto. Quaisquer estudos futuros envolvendo o uso dessas amostras serão analisados eticamente por um comité de ética competente.

Que tipo de informação pessoal devo fornecer e porquê?

Sabe-se que a dieta, medicamentos, tabagismo e elementos aos quais pode estar exposto no seu trabalho diário podem ter um grande impacto na composição dos seus micróbios intestinais. O peso e altura também podem refletir

Pacote Informativo para o Participante

diferenças na composição dos seus micróbios intestinais, já que condições de magreza e obesidade estão associadas a abundâncias maiores ou menores de determinadas bactérias intestinais. O país e local de nascimento e a residência são informações importantes porque o microbioma é influenciado pelo ambiente.

Neste sentido, ao participar no estudo ser-lhe-á solicitado que responda a inquéritos para nos fornecer alguns dados pessoais e informações sobre a sua saúde e estilo de vida como ano e local de nascimento, local e país de residência, sexo, peso, altura, hábitos alimentares, tabagismo, ocupação, medicação regular.

Opcionalmente, poderá também fornecer informações pessoais adicionais, incluindo modo de nascimento e tipo de fezes, entre outras informações.

Como será mantida a semi-confidencialidade dos meus dados pessoais?

Quando for incluído no estudo, os seus dados e as suas amostras fecais serão associados a um código gerado especificamente para o estudo. Apenas o coordenador e um membro da equipa de investigação terão acesso à identificação dos participantes. O semi-anonimato no tratamento de dados é garantido. Todos os dados resultantes do estudo serão publicados de forma anónima.

O Promotor deste estudo é o responsável pelo tratamento dos seus dados pessoais no âmbito do mesmo.

Os seus dados pessoais serão tratados com base no seu consentimento explícito, para as finalidades de realização de investigação científica, contactos no âmbito do projeto de Ciência Cidadã e cumprimento das obrigações e requisitos legais e regulamentares aplicáveis.

Com exceção dos dados pessoais de identificação e contacto que sejam estritamente necessários de acordo com o processo descrito neste documento, todos os dados pessoais são tratados de forma codificada, utilizando um código de identificação em vez do seu nome, e todos os dados e amostras recolhidos sobre si serão protegidos através da utilização desse código. Apenas a equipa de investigação controlará a chave de códigos.

Posso ter acesso aos dados do estudo das minhas amostras fecais?

De acordo com a legislação em vigor, é garantido o acesso, retificação ou cancelamento do acesso aos seus dados. Para cancelar deverá enviar um e-mail para o e-mail microbiomapt@gimm.pt.

A propriedade das amostras humanas dependerá sempre do participante. Todos os direitos de propriedade intelectual decorrentes do projeto de investigação pertencem à Entidade Promotora do projeto. Esses direitos de propriedade intelectual incluem, mas não estão limitados, a descobertas, "know-how", invenções, direitos autorais, marcas registadas, pedidos de marcas registadas e pedidos de patentes. A Instituição Promotora compromete-se a estabelecer uma comunicação regular com os participantes inscritos e a informá-los antecipadamente sobre os resultados científicos do projeto.

Pacote Informativo para o Participante

Posso interromper a minha participação no estudo Microbioma Comunidade Portugal a qualquer momento?

Sim, tem o direito a retirar a sua participação do estudo e solicitar que a sua amostra fecal seja descartada e os seus dados eliminados, incluindo os dados obtidos no estudo dos seus micróbios intestinais, a qualquer momento. No entanto, se os dados fizerem parte de um artigo científico publicado ou fizerem parte de um artigo aceite para publicação, não será possível retirar ou suspender a publicação dos dados.

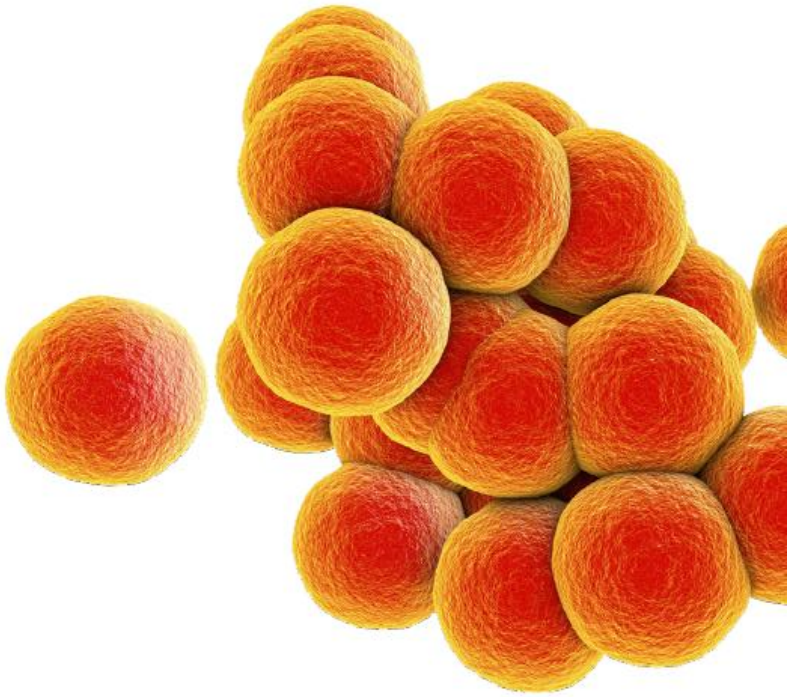
Se concordar em participar no estudo e depois mudar de ideias, pode desistir do mesmo a qualquer momento, sem necessidade de indicar o motivo, através do e-mail microbiomapt@gimm.pt.

Com quem posso entrar em contacto para tirar dúvidas?

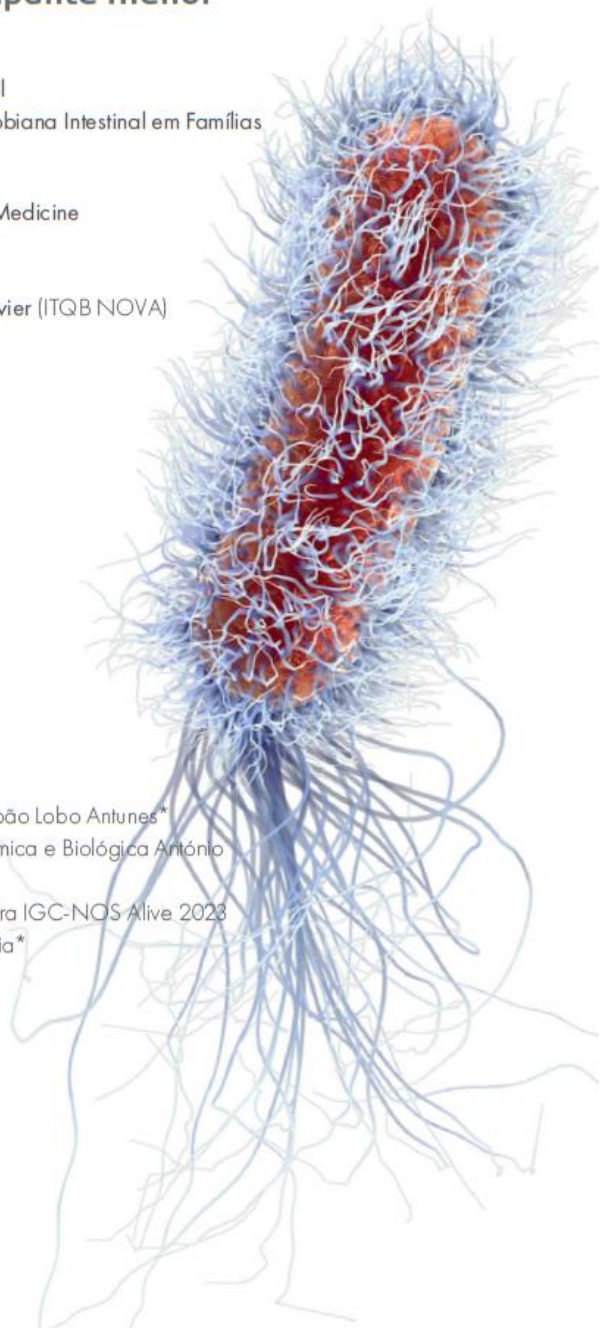
Se tiver alguma dúvida ou questão, ou em caso de emergência, deverá contactar a equipa de coordenação do projeto de investigação através do e-mail microbiomapt@gimm.pt.

Serão também organizadas sessões de esclarecimento com os participantes no projeto.

Também poderá entrar em contacto com a comissão de ética IHMT NOVA – ITQB NOVA para esclarecer dúvidas relacionadas aos seus direitos e obrigações como participante do estudo Microbioma Comunidade Portugal: comissaodeetica@ihmt.unl.pt.



Pacote Informativo para o Participante Menor



Título do Estudo

Microbioma Comunidade Portugal – Intestino Saudável
Ciência Cidadã em Estudo pioneiro da Dinâmica Microbiana Intestinal em Famílias

Instituição Promotora

Fundação GIMM - Gulbenkian Institute for Molecular Medicine

Parceiros

Instituto de Tecnologia Química e Biológica António Xavier (ITQB NOVA)
Católica Biomedical Research Centre (CBR)
Everything is New
Programa Ciência + Cidadã
Município de Oeiras

Coordenação da Equipa de Investigação

Isabel Gordo, Instituto Gulbenkian de Ciência*

Coordenação Ciência Cidadã

Maria João Leão, Programa Ciência + Cidadã

Equipa de Investigação

Isabel Gordo, Instituto Gulbenkian de Ciência*
Karina Xavier, Instituto Gulbenkian de Ciência*
Luís Teixeira, Católica Biomedical Research Centre
Ana Santos Almeida, Instituto de Medicina Molecular João Lobo Antunes*
Sarela Garcia-Santamarina, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa
Patrícia Morais, Instituto Gulbenkian de Ciência*/Bolseira IGC-NOS Alive 2023
António Gomes da Costa, Instituto Gulbenkian de Ciência*
Maria João Leão, Programa Ciência + Cidadã

Comissão de Ética Responsável

Comissão de Ética conjunta do IHMT-ITQB-NSL-IGC

Data de Aprovação pela Comissão de Ética

13 de março de 2024

---

* O Instituto Gulbenkian de Ciência (IGC) e o Instituto de Medicina Molecular João Lobo Antunes (IMM) uniram-se para criar a Fundação GIMM – Gulbenkian Institute for Molecular Medicine.

Pacote Informativo para o Participante Menor

O teu microbioma intestinal é o conjunto dos biliões de bactérias, fungos e outros seres vivos, e dos seus genes, que são importantes para a digestão dos alimentos, produção dos nutrientes e para o teu crescimento e saúde.

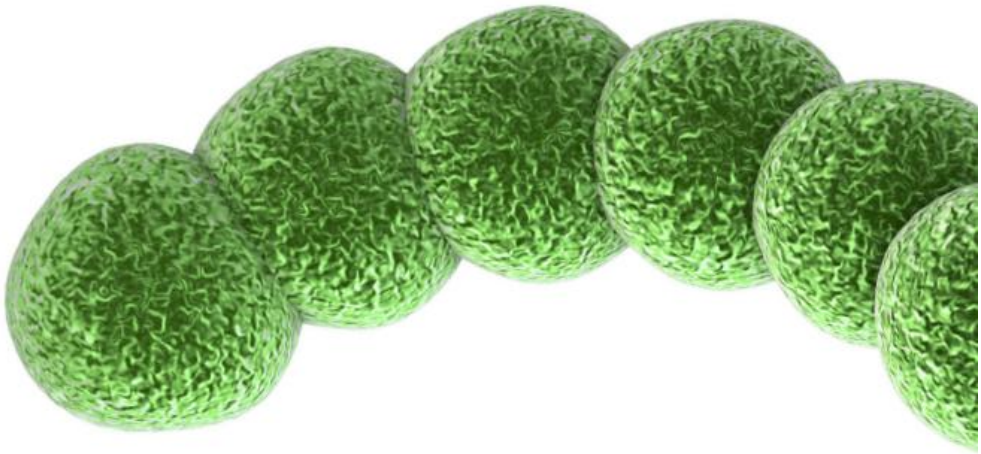
Sabias que o teu intestino é único, assim como tu? Cada pessoa tem um microbioma diferente e ele muda ao longo do tempo, dependendo de vários fatores como o ambiente em que vives, os teus hábitos alimentares e os teus genes.

O objetivo deste projeto é descobrir mais sobre os micróbios que vivem nos nossos intestinos. Queremos saber quais é que estão lá e ver como mudam ao longo do tempo, comparando com os da tua família e com os de outras pessoas, na tua comunidade.

Tu e a tua família vão receber um kit para recolher amostras de fezes e responderem a algumas perguntas sobre a vossa saúde e estilos de vida.

Ao fazeres parte do Microbioma Comunidade Portugal vais poder aprender muitas coisas novas e ao mesmo tempo contribuir para a produção de conhecimento como cientista cidadão/ã.

Vamos desvendar juntos os segredos do Microbioma Intestinal Humano?



Pacote Informativo para o Participante

Tão único como tu!

O teu Microbioma é único, mas é com a tua participação que conseguiremos definir os componentes essenciais de um microbioma intestinal humano universal.

SÊ CIENTISTA CIDADÃO/Ã.
JUNTA-TE A NÓS!

Appendix C – Informed Consent of the Project

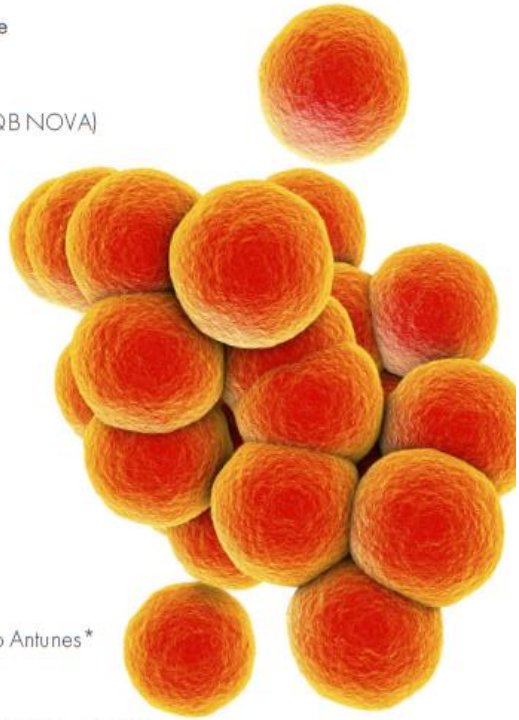
Microbioma Comunidade Portugal | município Oeiras

Formulário de Consentimento Informado

Título do Estudo
Microbioma Comunidade Portugal – Intestino Saudável
Ciência Cidadã em Estudo pioneiro da Dinâmica Microbiana Intestinal em Famílias

Instituição Promotora
Fundação GIMM - Gulbenkian Institute for Molecular Medicine

Parceiros
Instituto de Tecnologia Química e Biológica António Xavier (ITQB NOVA)
Católica Biomedical Research Centre (CBR)
Everything is New
Programa Ciência + Cidadã
Município de Oeiras



Coordenação da Equipa de Investigação
Isabel Gordo, Instituto Gulbenkian de Ciência*

Coordenação Ciência Cidadã
Maria João Leão, Programa Ciência + Cidadã

Equipa de Investigação
Isabel Gordo, Instituto Gulbenkian de Ciência*
Karina Xavier, Instituto Gulbenkian de Ciência*
Luís Teixeira, Católica Biomedical Research Centre
Ana Santos Almeida, Instituto de Medicina Molecular João Lobo Antunes*
Sarela Garcia-Santamarina, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa
Patrícia Morais, Instituto Gulbenkian de Ciência/Bolseira IGC-NOS Alive 2023
António Gomes da Costa, Instituto Gulbenkian de Ciência*
Maria João Leão, Programa Ciência + Cidadã

Comissão de Ética Responsável
Comissão de Ética conjunta do IHMT-ITQB-NSL-IGC

Data de Aprovação pela Comissão de Ética
13 de março de 2024

*
O Instituto Gulbenkian de Ciência (IGC) e o Instituto de Medicina Molecular João Lobo Antunes (IMM) uniram-se para criar a Fundação GIMM – Gulbenkian Institute for Molecular Medicine.

Formulário de Consentimento Informado

Por favor, leia com atenção todo o conteúdo deste documento. Não hesite em solicitar mais informações se não estiver completamente esclarecido/a. Verifique se todas as informações estão corretas.

Confirmo que fui informado/a pela equipa de investigação sobre o projeto de Ciência Cidadã Microbioma Comunidade Portugal - Intestino Saudável para o qual fui convidado/a a participar. Confirmo que depois de ler o "Pacote informativo para o/a participante" do estudo tive tempo para refletir, ter todas as minhas dúvidas esclarecidas e que consinto voluntariamente em participar neste projeto. Confirmo também que tomei conhecimento que posso cancelar a minha participação a qualquer momento e por qualquer motivo. Para isso, basta informar por escrito a coordenadora da equipa de investigação Doutora Isabel Gordo.

Concordo que a equipa de investigação terá acesso aos dados que fornecerei para o estudo. A minha identidade, no entanto, permanecerá sempre semi-anonimizada e confidencial, e somente a coordenadora científica e um membro da equipa de investigação terão acesso aos códigos que protegem a minha identidade.

Ao participar neste estudo, concordo em fornecer as informações mínimas requeridas no Inquérito sobre dados pessoais, saúde e estilos de vida: ano de nascimento, sexo, peso, altura, índice de massa corporal, hábitos alimentares, hábitos tabágicos, ocupação, medicação regular, país e local de nascimento e de residência. Opcionalmente, posso fornecer informações pessoais adicionais, incluindo modo de nascimento ou gravidez, entre outras informações.

Confirmo que a amostra fecal é minha, mas a propriedade intelectual dos resultados da investigação nela baseada pertence à Instituição Promotora deste estudo. Esta instituição também poderá utilizar o material para outras investigações relacionadas ao objeto deste estudo, depois de ser eticamente revisto pelo Comité de Ética competente. As amostras fecais serão armazenadas ultracongeladas na Fundação GIMM durante o período deste estudo sendo posteriormente armazenadas no Biobanco da Fundação GIMM. Os micróbios ou coleções de micróbios isolados a partir de amostras fecais serão armazenados indefinidamente na instituição e serão usados para fins de investigação no estudo Microbioma Comunidade Portugal - Intestino Saudável ou noutros estudos relacionados, após ser revisto por um Comité de Ética competente.

A equipa de investigação será responsável por manter os meus dados armazenados, protegidos por código e bloqueados num arquivo. Da mesma forma, a equipa de investigação será responsável por manter os formulários de consentimento informado num arquivo bloqueado por 3 anos após o término do estudo. Após esse período, os formulários de consentimento informado serão destruídos.

Receberei uma cópia deste formulário assinada e datada por mim e pela coordenadora da equipa de investigação, Doutora Isabel Gordo, e manterei este registro até o final do estudo.

Parte declarativa da pessoa que consente

Declaro:

- Estar ciente do carácter voluntário da participação no presente estudo e ter compreendido os objetivos do mesmo.
- Ter-me sido dada oportunidade de fazer todas as perguntas sobre o assunto e para todas elas ter obtido resposta esclarecedora. Ter-me sido dado tempo suficiente para refletir sobre esta proposta. Ter-me sido garantido que posso revogar o consentimento entretanto prestado.
- Compreender que o presente estudo tem uma duração de aproximadamente 2 (dois) anos.
- Ter lido e compreendido a informação disponibilizada sobre o tratamento dos meus dados pessoais, entendendo que a participação neste estudo requer o uso desses dados no sentido de caracterizar a dinâmica do microbioma no contexto da família e comunidade.

Formulário de Consentimento Informado

Identificação do participante

Nome: _____

Data de nascimento: ____/____/_____

Morada: _____

Tipo do documento de identificação: _____

Número do documento de identificação: _____

Data ou validade do documento de identificação: ____/____/_____

Se não for o próprio a submeter a participação

Nome do representante: _____

Tipo de representação: _____

Tipo do documento de identificação: _____

Número do documento de identificação: _____

Data ou validade do documento de identificação: ____/____/_____

Nome da coordenadora da equipa de investigação: **Isabel Gordo**

Assinatura do participante

_____

Data: ____/____/_____

Assinatura do representante, se o participante for menor

_____

Data: ____/____/_____

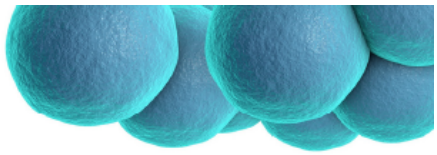
Assinatura da coordenadora da equipa de investigação

_____

Data: ____/____/_____

Appendix D – Informed Consent for Photos

Microbioma Comunidade Portugal | município Oeiras



Consentimento informado, livre e esclarecido para captação e utilização de imagens em fotografia ou vídeo e cedência dos direitos de imagem

1 Informação sobre as atividades

O projeto Microbioma Comunidade Portugal é um projeto Ciência Cidadã inovador na área da Microbiologia que tem como objetivo perceber a dinâmica do microbioma intestinal humano em famílias. Os participantes serão envolvidos em várias fases do projeto, durante aproximadamente 6 meses, e terão acesso a sessões de sensibilização com os investigadores e colaboradores, onde serão dadas a conhecer as várias fases do projeto, apresentados os resultados obtidos ao longo do tempo e esclarecidas as dúvidas. Os participantes serão também envolvidos na cocriação de uma publicação em formato de mini-livro ou brochura sobre o tema do microbioma e sobre a sua importância para a saúde humana.

2 Captação de imagens/vídeos

No âmbito das ações desenvolvidas durante este projeto de Ciência Cidadã, poderá haver lugar à captação e divulgação de imagens em redes sociais e outros meios de comunicação para efeitos de documentação e promoção junto da comunidade.

As fotografias e vídeos captados durante as sessões serão objeto de conservação durante 3 anos, sem prejuízo da possibilidade de reprodução por parte de terceiros.

Poderá a qualquer momento revogar o consentimento prestado em seu nome ou no nome do seu educando (em caso de menor de idade), bem como solicitar que sejam apagadas todas as fotografias e vídeos em que seja enquadrado, de todos os meios, suportes ou canais da responsabilidade direta da Comissão Organizadora, através do endereço microbiomapt@gimm.pt.

Consentimento

Eu,_____,

autorizo a captação e divulgação de fotografias e vídeos meus, nas condições acima referidas.

Assinatura_____

Local e data ____/____/_____, _____

Consentimento (em caso de menor de idade)

Eu, _____,

responsável pelo(a)_____

autorizo a captação e divulgação de fotografias e vídeos, nas condições acima referidas.

Assinatura_____

Local e data ____/____/_____, _____

GIMM Gulbenkian Institute for Molecular Medicine · nms nova · CATÓLICA BIOMEDICAL RESEARCH CENTRE · Everything is New · CIÊNCIA CIDADÃ · OEIRAS VALLEY | MUNICÍPIO OEIRAS

Appendix E – Questionnaire

Microbioma Comunidade Portugal | município Oeiras

Inquérito:
Dados Pessoais e Informações sobre Saúde e Estilos de Vida

A. Informações Obrigatórias

As respostas às perguntas desta seção são obrigatórias e os dados serão utilizados de forma semi-anónima.

Nota: Iremos pedir que este inquérito seja respondido de 2 em 2 meses, antes das recolhas das amostras.

Código: _____

Informações gerais

1 Ano de nascimento

Ano: _____

2 Peso (em kg) e Altura (em m)

Peso: _____kg

Altura: _____m

3 Sexo

- ☐ Masculino
- ☐ Feminino
- ☐ Outro

4 País e local de nascimento?

País: _____

Local: _____

5 País e local de residência?

País: _____

Local: _____

Desde quando mora neste local? _____

6 Grupo Sanguíneo _____

7 Família e grau de parentesco ou coabitantes

Número de membros da sua família ou coabitantes que também participam neste estudo (a contar consigo):

_____

Indique o código e o seu grau de parentesco com cada um dos membros da sua família que irá participar ou caso seja apenas coabitante:

_____

_____

_____

_____

_____

Razão científica:

Estas perguntas são realizadas para recolher informações que podem ajudar a descrever os resultados da investigação das suas amostras.
O local de nascimento é um parâmetro importante para identificar diferenças no conteúdo genético dos micróbios nas suas amostras relacionadas à sua área de nascimento. O peso e altura são parâmetros fisiológicos importantes para calcular o índice de massa corporal (IMC), que pode explicar a presença aumentada / reduzida de certos micróbios intestinais. O grupo sanguíneo permite relacionar o grupo sanguíneo com o microbioma.

Inquérito:
Dados Pessoais e Informações sobre Saúde e Estilos de Vida

8 Qual é a sua dieta habitual? (selecione uma opção)

- ☐ Dieta vegan (vegetariana estrita)
- ☐ Dieta vegetariana (ovo-lacto-vegetariana, ovo-vegetariana ou lacto-vegetariana)
- ☐ Dieta de base vegetal (sem consumo de carne, mas com consumo de peixe)
- ☐ Dieta de base vegetal (com consumo moderado de carne e peixe)
- ☐ Dieta macrobiótica
- ☐ Dieta paleolítica
- ☐ Dieta cetogénica (com elevada restrição de hidratos de carbono)
- ☐ Dieta isenta de glúten
- ☐ Jejum intermitente
- ☐ Não sigo nenhum tipo de dieta especial ou restrita

9 Consome atualmente bebidas alcoólicas (como cerveja, vinho, licor ou álcool local)?

- ☐ Sim, frequentemente
- ☐ Sim, esporadicamente
- ☐ Não (atualmente)
- ☐ Não (nunca bebi)

10 Quantas porções de frutas e vegetais come em média por dia (porção significa 1 banana, 1 maçã ou 1 tangerina)?

- ☐ Menos de 1 porção
- ☐ Entre 2 e 3
- ☐ 4 ou mais

11 Quantas porções de lacticínios consome em média por dia?

- ☐ Menos de 1 porção
- ☐ Entre 2 e 3
- ☐ Entre 3 e 4
- ☐ 5 ou mais

12 É fumador atualmente?

- ☐ Sim
- ☐ Não (atualmente)
- ☐ Não (nunca fumei)
- ☐ Prefiro não dizer

13 Está a tomar algum antibiótico atualmente?

- ☐ Sim
- ☐ Não

Se sim, há quanto tempo? E qual?

_____

Tomou algum antibiótico há menos de um ano?

- ☐ Sim
- ☐ Não

14 Está a tomar algum probiótico atualmente?

- ☐ Sim
- ☐ Não

Se sim, há quanto tempo? E qual?

_____

Tomou algum probiótico há menos de um ano?

- ☐ Sim
- ☐ Não

Inquérito:
Dados Pessoais e Informações sobre Saúde e Estilos de Vida

15 **Tem alguma doença?**

- ☐ Sim
- ☐ Não

Se sim, qual/quais?

_____

_____

_____

16 **Está a tomar medicação para alguma doença diagnosticada?**

- ☐ Sim
- ☐ Não

Se sim, qual a medicação? E há quanto tempo?

_____

_____

_____

17 **Qual e a sua ocupação profissional?**

_____

_____

- ☐ Prefiro não dizer

Razão científica:
Estas perguntas são realizadas para recolher informações sobre os principais fatores conhecidos que afetam a composição dos micróbios presentes dentro de seu intestino. Sabe-se que a dieta, os medicamentos, o tabagismo e fatores ambientais aos quais pode estar exposto na sua profissão podem ter um grande impacto na composição dos micróbios presentes no seu intestino.

B. Informação Opcional

Por favor, responda às seguintes perguntas apenas se tiver a certeza. Quantas mais perguntas responder, mais refinada será a análise da sua amostra.

1 **Pode indicar o seu modo de nascimento? (selecione um)**

- ☐ Cesariana
- ☐ Vaginal

Onde se realizou?

- ☐ Hospital /Clínica
- ☐ Casa
- ☐ Não sei

2 **Pode indicar se foi amamentado/a na infância?**

- ☐ Sim
- ☐ Não
- ☐ Não sei

Inquérito:
Dados Pessoais e Informações sobre Saúde e Estilos de Vida

3 Pode descrever o tipo das suas fezes (segundo a Bristol Stool Chart)?

- ☐ **Tipo 1** - Grânulos duros e separados, tipo nozes (difíceis de passar)
- ☐ **Tipo 2** - Em forma de salsicha, mas granuloso
- ☐ **Tipo 3** - Em forma de salsicha, mas com fendas na superfície
- ☐ **Tipo 4** - Semelhante a cobra, liso e macio
- ☐ **Tipo 5** - Bolhas moles com bordas bem definidas (passa facilmente)
- ☐ **Tipo 6** - Pedaços fofos com bordas irregulares
- ☐ **Tipo 7** - Aguado, sem bordas (completamente líquido)

Nota: Tipo 1-2 indicam obstipação. Tipo 3-4 são fezes ideais. Tipo 5-7 pode indicar diarreia.

4 Durante a colheita das suas fezes, como considera a cor das suas fezes?

- ☐ Castanho claro
- ☐ Pretas ou mais escuras
- ☐ Verdes
- ☐ Vermelhas (ou com listas vermelhas)
- ☐ Amarelas (cor amarelada)
- ☐ Mais claras ou esbranquiçadas

5 Pode indicar sua frequência média de evacuação (selecione uma opção)?

- ☐ Menos de uma vez por dia
- ☐ Uma vez por dia
- ☐ Mais de 1 vez por dia

6 Considerando os últimos 5 dias, com que frequência evacuou?

_____

Razão científica:
O tipo de fezes que produz depende de quanto tempo elas passam no cólon. Isso depende da sua dieta, ingestão de líquidos, medicamentos e estilo de vida. O Bristol Stool Chart está dividido em 7 categorias de fezes diferentes conforme descrito em cima.

7 Se for mulher, pode indicar:

Está grávida?

- ☐ Sim
- ☐ Não

O seu período/cido menstrual é regular?

- ☐ Sim
- ☐ Não

Ciência Cidadã em Estudo pioneiro da
Dinâmica Microbiana Intestinal em Famílias

Appendix F – Protocol *Microbioma Comunidade Portugal* for Sample Collection

Passo 1: Preparação e montagem do kit

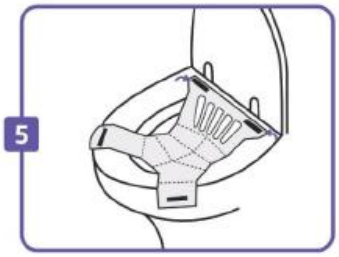
Urinar.

Levantar o assento da sanita.

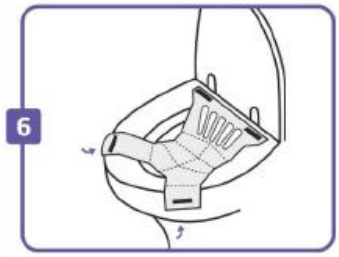
Secar a sanita com papel higiénico para que a base de recolha possa ser montada numa superfície limpa e seca.

Retirar a base de recolha.

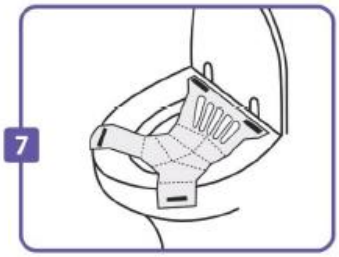
Nota:
A base de recolha não deve tocar na superfície da água.



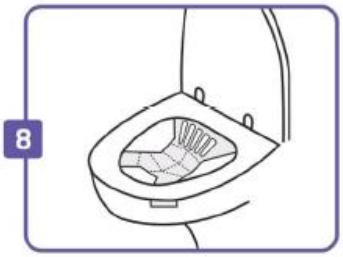
Retirar as proteções de duas fitas da base de recolha e montar na parte de trás da sanita.



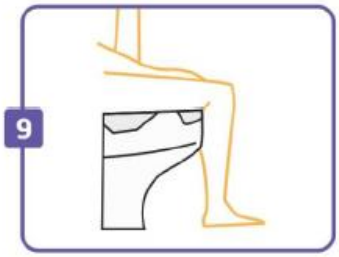
Remover a restante proteção da fita e colocar a parte da frente da base de recolha em cada um dos lados da sanita. O dispositivo deve ficar no centro da sanita, sem tocar na água, e permitindo que haja espaço para deitar fora o papel higiénico pela frente da base de recolha.



A base de recolha é montada de modo a criar uma área de amostra, ficando com um formato de tigela, caso contrário, reorientar as abas para o posicionamento correto.

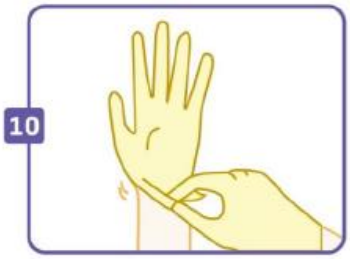


Por último, baixar o tampo da sanita. A base de recolha está pronta a ser utilizada.

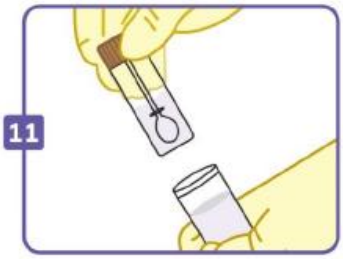


Evacuar. É importante que as fezes não entrem em contacto com a água da sanita ou com a urina.

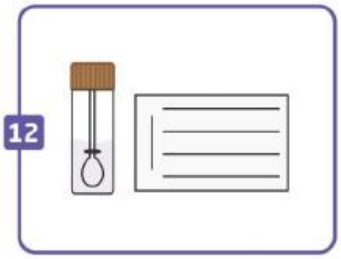
Passo 2.1: Recolha da amostra das fezes para o tubo de colheita 1



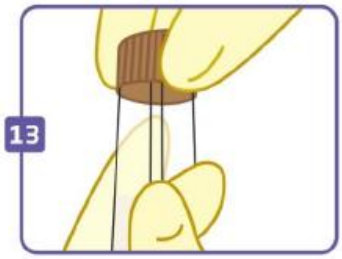
Colocar as luvas descartáveis.



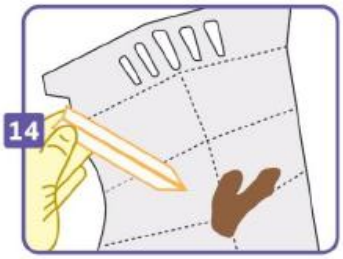
Abrir o recipiente exterior com tampa branca e remover do interior o tubo de colheita 1 com tampa castanha sem o abrir. Deixar o material absorvente no interior do recipiente exterior.



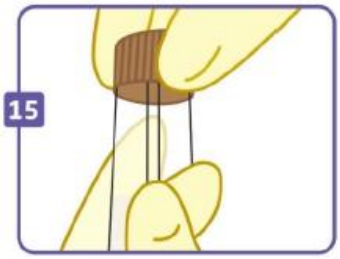
Etiquetar com um dos autocolantes de tamanho mais pequeno o recipiente interior de recolha de amostras, de acordo com o apresentado na imagem, e preencher com o código, a data e a hora da recolha das amostras.



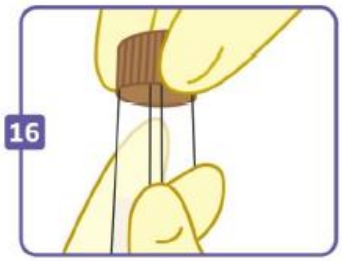
Abrir o tubo de colheita 1 com cuidado para o líquido no interior não verter.



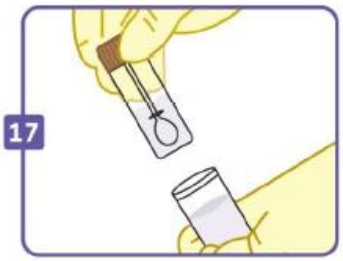
Recolher uma pequena amostra de fezes usando uma das colheres de papel fornecidas no kit, dobrando o papel ao meio para facilitar a recolha da amostra.



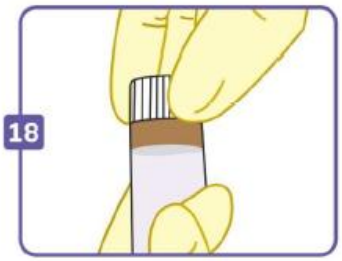
Usar a espátula do tubo de colheita 1 para retirar da colher de papel uma quantidade correspondente ao tamanho de **uma ervilha**. Voltar a colocar a espátula com a amostra das fezes dentro do tubo de colheita 1 com líquido no interior.



Enroscar bem a tampa castanha no recipiente de colheita 1 para evitar fugas da amostra durante o transporte.

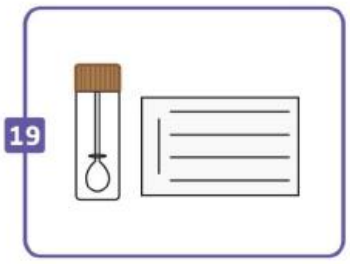


Colocar o tubo de colheita 1 no recipiente exterior de tampa branca, mantendo o material absorvente entre os dois recipientes.

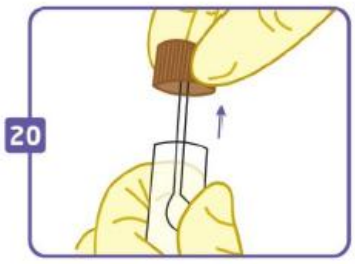


Enroscar a tampa branca no recipiente exterior.

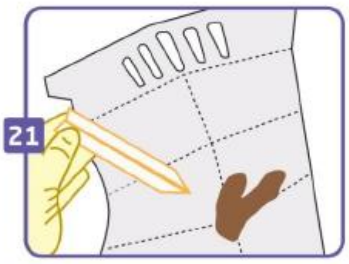
Passo 2.2: Recolha da amostra das fezes para o tubo de colheita 2



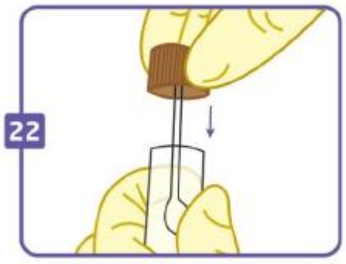
Etiquetar com o autocolante de tamanho pequeno o tubo de colheita 2 com tampa castanha e sem líquido (sem recipiente exterior) e preencher com o código, a data e a hora da recolha das amostras.



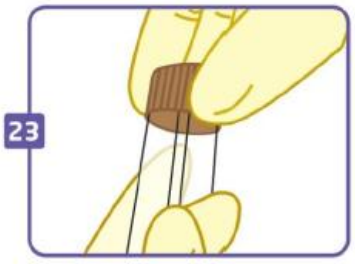
Abrir o tubo de colheita 2.



Recolher nova amostra de fezes da base de recolha na sanita usando a outra colher de papel fornecidas no kit, dobrando o papel ao meio para facilitar a recolha da amostra. Usar a espátula do tubo de recolha 2 para retirar da colher de papel uma quantidade correspondente ao tamanho de **três ervilhas**.

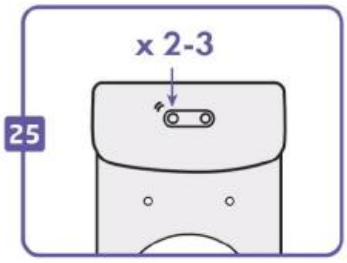


Voltar a colocar a espátula com a amostra das fezes dentro do tubo de colheita 2.



Enroscar bem a tampa no tubo de colheita 2 para evitar fugas da amostra durante o transporte.

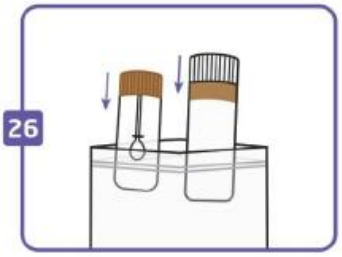
Segurar as colheres usadas na palma de uma mão. Retire a luva sobre as colheres para que o material usado fique contido dentro da luva. Retirar a luva da outra mão e deite fora em conjunto com os outros resíduos domésticos.



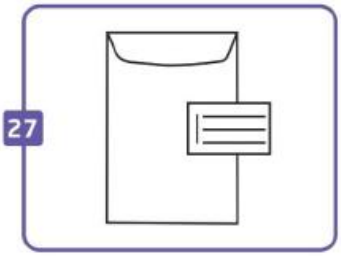
Remover as abas do EasySampler da sanita e deite o EasySampler com as fezes na sanita e descarregue o autoclismo.

Nota:
Quando usar sanitas com poupança de água usar a descarga máxima do autoclismo. Repita até ter descarregado 10-15 litros de água.
Os resíduos da cola do EasySampler na sanita podem ser removidos com álcool.

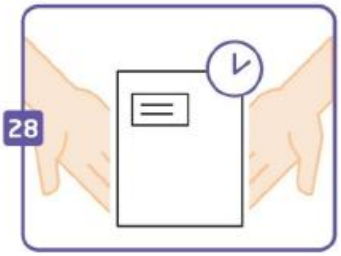
Passo 3: Embalar e enviar as amostras de fezes



Colocar os tubos de colheita 1 e 2 no saco de biossegurança. Feche o zip do saco.

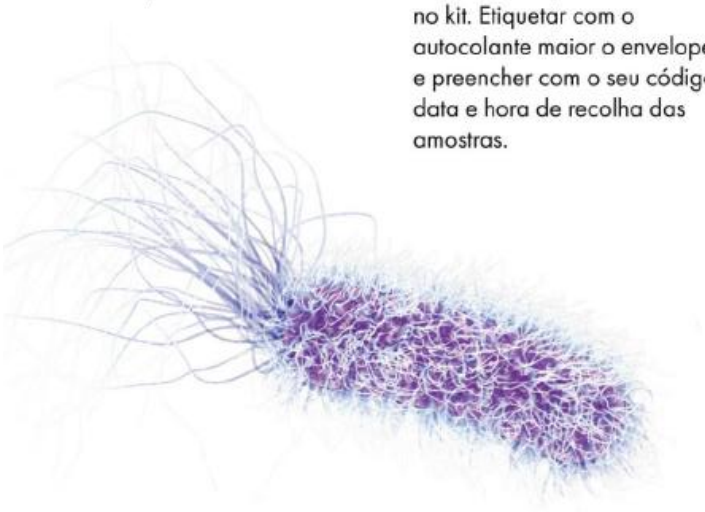


Colocar o saco de biossegurança com os tubos no envelope almofadado que se encontra no kit. Etiquetar com o autocolante maior o envelope e preencher com o seu código, data e hora de recolha das amostras.



Guardar o envelope almofadado com as amostras a 4ºC no frigorífico, por um período máximo de 24 horas.

Nota Importante:
A recolha do envelope com as amostras será feita de acordo com o agendamento prévio.



Saiba mais
QR Code para o Linktree do Programa C+C

Precauções:

Não realizar esta colheita durante a **menstruação** ou se tiver algum **sangramento na zona pélvica** (por exemplo devido a hemorróidas ou outras causas);

Impedir que o líquido dos tubos de colheita seja ingerido ou entre em contacto com os olhos e outras mucosas[1];

Manter os tubos de colheita **fora do alcance das crianças.**

[1]Provoca irritação ocular grave. Se entrar em contacto com os olhos, enxaguar cuidadosamente com água durante vários minutos. Se usar lentes de contacto, retirar e continuar a enxaguar.

Este kit contém:

A

A. EasySampler (Base de recolha)

B

B. Colheres de papel

C

C. Luvas

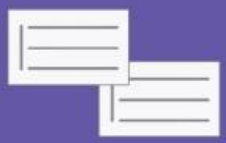
D

D. Tubo de colheita 1 com líquido no interior e tampa castanha; recipiente exterior com tampa branca

E

E. Tubo de colheita 2 sem líquido no interior e sem recipiente exterior

F



F. Autocolantes

G

G. Saco de biossegurança e envelope alcochoado

H

H. Protocolo com instruções do Kit Microbioma Comunidade Portugal

I

I. Folheto sobre o Microbioma

Appendix G – Informative Leaflet about the Importance of the Microbiome

Microbioma Comunidade Portugal | município Oeiras

Projeto de Ciência Cidadã inovador em Portugal para caracterização temporal do Microbioma Intestinal Humano em 30 famílias no Município de Oeiras.

Fomos educados a ter medo dos micróbios (vírus, bactérias e fungos) e falamos muito mais sobre os que nos fazem mal porque são estes que nos trazem preocupações imediatas. Mas a realidade é que a grande maioria dos micróbios com que interagimos são necessários para termos uma vida saudável. É muito importante aprendermos como preservar e beneficiar mais destes micróbios bons.

Temos biliões de microrganismos, na sua maioria bactérias, sendo que a maior parte desses microrganismos encontram-se no trato digestivo, constituindo a microbiota intestinal. Esta imensidão de microrganismos coabita connosco desde que nascemos e, se tudo correr bem, acompanham-nos toda a vida. O microbioma intestinal é o conjunto da microbiota com os genes que a constituem.

Se nos lembrarmos que a espécie humana evoluiu durante milhares e milhares de anos em simbiose com micróbios, então não é assim tão surpreendente que muitas das nossas funções fisiológicas dependam destes seres microscópicos. Evoluímos para contarmos com eles na degradação de muito dos alimentos que ingerimos, na obtenção de compostos essenciais, como vitaminas e alguns aminoácidos, e no treino do sistema imunitário contra agentes microbianos invasores e causadores de doenças.

Todos sabemos que uma alimentação diversificada é importante para a saúde e a nossa microbiota tem um papel essencial ao garantir que tiramos o melhor proveito dos alimentos. Os órgãos humanos não produzem enzimas necessárias para degradar as fibras dos vegetais que ingerimos. São as bactérias nos nossos intestinos que degradam vegetais através de mecanismos especializados e os compostos resultantes são absorvidos pelo nosso organismo e utilizados como fonte de energia. Alguns destes compostos produzidos pela microbiota circulam pelo corpo humano e são detetados pelo chamado eixo cérebro-intestino; estudos recentes mostram que podem influenciar o nosso comportamento.

A nossa microbiota tem um papel essencial na nutrição, na proteção contra agentes infeciosos e até na nossa saúde mental. Alimentos processados, excesso de açucares, baixo teor de fibras e uso excessivo de antibióticos prejudicam a nossa microbiota.

No entanto, o nosso estilo de vida pode prejudicar a microbiota humana. Quando a nossa alimentação inclui demasiados alimentos processados, um excesso de açúcares simples e um baixo teor de fibras, o resultado é uma deficiência nos nutrientes que alimentam a nossa microbiota. Também os antibióticos, apesar de ainda serem a melhor forma de combater infeções bacterianas, têm o efeito secundário de afetar a nossa microbiota intestinal quando usados em excesso, pois estes levam a uma diminuição das bactérias benéficas.

Investigar sobre a composição e alteração do microbioma intestinal saudável ajuda-nos a perceber como se adaptam os microrganismos que colonizam os nossos intestinos e quais os fatores que levam à alteração da dinâmica da microbiota intestinal.

Nota:
Texto de Karina Xavier.

Faça parte de um projeto pioneiro de Ciência Cidadã em Portugal.

Seja cientista cidadão/ã na descoberta do fascinante mundo do microbioma intestinal!

JUNTE-SE A NÓS!

Tão único como tu!

Fase do projeto

Queremos partilhar consigo os progressos que estamos a fazer no estudo do microbioma intestinal e as etapas do processo.

Cada participante entregou dois tubos com amostras fecais. O ADN microbiano das amostras fecais num dos tubos foi preparado e armazenado para sequenciação, que será realizada nos próximos meses. Depois de sequenciadas os resultados serão analisados informaticamente pela equipa científica.

O segundo tubo foi processado e armazenado para estudos de metabolitos produzidos por bactérias e outros futuros estudos envolvendo a cultivo de bactérias, sempre sujeito à aprovação do Comité de Ética.

O que vai receber?

Os participantes vão receber um folheto informativo sobre os resultados obtidos relativos ao microbioma intestinal da comunidade analisada e informações sobre o seu microbioma intestinal e o de suas famílias.

A importância de cada um!

A sua participação tem sido essencial para este projeto e a sua opinião é fundamental! Com base no feedback que recolhermos, ajustaremos os métodos e a comunicação deste projeto para torná-lo mais eficiente e acessível a todos.

Agradecemos o seu compromisso e entusiasmo por fazer parte desta iniciativa inovadora. Juntos, podemos descobrir mais sobre este fascinante universo microscópico que coexiste connosco e que pode ser uma chave para melhorar a saúde de todos.

Obrigado por fazer parte deste projeto pioneiro em Portugal!

(1) Vatanen, T., Franzosa, E.A., Schwager, R. et al. The human gut microbiome in early-onset type 1 diabetes from the TEDDY study. Nature 562, 589–594 (2018).
(2) Lloyd-Price, J., Arze, C., Ananthakrishnan, A.N. et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. Nature 569, 655–662 (2019).
(3) Zhou et al 2024, Cell Host and Microbe, https://doi.org/10.1016/j.chom.2024.02.012

Microbioma Comunidade Portugal | município Oeiras

Relatório

Ciência Cidadã em Estudo Pioneiro da Dinâmica Microbiana Intestinal em Famílias

Saiba mais QR Code para o Linktree do C+C

GIMM | nms nova | Católica | Everything a New | Ciência Cidadã | Oeiras Valley | Município Oeiras

Investigar o Microbioma Intestinal Humano

A investigação do microbioma humano explodiu na última década. Inúmeros estudos revelaram a complexa influência dos micróbios que habitam no nosso corpo na saúde e em várias doenças humanas. Sabemos que o microbioma pode influenciar a saúde intestinal e a saúde do cérebro.

Até à data, a maioria dos estudos sobre microbiomas humanos têm sido transversais, explorando apenas um instante temporal da composição do microbioma. No entanto, os microbiomas humanos podem mudar rapidamente em resposta a diferentes alimentos, medicamentos e ao seu ambiente, e é essa variação ao longo do tempo que nos podem ajudar a compreender a influência do microbioma humano na saúde e na doença.

Informação de artigos

No contexto de uma série de doenças inflamatórias, incluindo doença inflamatória intestinal, asma e artrite, têm sido encontradas diferenças na composição do microbioma intestinal quando comparadas com pessoas saudáveis. (1)

Evidências mais recentes de estudos longitudinais também mostraram alterações no microbioma intestinal que ocorrem mesmo antes do aparecimento de doenças ou dos seus sintomas, incluindo antes do aparecimento de diabetes tipo 1 em crianças e de surtos de doenças inflamatórias intestinais em adultos. (1)

No caso de doenças inflamatórias intestinais, que incluem a doença de Crohn e a colite ulcerosa, que afetam vários milhões de indivíduos em todo o mundo, foi observado que flutuações da composição do microbioma aumentam durante os períodos de atividade da doença. (2)

Num estudo publicado este ano foram seguidos 86 indivíduos durante 6 anos e a dinâmica dos microbiomas do intestino, da pele e das cavidades orais e nasais foi caracterizada. O estudo não só revelou que o microbioma humano varia entre indivíduos e locais do corpo, mas também que em cada local há uma coordenação entre as dinâmicas dos micróbios em resposta à insulina. (3)

1

Microbioma Comunidade Portugal

Projeto Ciência Cidadã num estudo pioneiro sobre a dinâmica microbiana intestinal em 30 famílias, da região Metropolitana de Lisboa, ao longo do tempo

Como é expectável a logística necessária para estudos longitudinais, ou seja, estudos que seguem um individuo ao longo do tempo, é muito mais exigente, do que a logística para estudos de caracterização pontual. Não só a capacidade de manter cada individuo motivado para participar é mais difícil, mas também os orçamentos associados à recolha de amostras, ao trabalho laboratorial de biologia molecular e genética, bem como de análise de dados são muito mais elevados que noutros estudos cujo objetivo não contempla caracterizar flutuações temporais.

Tendo consciência do aumento de poder observado nos poucos estudos longitudinais feitos noutros países, esta equipa de investigação decidiu iniciar um estudo piloto na região da grande Lisboa para obter uma primeira caracterização do perfil da composição do microbioma intestinal, em 30 famílias de, pelo menos, dois indivíduos em 3 instantes de tempo.

O que queremos alcançar com este estudo?

1. Compreender como a composição do microbioma intestinal varia ao longo do tempo
2. Consciencializar a comunidade sobre a importância do microbioma e como ele pode ser uma ferramenta para melhorar a saúde
3. Identificar principais desafios e limitações deste tipo de projeto para, no futuro, podermos estender o estudo a todo o país

2

Appendix I – Personalized Microbiota Report – Provisional Vesion

RELATÓRIO DA COMPOSIÇÃO DO MICROBIOMA

MISSÃO DO PROJETO MICROBIOMA COMUNIDADE PORTUGAL

Caracterização da microbiota intestinal humana em famílias na região de Lisboa, em particular no Município de Oeiras, e analisar a sua variação temporal.

O projeto de Ciência Cidadã Microbioma Comunidade Portugal está a ser desenvolvido, através de uma parceria entre o GIMM, o ITQB NOVA, o CBR, a *Everything is New* e o Município de Oeiras e no âmbito do Programa Cidadã + Cidadã.

EQUIPA CIENTÍFICA

- Isabel Gordo, GIMM
- Karina Xavier, GIMM
- Patrícia Morais, GIMM
- Luís Teixeira, CBR
- Ana Santos Almeida, GIMM
- Sarela Santamarina, ITQB NOVA
- António Gomes da Costa, Fundação Calouste Gulbenkian
- Maria João Leão, Programa Ciência + Cidadã (ITQB NOVA e Município de Oeiras)

CIENTISTAS CIDADÃOS

Muito obrigado por fazer parte de um projeto pioneiro de Ciência Cidadã em Portugal e por ser cientista cidadão/ã na descoberta do fascinante mundo do microbioma intestinal!

MICROBIOMA COMUNIDADE PORTUGAL - CIÊNCIA + CIDADÃ	
6	**INVESTIGADORES** ENVOLVIDOS
32	**FAMÍLIAS ENVOLVIDAS**
119	**CIENTISTAS CIDADÃOS** A PARTICIPAREM ATIVAMENTE
1000	**VISITANTES EM EVENTOS** EXTERNOS

Seleção dos participantes:

- Critérios de inclusão e de exclusão;
- Ordem de inscrição;
- Preferência por residência no Município de Oeiras.

Recolha de amostras

- Período: 6 meses
- Frequência: 3 amostras por participante, de 2 em 2 meses
- Tipo: amostras fecais

Envolvimento das famílias nas fases do processo científico:

- Montagem dos Kits Microbioma Comunidade Portugal;
- Recolha das amostras;
- Envio de sugestões para melhoria das várias fases do projeto;
- Sessões de informações sobre o projeto;
- Visitas aos laboratórios das instituições parceiras;
- Participação como embaixadores em ações de sensibilização nas comunidades locais.

TÃO ÚNICO COMO TU!

A. O QUE É A CIÊNCIA CIDADÃ

"A ciência cidadã é a participação pública em atividades de investigação científica quando os cidadãos contribuem ativamente para a ciência, seja com o seu esforço intelectual ou conhecimento de contexto, seja com os seus instrumentos e recursos" (Comissão Europeia, 2014).

O Programa Ciência + Cidadã (Programa C+C) está enquadrado na estratégia "Ciência Aberta a Oeiras" do Município de Oeiras, é coordenado no ITQB NOVA em estreita colaboração com várias instituições científicas, e pretende implementar uma abordagem de ciência cidadã com os investigadores e comunidades locais de forma, transdisciplinar e intergeracional. Atualmente, estão já a decorrer ativamente 9 projetos de ciência cidadã nas áreas da agricultura sustentável, microbiologia, saúde, ambiente e alterações climáticas. Até à data, o Programa C+C envolveu mais de 25 investigadores, 400 cientistas cidadãos e mais de 2.500 participantes em atividades públicas. Os resultados obtidos já se traduziram em publicações científicas internacionais com revisão por pares[1,2], bem como em financiamento e prémios da União Europeia[3], demonstrando o potencial transformador da ciência partilhada.

Missão Do Programa C+C

I. Ciência Cidadã, implementar projetos de investigação científica com envolvimento e participação ativa dos cidadãos;

II. Cocriação, pensar a ciência com as pessoas e aliar a educação, a arte e o desporto;

III. Consulta pública, tomada de decisões para a ciência.

B. SOBRE O MICROBIOMA INTESTINAL HUMANO - INTRODUÇÃO

Fomos educados a ter medo dos micróbios (vírus, bactérias, parasitas e fungos) e falamos muito mais sobre os que nos fazem mal porque são estes que nos trazem preocupações imediatas. Mas a realidade é que a grande maioria dos micróbios com que interagimos são necessários para termos uma vida saudável. É muito importante aprendermos como preservar e beneficiar mais destes micróbios bons.

Temos biliões de microrganismos no nosso corpo, na sua maioria bactérias, sendo que a maior parte desses microrganismos encontram-se no trato digestivo, constituindo a microbiota intestinal. Esta imensidão de microrganismos coabita connosco desde que nascemos e, se tudo correr bem, acompanha-nos toda a vida. O microbioma intestinal é o conjunto da microbiota com os genes que a constituem.

Se nos lembrarmos de que a espécie humana evoluiu durante milhares e milhares de anos em simbiose com micróbios, então não é assim tão surpreendente que muitas das nossas funções fisiológicas dependam destes seres microscópicos. Evoluímos para contarmos com eles na degradação de muito dos alimentos que ingerimos, na obtenção de compostos essenciais, como vitaminas e alguns aminoácidos, e no treino do sistema imunitário contra agentes microbianos invasores e causadores de doenças.

Todos sabemos que uma alimentação diversificada é importante para a saúde e a nossa microbiota tem um papel essencial ao garantir que tiramos o melhor proveito dos alimentos. Os órgãos humanos não produzem as enzimas necessárias para degradar as fibras dos vegetais que ingerimos. São as bactérias nos nossos intestinos que degradam vegetais através de mecanismos especializados e os compostos resultantes são absorvidos pelo nosso organismo e utilizados como fonte de energia. Alguns destes compostos produzidos pela microbiota circulam pelo corpo humano e são detetados

pelo chamado eixo cérebro-intestino; estudos recentes mostram que podem influenciar o nosso comportamento.

A nossa microbiota intestinal tem um papel essencial na nutrição, na proteção contra agentes infeciosos e até na nossa saúde mental. No entanto, o nosso estilo de vida pode prejudicar a microbiota humana. Quando a nossa alimentação inclui alimentos ultraprocessados, excesso de açúcares simples e um baixo teor de fibras, o resultado é uma deficiência nos nutrientes que alimentam a nossa microbiota. Também os antibióticos, apesar de ainda serem a melhor forma de combater infeções bacterianas, têm o efeito secundário de afetar a nossa microbiota intestinal quando usados em excesso, pois estes levam à destruição da microbiota intestinal e como consequência à diminuição de bactéria.

Investigar sobre a composição e alteração do microbioma intestinal saudável ajuda-nos a perceber como se adaptam os microrganismos que colonizam os nossos intestinos e quais os fatores que levam à alteração da dinâmica da microbiota intestinal.

Nota: Para fins de simplificação, o termo microbioma foi usado como referência tanto à comunidade microbiana (microbiota) quanto ao seu conteúdo genético.

CLASSIFICAÇÃO DAS BACTÉRIAS

As bactérias são classificadas em níveis hierárquicos como reino, filo, classe, ordem, família, gênero e espécie. Na figura seguinte pode ver essa organização.

Figura 1. Diagrama ilustrativo dos níveis hierárquicos da classificação bacteriana, desde o reino até a espécie.

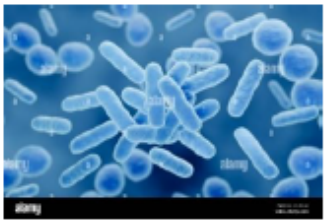
A identificação e caracterização das bactérias presentes no microbioma intestinal dos participantes foram realizadas ao nível do filo.

"CARTÃO DE IDENTIFICAÇÃO" DOS FILOS DE BACTÉRIAS IDENTIFICADOS NESTE ESTUDO

Os filos bacterianos apresentados em baixo atuam em conjunto para preservar o equilíbrio do nosso intestino e, consequentemente, da nossa saúde. A diversidade e o equilíbrio entre os filos do microbioma são indicativos de um sistema saudável, e fatores

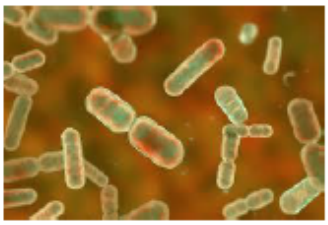
como alimentação, estilo de vida e ambiente exercem uma grande influência na sua composição.

BACILLOTA



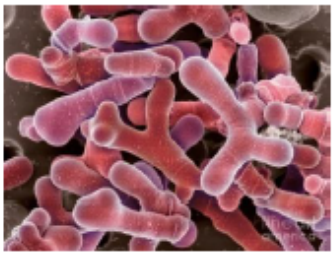
O filo **Bacillota** (anteriormente designado *Firmicutes*) inclui bactérias responsáveis pela fermentação das fibras da nossa alimentação, que não podem ser digeridas pelas células humanas. Durante este processo, produzem metabolitos como o butirato, que serve como fonte principal de energia para as células do intestino e possui efeito anti-inflamatório, contribuindo para a saúde do sistema digestivo. As bactérias deste filo são também importantes na digestão e absorção de lípidos (gorduras). As espécies *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, *Clostridium butyricum* e *Lactobacillus rhamnosus* pertencem a este importante filo.[4]

BACTEROIDOTA



O filo **Bacteroidota** (anteriormente designado *Bacteroidetes*) é constituído por bactérias que ajudam na digestão dos vegetais da nossa alimentação, contribuindo para a obtenção de energia e para a produção de vitaminas. Desempenham também um papel importante no sistema imunitário, ao participar na produção de proteínas chamadas citocinas, que promovem uma resposta adequada contra agentes infeciosos e ajudam a manter o equilíbrio do ambiente intestinal.[5]

ACTINOMYCETOTA

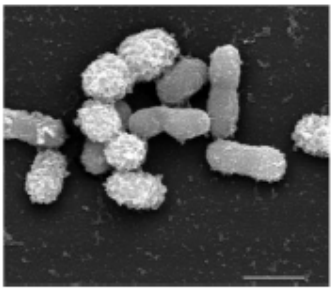


O filo **Actinomycetota** (anteriormente designado *Actinobacteria*) é composto por bactérias com uma forma filamentosa, semelhante a fios ou raízes. Estas bactérias produzem diversas vitaminas e acetato, substâncias essenciais para o metabolismo, o funcionamento do sistema imunitário e a produção de energia nas nossas células. Um dos géneros mais conhecidos deste filo é o *Bifidobacterium*, frequentemente utilizado em probióticos e associado à manutenção de um intestino saudável.[6]

PSEUDOMONADOTA

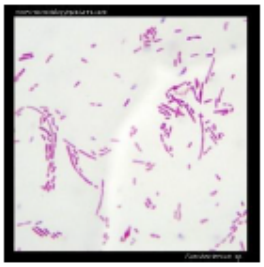
O filo **Pseudomonadota** (anteriormente designado *Proteobacteria*) faz parte do nosso microbioma desde o nascimento. Embora geralmente presente em baixa abundância, as bactérias deste grupo desempenham funções importantes no intestino, incluindo a produção de fatores antimicrobianos, e algumas espécies são utilizadas como probióticos.[7] Entre elas encontra-se a *Escherichia coli*, que habitualmente vive no intestino de forma inofensiva ou até benéfica. Contudo, quando se desloca para fora do intestino, como para o sistema urinário, ou é ingerida através de água ou alimentos contaminados, pode causar infeções urinárias ou gastrointestinais.[8]

VERRUCOMICROBIOTA



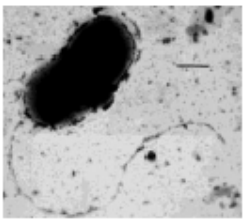
O filo **Verrucomicrobiota** (anteriormente designado *Verrucomicrobia*) tem como principal representante a espécie *Akkermansia muciniphila*, uma bactéria que se alimenta do muco intestinal de forma controlada e benéfica. Esta atividade contribui para a manutenção da saúde do intestino e para a diminuição da inflamação.[9]

FUSOBACTERIOTA



O filo **Fusobacteriota** (anteriormente designado *Fusobacteria*) é constituído por bactérias associadas à mucosa intestinal e à fermentação dos hidratos de carbono presentes na nossa alimentação com a produção de metabolitos benéficiais para a saúde dos nossos intestinos. A sua quantidade nas fezes é influenciada pela dieta, principalmente pela redução no consumo de açúcares refinados.[8]

THERMODESULFOBACTERIOTA



O filo **Thermodesulfobacteriota** (anteriormente incluído no filo *Proteobacteria*) é composto por bactérias capazes de utilizar o sulfato proveniente da nossa alimentação, o qual serve como fonte de alimento para outras bactérias e contribui para a manutenção do equilíbrio e da diversidade do microbioma intestinal. A acumulação de sulfato, caso não seja degradado, pode alterar o ambiente intestinal e favorecer o crescimento de microrganismos potencialmente prejudiciais.[9]

CAMPYLOBACTEROTA

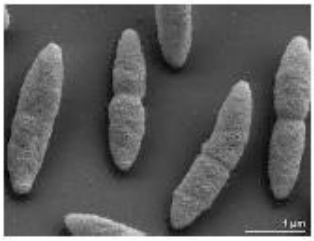
O filo **Campylobacterota** (anteriormente incluído no filo *Proteobacteria*) pode estar presente em pequenas quantidades no microbioma humano. Algumas das suas espécies bacterianas são conhecidas por causar infeções quando atingem determinadas concentrações, como a *Helicobacter pylori*, mas na maioria das vezes surgem em níveis muito baixos sem provocar problemas de saúde. Investigações recentes procuram compreender melhor o seu papel no organismo, dado que também são encontradas no intestino de indivíduos saudáveis.[10]

CYANOBACTERIOTA

Nota: A ilustração não representa as bactérias presentes no nosso intestino, mas sim as do ambiente, já que as bactérias intestinais não são cultiváveis.

O filo **Cyanobacteriota** (anteriormente designado *Cyanobacteria*) é composto por bactérias geralmente capazes de realizar a fotossíntese e de produzir oxigénio, sendo por isso consideradas prováveis ancestrais das algas e das plantas. Contudo, no intestino humano foi identificado o género *Melainabacteria*, pertencente a este filo, que, apesar de não conseguir realizar a fotossíntese como as espécies presentes no ambiente, desempenha um papel importante na fermentação dos vegetais consumidos na nossa alimentação.[11]

SYNERGISTOTA



O filo **Synergistota** (anteriormente designado *Synergistetes*) é constituído por bactérias que habitam ambientes desprovidos de oxigénio, como o intestino humano. Estas bactérias têm a capacidade de degradar aminoácidos e compostos sulfurados provenientes da nossa alimentação, contribuindo para a fermentação de nutrientes que as nossas células não conseguem digerir, auxiliando assim na manutenção do equilíbrio intestinal.[12]

C. METODOLOGIAS E TECNOLOGIAS UTILIZADAS – RECOLHA DE AMOSTRAS E SEQUENCIAÇÃO DAS BACTÉRIAS PRESENTES NO MICROBIOMA INTESTINAL DOS PARTICIPANTES

Materiais dos Kits de Recolha

- Kits de auto-recolha de fezes
- Folheto com instruções sobre a recolha de amostras
- Folheto com informação sobre o microbioma intestinal humano
- Consentimentos informados
- Pacote informativo ao participante
- Questionários sobre estilos de vida

Participação das Famílias

- Montagem dos kits Microbioma Comunidade Portugal e entrega aos participantes
- Recolha de amostras fecais nas suas casas
- Entrega das amostras aos investigadores
- Resposta aos questionários
- Participação em ações de sensibilização nas comunidades locais, contribuindo para a literacia científica e confiança na ciência

Comunidade Estudada

Nº de famílias	Nº de cidadãos	Faixa etária	Local de residência
32	119	6 a 95 anos	Maioritariamente no Município de Oeiras (66.39%)

Recolhas de Amostras

Recolha	Período	Nº de amostras recolhidas	Observações
T1 (Tempo 1)	Agosto – Outubro 2024	119	7 amostras descartadas por concentração insuficiente

Recolha	Período	Nº de amostras recolhidas	Observações
T2 (Tempo 2)	Outubro – Dezembro 2024	115	97 amostras enviadas a 15 de janeiro; 17 descartadas por baixa concentração; 18 recolhidas fora do prazo, mas armazenadas
T3 (Tempo 3)	Dezembro – Fevereiro 2024	112	Sequenciação adiada; amostras congeladas e armazenadas

Extração e Qualidade do ADN

Objetivo	Analisar o ADN bacteriano presente nas amostras fecais
Avaliação	Quantidade e qualidade do ADN verificadas para garantir sequenciação
Limitações	3 participantes sem dados de microbioma devido a ADN insuficiente
Nota	Falhas na extração são comuns neste tipo de estudo

Sequenciação do ADN Bacteriano

Método	Sequenciação do gene 16S rRNA, funcionando como "impressão digital" bacteriana
Processo	Leitura da sequência genética (A, T, C, G) e comparação com bases de dados
Métrica utilizada	Mediana em vez da média, por ser menos sensível a variações extremas (1)
Amostras incluídas no estudo	216 das 347 recolhidas (T1 + T2 até 21 de novembro de 2024) (2)

(1) A mediana é o valor central de um conjunto de dados ordenado e, no estudo do microbioma intestinal, representa melhor a composição dos filos do que a média, por ser menos influenciada por valores extremos.

(2) Para os resultados apresentados neste estudo, foram incluídas apenas as amostras das primeiras e segundas recolhas (T1 e T2) entregues até 21 de novembro de 2024, totalizando 216 das 347 amostras recolhidas. As amostras da segunda recolha entregues após esse prazo, bem como as da terceira recolha (T3), não foram analisadas neste estudo e poderão ser utilizadas no futuro.

D. CARACTERIZAÇÃO DO MICROBIOMA INTESTINAL DOS PARTICIPANTES – RESULTADOS OBTIDOS

Os resultados aqui apresentados têm carácter exploratório e destinam-se exclusivamente à investigação científica, não sendo aplicáveis para fins clínicos ou de diagnóstico.

Relativamente à composição geral do microbioma intestinal na comunidade que participou no estudo, serão mostrados os resultados da mediana dos filos bacterianos.

D.1. CARACTERIZAÇÃO DA COMUNIDADE DE PARTICIPANTES ATRAVÉS DOS QUESTIONÁRIOS

PERFIL DOS PARTICIPANTES: DADOS DEMOGRÁFICOS E FÍSICOS

(valores medianos com intervalos, peso em kg, altura em m e IMC em kg/m²)

- **Idade (mediana):**
  Crianças/Adolescentes: **12 anos**
  Adultos: **46 anos**
  Seniores: **77 anos**
- **Peso (mediana):**
  Crianças/Adolescentes: **42 kg**
  Adultos: **73 kg**
  Seniores: **67 kg**
- **Altura (mediana):**
  Crianças/Adolescentes: **1,48 m**
  Adultos: **1,72 m**
  Seniores: **1,67 m**
- **Índice de Massa Corporal (IMC, mediana):**
  Crianças/Adolescentes: **18,4**
  Adultos: **24,7**
  Seniores: **24,5**

ESTILO DE VIDA E SAÚDE DOS PARTICIPANTES

(número de participantes e percentagens por categoria)

- **Sexo:** 48% homens | 52% mulheres
- **Residência:** Maioria em Oeiras (66%)
- **Nascimento:** 90% nasceram em Portugal
- **Grupo sanguíneo mais comum:** A (40%) e O (34%)
- **Alimentação:**
  Dieta normal (88%)
  Vegetariana (8%)
  Outras dietas específicas (4%)
- **Consumo de álcool:**
  Nunca (29%)
  Às vezes (45%)
  Frequentemente (10%)
- **Tabaco:**
  Nunca fumaram (67%)
  Ex-fumadores (26%)
  Fumadores atuais (7%)

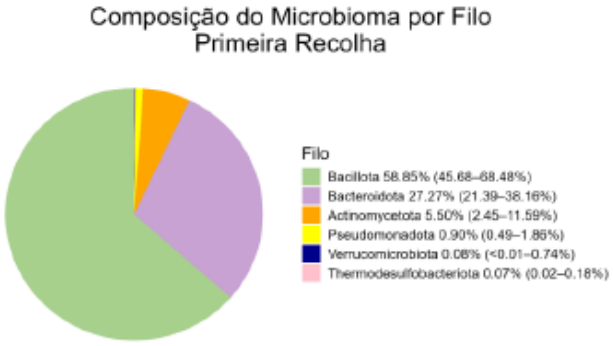
- **Consumo diário:**
  Frutas/vegetais: maioria (67%) consome 2–3 porções/dia
  Lacticínios: maioria (56%) consome 2–3 porções/dia
- **Antibióticos e probióticos:**
  Uso recente de antibióticos: 3%
  Consumo de probióticos: 6%
- **Saúde geral:**
  58% têm alguma doença
  60% tomam medicação regularmente
- **Nascimento:**
  Parto vaginal (75%)
  Parto por cesariana (25%)
  Local: 92% nasceram em hospital
- **Aleitamento materno:** 87% foram amamentados
- **Saúde intestinal:**
  Fezes mais comuns: Tipo 3 (38%) e Tipo 4 (36%) – considerados normais
  Frequência: 50% evacuam 1 vez/dia, 23% mais de 1 vez/dia, 25% menos de 1 vez/dia
  Cor mais comum: castanho-claro (66%)
- **Menstruação (apenas mulheres):**
  Ciclos irregulares: 61%
  Ciclos regulares: 39%

D.2. CARACTERIZAÇÃO DO MICROBIOMA INTESTINAL DA COMUNIDADE TOTAL DE PARTICIPANTES

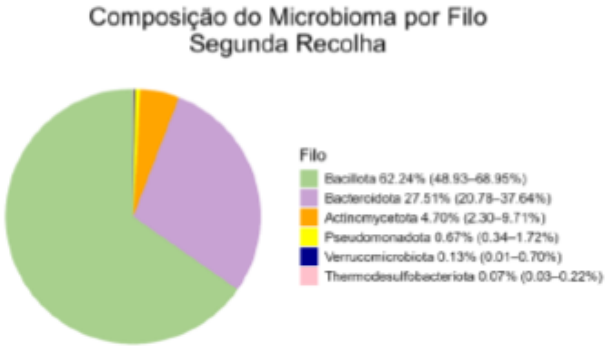
Resultados Observados

As **Figuras 1A e 1B** apresentam a análise global da diversidade microbiana da comunidade estudada, evidenciando as percentagens dos filos bacterianos observados na primeira e na segunda recolha. A amostragem incluiu 112 participantes na primeira recolha e 80 na segunda, pertencentes a 32 famílias.

A)



B)



FIGURAS 1A e 1B. Composição do microbioma intestinal da comunidade estudada ao nível do filo, representada pela mediana dos filos bacterianos da primeira recolha (A) e segunda recolha (B). A legenda identifica os filos bacterianos e a sua composição com respetiva cor.

Através das **Figuras 1 A e 1B** pode observar-se que:

1. O microbioma intestinal da comunidade estudada é constituído na sua maioria pelos **filos Bacillota e Bacteroidota,** seguidos em muito menores percentagens pelos filos **Actinomycetota, Pseudomonadota, Verrucomicrobiota e Thermodesulfobacteriota;**
2. A composição de filos manteve-se relativamente estável entre as duas recolhas temporais realizadas (1ª e 2ª recolhas). Os principais filos bacterianos estão presentes em percentagens semelhantes, com pouca variação ao longo do tempo analisado.

A Figura 2 mostra o gráfico com a composição de Filos Bacterianos do microbioma intestinal nas amostras da primeira recolha de todos os participantes, representados de forma individual e organizados por famílias. Esta recolha inclui os dados de 112 pessoas pertencentes a 32 famílias, permitindo uma visão mais abrangente da diversidade de filos bacterianos presentes na comunidade participante. Optou-se por representar apenas a 1ª recolha, pois é a única em que todas as famílias estão representadas e devido a não se observarem alterações significativas entre a 1ª e a 2ª recolhas como já mencionado nos resultados da Figura 1.

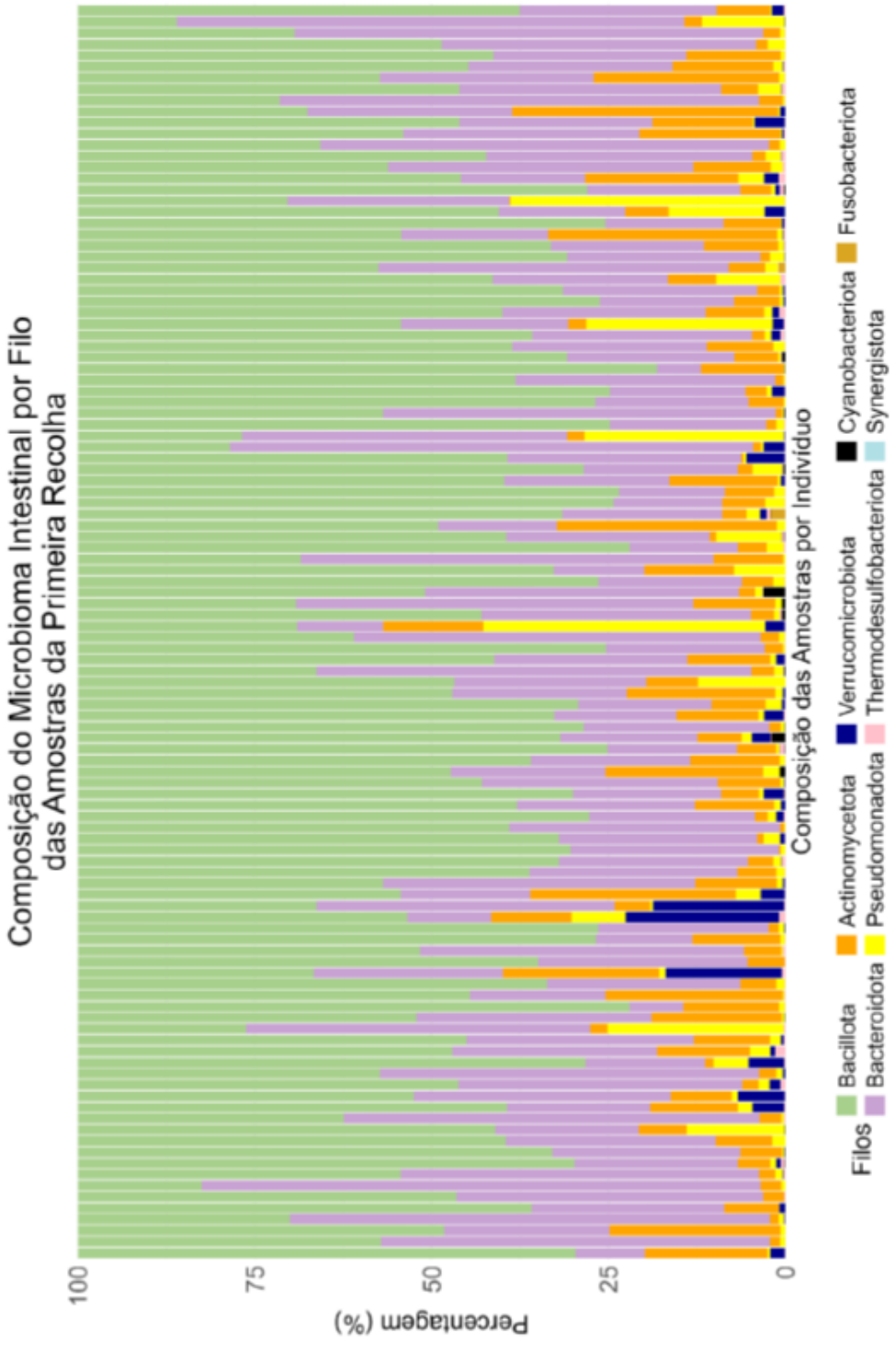


Figura 2. Composição do microbioma intestinal da comunidade, representada pela percentagem relativa dos filos bacterianos da primeira recolha. Cada barra corresponde a um participante, estando agrupados por família. A legenda identifica os filos bacterianos presentes por cor.

A **Figura 2** mostra que:

1. Como seria de esperar pela análise global da Comunidade na 1ª recolha (Figura 1 A) os filos Bacillota e Bacteroidota são dominantes na maioria das amostras;
2. Observam-se também a presença dos filos bacterianos menos frequentes já identificados nas figuras anteriores: **Actinomycetota**, **Pseudomonadota**, **Verrucomicrobiota** e **Thermodesulfobacteriota** e ainda os Filos **Cyanobacteriota**, **Synergistota** e **Fusobacteria**, o que contribui para a diversidade microbiana individual.

Interpretação dos resultados das Figuras 1 e 2

1. A composição do microbioma intestinal da comunidade total que participou neste estudo está de acordo com diversos estudos realizados a nível mundial, refletindo um padrão comum do microbioma intestinal humano, com os filos Bacillota e Bacteroidota como dominantes. A grande abundância destes filos é importante porque desempenham papéis essenciais para a nossa saúde;
2. A composição do microbioma intestinal da comunidade total neste estudo em Portugal (maioritariamente residente no Município de Oeiras) mostra uma maior semelhança com estudos realizadas em populações espanholas[13] em relação a estudos realizados com comunidades chinesas e africanas.[14, 15] As variações na composição do microbioma intestinal encontradas entre populações são comuns devido às diferenças genéticas, ao ambiente e à cultura de cada país;
3. A semelhança na composição do microbioma intestinal ao nível do filo entre a 1ª e a 2ª recolhas está de acordo com estudos que mostram que o microbioma intestinal é caracterizado pela sua estabilidade e resiliência, ou seja, a capacidade de voltar a um estado de equilíbrio e se restabelecer após pequenas alterações devidas a diferenças na alimentação ou no ambiente.[16]

D.3. CARACTERIZAÇÃO DO MICROBIOMA INTESTINAL DOS MEMBROS DA SUA FAMÍLIA

Resultados Observados

A **Figura 3** apresenta a composição do microbioma intestinal de cada membro da família que participou no estudo, em ambas as recolhas. Nem todos os membros da sua família têm a caracterização das duas primeiras recolhas do microbioma intestinal, uma vez que a quantidade de ADN não foi suficiente para a sequenciação ou devido à entrega ter sido posterior à data anteriormente referida. Cada barra corresponde a um participante, identificado por um código que inclui as duas primeiras letras da família, quatro números individuais e a abreviatura "_T1" ou "_T2", que indica o momento da recolha (primeira ou segunda). As cores representam os diferentes filos bacterianos, e a percentagem relativa de cada filo encontra-se indicada na **Tabela 1**.

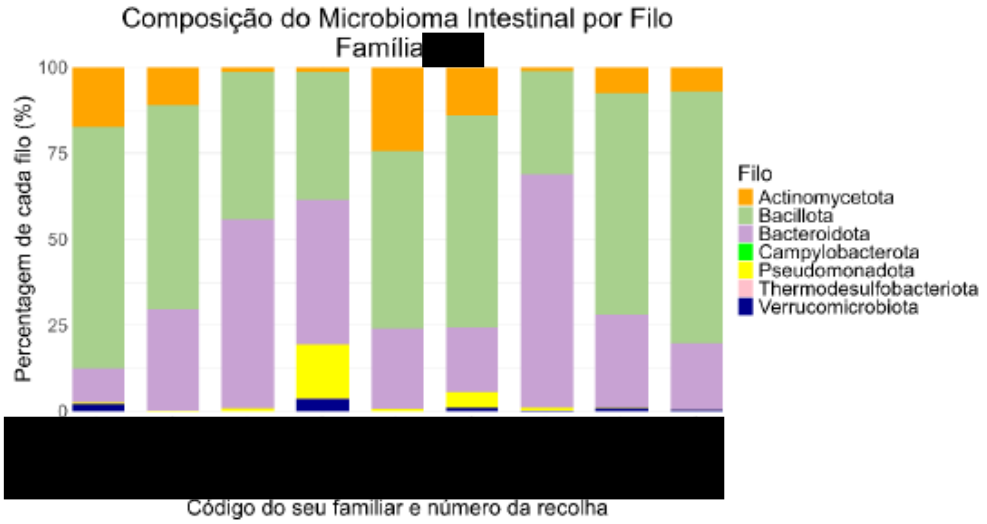


Figura 3. Composição do microbioma intestinal dos membros da sua família, representada pela percentagem relativa dos filos bacterianos. Cada código indica a família e o número da recolha (T1 = primeira recolha; T2 = segunda recolha). A legenda identifica os filos presentes.

Tabela 1. Percentagem de cada um dos filos bacterianos encontrados na microbiota intestinal da sua família. Cada código indica a família e o número da recolha (T1 = primeira recolha; T2 = segunda recolha).

Bacillota	70.31	59.42	42.76	37.13	51.75	61.57	29.96	64.15	73.30
Bacteroidota	9.84	29.44	55.13	41.99	23.36	18.77	67.94	27.27	19.34
Actinomycetota	17.35	10.95	1.40	1.40	24.25	14.12	1.13	7.63	6.98
Pseudomonadota	0.42	0.13	0.69	15.73	0.64	4.51	0.79	0.09	0.13
Verrucomicrobiota	2.01	0.05	0.02	3.60	0.00	1.03	0.13	0.86	0.25
Thermodesulfobacteriota	0.06	0.00	0.00	0.16	0.00	0.00	0.05	0.00	0.00
Campylobacterota	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Nota: A soma das percentagens pode não corresponder a exatamente 100% devido aos arredondamentos das casas decimais.

A Figuras 3 e a Tabela 1 mostram os seguintes pontos:

1. À semelhança da composição da microbiota intestinal na comunidade total de participantes os filos Bacillota e Bacteroidota são dominantes na maioria dos participantes seguindo-se de filos com presenças em percentagens muito mais reduzidas;
2. São observadas variações do microbioma intestinal entre indivíduos, mesmo dentro da sua família. Por exemplo, são encontrados filos bacterianos apenas em certos membros da sua família;
3. A variação da composição do microbioma intestinal entre a primeira e a segunda recolha é geralmente menor dentro da mesma pessoa do que entre pessoas diferentes. Ou seja, a variação intraindividual é inferior à variação interindividual.

Interpretação dos resultados da Figura 3 e Tabela 1

1. Os resultados da caracterização do microbioma intestinal dos membros da sua família confirmam, que à semelhança dos resultados observados na caracterização da comunidade total, os filos Bacillota e Bacteroidota são dominantes seguidos de outros filos bacterianos, com percentagens muito mais reduzidas;
2. Apesar disto, observam-se variações nas percentagens dos respetivos filos entre os membros da sua família o que demonstra o carácter altamente individual do microbioma intestinal, influenciado por fatores como alimentação, estilo de vida, idade e uso prévio de antibióticos;[16]
3. Por outro lado, observa-se também que a variação temporal entre os dois pontos de recolha é inferior à variação temporal entre diferentes membros da família o que evidencia mais uma vez a estabilidade e resiliência do nosso microbioma, que, apesar de pequenas mudanças ao longo do tempo, tende a retornar ao seu estado de equilíbrio. Pequenas flutuações na composição dos filos bacterianos do nosso microbioma intestinal são comuns e esperadas, influenciadas por fatores como a dieta ou o stress, não evidenciam doenças e tendem a voltar a um equilíbrio; [16]
4. O presente estudo não teve poder estatístico para confirmar alterações significativas através os dados recolhidos pelo questionário. A continuação do estudo com a contribuição de mais amostras e com um questionário mais pormenorizado poderá ser um fator essencial para a compreensão mais detalhada da dinâmica do nosso microbioma intestinal tendo em conta fatores como a alimentação e estilos de vida.

E. CONCLUSÕES DO ESTUDO E IMPACTO DO PROJETO MICROBIOMA COMUNIDADE PORTUGAL

Em conclusão, o Projeto Microbioma Comunidade Portugal permitiu realizar a primeira análise longitudinal da microbiota intestinal portuguesa, com uma taxa de desistência de apenas 6%. Os resultados obtidos sugerem que a microbiota intestinal da comunidade portuguesa que participou neste estudo apresenta um perfil microbiano ocidental, mais semelhante ao de populações espanholas do que ao de populações africanas ou chinesas, reforçando a ideia de que esta população segue hábitos alimentares e estilos de vida comuns em países industrializados.
De destacar também que este projeto resultou na Tese de Mestrado de Patrícia Morais, evidenciando o valor do envolvimento dos cidadãos na investigação do nosso microbioma através de projetos de ciência cidadã.

Contributo dos Cientistas Cidadãos	32 famílias colaboraram ativamente com feedback e sugestões, contribuindo de forma decisiva e imprescindível para a tese de mestrado e futuros estudos nesta área. **Sugestões e Comentários:** As sugestões e comentários enviados vão melhorar futuros projetos e aprofundar o conhecimento sobre o microbioma. **Participação em Eventos:** Vários participantes foram embaixadores do projeto, em eventos como: • Dia do Microrganismo • Noite Europeia dos Investigadores (Oeiras) • Dia Aberto do ITQB NOVA **Participação em Conferência:** • Congresso SciComPT 2025
Impacto	• O projeto representa um passo importante na aproximação entre ciência e comunidade, promovendo literacia científica e gerando conhecimento. • Foi realizada uma tese de mestrado no âmbito deste projeto (Patrícia Morais)
Sucesso	A realização em contexto familiar foi essencial, proporcionando apenas 6% de desistências ao longo do projeto.
Próximos Passos	• As amostras recolhidas e que não foram utilizadas no estudo apresentado neste relatório estão a ser utilizadas noutros estudos e também armazenadas para estudos futuros. • Os resultados serão partilhados com os participantes quando publicados em artigos científicos, sempre com agradecimento ao grupo de participantes.

Legenda: Eventos onde o projeto Microbioma Comunidade foi divulgado com a participação de cientistas cidadãos

AGRADECIMENTOS

O Microbioma Comunidade Portugal só foi possível graças à participação ativa de todos os cientistas cidadãos envolvidos, ao trabalho da equipa de investigação e à Patrícia Morais, aluna do Mestrado em Microbiologia Médica da Universidade Nova de Lisboa, que está a desenvolver a sua tese no âmbito do Microbioma Comunidade Portugal.

MUITO OBRIGADA POR SEREM CIENTISTAS CIDADÃOS DO MICROBIOMA COMUNIDADE PORTUGAL!

Partilhe connosco a sua experiência ao longo do projeto!

A sua resposta é totalmente anónima e todas as sugestões são muito bem-vindas.

Bibliografia

1. Henriques B, et al. MicroMundo@Oeiras: citizen science promoting antibiotic stewardship, discovery of new antimicrobials, and monitoring of soil resistance. *FEMS Microbiol Lett*, 372, fnaf061. (2025).
2. Gil-Serna, J., et al. Citizen Science to Raise Antimicrobial Resistance Awareness in the Community: The MicroMundo Project in Spain and Portugal. *Microbial biotechnology*, *18*(3), e70123. (2025).
3. Oeiras Experimenta: Climate-Smart Crops for Sustainable Food Production. (2025). European Union Prize for Citizen Science – Honorary Mention. Prix Ars Electronica. Retrieved September 2, 2025, from Ars Electronica website: https://calls.ars.electronica.art/2025/prix/winners/17245/
4. Sun, Y. et al. Gut firmicutes: Relationship with dietary fiber and role in host homeostasis. Critical reviews in food science and nutrition, 63(33), 12073–12088. (2023).
5. Tufail, M.A. et al. Exploring the Probiotic Potential of Bacteroides spp. Within One Health Paradigm. Probiotics & Antimicro. Prot. 17, 681–704 (2025).
6. Binda, C. et al. Actinobacteria: A relevant minority for the maintenance of gut homeostasis. Digestive and liver disease: official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver, 50(5), 421–428. (2018).
7. Sorbara, M.T., Pamer, E.G. Microbiome-based therapeutics. Nat Rev Microbiol 20, 365–380 (2022).
8. Nalage, D. et al. Bacterial phyla: microbiota of kingdom animalia. Acad. Biol. 2, (2024).
9. Figliuolo, V. R., Coutinho-Silva, R., & Coutinho, C. M. L. M. Contribution of sulfate-reducing bacteria to homeostasis disruption during intestinal inflammation. *Life sciences*, *215*, 145–151. (2018).
10. Cornelius, A. J. et al. Epsilonproteobacteria in humans, New Zealand. Emerging infectious diseases, 18(3), 510–512. (2012).
11. Di Rienzi, S. C. et al. The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new candidate phylum sibling to Cyanobacteria. *eLife*, *2*, e01102. (2013).
12. Rajilić-Stojanović, M. et al. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS microbiology reviews, 38(5), 996–1047. (2014).
13. Latorre-Pérez, A. et al. The Spanish gut microbiome reveals links between microorganisms and Mediterranean diet. Scientific reports, 11(1), 21602. (2021).
14. Qu, Q. et al. Population-level gut microbiome and its associations with environmental factors and metabolic disorders in Southwest China. npj Biofilms Microbiomes 11, 24 (2025).
15. Doumatey, A. et al. Gut Microbiome Profiles Are Associated With Type 2 Diabetes in Urban Africans. Frontiers in cellular and infection microbiology, 10, 63. (2020).
16. Lozupone, C. et al. Diversity, stability and resilience of the human gut microbiota. Nature 489, 220–230 (2012).