Liliane Miguel Dias Pinto Ferrador

The Gut Bacteriome and *Bacteroides* spp. diversity in Healthy Ageing

Faculdade de Ciências e Tecnologia

2022

Liliane Miguel Dias Pinto Ferrador

The Gut Bacteriome and *Bacteroides* spp. diversity in Healthy Ageing

Mestrado em Biologia Molecular e Microbiana

Trabalho efetuado sob a orientação de:

Maria Leonor Faleiro

UAlg
UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

2022

The Gut Bacteriome and *Bacteroides* spp. diversity in Healthy Ageing

Authorship Statement

I hereby declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

Acknowledgements

Thanks are due to everyone who somehow contributed to the conclusion of this work and who in the most difficult moments showed unparalleled support. First, a huge thanks to my thesis supervisor Professor Maria Leonor Faleiro from Universidade do Algarve for all the support and trust placed along this path. Prof. Leonor was the one who was always open to doubts, and questions and above all who was always around when it was most needed, for all the words of encouragement and support in the laboratory work and writing process, thank you very much.

To the people who passed through the laboratory and always ended up leaving a tip, advice, or a word of support, namely, Master José Matos and Master Isabel Matos, thank you for their help in adaptation to the laboratory, for their willingness to teach and help and for all the conversations and advice shared in the hours spent in the lab. I would also like to thank the Laboratory Technician Bruno Garcia who, with his good mood and, despite not being part of our laboratory, was untiring with the help provided whenever necessary.

I acknowledge the support of the project "ALgarve Fit Aging Score (ALFA Score): a new integrated tool to improve quality of life and healthy ageing".

To all those who, sometimes without even realizing what the work was about, were always around to support me on this journey, Family and Friends, a giant thank you, without you, this journey would be thousand times more difficult.

A special reference to two colleagues and above all good friends who more than anyone understood the experience, the journey and the difficulty, Bárbara Vieira, and Inês Leal, thank you for all the support and all the moments shared until today. A thank you to the person who never let me give up or get discouraged, which was the untiring company, Guilherme Gusmão.

To all those who I did not name, but who somehow facilitated my journey and supported me during these months, a huge thank you.

Thank you.

Abstract

Ageing is essentially a biological process that leads to the malfunction of cells and organs, dysregulation of body systems, and generally, age-related illnesses that ultimately result in the individual's death. These age-related medical conditions can have several consequences for the organism affecting several systems in the body. One of the elements between disease and body response is the gut microbiota. Several factors can affect the microbiota composition, including age and health status. An important genus in the gut is *Bacteroides* and its abundance in the elderly have been targeted with different outcomes. Therefore, studying the alterations of the microbiome in the elderly population can constitute a valuable tool to help understand the non-healthy and healthy status of old individuals, and also establish interventions in the gut microbiota to promote health in the elderly people.

In the current study, twelve faecal samples from 7 healthy and 5 non-healthy individuals aged between 60 and 90 years old, including individuals from both genders from the Algarve region were analysed regarding their bacteriome using the Oxford Nanopore system. The isolation of *Bacteroides* species was performed using the culture medium *Bacteroides vulgatus* Selective Agar (BVSA). Isolates from representative characteristic and uncharacteristic colonies were identified by sequencing the *16S rRNA* gene and the Gram-negative isolates from uncharacteristic colonies were first identified using the RapID ONE system (Thermo Fisher Scientific).

The individual bacteriome profile revealed that the phylum *Firmicutes* was prevalent in all samples except for one that showed the dominance of the phylum *Bacteroidetes.* The bacteriome pattern regarding the abundance of the different genera and species evidence a tendency for a unique pattern, but *Oscillibacter valericigenes* was abundant in the majority of the samples. Differences between gender were noticed, namely, the phylum *Proteobacteria* was more abundant in males, and several species were unique for each group. The results of the bacteriome profile according to the health status evidence that *Bacteroides vulgatus* (*Phocaeicola vulgatus*) was more abundant in the non-healthy group, whereas *Prevotella copri* was more abundant in the healthy group. It was possible to recover in culture the different *Bacteroides* species, even those reported at very low numbers in the bacteriome.

Key words: ageing, health status, gut microbiota, *Bacteroides* spp., microbiome, elderly.

Resumo

Uma microbiota intestinal funcional é atualmente reconhecida como um elemento essencial na prevenção de doenças atribuídas ao processo de envelhecimento. O papel da microbiota intestinal em doenças relacionadas com a idade, tais como diabetes tipo 2, demência e Alzheimer, Parkinson, doenças cardiovasculares, obesidade, depressão, entre outras tem sido nos últimos anos reconhecido pela comunidade científica. É importante realçar que nos últimos anos, um número significativo de estudos evidenciou a forte associação entre a composição do microbiota intestinal e os vários parâmetros utilizados na avaliação do desempenho físico e no estado de fragilidade. Em particular, as alterações na abundância do género *Bacteroides* no envelhecimento têm sido reportadas em alguns países. O presente estudo preliminar tem como objetivo a avaliação do bacterioma intestinal de indivíduos idosos com um padrão de envelhecimento saudável e não saudável de diferentes locais da região do Algarve, conjuntamente com a quantificação e identificação de espécies cultiváveis de *Bacteroides* do trato gastrointestinal destes indivíduos. Neste estudo, foram recolhidas 12 amostras fecais incluindo 7 indivíduos saudáveis e 5 não saudáveis com idades compreendidas entre os 60 e os 90 anos, abrangendo indivíduos de ambos os géneros. O perfil bacteriano da microbiota intestinal de cada indivíduo foi analisado com recurso à plataforma de sequenciação de quarta geração Nanopore MinION (MinION Mk1C). Assim sendo, foi realizada a sequenciação do gene *16S rRNA* (região V1-V9, com um comprimento ~1.5 kpb) a partir do DNA total de cada amostra fecal. Previamente à preparação da biblioteca a concentração do DNA foi determinada com recurso ao fluorómetro Qubit 2.0. Na preparação da biblioteca foram utilizados o kit 16S barcode (SQK-RAB204) e a célula de fluxo R9.4.1. Os resultados da sequenciação foram analisados com recurso à plataforma EPI2ME da Oxford Nanopore Technologies. A segunda abordagem (cultura-dependente) foi direcionada para a quantificação e identificação de espécies de *Bacteroides*. Com este objetivo, foi utilizado o meio seletivo *Bacteroides vulgatus* Selective Agar (BVSA) para inoculação das amostras, todas as colónias características foram contabilizadas e sujeitas a purificação em meio Brain Heart Infusion (BHI) suplementado com hemina (0,01% p/v). Colónias não características foram igualmente isoladas. Posteriormente a identificação dos isolados foi realizada através da sequenciação do gene *16S rRNA*. Os resultados da análise do bacterioma intestinal evidenciaram que os filos mais abundantes nos indivíduos idosos

mais saudáveis e menos saudáveis são similares diferindo no quarto e quinto filo mais abundante. Os três filos mais abundantes quer nos indivíduos saudáveis, quer nos indivíduos menos saudáveis foram *Firmicutes, Bacteroidetes, Proteobacteria,* o quarto e quinto filo variaram entre os indivíduos havendo, nomeadamente o filo *Fusobacteria*, *Lentisphaerae*, *Verrucomicrobia*, *Cyanobacteria*, *Actinobacteria* e *Tenericutes*.

O perfil dos idosos com envelhecimento menos saudável, a nível do género, foi caraterizado por níveis elevados de *Bacteroides, Acidaminococcus* e *Oscillibacter*, este grupo de amostras possui ainda dois géneros que não se observaram no grupo saudável, nomeadamente o género *Kineothrix* e *Klebsiella*. Nos idosos com perfil saudável, o género bacteriano mais abundante foi *Prevotella,* seguido de *Oscillibacter*. Os géneros *Lactobacillus* e *Streptococcus* foram apenas observados no grupo de idosos saudáveis.

Nos idosos menos saudáveis as espécies mais abundantes foram *Bacteroides vulgatus, Acidominococcus intestini, Oscillibacter valericigenes, Bacteroides faecis* e *Haemophilus parainfluenzae*, enquanto nos mais saudáveis as espécies mais abundantes foram *Prevotella copri, O. valericigenes, Dialister invisus, Lactobacillus mucosae* e *Agathobacter rectale*.

A recuperação em cultura das espécies de *Bacteroides* a partir das amostras de idosos saudáveis e menos saudáveis permitiu a identificação de trinta e quatro isolados, destes, dezoito pertencem à ordem *Bacteroidales*. Com esta técnica, foi ainda observada a recuperação de um número significativo de enterobactérias com menor suscetibilidade à colistina.

Os resultados obtidos evidenciam um bacterioma intestinal diferenciado de acordo com o estado de saúde dos idosos, embora com uma tendência de perfil individual único.

A análise entre géneros também demonstrou diferenças entre os perfis do bacterioma intestinal feminino e masculino, com valores mais elevados de *Bacteroidetes* e *Proteobacteria* nos indivíduos do género masculino em comparação com as amostras provenientes de indivíduos do género feminino que apresentaram no seu bacterioma as espécies *H. parainfluenzae* and *D. invisus.*

Os resultados do corrente estudo apesar de preliminares evidenciam diferenças na composição do bacterioma entre idosos com um envelhecimento saudável e idosos com um envelhecimento menos saudável, bem como foram observadas diferenças no

bacterioma intestinal entre idosos do género masculino e do género feminino, reforçando a ideia de que vários fatores podem ter influência na composição da microbiota intestinal.

Palavras-chave: envelhecimento saudável, envelhecimento não saudável, microbiota intestinal, bacterioma espécies de *Bacteroides,* microbioma, idosos

Contents

List of Figures viii

List of Tables ix

Glossary x

1. Introduction 1

1.1. The Gut Microbiota 1

1.2. Factors That Potentiate Variations in the Intestinal Microbiota 3

1.2.1. Ageing 4

1.2.2. "Inflammaging" 5

1.2.3. Geographic provenance 6

1.2.4. Diet 7

1.2.5. Medication Intake and Hospitalization Periods 7

1.3. Alterations in the Gut Microbiota: Health and Disease 8

1.3.1. Type 2 Diabetes (T2D) 9

1.3.2. Obesity 10

1.3.3. Cardiovascular pathologies 10

1.3.4. Colorectal Cancer 11

1.3.5. Alzheimer's disease (AD) 12

1.3.6. Parkinson's disease (PD) 13

1.3.7. Sarcopenia and Frailty 14

1.3.8. Depression 16

1.3.9. Inflammatory Bowel Disease (IBD) 17

1.3.10. Chronic Kidney Disease (CKD) 17

1.4. Alterations in the Gut Microbiota: Epigenetics 18

1.5. *Bacteroides* spp. in the Gut of the Elderly 20

2. Objectives 22

3. Materials and Methods 23

3.1. Equipment 23

3.2. Culture Media 24

3.3. Solutions 24

3.4. Faecal Samples 25

3.5. Bacterial Culture Conditions 26
3.6. DNA Extraction from Faecal Samples 27
3.7. DNA Extraction from Bacterial Cultures 27
3.8. DNA Purification 28
3.9. Identification of Bacterial Isolates 29
3.10. Antibiotic Susceptibility 30
3.10.1. Antibiotic Susceptibility of *Escherichia coli* Isolates 30
3.11. Microbiome Analysis 30
3.11.1. Library Preparation 31
3.11.2. Sequencing 31
3.11.3. Data Acquisition and Analysis 31
4. Results 33
4.1. Isolation and Identification of *Bacteroides* spp. 33
4.2. Antibiotic Susceptibility 42
4.3. Microbiome Analysis 42
4.3.1. Individual Profile 42
4.3.2. Patterns within Age, Gender, and Health Status 45
4.3.3. Gender on Gut Microbiota Composition 46
4.3.4. State of Health on Gut Microbiota Composition 50
4.4. Representativeness of the Recovered Bacterial Isolates including *Bacteroides* spp. from the Faecal Samples in the Microbiome 54
5. Discussion 56
6. Conclusion 61
7. Future Perspectives 62
8. References 63
9. Appendix 1 73

List of Figures

1.2.1. Factors that impact the gut microbiota and diseases related with alterations in the gut microbiota composition. 3

3.11.1. Schematic of the bacteriome analysis workflow performed in the current study. 32

4.1.1. BVSA plates with characteristic colonies of Bacteroides spp. from faecal samples. Potential *Bacteroides* colonies are indicated with blue arrows. Plate A) 10^{-4} dilution and B) Section of a plate inoculated with the 10^{-5} dilution C) Isolate MHB PEB2 identified as *B. fragilis* in BHI+H D) Isolate FHB6 identified as *B. caccae* in BHI+H. 33

4.1.2. Diversity of Bacteroides spp. in faecal samples of healthy and non-healthy elderly individuals, according to the 16S rRNA gene sequencing. 41

4.3.1. The five most abundant phyla in the analysed faecal samples of old adults from Algarve region, Portugal. 43

4.3.2. A heat map shows A) the relative genus abundance (%) of classified reads, B) the relative species abundance (%) of classified reads. 44

4.3.3. The five most abundant phyla in male and female faecal samples. 47

4.3.4. Phylogenetic representation of the genera identified in A) male and B) female faecal samples. 48

4.3.5. Phylogenetic representation of the species identified in A) male and B) female faecal samples. 49

4.3.6. The five most abundant phyla in healthy and non-healthy faecal samples. 50

4.3.7. Phylogenetic representation of the genera identified in A) Non-Healthy and B) Healthy faecal samples. 52

4.3.8. Phylogenetic representation of the bacterial species identified in A) Non-Healthy and B) Healthy faecal samples. 53

A1. Phylogenetic representation of the genera (A) and species (B) found in all samples. 78

List of Tables

3.4.1. Participant's general information. 26

4.1.1. Determination of *Bacteroides* spp. counts in faecal samples of healthy and non-healthy elderly individuals. 35

4.1.2. Results of the identification of the bacterial isolates by sequencing the *16S rRNA* gene. 37

4.1.3. Identification of isolates using the RapID ONE. 40

4.4.1. Cultured bacterial isolates from each faecal sample and their observed percentages in the microbiome. 54

A1. Similarity scores obtained with NCBI and RDP databases for the identification of each isolate. 73

A2. Inhibition zone diameters for each tested bacterial isolate by Kirby-Bauer test. 77

Glossary

AD – Alzheimer's Disease

BHI – Brain Hearth Infusion

BHI+H – Brain Hearth Infusion with Hemin

BVSA – *Bacteroides vulgatus* Selective Agar

CKD – Chronic Kidney Disease

CRD – Crohn's Disease

CVD – Cardiovascular Diseases

DNA – Deoxyribonucleic Acid

DOPAC – 3,4-dihydroxyphenylacetic acid

HDAC – Histone deacetylase

IBD – Inflammatory Bowel Disease

lncRNA – long noncoding RNA

miRNA – micro RNA

ncRNA – noncoding RNA

OMVs – Outer Membrane Vesicles

PBS – Phosphate-Buffered Salin

PCR – Polymerase Chain Reaction

PD – Parkinson's Disease

PULs – Polysaccharide Utilization Loci

RNA – Ribonucleic Acid

SAM – S-adenosylmethionine

SCFA – Short-Chain Fatty Acid

TMAO – Trimethylamine-N-oxide

TNF-α – Tumor Necrosis Factor-α

T2D – Type 2 Diabetes

UC – Ulcerative Colitis

1. Introduction

1.1. The Gut Microbiota

The intestinal microbiota is a complex microbial community that inhabits our gastrointestinal (GI) tract, being composed of bacteria, archaea, fungi, and viruses (Haran, J. P. & McCormick, B. A., 2021), it holds the largest bacterial community in the human body. Although the entire GI tract harbours a microbiota, the different conditions between the small and large intestines lead to different bacterial communities (Martinez-Guryn, K., Leone, V., & Chang, E. B., 2019). The colonic environment supports a large and diverse bacterial population while in the small intestine high levels of oxygen, acids and antimicrobials, and a short transit time contribute to the survival of only the resistant bacteria to such conditions, which usually results in an environment constituted by facultative rapidly growing anaerobes with the ability to adhere to the intestinal epithelia (Thursby, E. & Juge, N., 2017). In the large intestine is observed an abundance of nutrients and complex carbohydrates, and a robust mucus layer, these are responsible for the establishment of several groups of bacteria, creating a massive bacterial community that brings many benefits to the host, generating a mutually favourable relationship between the host and the gut microbiota (Finlay, B. B. *et al.*, 2019). The bacterial community helps with the modulation of the gut epithelia, protects against pathogens, contributes to the development of the immune system, and has a crucial role in digestion, producing metabolites that affect other systems in the organism, while the host provides a favourable environment to the bacterial growth (Rowland, I. *et al.*, 2018; Thursby, E. & Juge, N., 2017; Yoo, J. Y. *et al.*, 2020).

The composition of the gut microbiota has been studied over the years, revealing that the most predominant phyla in the intestinal microbiota are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* (Rinninella, E. *et al.*, 2019).

One of the main advantages of colonic bacteria is their ability to ferment complex carbohydrates, obtained from food intake or the intestinal mucus, generating metabolites, such as short-chain fatty acids (SCFAs). Propionate, butyrate, and acetate are three of the main SCFAs produced in the gut, being absorbed by the epithelial cells in the GI tract. In the human gut, acetate is produced by most gut anaerobes, whereas propionate and butyrate are mainly produced by *Bacteroidetes* and *Firmicutes*, respectively. These

SCFAs have an important role in the human body, propionate is absorbed in the liver and can activate gluconeogenesis, act on β-cell function and attenuate reward-based eating behaviour, acetate is released into peripheral tissues and is lipogenic and butyrate has anti-inflammatory and anticancer activities, is an energy source for colonocytes, attenuates bacterial translocation and enhance gut barrier function. Both propionate and butyrate are also histone deacetylase inhibitors, having a role in human epigenetics. SCFAs appear to regulate hepatic lipid and glucose homeostasis, playing a role in regulation of the immune system and inflammatory response and in the modulation of appetite regulations and energy intake (Al Bander, Z. *et al.*, 2020; Czajkowska, A. *et al.*, 2020; Rowland, I. et al., 2018; Thursby, E. & Juge, N., 2017; Yoo, J. Y. et al., 2020). These SCFAs represent just a fraction of the metabolites of bacterial origin produced in the gut that help modulate and regulate the entire human body (Rowland, I. *et al.*, 2018).

Another important aspect resulting from gut colonization is the barrier that is created against pathogenic bacteria. Bacteria in the gut, by competing for attachment sites or nutrient sources and producing antimicrobial products, control the colonization of the GI tract by pathogenic bacteria (Li, Z. *et al.*, 2018; Thursby, E. & Juge, N., 2017).

Over the years, the role of the intestinal microbiota in the human body has been gaining prominence, and characteristics like composition, metabolite production and interactions within and without the gut became major areas for research. Consequently, differences in the intestinal microbiota between individuals and within the individual became related to factors, such as type of delivery, the ageing process, genetic inheritance, individual's lifestyle, diet, medication intake, hospitalization periods and geographic origin (Gupta, V. K., Paul, S., & Dutta, C., 2017; Leite, G. *et al.*, 2021; Nagpal, R. *et al.*, 2018; Rinninella, E. et al., 2019; Thomson, C., Garcia, A. L., & Edwards, C. A., 2021). Therefore, it is established that the gut microbiota develops from birth and undergoes constant fluctuations during the individual life time, resulting in a bacterial community that is unique to each person (Dominguez-Bello, M. G. *et al.*, 2019; Xu, C., Zhu, H., & Qiu, P., 2019).

When intense these bacterial composition fluctuations are considered dysbiosis, which means a major alteration in the composition of the gut microbiota that usually leads to a modification of the core bacterial groups present in our intestine at a certain moment in life. This dysbiosis state is responsible for an increase in susceptibility to disease and a

higher propensity to external infection but can also create a propitious environment for opportunistic gut pathogenic bacteria to thrive (Buford, T. W., 2017).

1.2. Factors That Potentiate Variations in the Intestinal Microbiota

The gut microbiota develops from birth (and probably in the womb) and changes during an individual lifetime (Dominguez-Bello, M. G. et al., 2019). Several factors can exert a striking effect on the intestinal microbiota, namely the ageing process itself, the state of chronic inflammation (Inflammaging), the geographic location, diet, and medication and hospitalization. Following, each one of these factors will be addressed in the next sections.

Figure 1.2.1. illustrates the factors that impact the gut microbiota and the diseases that are associated with alterations in the composition of the microbiota.

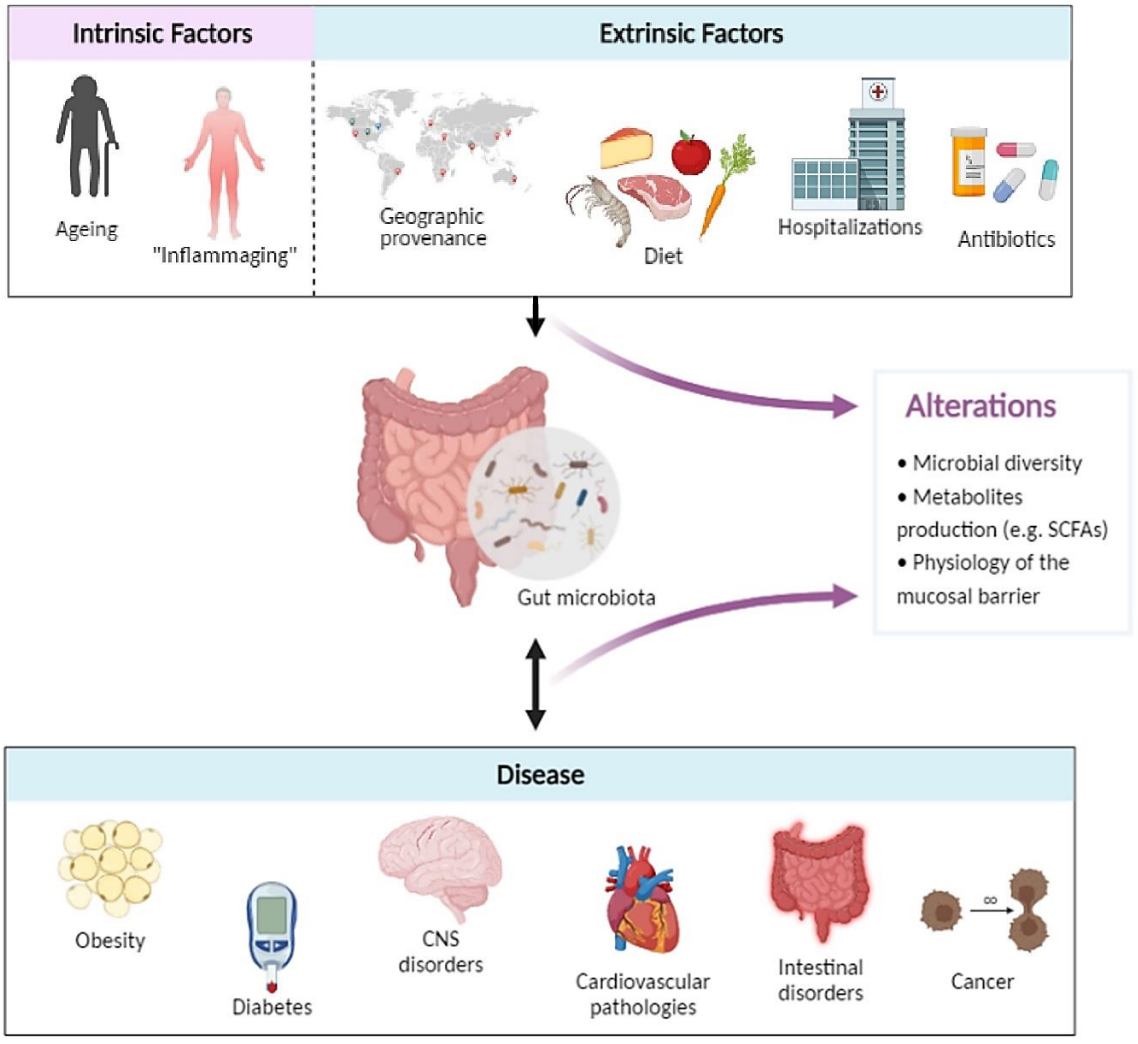


Figure 1.2.1. Factors that impact the gut microbiota and diseases related with alterations in the gut microbiota composition. Image created using BioRender (biorender.com).

1.2.1. Ageing

Ageing is the biological process that leads to psychological and physiological changes, malfunction of cells and organs, dysregulation of body systems and, generally, age-related diseases that end up in the individual's death (Finlay, B. B. *et al*., 2019; Rinninella, E. *et al*., 2019). Among some of the global physiological changes is loss of muscle mass, increase in body fat and cardiac and respiratory dysfunctions (Stachura, A. *et al.*, 2020). These physiological changes are unavoidable, and the ageing process is categorized based on their degree. Successful ageing is characterized by minimal physiological, psychological, and social deficits attributed to age. Typical ageing brings some deficits to the individual without coexisting diseases, in contrast, pathological ageing is defined by a disease pattern (Stachura, A. *et al*., 2020).

One of the main systems that undergo changes in both physiology and functionality during an individual's lifetime is the gastrointestinal tract. The ageing process is responsible for a reduction in basic metabolism and enzymatic activity, for decreasing the acidity of the gastric fluid and reducing intestinal peristalsis, and also for the reduction of the total area of the mucous membranes of the entire gastrointestinal tract, consequently reducing absorption (Stachura, A. *et al*., 2020). All these changes interfere with the microbial community that inhabits our gut.

Hence, the intestinal microbiota of a new-born is less complex, with the level of complexity increasing with age (Dominguez-Bello, M. G. *et al*., 2019). In an overview, from birth to adulthood there is an increase in the number and diversity of bacterial members of the gut microbiota. In the transition from adulthood to old age, the gut microbiota generally decreases in diversity and increases in quantity (Bana, B. & Cabreiro, F., 2019).

The intestinal microbiota identified in a new-born is acquired from the mother's microbiota and the surroundings during labour and undergoes minimal changes until the incorporation of solid foods into the diet (Rinninella, E. *et al*., 2019). Different types of delivery influence a baby's gut microbiota, the main phylum present in a new-born, who had a vaginal delivery, is usually bacteria of the genus *Bifidobacterium*, in contrast, babies born by caesarean section show the dominance of *Corynebacterium* and *Propionibacterium* and, in general, a lower bacterial diversity (Rinninella, E. *et al*., 2019). In adulthood, the gut microbiota composition tends to stabilize, forming a community in

which five phyla are usually the most abundant, namely *Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia* and *Tenericutes* (Rinninella, E. *et al.*, 2019). *Firmicutes* and *Bacteroidetes* are the two main ones, forming an important element within the gut microbiota, their rate is accounted for in most studies about gut microbiota composition. The *Firmicutes/Bacteroidetes* ratio also changes during an individual's lifetime, being low during childhood and old age and increasing during adulthood (Mariat, D. *et al.*, 2009). In old age, the gut microbiota is generally distinct from adulthood, changing towards a more unique composition (Wilmanski, T. *et al.*, 2021). In general, the microbiota of the elderly shows a depletion of aerobic bacteria with an increase in the strictly anaerobic groups (Rinninella, E. *et al*., 2019). Despite the greater number of *Bacteroidetes*, *Bacteroides* spp. are generally less abundant (Leite, G. *et al*., 2021), as are *Bifidobacteria* and *Lactobacilli*, on the contrary, *Escherichia coli* seem to be more abundant in the elderly population (Kong, F. *et al.*, 2019; Mariat, D. *et al.*, 2009). Regarding metabolites, the levels of SCFAs in the elderly population are lower in comparison with younger subjects (Nagpal, R. et al., 2018).

1.2.2. "Inflammaging"

"Inflammaging" is a chronic inflammation occurring in the absence of noticeable infection that represents a risk factor for morbidity, disability, frailty, cardiovascular diseases, and mortality in the elderly population. This type of inflammation develops with age, and it is characterized by elevated levels of blood inflammatory markers and mediators (Chung, H. Y. *et al.*, 2019; Ferrucci, L. & Fabbri, E., 2018; Shintouo, C. M. *et al.*, 2020). Although the permeability of the gut barrier is an established contributively factor, the gut microbiota composition also plays a role in inflammation (Shintouo, C. M. *et al*., 2020). In general, there is a reduction in bacteria that are responsible for maintaining the immune tolerance in the gut, alongside an increase of the opportunistic bacteria that usually stimulate intestinal inflammation (Shintouo, C. M. *et al*., 2020). Animal studies showed that *Parabacteroides*, *Mucispirillum*, *Clostridium* and *Sarcina* positively associate with the pro-inflammatory marker MCP-1 while *Akkermansia*, *Oscillospira, Blautia* and *Lactobacillus* negatively correlate with it. In humans, the phylum *Proteobacteria* exhibited a positive correlation with IL-6 and IL-8, while *Ruminococcus lactaris* et rel. revealed a negative correlation with IL-8 (Shintouo, C. M. *et al*., 2020).

It is also established that the elderly population has a decreased masticatory function together with a compromised intestinal mucosal immune system (An, R. *et al.*, 2018), with reduced mucin production by epithelial cells, which causes an increase in the permeability of the mucous membranes (Yoo, J. Y. *et al*., 2020), contributing to a state of inflammation in the body.

Moreover, an aged-type microbiota was related to increased levels of several pro-inflammatory markers and in a group of semi-centenarians, inflammation can predict successful ageing (Chung, H. Y. et al., 2019). Centenarians often have an increase in *Akkermansia*, *Bifidobacterium*, and *Christensenellaceae* in their intestinal microbiota, which promotes a healthy immune function and increased anti-inflammatory activity (Ferrucci, L. & Fabbri, E., 2018).

1.2.3. Geographic Provenance

Many factors significantly vary from one country to another, with the composition of the gut microbiota happening the same way. Several differences were identified between cohorts of different origins. These differences are essentially linked to lifestyle, food, health conditions and access to basic sanitation (Gupta, V. K. et al., 2017).

Studies revealed that foraging communities have greater diversity in their gut microbiota than Western populations, with the people living in the Western countries having a gut microbiota enriched in *Firmicutes*, *Actinobacteria*, *Verrucomicrobia* and *Bacteroidetes* (Gupta, V. K. et al., 2017; Senghor, B. *et al.*, 2018). The gut microbiota diversity of the Asian populations, for instance, is usually a transitional composition between the diversity of Western populations and African communities, with a dominance of *Bacteroidetes* and *Firmicutes* (Senghor, B. et al., 2018). The differences have been studied all around the globe, being reported that *Firmicutes* are usually higher in the American population when compared with other countries, contrarily to *Bacteroidetes* that are usually higher in Korean and Japanese individuals in comparison with American individuals (Senghor, B. *et al*., 2018).

At the genus level, *Bifidobacterium* and *Clostridium* abundance is usually higher in Japanese populations. Regarding *Bacteroides* spp., levels are increased in the gut microbiota of the Western countries' populations (enterotype *Bacteroides*) while in the

non-Western countries the most common is the *Prevotella* enterotype, except for China, which has a prevalence of *Bacteroides* higher than in other countries (Senghor, B. *et al.*, 2018).

1.2.4. Diet

Differences associated with geographic origin are commonly linked to the dietary habits of each region, with the Western diet high in fat and protein and non-Western populations consuming higher amounts of fibre, for example, the African gut microbiota has a *Prevotella* enterotype with a diet containing very little protein and animal lipids, as opposed to a Western diet that is enriched in these two components (Al Bander, Z. et al., 2020; Rinninella, E. et al., 2019). The intake of fibre is also associated with higher levels of *Faecalibacterium prausnitzii* (now classified as *F. duncaniae* (Sakamoto, M. *et al.*, 2022) and lower abundances of *Eubacterium dolichum* and *Bacteroides uniformis* (Lin, D. *et al.*, 2018).

Regarding specific types of diet (vegan, vegetarian and omnivorous), different species of *Bacteroides* colonize the intestine in each situation. Vegans and vegetarians have a lower number of *B. fragilis* when compared to omnivores. On the other hand, the omnivorous diet leads to a prevalence of *B. salanitronis* and *B. coprocola*, while *B. vulgatus* (currently *Phocaeicola vulgatus*, (García-López, M. *et al.*, 2019)) is present only in vegetarians and *B. salyersiae* is prevalent in vegans. The predominance of *Bacteroides* spp. has been associated with animal-based diets, however, *B. thetaiotaomicron* is prevalent in vegans and vegetarians (Zafar, H. & Saier, M. H., Jr., 2021).

1.2.5. Medication Intake and Hospitalization Periods

Extensive periods of hospitalization lead to structural differences in this bacterial community, usually linked to medication intake, a poor diet and hospital-associated microorganisms (Haran, J. P. & McCormick, B. A., 2021; Ravi, A. *et al.*, 2019; Ticinesi, A. *et al.*, 2017). The hospital environment is enriched with antibiotic-resistant pathogens, responsible for serious hospital infections, that thrive in weakened gut microbiota (Milani, C. *et al.*, 2016). In the elderly, these alterations can be responsible for early death in hospitals (Ticinesi, A. *et al*., 2017). Differences between long-term and short-term

hospitalizations have been reported, with patients in long-term hospitalizations showing a predominance of the phylum *Bacteroidetes* contrarily to healthy patients that have a predominance of *Firmicutes*. The alterations at the phylum level involving short-term hospitalizations are variable (Zapata, H. J. & Quagliarello, V. J., 2015).

Antibiotics are commonly used to treat bacterial infections and stop the proliferation of pathogens. Overuse of broad-spectrum antibiotics around the world triggered the development of several resistance mechanisms by bacteria leading to an emergence of multi-resistant infections (Ramirez, J. *et al.*, 2020; Yang, L. *et al.*, 2021). Broad-spectrum antibiotics are also responsible for the depletion of large amounts of bacteria from our intestines, and the longer the exposure the bigger the damage (Korpela, K. *et al.*, 2020; Yang, L. *et al*., 2021). The depth of the perturbation that the antibiotic will cause depends on several factors, including the antimicrobial spectrum of action and the composition of the gut microbiota at pre-treatment (Rashidi, A. *et al.*, 2021).

Antibiotic administration can occur at several stages during the individual lifetime. When administered in new-borns, generally there is a depletion in *Bifidobacterium* spp. and an increase in *Clostridium* spp. and *Enterobacteriaceae*, these changes at a such young age can cause long-term dysfunctions of the immune system and in the metabolic pathways (Korpela, K. *et al.*, 2020). When administered during adulthood, unsettle short- and long-term microbial balance, with decreases in diversity and richness in the community. Some beneficial bacteria are depleted, leading to a fragile state in which the gut microbiota is susceptible to opportunistic pathogens and antibiotic-resistant bacteria (Thursby, E. & Juge, N., 2017). In the elderly, hospitalizations alongside antibiotic intake tend to increase, which usually leads to a decrease in richness and diversity in the gut microbiota (Lakshminarayanan, B. *et al.*, 2014; Ramirez, J. *et al.*, 2020), this can contribute to the frailty state of the individual by the disruption of a previously balanced gut microbiota.

1.3. Alterations in the Gut Microbiota: Health and Disease

During an individual's lifetime, patterns of illness will occur. However, the ageing process is responsible for an overall decline in the body's fitness, and this decline is often accompanied by the development of disease.

Some age-related disorders have been associated with dysbiosis, including those with an impact on the brain. With increasing evidence that the gut microbiota influences and is influenced by the entire organism, attention has turned to the possible link between the nervous system and the gut microbiota, establishing the Microbiota-Gut-Brain axis. This axis is a multidirectional system in which the brain affects the gut microbiota through the Autonomic Nervous System and the gut affects the Central Nervous System (CNS) through neuroendocrine, neuronal, and immune-mediated pathways (Dieterich, W., Schink, M., & Zopf, Y., 2018; Santoni, M., Miccini, F., & Battelli, N., 2021; Zheng, S. Y. *et al.*, 2021). From this perspective, several studies have begun to identify gut microbiota dysbiosis in patients with CNS-related disorders in comparison with healthy controls. Some of these disorders are largely associated with the elderly and affect a large percentage of the world population, however, intestinal dysbiosis may be associated not only with the onset of these disorders but also with their progression and severity (Zheng, S. Y. *et al.*, 2021).

Therefore, some of the most common age-related disorders already associated with dysbiosis are type 2 diabetes, Alzheimer's disease, Parkinson's disease, certain cardiovascular pathologies (atherosclerosis), obesity, cancer, and even the individual's frailty state (Buford, T. W., 2017; Gemikonakli, G., Mach, J., & Hilmer, S. N., 2021; Leite, G. et al., 2021; Nagpal, R. et al., 2018; Rinninella, E. et al., 2019).

In addition, other disorders, unrelated to the ageing process, are also commonly associated with dysbiosis in the gut, such as autism spectrum disorders, depression, celiac disease, irritable bowel syndrome and inflammatory bowel disease (Du, Y. *et al.*, 2020; Rinninella, E. et al., 2019; Xiao, L. *et al.*, 2021).

1.3.1. Type 2 Diabetes (T2D)

T2D is a metabolic disorder characterized by hyperglycemia i.e., high levels of insulin in the bloodstream, associated with insulin resistance or deficient insulin production (Cunningham, A. L., Stephens, J. W., & Harris, D. A., 2021). Approximately 422 million people worldwide have diabetes and more than 95% of that group have type 2 diabetes (World Health Organization (WHO), 2021). Diabetes in the elderly population occurs with functional loss, various comorbidities, and premature death (LeRoith, D. *et al.*, 2019).

Studies involving type 2 diabetes patients reported that the composition of the gut microbiota is altered in comparison with healthy controls. At the phylum level, there is a reduction in *Firmicutes*, with a consequent decrease in the *Firmicutes/Bacteroidetes* ratio, and an increase in *Negativicutes* (correlated with inflammation in these patients). Regarding genus-level proportions, a reduction in the genus *Clostridium* and SCFAs producing bacteria, such as *Roseburia, Bacteroides, Prevotella, Akkermansia* and *Bifidobacterium* species is noticeable, alongside an increase in *Lactobacillus* and the opportunistic bacteria *Bacteroides caccae* (Cunningham, A. L. et al., 2021; Rinninella, E. et al., 2019; Santacroce, L. *et al.*, 2021; Umirah, F. *et al.*, 2021).

1.3.2. Obesity

Obesity is defined as an abnormal accumulation of fat that poses a health risk. This condition is a risk factor for several diseases, such as musculoskeletal disorders, cardiovascular diseases, and some types of cancer, being responsible for 4 million deaths each year (WHO, 2021). In Portugal in 2015, according to data from the 1st National Health Survey with Physical Examination (INSEF), there was a prevalence of 28.7%, with overweight reaching 38.9%, values that near doubled since they were first reported in 1998 (Gaio, V. *et al.*, 2018).

Besides the mentioned diseases, obese individuals are at risk of developing T2D, 86% of T2D patients are obese or overweight, and it is expected that the prevalence of obesity-related diabetes will double to 300 million by 2025 (Dyson, P. A., 2010).

Obese people generally have decreased overall bacterial diversity in their gut, with a lower relative abundance of *Bacteroidetes* (higher *Firmicutes/Bacteroidetes* ratio), at the phylum level. Their gut microbiota shows enrichment in *Prevotella* and SCFAs-producing bacteria along with a decrease in *Bacteroides* spp. particularly *B. thetaiotaomicron* (Dong, T. S. *et al.*, 2022; Ejtahed, H. S. *et al.*, 2020; Liu, R. *et al.*, 2017; Rinninella, E. *et al*., 2019; Yoshida, N. *et al.*, 2021).

1.3.3. Cardiovascular pathologies

Cardiovascular pathologies are responsible for 32% of deaths worldwide each year (WHO, 2021). Although the information on gut microbiota and cardiovascular diseases

(CVD) is still limited, in expansion an association between CVD phenotypes and changes in the relative abundance of specific microbial groups has been reported (Anderson, K. M. *et al.*, 2022). Patients with cardiovascular pathologies tend to have altered levels of specific intestinal microbiota-dependent metabolites that will influence CVD phenotypes. These changes are detected in plasma and have been linked to an impaired intestinal barrier that allows bacteria and metabolites to leak into the bloodstream. The bacteria in the gut microbiota are responsible for the production of the metabolites alongside the impaired gut barrier (Tang, W. H. & Hazen, S. L., 2017; Witkowski, M., Weeks, T. L., & Hazen, S. L., 2020). The primarily trimethylamine-N-oxide (TMAO) is the most associated gut metabolite with heart failure (Chioncel, O. & Ambrosy, A. P., 2019; Dong, Z. *et al.*, 2021; Heianza, Y. *et al.*, 2020; Israr, M. Z. *et al.*, 2021). TMAO is produced during the digestion of nutrients, such as phosphatidylcholine (lecithin), choline and L-carnitine by the action of intestinal bacteria, and then is transported to the bloodstream (Cui, X. *et al.*, 2018). The levels of TMAO in the plasma of patients that suffer heart failure are very consistent with age, brain natriuretic peptide (BNP), and inversely correlated with values of renal insufficiency, such as estimated glomerular filtration rate (eGFR) (Suzuki, T. *et al.*, 2019; Trøseid, M. *et al.*, 2015; Wei, H. *et al.*, 2022; Zhou, X. *et al.*, 2020). However, the use of TMAO levels as a biomarker is still in debate (Anderson, K. M. et al., 2022).

1.3.4. Colorectal Cancer

Presently, colorectal cancer is one of the main causes of death among cancer patients worldwide, being reported more than 600,000 deaths per year, which makes this type of cancer the most challenging in sustaining the health of populations (Brenner, H., Kloor, M., & Pox, C. P., 2014).

In Portugal, the data about the incidence of colorectal cancer evidence that between 2007 and 2011, colorectal cancer was implicated in about 10,250 deaths in men and 7,410 deaths in women, and about 37,500 new cases of this cancer were recorded during that period (Roquette, R., Painho, M., & Nunes, B., 2019).

The start of colorectal cancer is associated with the development of polyps, the unregulated growth of cells lining the inner layers of the intestinal walls. Some are quite common and have low malignant potential, these are called hyperplastic polyps. These

polyps are not recognized as colorectal cancer promoters. Other polyps, denominated by adenomas, are the main indicators of cancer and these are called precancerous polyps (Meseeha, M. & Attia, M., 2022).

Around 70% of all colorectal cancer cases are influenced by environmental factors rather than genetic, factors, including lifestyle, diet, metabolism and even gut microecology. Diseases, such as Diabetes mellitus, inflammatory bowel disease, obesity, and high-fat diets are among the main factors that contribute to the onset of colorectal cancer. Other risk factors for the development of this type of cancer are unhealthy habits, such as smoking and alcohol consumption (Arvelo, F., Sojo, F., & Cotte, C., 2015; Kato, I. *et al.*, 2013).

The role of the gut microbiome in the development and progression of colorectal cancer has evoked great interest from both clinicians and researchers (Bamola, V. D. *et al.*, 2022; Gao, Z. *et al.*, 2015; Mira-Pascual, L. *et al.*, 2015; Shen, X. J. *et al.*, 2010; Wang, T. *et al.*, 2012).

The bacteria of the phylum *Proteobacteria* are more abundant in patients with colorectal cancer in comparison with healthy individuals. The genera of this phylum more implicated in colorectal cancer are *Salmonella* spp. and *Escherichia* spp. (Bonnet, M. *et al.*, 2014; Iftekhar, A. *et al.*, 2021; Lu, R. *et al.*, 2017).

Patients with this type of cancer have decreased quantities of *Bacteroidetes*, at the phylum level. However, their gut microbiota is enriched in *Bacteroides*, particularly *Bacteroides fragilis*. Different species of this genus produce metabolites, such as carboxylic acids and simple sugars, which promote colorectal cancer (Coker, O. O. *et al.*, 2022). Other genera associated with colorectal cancer are *Enterococcus, Escherichia/Shigella, Klebsiella, Lactobacillus, Streptococcus* and *Peptostreptococcus*. While presenting lower proportions of *Roseburia* and other butyrate producers (DeJong, E. N., Surette, M. G., & Bowdish, D. M. E., 2020; Rinninella, E. et al., 2019; Santacroce, L. et al., 2021).

1.3.5. Alzheimer's disease (AD)

Alzheimer's is a multifactorial neurodegenerative disease, clinically characterized by an insidious onset of memory and cognitive impairment, the onset of psychiatric symptoms and behavioural disorders, and impairment of activities of daily living (Wu, S. *et al.*,

2021). In a mice model of AD-like pathology with amyloid and neurofibrillary tangles ($ADLP^{APT}$) mice was observed a modified intestinal microbiota, lower epithelial barrier integrity, and pronounced intestinal and systemic inflammation (Kim, M. S. *et al.*, 2020). The frequent transfer and transplantation of the intestinal microbiota of healthy wild-type mice to the $ADLP^{APT}$ mice over a long period of time weakened the beta-amyloid (Aβ) settling, tau pathology, reactive gliosis, and memory impairment in this mice model. Moreover, the authors during the transfer of the intestinal microbiota over a long period, observed that intestinal macrophage activity and the circulating blood Ly6Chi monocyte population achieved comparable levels with those of wild-type mice (Kim, M. S. et al., 2020). With this model, the authors evidenced the interaction between the gut (through the evaluation of the gut permeability and colonic gene expression), blood (studying the blood immune cell population) and the brain (the injury observed) axis and AD.

The gut microbiota of people with AD is characterized by an overall decreased microbial richness and diversity, with a reduction in some proportions of phyla, such as *Firmicutes* and *Actinobacteria*, while an increase in others, such as *Bacteroidetes* and *Proteobacteria* (Rinninella, E. *et al*., 2019; Romanenko, M. *et al.*, 2021; Wu, S. *et al*., 2021). In addition, there is a decrease in the proportions of three main families *Clostridiaceae*, *Turicibactereaceae* and *Bifidobacteriaceae*, with a consequent decrease in genera, namely *Turicibacter*, *Clostridium* and *Bifidobacterium* (Vuotto, C. *et al.*, 2020; Wu, S. *et al.*, 2021). Other studies have also recorded a decrease in bacteria, such as *Dialister*, the anti-inflammatory *Eubacterium rectale* (currently *Agathobacter rectalis*) (Vuotto, C. et al., 2020) and butyrate-producing bacteria (Wu, S. *et al.*, 2021). These patients also have increased proportions of members of the families *Gemellaceae* (*Gemella* and *Blautia*), *Bacteroidaceae* (*Bacteroides*) and *Rikinellaceae* (*Alistipes*), and the genera *Phascolarctobacterium* and *Bilophila*. At the species level, these patients show a greater abundance of taxa responsible for pro-inflammatory states, such as *Odoribacter splanchnicus*, *Bacteroides vulgatus* and species of the genus *Escherichia/Shigella* (Vuotto, C. *et al.*, 2020; Wu, S. *et al.*, 2021).

1.3.6. Parkinson's disease (PD)

Parkinson's disease (PD) is a neurodegenerative disease with a global incidence and increasing prevalence and mortality rates worldwide. This disease is clinically

characterized by a vast number of symptoms, but the pathohistological hallmark is the presence of Lewy bodies (aggregated proteins) in the nervous system (Unger, M. M. *et al.*, 2016). The intestinal microbiota of patients with PD is characterized by a decrease in the phylum *Bacteroidetes* when compared to healthy controls (Unger, M. M. et al., 2016). Bacterial families, such as *Prevotellaceae, Lactobacillaceae, Enterococcaceae* and *Lachnospiraceae* also decrease, while there is an increase in *Enterobacteriaceae, Verrucomicrobiaceae, Bifidobacteriaceae, Christensenellaceae* and *Ruminococcaceae* (Unger, M. M. *et al*., 2016; Zheng, S. Y. *et al*., 2021). In addition, there is a decrease in butyrate-producing bacteria, such as those belonging to the genera *Faecalibacterium, Coprococcus, Blautia, Prevotella* and other members of the *Prevotellaceae* family. In contrast, several genera, such as *Lactobacillus, Bifidobacterium, Akkermansia* and the opportunistic pathogens *Porphyromonas* and *Corynebacterium* are generally detected at higher levels (Vuotto, C. *et al.*, 2020; Zheng, S. Y. et al., 2021). At the species level, lower abundances of *F. prausnitzii* and higher levels of *Akkermansia muciniphila* were reported (Unger, M. M. *et al*., 2016).

In patients with PD, the genus *Bacteroides* was associated with increased plasma levels of tumour necrosis factor- α (TNF-α), while *Verrucomicrobia* was associated with increased plasma concentrations of IFN-γ, which can lead to auto-inflammatory states (Zheng, S. Y. et al., 2021). Some bacterial groups were positively related to disease severity, such as members of the family *Enterobacteriaceae* including *Escherichia, Shigella* and *Proteus,* members of the family *Lactobacillaceae,* and the genus *Enterococcus,* (Zheng, S. Y. *et al.,* 2021).

Importantly, in these patients, the integrity of the intestinal barrier is disrupted, and intestinal bacterial counts decrease as the disease progresses (Vuotto, C. *et al*., 2020).

1.3.7. Sarcopenia and Frailty

Sarcopenia is a continuous impairment in muscle mass and strength that is observed during ageing (Marzetti, E. *et al.,* 2017). This condition is particularly harmful to the elderly since occurs a higher probability of them being exposed to events that can compromise their health and even their life, namely disfunction, loss of their independence, risk of institutionalization, and ultimately death (Marzetti, E. et al., 2017). So far, sarcopenia has not been recognized as a single event because of the difficulty in

establishing a clear pathophysiology and the occurrence of many overlaps with other age-related changes (Calvani, R. *et al.*, 2018). Attempts to find an unambiguous functional definition of sarcopenia have been made, but so far, this plenty clear definition is still absent (Landi, F. *et al.*, 2018). Nevertheless, the sarcopenia guidelines (last reviewed in 2018), recognized the prime importance of muscle strength affection on the amount of muscle mass as a predictor of the sarcopenia state (Cruz-Jentoft, A. J. *et al.*, 2019). Regarding the prevalence of sarcopenia, it is estimated that in the population aged 60 years and over the prevalence value is around 10%, with similar values between genders (Shafiee, G. *et al.*, 2017).

As mentioned above sarcopenia is mainly associated with age, but can also be observed at younger ages, being in this case designated by pre-sarcopenia, to which other factors besides age are linked, namely obesity and lack of physical activity (Finlay, B. B. et al., 2019; Marzetti, E. et al., 2017). In terms of clinical aspects, an overlap occurs between sarcopenia and frailty and also with the characteristic decline associated with age in terms of physiological limitations and difficulties in maintaining the whole organism in equilibrium, which facilitates the occurrence of life-threatening events in older individuals, such as falls, hospitalization, disability, institutionalization, and mortality (Cesari, M., Calvani, R., & Marzetti, E., 2017; Haran, J. P. & McCormick, B. A., 2021).

The first study that investigated the gut microbiota in frailty was conducted in 2005 by Van Tongeren *et al*., (2005). In this study for individuals with high frailty scores, it was reported a lower proportion of *Lactobacilli*, *Bacteroides*/*Prevotella* and *F. prausnitzii*, and an increase in the proportion of *Ruminococcus*, *Atopobium* and members of the family *Enterobacteriaceae* (van Tongeren, S. P. *et al.*, 2005). More recent studies also observed a lower proportion of *F. prausnitzii* along with members of the families *Lachnospiraceae, Ruminococcaceae* and *Erysipelotrichaceae,* in contrast with a higher proportion of members of *Clostridiales* and *Coriobacteriaceae*, specifically two species, the *Eubacterium dolichum* and *Eggerthella lenta* (Jackson, M. A. *et al.*, 2016; Stachura, A. *et al*., 2020). The fact that *F. prausnitzii,* a butyrate-producing bacteria is at lower numbers in frailty individuals suggests that the tight junctions of the intestinal epithelial cells with the consequent prevention of microbial translocation into system circulation is compromised in frailty individuals (Peng, L. *et al.*, 2009; Vital, M., Howe, A. C., & Tiedje, J. M., 2014).

1.3.8. Depression

Depression is a prevalent neuropsychiatric disease that affects around 280 million people worldwide, and currently, the World Health Organization anticipates that depression will be the leading factor toward disease overburden by 2030 (WHO, 2021). This disease exerts heavy public health and economic burdens and has an incredibly high recurrence rate. The elderly are highly affected by depression, being this condition one of the main causes of disability, decline and comorbidity in older adults (Alexopoulos, G. S., 2005; Almeida, O. P., 2014). It is estimated a high prevalence of depression among the geriatric population, reaching about 10 to 20 % (WHO, 2017). The causes of depression are not yet clarified.

In the study conducted by Valles-Colomer *et al*., (2019) it was reported enrichment of *Bacteroides* 2 enterotype (a pattern with a lower relative abundance of the genus *Faecalibacterium*) in individuals suffering from depression and with poor quality of life, and also in patients with Crohn's disease. In contrast, butyrate-producing bacteria, such as *Faecalibacterium* and *Coprococcus* showed to be positively correlated with better quality of life indicators. Moreover, the authors observed that the synthesis of the compound 3,4-dihydroxyphenylacetic acid (DOPAC) was strongly correlated with the abundance of *Coprococcus*. However, the ability of *Coprococcus* to synthesize DOPAC is not clear. These results reinforce the idea that not only butyrate can be implicated in a higher quality of life, but also suggest that other compounds can contribute to the association of *Coprococcus* with a higher quality of life and lack of depression (Valles-Colomer, M. *et al.*, 2019).

Another study also reported gut microbiota alterations in individuals suffering from depression, results showed an underrepresentation of *Firmicutes* and an overrepresentation of *Bacteroidetes, Proteobacteria,* and *Actinobacteria*. Moreover, the gut microbiota composition of these patients is characterized by lower levels of *Lachnospiraceae, Ruminococcaceae, Faecalibacterium, Lactobacillus* and *Bifidobacterium,* while having a higher proportion of *Bacteroidales* (Du, Y. et al., 2020). The findings of these two mentioned studies consolidate the positive role of butyrate-producing bacteria in preventing mental disorders.

1.3.9. Inflammatory Bowel Disease (IBD)

IBD is a chronic inflammatory disease of the gastrointestinal tract, which includes ulcerative colitis (UC) and Crohn's disease (CRD), that can appear at any age (Ananthakrishnan, A. N., Nguyen, G. C., & Bernstein, C. N., 2021; Guan, Q., 2019). In the elderly, it represents a challenge in terms of diagnosis and treatment, due to other comorbidities that are usually present (Ananthakrishnan, A. N. et al., 2021). About 1 in 160 elderly people is affected by IBD, and the prevalence of the disease among the elderly is increasing by 5.2% per year (Ananthakrishnan, A. N. et al., 2021). In addition, premature development of sarcopenia has been reported in patients with IBD, and these patients are more likely to require surgery (Ryan, E. *et al.*, 2019).

In this case, intestinal changes are often linked to the severity of the disease (Guan, Q., 2019). The changes in UC and CRD are different, in the former, there is a decrease in the levels of butyrate-producing bacteria while in CRD there is a reduced global diversity, decreased levels of *F. prausnitzii* (butyrate-producing strain) and increased levels of *Mycobacterium avium* subsp. *paratuberculosis* and adherent-invasive *E. coli* (Guan, Q., 2019; Rinninella, E. et al., 2019; Santacroce, L. et al., 2021). Furthermore, the presence of *Clostridium difficile* is increased in both (Guan, Q., 2019).

1.3.10. Chronic Kidney Disease (CKD)

Kidney function suffers a decline with ageing, as such, diseases like CKD have a high prevalence in the elderly population (Mallappallil, M. *et al.*, 2014). Some of the risk factors of CKD are other ageing-associated disorders, such as obesity, diabetes, hypertension, frailty, and sarcopenia (Mallappallil, M. et al., 2014; Margiotta, E. *et al.*, 2020).

The gut microbiota of CKD patients shows an increased abundance of the genera *Citrobacter, Anaerotruncus, Coprobacillus* and strains of *Ruminococcus torques* accompanied by a decrease in saccharolytic and butyrate-producing bacteria, such as *Prevotella, Roseburia* strains and *F. prausnitzii* (Margiotta, E. et al., 2020), Regarding patients with a diagnostic of frailty alongside CKD, an increase in abundance of several bacteria was observed in comparison with CKD patients alone, namely bacteria from the family *Coriobacteriaceae*, the genera *Anaerotruncus, Coprobacillus* and *Dorea* and the species *Eubacterium dolichum* and *Eggerthella lenta* (Margiotta, E. et al., 2020). These

findings evidence how the existence of more than one comorbidity can influence the alterations in the gut.

Even though all these profiles of the gut microbial composition were detected over the years, there are numerous inconsistencies between the studies regarding each scenery, this, alongside the absence of a line of causation are two of the main difficulties in studying the microbiome and diseases.

1.4. Alterations in the Gut Microbiota: Epigenetics

Epigenetic modifications are mechanisms that allow the cells to alter their gene expression without modifying the genetic makeup of each cell. The mechanisms underlying epigenetic changes are based on DNA methylation and post-translational histone modification, which are monitored by epigenetic modifying enzymes (Woo, V. & Alenghat, T., 2022). The role of this epigenetic regulation in the human body is being unveiled and its involvement as a tool for the microbiota to influence the physiology of the host is already recognized. DNA methylation is already considered a promising marker for the study of human development and ageing. Its applicability to different tissues and cell types is the main advantage of its use as a biomarker (Horvath, S., 2013).

Thus, the mechanisms underlying this are being identified and are based on the principle that microbial metabolism influences the availability of chemical donors for DNA or histone modifications, the regulation of the expression and/or activity of epigenetic modifying enzymes and the activation of intrinsic host cell processes than direct epigenetic pathways (Peery, R. C. *et al.*, 2021; Woo, V. & Alenghat, T., 2022).

One of the binding elements in these systems is the primary substrate for DNA and histone methylation, S-adenosylmethionine (SAM). This substrate is metabolized using folate, which is generated by the intestinal microbiota, mainly by *Bifidobacterium* and *Lactobacillus* (Woo, V. & Alenghat, T., 2022). Changes in the composition of the gut microbiota can influence the production of SAM and alter the status of DNA and histone methylation. In addition, high levels of *Fusobacterium* were already associated with

impaired DNA methylation in patients with UC, and an increase in DNA methylation in colorectal cancer-related genes was observed (Aleksandrova, K., Romero-Mosquera, B., & Hernandez, V., 2017; Woo, V. & Alenghat, T., 2022).

Ethionine, another microbiota-derived metabolite produced by *Lactobacillus reuteri*, is known to inhibit histone deacetylases (HDACs)(Woo, V. & Alenghat, T., 2022). As mentioned above, SCFAs, such as propionate and butyrate are also HDAC inhibitors that can induce chromatin changes resulting in impaired gene expression. Studies have shown that children with untreated IBD have differences in methylation status and gene expression in the gut (Howell, K. J. *et al.*, 2018). *B. thetaiotaomicron*, a strain producer of SCFAs, was able to increase HDAC expression in germ-free mice, suggesting that one species can largely influence the host epigenome. Therefore, the gut microbiota has been found to stimulate various types of histone modifications, including histone acetylation and methylation, in various human tissues (Aleksandrova, K. et al., 2017; Woo, V. & Alenghat, T., 2022). Thus, dysbiosis, by depleting commensal SCFAs producers, can lead to changes in the epigenome, which can result in serious consequences for the host (Peery, R. C. et al., 2021).

Other molecules that can help to bridge the gap between the epigenome and the gut microbiota are non-coding RNAs (ncRNAs), which break down into long and small non-coding RNAs, these molecules can regulate gene expression at the chromosome or gene level (Woo, V. & Alenghat, T., 2022).

Long non-coding RNAs (lncRNAs) regulate gene expression by acting directly on DNA scaffolds by modifying chromatin and can be induced by the gut microbiota but have been found to be expressed in other organs (spleen and thymus). As a result, the microbiota regulates the expression of lncRNAs in the intestine, but also remotely in other tissues (Woo, V. & Alenghat, T., 2022).

Micro RNAs (miRNAs) are short, single-stranded ncRNAs that are expressed in cells and circulate systemically (Woo, V. & Alenghat, T., 2022). These molecules have been shown to control the integrity of the intestinal barrier in patients with UC, in addition, a different expression of specific miRNAs was detected in the mucosa of patients with IBD when compared to controls. (Aleksandrova, K. et al., 2017).

Although the number of studies is limited, these studies encourage further research in this field. The link between microbiota and epigenome also creates the possibility that new

epigenetic biomarkers will emerge alongside those already linked to the ageing process (Levine, M. E. *et al.*, 2018).

1.5. *Bacteroides* spp. in the Gut of the Elderly

Bacteroides are Gram-negative, anaerobe, non-motile, non-spore-forming rods, that are known to inhabit the human gut (Wexler, H. M., 2007). They account for a major proportion of the gut microbiota community, and play several roles in the gut, maintaining the microbial food web, acting as nutrient providers for the host and other gut bacteria, and working as primary producers of SCFAs, *Bacteroides* spp., produce mainly acetate and propionate, which are powerful anti-inflammatory mediators (Dong, T. S. *et al.*, 2022; Zafar, H. & Saier, M. H., Jr., 2021).

Species, such as *B. thetaiotaomicron* and *B. fragilis* carry, in their genomes, polysaccharide utilization loci (PULs) that are activated in the presence of the glycans which allow an interspecies cross-feeding relationship with other microorganisms (Tan, H., Zhai, Q., & Chen, W., 2019). For example, *B. thetaiotaomicron* metabolizes starch, providing both maltose and glucose to *Eubacterium ramulus*, which in turn ferments glucose into butyrate (Zafar, H. & Saier, M. H., Jr., 2021). These types of interactions are not only beneficial to the bacterial community, but they also play beneficial roles in human health. As providers, *Bacteroides* spp. are also major sources of outer membrane vesicles (OMVs), which usually contain glycosidases, lipid hydrolases and proteases, that are shared with other species that without it would not be capable of degrading complex polysaccharides (Zafar, H. & Saier, M. H., Jr., 2021). By providing the inheritor bacteria with the required tools to obtain nutrients, the OMVs support the growth of other bacteria in the gut contributing to overall gut homeostasis. Moreover, this support also happens between intra-genus, namely with *B. ovatus* that by breaking important glycans allows their utilization by *B. vulgatus* (Zafar, H. & Saier, M. H., Jr., 2021).

Although their role as providers in the gut is a major characteristic of this genus, the environment in the gut requires competition advantages between microorganisms. For that, some *Bacteroides* spp. have specific transporters for some of their partially degraded

polysaccharides and secrete antimicrobial toxins in a contact-independent manner, resorting to the OMVs (Zafar, H. & Saier, M. H., Jr., 2021).

Even though they normally play a beneficial role in the human gut, when translocated into other parts of the human body they can trigger disease, turning out to be opportunistic pathogens (Wexler, H. M., 2007). The translocation of *Bacteroides* spp. to sterile tissues through the intestinal mucosa may be assigned to several factors, particularly the disruption of the gut barrier, surgical injuries, compromised immune system, excessive antibiotic usage, and ageing. It is also known that some *Bacteroides* spp., such as *B. caccae*, are responsible for the degradation of the mucus that leads to thinner layers of the intestinal mucosa, which compromises its function as a barrier to the movement of pathogens and opportunistic pathogens to other organs. When translocation occurs, aerobic bacteria are the first responsible for tissue damage. Then, the redox potential of the oxygenated tissues reduces and anaerobes, such as *Bacteroides* have the chance to thrive. The spread of *Bacteroides* spp. outside the intestine can thus lead to bacteraemia (bacterial presence in the bloodstream) and abscess formation in different parts of the body. Other elements responsible for the *Bacteroides* pathogenicity are their virulence factors, this genus possesses a complex capsular system, and some strains are capable of producing toxins like the fragilysin from *B. fragilis* and some hemolysins produced by both *B. fragilis* and *B. thetaiotaomicron*, additionally, they can survive oxidative stress resorting to the action of a variety of oxidoreductases (Zafar, H. & Saier, M. H., Jr., 2021).

This genus and its abundance in the gut have been targeted in some studies, revealing that lower quantities of *Bacteroidaceae* members are observed in cases of obesity (Li, Z. et al., 2018), atherosclerosis (Yoshida, N. *et al.*, 2018), and dementia (Saji, N. *et al.*, 2019). Moreover, *Bacteroides* spp. are also diminished in mice exposed to stress (Rinninella, E. *et al.*, 2019). In contrast, higher levels of *Bacteroidaceae* members are associated with healthy colons (DeJong, E. N. et al., 2020), but also insulin resistance, Alzheimer's disease (Vogt, N. M. *et al.*, 2017), and autism spectrum disorders (Garcia-Gutierrez, E., Narbad, A., & Rodríguez, J. M., 2020). In some diseases, such as colorectal cancer, some species of *Bacteroides*, namely *B. vulgatus* are in lower concentrations while *B. fragilis* are in higher concentrations (DeJong, E. N. et al., 2020).

The amount of *Bacteroides* spp. in the gut of older individuals is not consistent across countries, and this may be associated with environmental factors, particularly diet

changes and alterations in the physiological functions of the gastrointestinal system (Ghosh, T. S., Shanahan, F., & O'Toole, P. W., 2022).

2. Objectives

Being established the symbiotic relationship between the gut microbiota and the host, alongside the importance of the ageing process and taking into account the uneven reports on the abundance of *Bacteroides* spp. in the gut of older individuals the main objective of the current study was to evaluate the gut bacteriome of elderly from Algarve region using the Oxford Nanopore system and examine the abundance and diversity of *Bacteroides* species using a culture-dependent approach. To achieve these goals, faecal samples from healthy and non-healthy old individuals (over 60 years old) were collected and the faecal DNA extracted, then the amplification of the *16S rRNA* gene was performed, and for sequencing the MinION MK1C device from Oxford Nanopore Technologies was used.

For the recovery of *Bacteroides* spp. from the old adults' faecal samples the *Bacteroides vulgatus* Selective Agar (BVSA) culture medium was used. The characteristic colonies were purified, and the identification of the isolates was performed by sequencing the *16S rRNA* gene.

3. Materials and Methods

3.1. Equipment

- Anaerobic Jar, Oxoid (UK).
- AnaeroGen 2.5 L, Atmosphere Generation Systems, Oxoid (UK).
- Analytical Balance AE 200, Mettler (USA).
- Analytical Balance XS-410, Fisher Scientific (Portugal)
- Autoclave Uniclave 88, AJC (Portugal).
- Binder Incubator, Binder (Germany)
- Electrophoresis Power Supply, EPS 301 (USA)
- Gel Electrophoresis Apparatus GNA-100, Pharmacia Biotech (USA)
- GeneFlash, Syngene (India)
- Heating and agitation plate, Selecta, Agimatic-E (Spain)
- High Pure PCR Template Preparation Kit, F. Hoffmann-La Roche (Switzerland)
- Microscope, Leitz, Laborlux 11.
- Leitz LaborLux 11, Leitz (Germany)
- Mikro 200R Centrifuge, Hettich Zentrifugen (UK).
- MinION MK1C, Oxford Nanopore Technologies (UK).
- Mini-V / PCR PCR camera, Telstar (Spain)
- Nanodrop 2000, Thermo Fisher Scientific (USA).
- Qubit 2.0 Fluorometer, Thermo Fisher Scientific (USA).
- SpectraMax iD3 Multi-Mode Microplate Reader, Molecular Devices (USA)
- Stereomicroscope SZ, Olympus (Japan)
- Thermocycler T-personal, Biometra (Germany)
- Thermocycler T1, Biometra (Germany)
- Ultra-low Temperature Freezer -80ºC U725, Innova New Brunswick Scientific (USA)
- Vortex L46, Labinco (The Netherlands)
- 16S Barcoding Kit (SQK-RAB204), Oxford Nanopore Technologies (UK)

3.2. Culture media

- *Bacteroides Vulgatus* Selective Agar (BVSA) prepared with 30g/L of Trypto-Casein Soy Broth (TSB)(Biokar Diagnostics, France), 2 g/L yeast extract (Biokar Diagnostics), 2 g/L dehydrated ox-bile (Sigma-Aldrich, USA), 1 g/L esculin (Merck), 0.5 g/L ferric ammonium citrate (Merck, Germany) supplemented with vitamin K1 (1%, v/v, Sigma-Aldrich), hemin (0.1%, v/v, Honeywell Fluka™), kanamycin (0.2%, w/v, Sigma-Aldrich), vancomycin (0.075%, w/v, Sigma-Aldrich), colistin (0.015%, w/v, Sigma-Aldrich), and 1.5% (w/v) agar (Biokar Diagnostics), pH 7.1.

- Brain Heart Infusion (Thermo Scientific™) prepared according to the manufacturer's instructions, supplemented with hemin (0.1%, v/v, Honeywell Fluka™) and L-cysteine (0.1%, w/v, Sigma-Aldrich) (BHI+ H). To obtain a solid medium, agar at a 1.5% concentration (Biokar Diagnostics) was added, pH 7.1.

- Brucella Agar (Liofilchem, Roseto d. Abruzzi, Italy) prepared according to the manufacturer's instructions, supplemented with vitamin K1 (1%, v/v) and hemin (0.1%, v/v). To obtain a solid medium, agar at a 1.5% concentration was added.

- Mueller-Hinton Agar (Biokar) prepared according to the manufacturer's instructions.

- Mueller-Hinton Broth (Biokar) prepared according to the manufacturer's instructions.

3.3. Solutions

The solutions used in the present study were as follows:

- Ammonium acetate solution 10 M: 77.05 g of ammonium acetate (Merck) in 100 mL of distilled water.
- Chloroform: Isoamyl alcohol (24:1) (v/v): 24 mL of chloroform (LAB-Scan) and 1 mL of isoamyl alcohol (Merck).

- Colistin solution (0.015 mg/mL) (Sigma-Aldrich). The antibiotic was dissolved in distilled water.
- Ethylenediamine tetra acetic acid (EDTA) 0.5 M, pH 8: 18.6 g EDTA in 100 mL distilled water.
- Lysozyme solution (100 mg/mL) (Merck).
- Guanidine, EDTA, Sarcosil (GES)–- 60 g of guanidine thiocyanate (Promega), 20 mL of 0.5 M EDTA solution pH8 (Sigma-Aldrich), 5 mL of sarcosyl, 100 mL of distilled water.
- Hemin solution 0.1%: 0.1 g hemin and 2 mL 1M NaOH in 100 mL of deionized water (light-sensitive solution).
- Kanamycin solution (50 mg/mL) (Sigma-Aldrich). The antibiotic was dissolved in distilled water.
- Lysis Buffer: 1 mL of 1M Tris HCl, pH 9 to 2.5 mL 1M KCl, 0.1 mL Triton-X, 93.9 mL of MilliQ water.
- Phosphate Buffered Saline (PBS): 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na2HPO4, 0.24 g/L KH2PO4.
- Tris-Acetate-EDTA buffer 50x: 242 g/L Tris base (Sigma), 57.1 mL Glacial acetic acid (Panreac), 100 mL 0.5 M EDTA pH 8.
- Vancomycin solution (7.5 mg/mL (Sigma-Aldrich). The antibiotic was dissolved in distilled water.
- 1 M NaOH solution.
- 10 mM Tris-HCl, pH 8.0, with 50 mM NaCl.

3.4. Faecal Samples

This preliminary study is in frame with the work of the funded Project "ALgarve Fit Aging Score (ALFA Score): a new integrated tool to improve quality of life and healthy ageing". Twelve faecal samples from 7 healthy and 5 non-healthy individuals aged between 60 and 90 years old, including individuals from both genders, were used. The exclusion criteria were the treatment with antibiotics one month before the study or hospitalization three months prior to sample collection. An informed consent for each participant was provided.

The faecal samples were collected from each participant and immediately transported to the laboratory in refrigerated conditions. The faecal samples were maintained in RNAlater (0,3 g/mL) at -80ºC until use. **Table 3.4.1.** summarizes the information about each participant.

Table 3.4.1. Participant's general information

Sample ID	Gender	Age	Health Status	Provenance
S1	Female	62	Non-healthy	Algarve, Portugal
MHA	Male	68	Healthy	Algarve, Portugal
MHB	Male	74	Healthy	Algarve, Portugal
MNA	Male	88	Non-healthy	Algarve, Portugal
MNB	Male	70	Non-healthy	Algarve, Portugal
FHA	Female	71	Healthy	Algarve, Portugal
FHB	Female	61	Healthy	Algarve, Portugal
FHC	Female	86	Healthy	Algarve, Portugal
FHD	Female	71	Healthy	Algarve, Portugal
FHF	Female	72	Healthy	Algarve, Portugal
FNA	Female	74	Non-healthy	Algarve, Portugal
FNB	Female	70	Non-healthy	Algarve, Portugal

3.5. Bacterial Culture Conditions

The isolation of *Bacteroides* species from faecal samples was carried out using the culture selective medium, *Bacteroides vulgatus* Selective Agar (BVSA) (Bacic, M. K. & Smith, C. J., 2008) supplemented with kanamycin (200 mg/L), vancomycin (0.75 mg/L) and colistin (0.015 mg/L). Briefly, 0.1-0.25 g of the stool were transferred into a sterile 2 mL Eppendorf tube and resuspended into 1 mL of Phosphate Buffered Saline (PBS) buffer. The mixture was homogenised using a vortex and centrifuged at 700 ×g, for 5 min. From that, 500 µL of the supernatant were retrieved and serial decimal dilutions were prepared using PBS. Afterwards, 100 µL of the dilutions 10^{-4} and 10^{-5} were inoculated into BVSA plates, in duplicate. The inoculated plates were incubated under anaerobic conditions using an anaerobic jar within an anaerobic sachet (AnaeroGen, Thermo Scientific™, UK) for 4 days at 37°C.

For quantification, characteristic colonies (small, black, shiny colonies with a surrounding dark halo zone) were identified and counted. Other cultural colony characteristics were also considered. Five colonies of each morphological type were transferred to Brain-Heart Infusion (BHI) agar supplemented with hemin (0.1%, v/v) and

L-cysteine (0.1%, w/v). The inoculated plates were incubated for 48 h at 37°C, under anaerobic conditions (as described above). The bacterial cells of the isolated colonies were subjected to Gram staining in order to confirm the presence of Gram-negative bacilli. The pure cultures were maintained in BHI supplemented with 25% (v/v) glycerol in cryovials at -80°C.

The differences between the bacterial counts in the healthy and non-healthy groups were analysed by performing an ANOVA with the software SPSS. Statistical significance was considered at $p < 0.05$.

3.6. DNA Extraction from Faecal Samples

For faecal DNA extraction, 0.3 g from each stool sample were used. DNA extraction was performed with the QIAmp PowerFecal DNA Kit (QIAGEN, Germany) following the manufacturer's instructions with minor modifications: after homogenization of the faecal sample with the PowerBead solution and the C1 solution, three cycles of 5 min. of heating (at 65°C) interspersed with three cycles of 5 min. of vortexing were done, and, in the final step, the solution C6 was left to act in the membrane for 2 min. All extracted DNA samples were stored at -20°C until further use.

3.7. DNA Extraction from Bacterial Cultures

Bacterial genomic DNA extraction from pure bacterial cultures was accomplished using the GES method (Pitcher, D. G., Saunders, N. A., & Owen, R. J., 1989). Briefly, bacterial cells were inoculated into 10 mL of BHI+H and incubated under anaerobic conditions (covered with 1 mL of sterile paraffin), for 2 days at 37°C. From this culture, 2 mL were retrieved and centrifuged at 5000 xg, for 10 min. Afterwards, each bacterial cell pellet was resuspended into 100 µL of lysis buffer combined with 25 µL of lysozyme (100 mg/mL) and incubated at 35°C for 90 min. Following, 500 µL of GES solution (guanidine thiocyanate, 0.5 M EDTA, 0.5% N-laurosarcosine) and 250 µL of 10 M ammonium acetate were added, followed by incubation on ice for 5 and 10 min., respectively. Afterwards, 500 µL of chloroform:isoamyl alcohol (24:1) were added and mixed by inversion, then the tubes were centrifuged (20 000 xg, for 10 min. at 4°C) and the supernatant recovered. From the collected supernatant DNA was precipitated by the addition of 0.5 volumes of cold isopropanol, followed by homogenization (by slow

inversion) and centrifugation (20 000 xg, for 5 min. at 4°C). The pellet was washed twice with 800 µL of 70 % ethanol and centrifuged at 20 000 xg, for 1 min., at 4°C. After being air-dried, the DNA was dissolved into 35 µL of Nuclease-Free Water and the tubes were maintained on ice for 1 h. The DNA samples were aliquoted and stored at -20°C until use. The quantity and quality of the extracted DNA were evaluated using the Nanodrop 2000 (Thermo Scientific™, UK).

To evaluate the DNA integrity an agarose gel was prepared with the addition of 6 µL of the dye "Green Safe" at a concentration of 1:100. In the wells of the gel were loaded 2 µL of DNA and 2 µL of Loading Buffer 6x concentrate (VWR). The marker "GeneRuler ™ DNA Ladder Brews 1 kb" was used. The gel electrophoresis was run in 1x Tris-Acetate EDTA buffer at 120V for 30-40 min.
2 µL of the isolated DNA were loaded into a 1% agarose gel electrophoresis, which was run at 120V for 40 min.

3.8. DNA Purification

Whenever required the DNA samples (both, faecal and bacterial) were purified using the High Pure PCR Template Preparation Kit (F. Hoffmann-La Roche, Switzerland) according to the manufacturer's instructions with minor modifications. Briefly, PBS was added to the DNA sample to achieve a volume of 200 µL. Afterwards, 200 µL of Binding Buffer were added into the tube, followed by 40 µL of reconstituted Proteinase K. The mixture was incubated at 70°C for 10 min. and afterwards for 1 min. on ice. After incubation, 100 µL of isopropanol are added and well mixed.
A High Pure Filter Tube was assembled into one Collection Tube and the sample was poured into the High Pure Filter Tube for a centrifugation step (8000 xg, for 1 min.). The Collection Tube was discarded, and the High Pure Filter Tube was assembled into a new one, 500 µL Inhibitor Removal Buffer was added into the upper reservoir of the Filter Tube, followed by centrifugation (8000 xg, for 1 min.). For the washing step, the Filter Tube was assembled into a new Collection Tube and 500 µL of Wash Buffer was added to the Filter Tube, followed by centrifugation (8000 xg, for 1 min.). This step was done twice, discarding the flow through between washes. After washing, an additional centrifugation step of 10 sec. was performed to withdraw all the remaining Wash Buffer. Finally, to elute the DNA the Filter Tube was placed into a 1.5 mL Eppendorf and 100

µL of prewarmed (70 °C) Elution Buffer was added to the filter followed by a 3 min. incubation at room temperature (optimized step). To retrieve the DNA onto the tube a final centrifugation step was performed (8000 xg, for 1 min.).

3.9. Identification of Bacterial Isolates

Uncharacteristic colonies (small with a brown centre surrounded by a blue light colour) showing Gram-negative bacilli were identified using the RapID ONE system (Thermo Fisher Scientific, USA) following the instructions of the manufacturer.

The identification of the recovered *Bacteroides* species was done by sequencing the *16S rRNA* gene. This gene was previously amplified by polymerase chain reaction (PCR), with the universal primers, 27F (5'AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'GGTTACCTTGTTACGACTT-3') (Bakir, M. A. *et al.*, 2006). Each 20 µL PCR reaction included 2.5 µL of 5x Colourless GoTaq® Flexi Reaction Buffer (Promega, USA), 1 µL of 25 mM $MgCl_2$ (Promega, USA), 0.5 µL dNTPs, 0.5 µL of each primer (10 pM), 0.125 µL of GoTaq® G2 Flexi DNA Polymerase (Promega, USA), 12.875 µL of nuclease-free water and 100 ng of genomic DNA. The PCR conditions were an initial denaturation step (94 ºC for 7 min.) followed by 29 cycles of denaturation, 94ºC for 1 min., an annealing step at 59 ºC for 1 min., an extension step at 72ºC for 1.5 min., and a final extension step at 72ºC for 5 min.
The amplicon of each reaction was visualized through gel electrophoresis as described in section 3.7.

The amplicons of the appropriate size were sequenced at the Molecular Biology Laboratory of the Centro Ciências Mar (CCMar) using standard Sanger sequencing procedures using the *1492R* primer. The 16S sequences were analysed and those with 750 -1200 bp were selected for analysis. The identification of the closest matches to sequence queries was performed using the National Center for Biotechnology Information (NCBI) Blastn (https://www.ncbi.nlm.nih.gov/) and also the Ribosomal Database Project (RDP) Sequence Match (Cole, J. R. *et al.*, 2013) (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

3.10. Antibiotic Susceptibility

3.10.1. Antibiotic Susceptibility of Escherichia coli Isolates

After the identification of the bacterial isolates, the antibiotic susceptibility of 4 isolates identified as *E. coli* was performed using the disk-diffusion method (Kirby-Bauer test). The determination of the minimum inhibitory concentration (MIC) of the antibiotic colistin was done by microdilution.

Briefly, the *E. coli* isolates were grown on BHI + H for 24 h at 37°C. Bacterial suspensions were prepared in PBS to a turbidity of McFarland 0.5. A volume of 100 µL was inoculated into Mueller-Hinton (MH) agar plates. The antibiotic disks (Oxoid, Basingstock, UK) of clindamycin (2 µg) (DA), imipenem (10 µg) (IMP) and moxifloxacin (10 µg) (MXF) were used. The inoculated plates were incubated at 35°C for 20 h. After incubation, the susceptibility zone diameters were measured, and the values were checked against the breakpoints established by EUCAST (2022).

For the microdilution method, a colony from each *E. coli i*solate was inoculated into 5 mL of MH broth and incubated at 37°C overnight. Then in a sterile 96-well flat-bottom microplate (Sarstedt Inc, Nümbrecht, Germany) 3 different concentrations of colistin were tested: 0.015 mg/L, 0.03 mg/L and 0.06 mg/L (which correspond to 1, 2 and 4 x colistin BVSA's concentration, respectively). Non-inoculated MH broth was used as blank and the culture of each isolate in MH broth was used as a control for bacterial growth. The microplate was incubated at 30°C, and the optical density ($OD_{600\ nm}$) was measured every hour for 9 h using the SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, USA).

3.11. Microbiome Analysis

The bacteriome of each faecal sample was established by the amplification of the *16S rRNA* gene using the 16S Barcoding Kit of the Oxford Nanopore (SQK-RAB204) following the instructions of the manufacturer.

The scheme of the bacteriome analysis performed in the current study is illustrated in **Figure 3.11.1**.

3.11.1. Library Preparation

Briefly, 10 ng of genomic DNA from each sample, previously quantified using Qubit 2.0 Fluorometer, was mixed with Nuclease-free water, into a final volume of 10 µL. Each PCR reaction contained 10 µL of DNA together with 1 µL of 16S Barcode (10 mM), 25 µL of LongAmp Taq 2X Master Mix (Biolabs, USA) and 14 µL of Nuclease-free water. Amplification was performed under the following conditions: an initial denaturation step at 95ºC for 1 min. followed by 25 cycles of a first denaturation step at 95ºC for 20 seconds, an annealing step at 55 ºC for 30 seconds and an extension step at 65ºC for 2 min., and the final extension step at 65ºC for 5 min. After amplification, all samples were transferred to new tubes and 30 µL of resuspended AMPure XP beads (Beckman Coulter, USA) were added and mixed into the reaction. The pellet with the beads was spun down in a magnet holder and the supernatant was discarded, following a wash step with fresh 70% ethanol and another spin down, in the magnet holder to retrieve most of the ethanol. The pellet is left to air-dry to evaporate all the ethanol. Afterwards, the pellet is resuspended in 10 µL of Tris-HCl (10 mM, pH 8.0) NaCl (50 mM) with an incubation period of 2 min. at room temperature. The mixture was then eluted by aggregating the spheres with the magnet holder, allowing 10 µL of the eluate to be recovered in a new tube. All eluted samples were quantified using the Qubit 2.0 Fluorometer (Thermo Scientific^{TM}, USA), and all barcoded libraries were pooled in the required ratios to a total of 50-100 fmoles in 10 µL of Tris-HCl (10 mM, pH 8.0) NaCl (50 mM). Finally, 1 µL of Rapid Adapter was added into the mix, and all tubes were incubated for 5 min. at room temperature.

3.11.2. Sequencing

Sequencing was done using a SpotON Flow Cell and a Flow Cell Priming Kit (Oxford Nanopore Technologies, UK), according to the manufacturer's instructions on the MinION MK1C device from Oxford Nanopore Technologies (UK).

3.11.3. Data Acquisition and Analysis

Live base calling was performed on MinION Mk1C with MinKNOW base calling whilst sequencing and running a fast base calling model and read filtering with a minimal score of 8. The base called Fastq files were uploaded to the EPIME platform via the software EPI2MEAgent (version 3.5.6) and 16S alignment was selected and the analysis results were collected. Assignment of the reads to their appropriate taxonomic group was

performed with the NCBI 16S database (Federhen, 2012; Coordinators, 2016). Taxa with low abundance (<0.01% of total reads) were not included in the analysis.

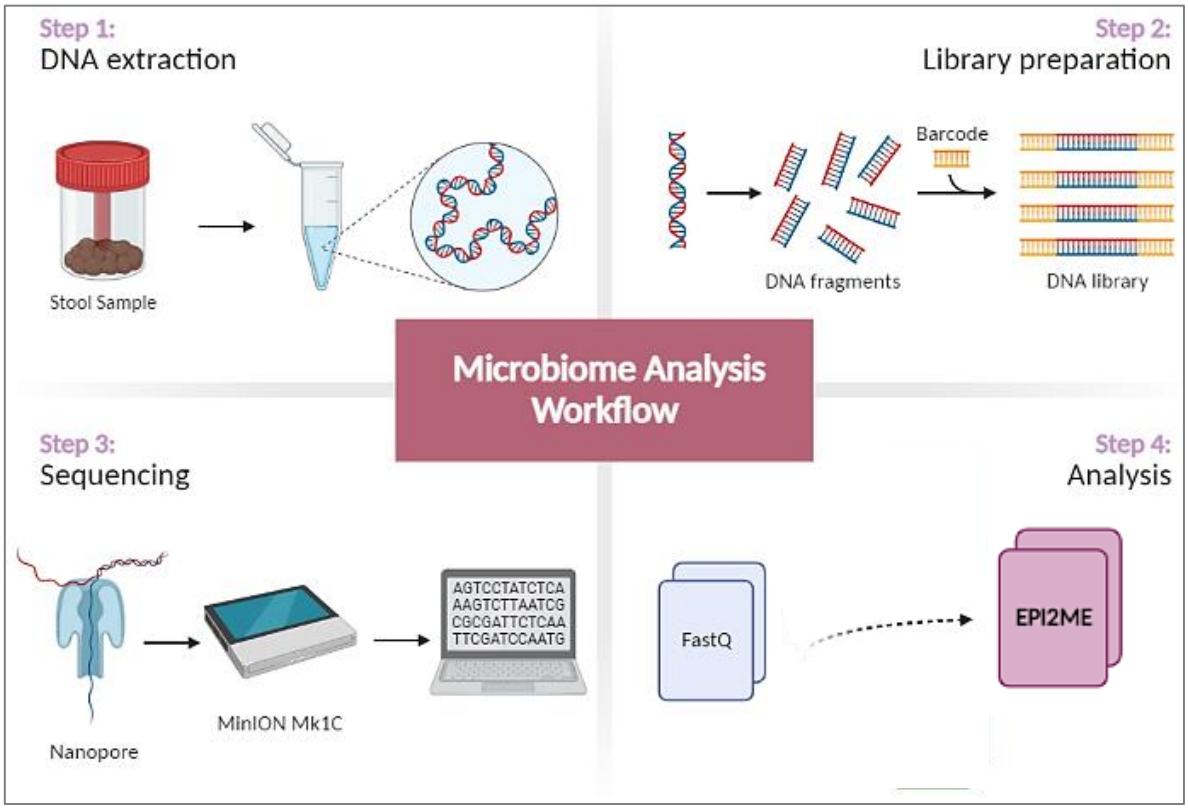
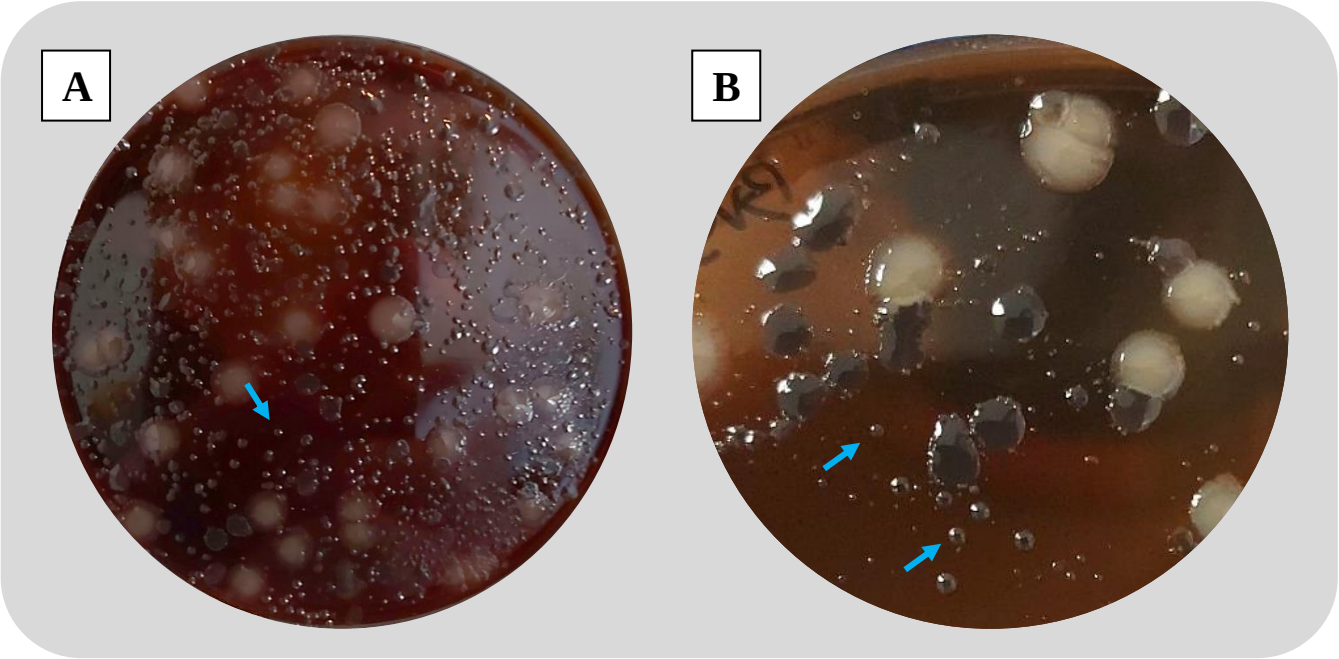


Figure 3.11.1. Schematic of the bacteriome analysis workflow performed in the current study. Image created resorting to BioRender (biorender.com).

4. Results

4.1. Isolation and Identification of Bacteroides spp.

As mentioned previously uneven reports about the abundance and diversity of *Bacteroides* spp. in the bacteriome of elderly people have been published over time. One of our objectives was to recover and identify the culturable species of the *Bacteroides* genus from the gut of healthy and non-healthy old individuals from Algarve, corroborating the abundance and diversity of the strains in their gut using a culture-independent approach. The isolation of *Bacteroides* spp. was performed by plating the selected dilutions of faecal samples in BVSA medium and picking the characteristic colonies from each sample. **Figure 4.1.1. A, B** illustrates a representative plate of BVSA with potential *Bacteroides* colonies (brown-black surrounded by a dark halo due to esculin hydrolysis). Other uncharacteristic colonies, which vary in size, colour, shape, edge pattern and degree of esculin hydrolysis can be observed. Representative colonies of *Bacteroides* spp. grown in BHI+H are depicted in **Figure 4.1.1. C, D.**



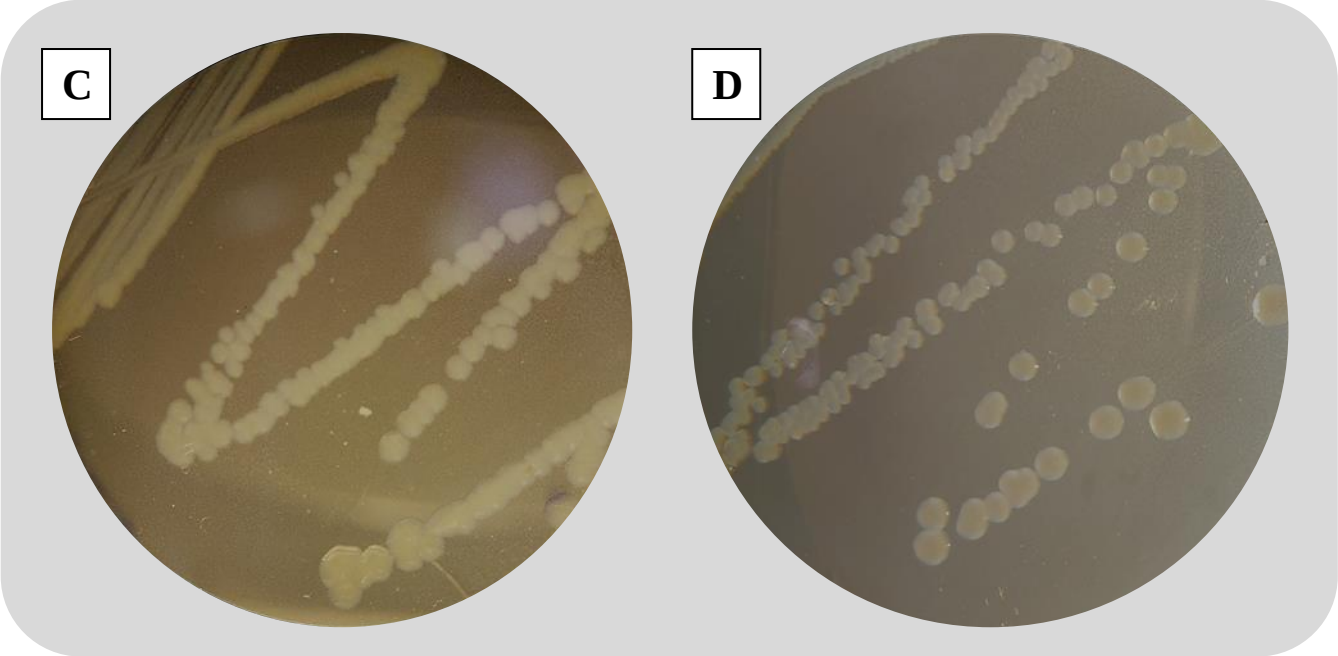


Figure 4.1.1. BVSA plates with characteristic colonies of *Bacteroides* spp. from faecal samples. Potential *Bacteroides* colonies are indicated with blue arrows. Plate A) 10^{-4} dilution and B) Section of a plate inoculated with the 10^{-5} dilution C) Isolate MHB PEB2 identified as B. fragilis in BHI+H D) Isolate FHB6 identified as B. caccae in BHI+H.

The counts of *Bacteroides* spp. (Log_{10} CFU/g faeces) are indicated in **Table 4.1.1**. The healthy group showed an amount of *Bacteroides* spp. of 6.92 ± 0.34 Log_{10} CFU/g faeces, in contrast, the non-healthy group showed an amount of 7.19 ± 0.28 Log_{10} CFU/g faeces.

In the healthy group, the sample that showed the highest number of *Bacteroides* spp. was MHA (7.43 ± 1.00 Log10 CFU/g faeces) whereas the MHB sample showed the lowest counts (6.30 ± 0.15 Log10 CFU/g faeces). In the non-healthy group, sample MNB had the highest number of *Bacteroides* (7.60 ± 0.15 Log10 CFU/g faeces), whereas the sample FNB showed the lowest number with 6.95 ± 1.03 Log10 CFU/g faeces. No significant differences ($P>0.05$) were observed between the counts of *Bacteroides* spp. in the healthy and non-healthy groups.

Table 4.1.1. Determination of *Bacteroides* spp. counts in faecal samples of healthy and non-healthy elderly individuals.

Healthy		Non-Healthy	
Sample	**Log10 CFU/g**	**Sample**	**Log10 CFU/g**
MHA	7.43 ± 1.00	S1	7.09 ± 0.75
MHB	6.30 ± 0.15	MNA	6.95 ± 1.15
FHA	6.82 ± 1.15	MNB	7.60 ± 0.15
FHB	6.90 ± 0.45	FNA	7.38 ± 0.55
FHC	7.11 ± 0.93	FNB	6.95 ± 1.03
FHD	6.90 ± 0.63		
FHF	6.97 ± 0.45		

Mean Log10 CFU/g	**Healthy**	**Non-Healthy**
	6.92 ± 0.34[a]	7.19 ± 0.28[a]

a - values not significantly different (P>0.05) as determined by ANOVA with the SPSS software.

The first step in the identification process was to perform a Gram stain, and the Gram-negative representatives of characteristic colonies were processed for identification by sequencing the *16S rRNA* gene. Gram-negative isolates from uncharacteristic representative colonies were previously identified using the RapID ONE system (Thermo Fisher Scientific, USA).

The *16S rRNA* gene sequences were analysed and those from 750-1200 bp were selected for analysis using the National Center for Biotechnology Information (NCBI) Blastn (https://www.ncbi.nlm.nih.gov/) and also the Ribosomal Database Project (RDP), (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). The results collected from NCBI Blastn and RDP Sequence Match databases are summarized in **Table 4.1.2**. In total, 20 bacterial isolates were identified from male samples, 19 using *16S rRNA* gene sequencing (**Table 4.1.2.**), 6 belonging to the *Bacteroidaceae* family (genus *Bacteroides* and *Phocaeicola*) and 2 belonging to the order *Bacteroidales* (genus *Parabacteroides* and *Dysgonomonas*). The remaining 11 (**Table 4.1.2.**), include the one identified using the RapID ONE system (**Table 4.1.3.**) that belongs to the *Enterobacteriaceae* family. Fourteen isolates were recovered from female faecal

samples. The identification performed by sequencing the *16S rRNA* gene on ten isolates (**Table 4.1.2.**) showed that 9 belong to the *Bacteroidaceae* family (genus *Bacteroides* and *Phocaeicola*) and 1 isolate belongs to the *Bacteroidales* order (genus *Parabacteroides*). The remaining 4 isolates were identified using the RapID ONE system, and all belong to the *Enterobacteriaceae* family (**Table 4.1.3.**).

Table 4.1.2. Results of the identification of the bacterial isolates by sequencing the *16S rRNA* gene.

Isolate	NCBI[a]	RDP[b]	
	Species	Genus	Species
S1 GBL1	*Escherichia coli*	*Escherichia/Shigella*	Uncultured Bacterium
	Uncultured *Shigella*		Uncultured Bacterium
	Escherichia coli		Uncultured Organism
	Cronobacter sakazakii		Uncultured Organism
	Escherichia coli		Uncultured Organism
S1 GBL2	*Escherichia fergusonii*	*Escherichia/Shigella*	*Escherichia fergusonii*
	Escherichia coli O157:H7		*Escherichia fergusonii*
	Escherichia coli O157:H7		*Escherichia fergusonii*
	Escherichia coli O157:H7		*Escherichia fergusonii*
	Escherichia coli O157:H7		Uncultured Bacterium
S1 GYR1	*Bacteroides koreensis*	*Bacteroides*	Uncultured Organism
	Uncultured organism clone		Uncultured Organism
	Uncultured organism clone		Uncultured Organism
	Uncultured organism clone		Uncultured Organism
	Uncultured organism clone		Uncultured Organism
S1 MYR1	Uncultured bacterium	*Parabacteroides*	Uncultured Bacterium
	Parabacteroides distasonis		Uncultured Organism
	Parabacteroides distasonis		*Parabacteroides distasonis*
	Parabacteroides sp.		Uncultured Organism
	Uncultured *Parabacteroides*		Uncultured Organism
S1 MBR1	Uncultured organism clone	*Escherichia/Shigella*	Uncultured Organism
	Uncultured organism clone		Uncultured Organism
	Uncultured organism clone		Uncultured Organism
	Uncultured organism clone		Uncultured Organism
	Escherichia coli		*Escherichia coli*
S1 PBR1	Uncultured organism clone	*Escherichia/Shigella*	Uncultured Organism
	Escherichia coli		*Escherichia coli*
	Shigella flexneri		*Escherichia coli*
	Escherichia coli		*Escherichia coli*
	Escherichia coli		Uncultured Organism
MHA GEHL	*Escherichia* spp.	*Escherichia/Shigella*	*Escherichia coli*
	Escherichia spp.		*Escherichia coli*
	Escherichia spp.		*Escherichia coli*
	Escherichia coli		*Enterobacteriaceae* bacterium
	Escherichia spp.		*Escherichia coli*
MHA GCEL	*Escherichia* spp.	*Escherichia/Shigella*	Uncultured Bacterium
	Escherichia spp.		Uncultured Organism
	Escherichia spp.		*Escherichia coli*
	Escherichia coli		*Escherichia coli*
	Escherichia spp.		*Escherichia coli*
MHB GCL	Uncultured bacterium clone	*Escherichia/Shigella*	*Escherichia coli*
	Escherichia coli		*Escherichia coli*
	Escherichia coli		*Cronobacter sakazakii*
	Escherichia coli		Uncultured *Escherichia* sp.
	Cronobacter sakasakii		Bacterium mkk1

The results are ordered from the higher to the lower values of similarity. The similarity score values are indicated in Table S1, Appendix 1.

[a] First five results for the closest known species, collected using Blastn software.

[b] First five results for the closest known species obtained with Ribosomal Database Project Sequence Match software.

Table 4.1.2. (continued).

Isolate	NCBI[a]	RDP[b]	
	Species	Genus	Species
MHB PEB1	Uncultured organism Uncultured organism Uncultured organism Uncultured organism Uncultured organism	*Dysgonomonas*	Uncultured organism Uncultured organism Uncultured organism Uncultured organism Uncultured organism
MHB PEB2	*Bacteroides fragilis* *Bacteroides fragilis* *Bacteroides fragilis* *Bacteroides fragilis* Uncultured *Bacteroides* spp.	*Bacteroides*	Uncultured organism Uncultured bacterium Uncultured bacterium *Bacteroides fragilis* *Bacteroides fragilis*
MHB PEB4	Uncultured organism *Phocaeicola dorei* *Phocaeicola dorei* *Phocaeicola dorei* *Phocaeicola dorei*	*Phocaeicola*	Uncultured bacterium Uncultured *Bacteroidetes* bacterium Uncultured *Bacteroidetes* bacterium Uncultured bacterium Uncultured bacterium
MHB MEB3	Uncultured bacterium Uncultured organism Uncultured organism Uncultured organism Uncultured organism	*Phocaeicola*	Uncultured organism Uncultured organism Uncultured organism Uncultured bacterium Uncultured bacterium
MNA MCL1	*Escherichia coli* *Escherichia coli* Bacterium EM-2014-85 *Escherichia marmotae* *Escherichia coli*	*Escherichia/Shigella*	Bacterium clone *Escherichia coli* Uncultured bacterium Uncultured bacterium *Escherichia coli*
MNA MCL2	*Shigella flexneri* Uncultured organism Uncultured organism *Escherichia coli* *Escherichia coli*	*Escherichia/Shigella*	Uncultured organism Uncultured organism Uncultured organism Uncultured bacterium Uncultured organism
MNA PEB1	*Escherichia* spp. *Escherichia coli* *Cronobacter sakasakii* *Escherichia coli* *Cronobacter sakasakii*	*Escherichia/Shigella*	Uncultured bacterium Uncultured *Shigella* *Escherichia coli* *Shigella sonnei* Uncultured *Shigella*
MNA PEB3	*Phocaeicola dorei* *Phocaeicola dorei* *Phocaeicola dorei* *Phocaeicola dorei* *Phocaeicola dorei*	*Phocaeicola*	Uncultured bacterium Uncultured bacterium Uncultured organism Uncultured organism Uncultured organism
MNA MEB3	Uncultured organism *Bacteroides caccae* *Bacteroides caccae* *Bacteroides caccae* *Bacteroides* spp.	*Bacteroides*	Uncultured organism Uncultured bacterium Uncultured bacterium Uncultured bacterium Uncultured organism
MNB GEL	*Klebsiella* spp. *Klebsiella pneumoniae* *Klebsiella pneumoniae* *Klebsiella pneumoniae* *Klebsiella pneumoniae*	*Klebsiella*	*Klebsiella pneumoniae* Uncultured bacterium *Klebsiella pneumoniae* Uncultured bacterium *Klebsiella pneumoniae*

Table 4.1.2. (continued).

Isolate	NCBI[a]	RDP[b]	
	Species	Genus	Species
FHA HEHP1	Uncultured organism	*Bacteroides*	Uncultured organism
	Uncultured organism		Uncultured organism
	Uncultured organism		Uncultured organism
	Uncultured organism		Uncultured organism
	Uncultured organism		Uncultured organism
FHA HEHC2	*Bacteroides ovatus*	*Bacteroides*	*Bacteroides* spp.
	Bacteroides koreensis		Uncultured bacterium
	Bacteroides ovatus		Uncultured organism
	Bacteroides ovatus		Uncultured organism
	Bacteroides ovatus		Uncultured organism
FHB2	Uncultured bacterium	*Parabacteroides*	Uncultured bacterium
	Uncultured bacterium		Uncultured bacterium
	Uncultured bacterium		Uncultured organism
	Uncultured organism		Uncultured organism
	Uncultured organism		Uncultured organism
FHB5	*Bacteroides koreensis*	*Bacteroides*	*Bacteroides ovatus*
	Bacteroides ovatus		Uncultured organism
	Bacteroides ovatus		Uncultured organism
	Bacteroides ovatus		Uncultured organism
	Bacteroides koreensis		*Bacteroides* spp.
FHB6	*Bacteroides caccae*	*Bacteroides*	Uncultured bacterium
	Bacteroides caccae		Uncultured bacterium
	Bacteroides caccae		Uncultured bacterium
	Bacteroides spp.		Uncultured bacterium
	Bacteroides spp.		Uncultured bacterium
FHD PEB3	Uncultured organism	*Phocaeicola*	Uncultured organism
	Uncultured organism		Uncultured organism
	Uncultured organism		Uncultured organism
	Uncultured *Bacteroides* spp.		Uncultured organism
	Uncultured organism		Uncultured organism
FNA GEL	*Bacteroides koreensis*	*Bacteroides*	Uncultured organism
	Bacteroides ovatus		Uncultured organism
	Bacteroides ovatus		Uncultured bacterium
	Bacteroides ovatus		Uncultured bacterium
	Bacteroides ovatus		Uncultured bacterium
FNB PEB4	*Bacteroides clarus*	*Bacteroides*	*Bacteroides clarus*
	Bacteroides clarus		*Bacteroides clarus*
	Bacteroides clarus		Uncultured bacterium
	Uncultured bacterium clone		Uncultured bacterium
	Uncultured bacterium clone		Uncultured bacterium
FNB MEB4	*Bacteroides dorei*	*Phocaeicola*	Uncultured bacterium
	Bacteroides dorei		Uncultured organism
	Phocaeicola dorei		Uncultured organism
	Phocaeicola dorei		Uncultured organism
	Phocaeicola dorei		Uncultured organism
FNB MEBH1	Uncultured organism	*Bacteroides*	Uncultured organism
	Uncultured organism		Uncultured organism
	Uncultured organism		*Bacteroides nordii*
	Uncultured organism		*Bacteroides thetaiotaomicron*
	Uncultured organism		Bacterium NLAE-zl-C182

Table 4.1.3. Identification of isolates using the RapID ONE.

Isolate	RapID ONE
FHA GCL	*Providencia alcalifaciens*
FHC MEB4	*Moellerella wisconsensis*
FHD PEB1	*Shigella* spp.
FHF PEBH1	*Shigella* spp.
MNB GCL	*Salmonella choleraesuis*

The distribution of culturable *Bacteroides* spp. recovered from the faecal samples of healthy and non-healthy old individuals are shown in **Figure 4.1.2**. The percentage of isolates in healthy individuals identified as *P. dorei* achieved 7%, in contrast with non-healthy individuals for which this percentage reached near the double, 13%. Interesting, the specie *B. fragilis* was only recovered from healthy individuals. The specie *B. ovatus* was also recovered in a higher number (15%) from healthy individuals whereas from non-healthy only 6% was retrieved. The specie *B. clarus* was only recovered from non-healthy individuals. In summary, the diversity of the *Bacteroides* species was higher in healthy individuals in comparison with non-healthy old adults (**Figure 4.1.2.**). It is also important to highlight that the "other genera" correspond to the isolates that belonged to the *Enterobacteriaceae* family identified by sequencing the *16S rRNA* gene.

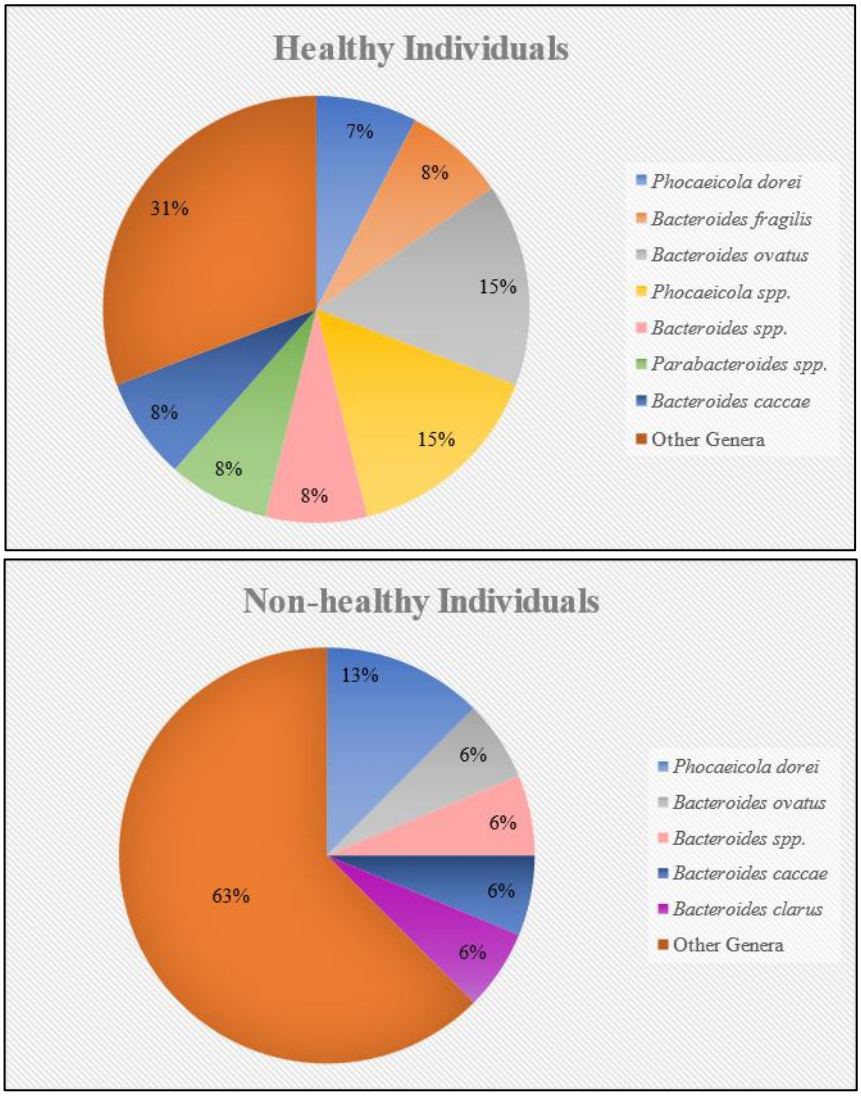


Figure 4.1.2. Diversity of *Bacteroides* spp. in faecal samples of healthy and non-healthy elderly individuals, according to the *16S rRNA* gene sequencing. "Other genera" include all isolates identified that belong to the *Enterobacteriaceae* family.

4.2. Antibiotic Susceptibility

Due to the high recovery of the *Enterobacteriaceae* isolates besides the supplementation of the culture medium with colistin, the antibiotic susceptibility of several of those isolates, namely the isolates S1 GBL1, S1 GBL2, MHA GCEL and MHA GEHL (identified as *E. coli*) was determined. Regarding the Kirby-Bauer test, the four isolates showed a resistant profile to the antibiotic clindamycin and a susceptible profile to moxifloxacin and imipenem. The inhibition zones are indicated in Table S2, appendix 1. The four *E. coli* isolates were able to grow at all colistin concentrations tested; 0.015 mg/L, 0.03 mg/L and 0.06 mg/L. However, they cannot be considered resistant since the resistant profile is observed when the MIC value is equal to 2 mg/L (EUCAST, 2022).

4.3. Microbiome Analysis

4.3.1. Individual Profile

All faecal DNA samples from the enrolled old individuals were sequenced using the Oxford Nanopore system and recorded by the MinKNOW software with further analysis using the EPI2ME platform, allowing the establishment of each profile. **Figure 4.3.1.** shows the percentages of the main phyla on each faecal sample.

The phyla *Firmicutes, Bacteroidetes* and *Proteobacteria* are observed in all samples, with the *Firmicutes* percentage being the highest in all samples except one, MNB, in which *Bacteroidetes* surpassed the phylum *Firmicutes*. The percentage of reads that belong to the phylum *Proteobacteria* varied between the samples, ranging between 1% and 24%.

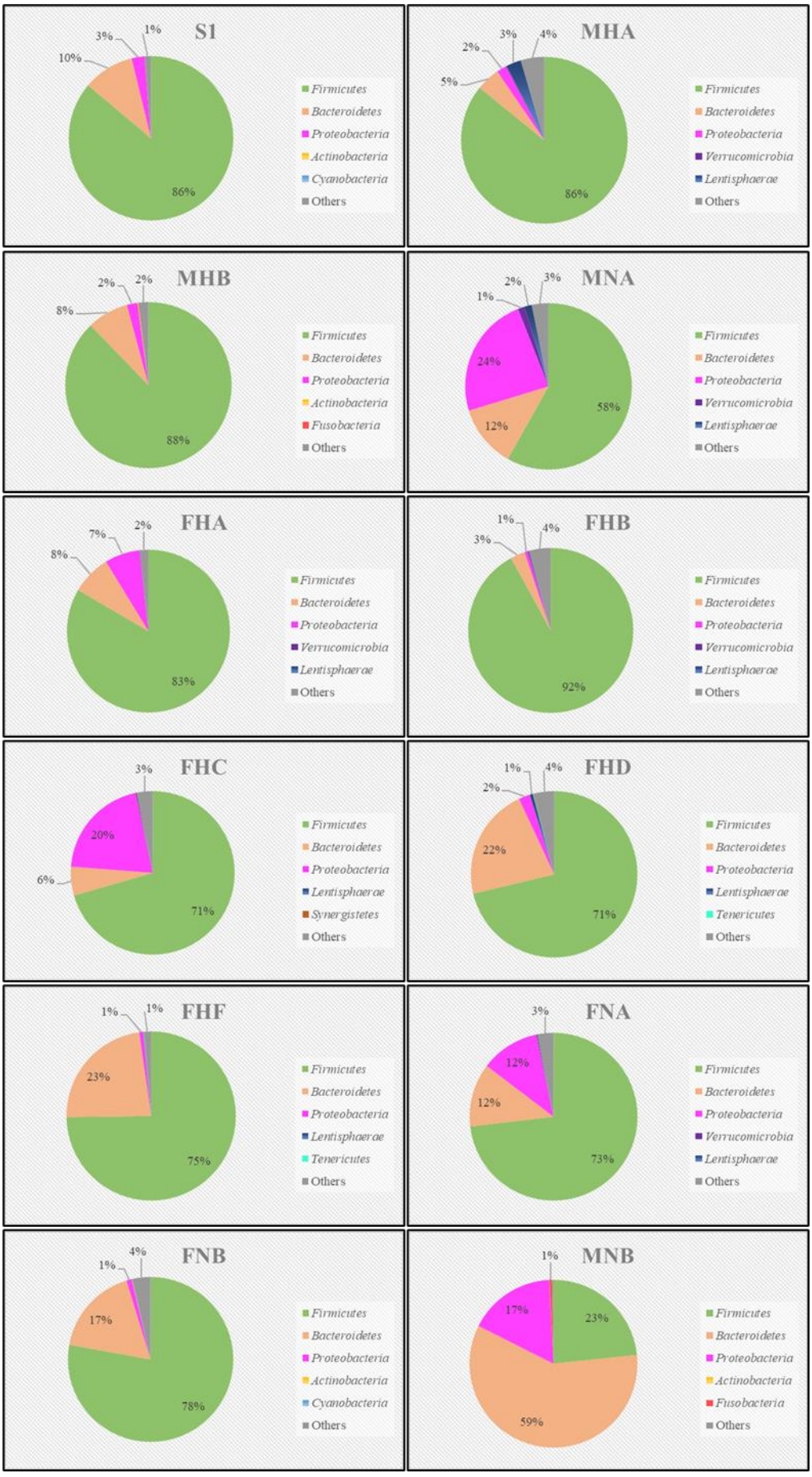
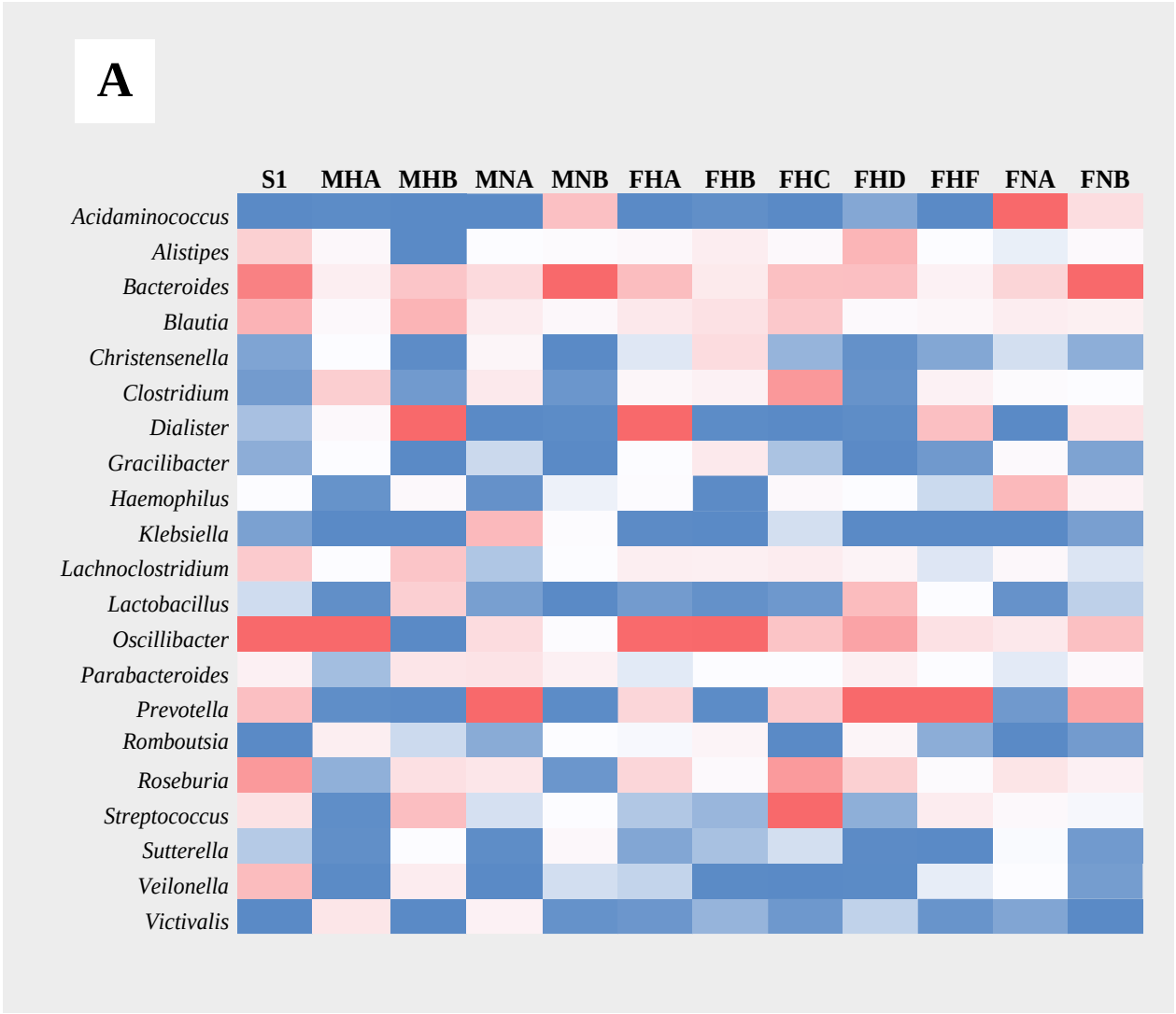


Figure 4.3.1. The five most abundant phyla in the analysed faecal samples of old adults from Algarve region, Portugal.

The heat map **(Figure 4.3.2.A, B)** shows the relative genus and species abundance (%) of classified reads on each faecal sample. *Bacteroides* and *Oscillibacter* were the two genera with higher representation in all samples (**Figure 4.3.2.A**), contrarily, *Christensenella, Gracilibacter, Klebsiella, Sutterella* and *Veilonella* had lower values in most samples (**Figure 4.3.2.A**). At the species level, *Oscillibacter valericigenes* is present in high values in all samples except MHB (**Figure 4.3.2.B**).



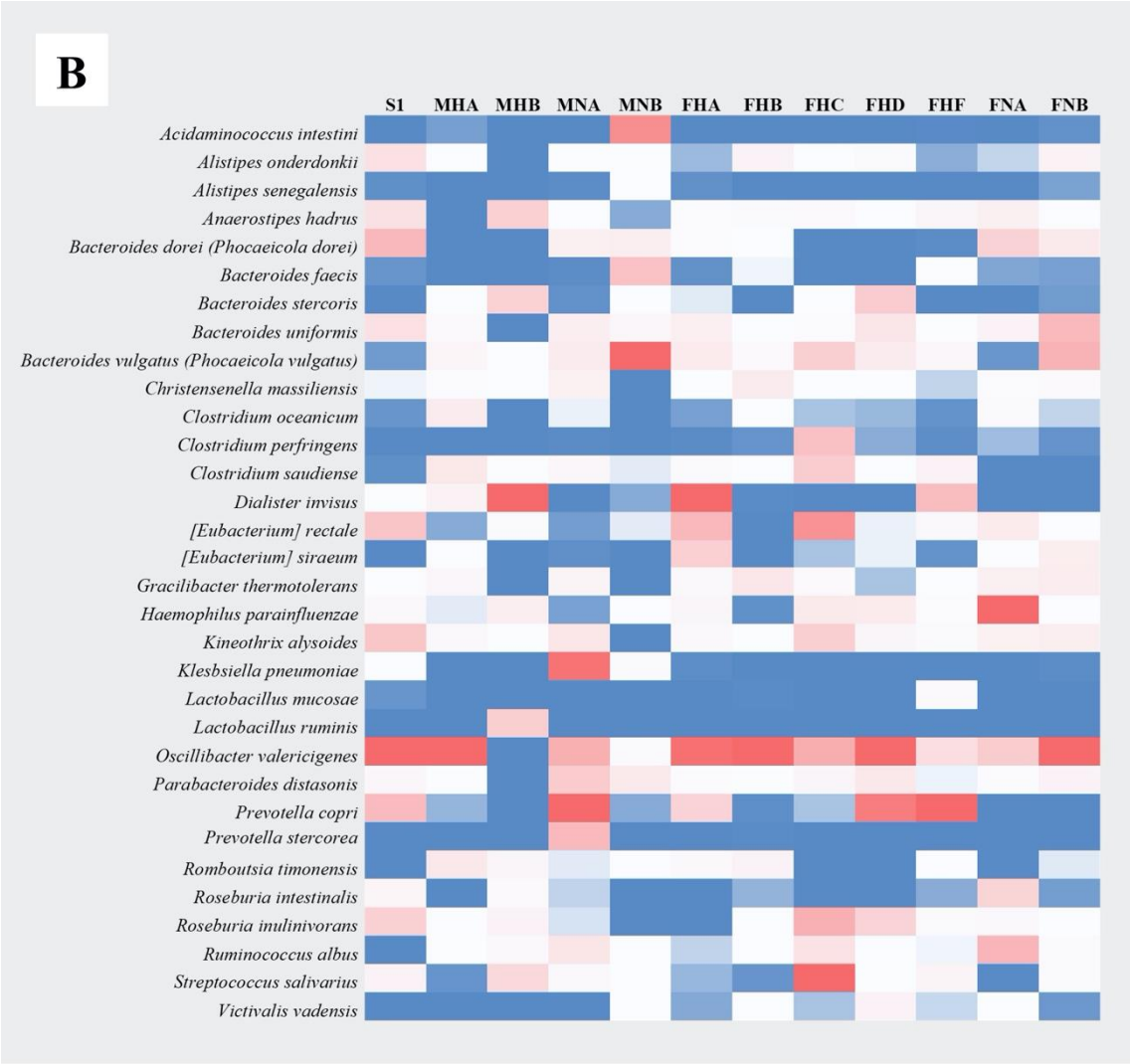


Figure 4.3.2. A heat map shows A) the relative genus abundance (%) of classified reads, B) the relative species abundance (%) of classified reads. The 21 most abundant taxa are shown. Range of values goes from dark blue (lower values) to dark red (higher values). Species in parentheses indicate the new classification (Gárcia-López et al., 2019).

4.3.2. Patterns within Age, Gender, and Health Status

We attempted to find faecal microbiome patterns between individuals of the same gender and age. For this evaluation, data from 3 healthy females (FHA, FHD, FHF) with 71, 71 and 72 years of age, respectively, were compared. FHA sample possesses, at the phylum level the highest percentage of *Firmicutes*, 83% followed by the sample FHF with 75% and the sample FHD with 71 %. Regarding the phylum *Bacteroidetes* the sample FHF showed the highest percentage reaching 23% followed by the sample FHD with 22%, and the sample FHA showed the lowest percentage, 8%. In contrast, the third more abundant

phylum, *Proteobacteria* was at the highest percentage in the sample FHA (8%) whereas the samples FHD and FHF showed low percentages, namely 2 and 1%, respectively (**Figure 4.3.1.**). Noteworthy, the phylum *Lentisphaerae* is present in the three samples, although the percentage value is lower than 1% (but higher than 0.5%).

Regarding the most abundant genera and species, *Prevotella copri* and *Oscillibacter valericigenes* are common to the three faecal samples. *Dialister invisus* is common to the samples FHF and FHA (**Figure 4.3.2.B**).

Interesting, the non-healthy female with 70 years old (FNB) showed similar abundances of *Firmicutes, Bacteroidetes* and *Proteobacteria* with the FHD and FHF samples (78%, 17%, 1%, respectively) (**Figure 4.3.1.**). However, members of the *Lentisphaerae* were not observed in the FNB faecal sample, instead, *Actinobacteria* were noticed. This non-healthy female showed high levels of *Bacteroides* and *Prevotella* in comparison with the healthy ones (**Figure 4.3.2.A**).

4.3.3. Gender on Gut Microbiota Composition

Gender-related differences in gut microbiota composition have been reported over time. Thus, the analysed faecal samples were grouped by gender and the microbiome patterns based on phyla, genera and species in both genders were examined. **Figure 4.3.3.** shows the percentages of the 5 most abundant phyla in male and female faecal samples and **Figure 4.3.4.** and **Figure 4.3.5.** show the phylogenetic representation of the most abundant genera and species, respectively.

The four most abundant phyla in both male and female groups were *Firmicutes, Bacteroidetes, Proteobacteria* and *Lentisphaerae*. The fifth phylum most abundant in the male gender was *Fusobacteria* whereas in females was the *Tenericutes*. Distinct percentages of the phyla *Firmicutes, Bacteroidetes, and Proteobacteria* between male and female gender were observed, namely the ratio *Firmicutes*/*Bacteroidetes* is very different between the two genders; in males, the ratio is equal to 1, in contrast with the female group that shows a ratio of 5 (**Figure 4.3.3.**). The phylum of *Proteobacteria* is much higher in males reaching 17%, whereas in the female group the percentage is 4% (**Figure 4.3.3.**).

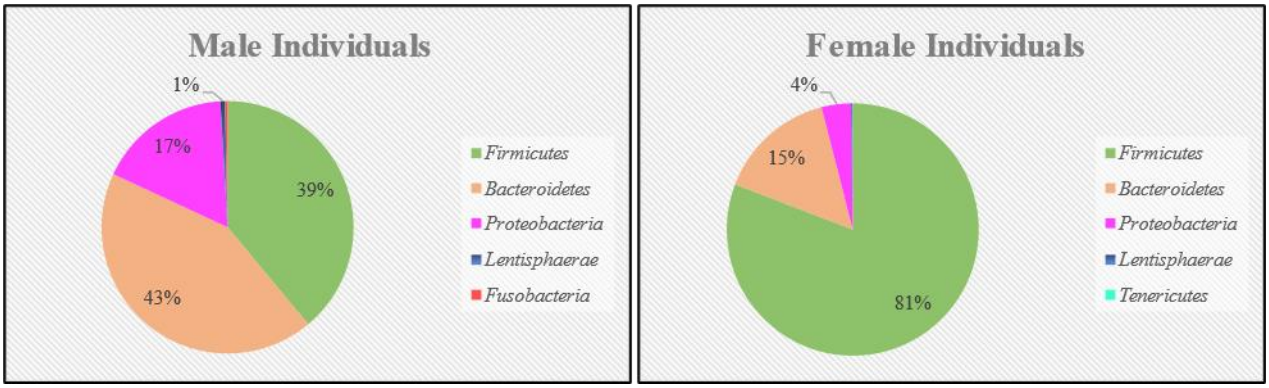


Figure 4.3.3. The five most abundant phyla in male and female faecal samples. Percentage values less than 1% were not included in the analysis.

Regarding the identified genera males showed a higher abundance of the genus *Bacteroides*, particularly *Parabacteroides* which was not detected in females (**Figure 4.3.4. A, B**). Several genera were only observed in males, namely *Lachnoclostridium*, *Clostridium*, *Acidaminococcus*, *Klebsiella* and *Sutterella* (**Figure 4.3.4.A**). The female group showed also unique genera, namely the genus *Haemophilus, Ruminococcus* and *Dialister* (**Figure 4.3.4.B**).

The species of *Bacteroides* are much more diverse in the male group in comparison with the female group (**Figure 4.3.5. A, B**), and *Bacteroides vulgatus* showed to be the more abundant (**Figure 4.3.5.A**) The species *O. valericigenes* and *P. copri* were common in both groups, but the abundance of *P. copri* was higher in the female group (**Figure 4.3.5. A, B**). The species *A. intestini*, *P. distasonis*, and *K. pneumoniae* were only identified in the male group, whereas *H. parainfluenzae* was only observed in the female group. (**Figure 4.3.5. A, B**).

Figure 4.3.4. Phylogenetic representation of the genera identified in A) male and B) female faecal samples. Relative abundance is represented by the thickness of each branch. Minimum abundance cut off 0.5%. Data obtained with the EPI2ME platform.

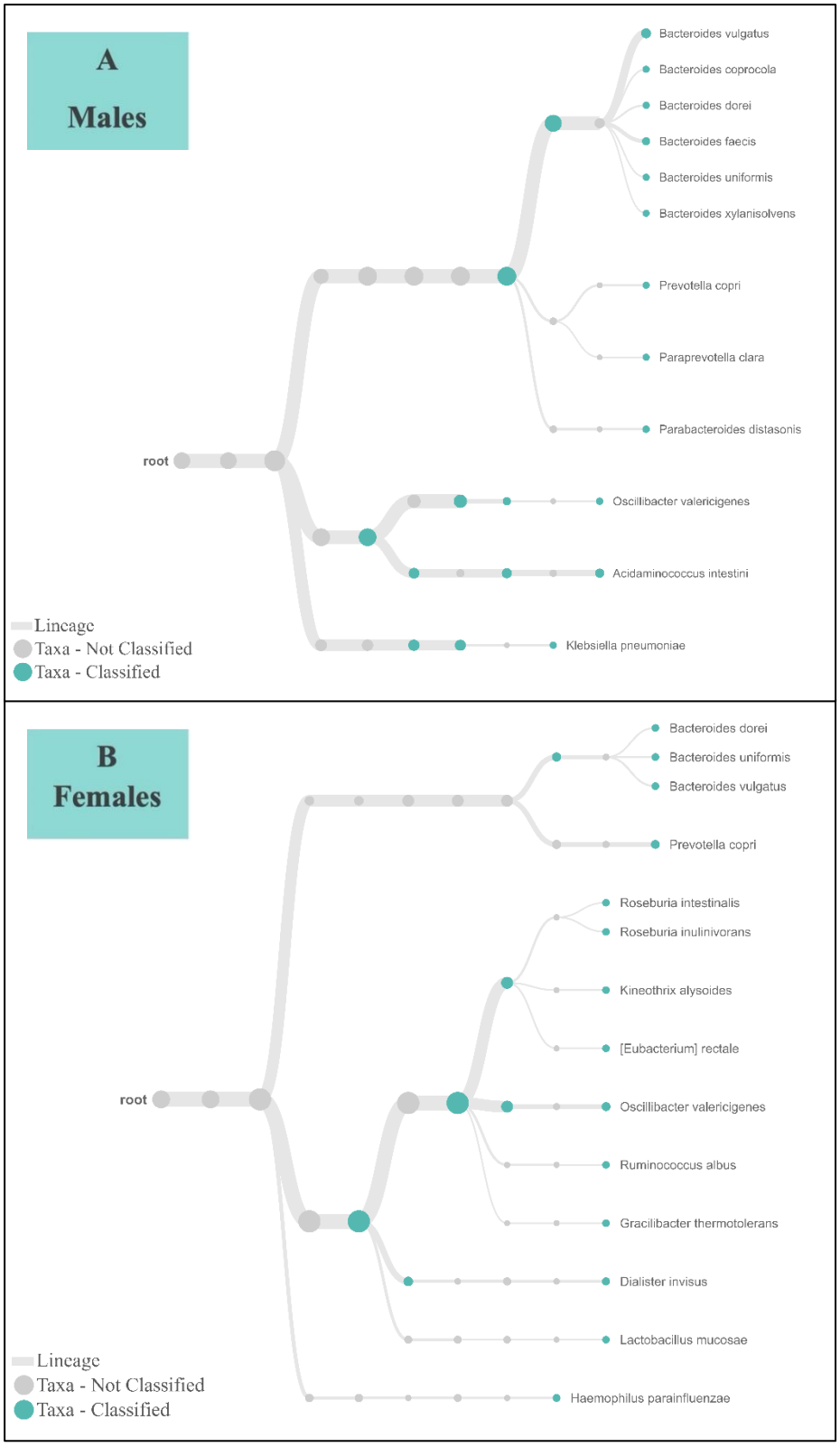


Figure 4.3.5. Phylogenetic representation of the species identified in A) male and B) female faecal samples. Relative abundance is represented by the thickness of each branch. Minimum abundance cut off 0.5%. Data obtained with the EPI2ME platform.

4.3.4. State of Health on Gut Microbiota Composition

As our primary objective was to profile the gut microbiota in healthy and non-healthy older adults, the faecal samples were grouped according to their health status, 7 faecal samples were allocated to healthy and 5 to non-healthy, and their intestinal microbiome patterns related to bacterial phyla, genera, and species were examined.

The 5 most abundant phyla in each group are illustrated in **Figure 4.3.6**. Non-healthy old individuals showed a ratio of *Firmicutes/Bacteroidetes* of 2, whereas for the healthy group this ratio was 5. The non-healthy group also showed a higher percentage (12%) of the phylum *Proteobacteria*, in comparison with the healthy group which showed 2%. *Verrucomicrobia* was the fourth most abundant phylum in the non-healthy group, whereas *Lentisphaerae* was the fourth most abundant phylum in the healthy group. The two groups also showed a difference in the fifth most abundant phylum, namely in the non-healthy was identified the phylum *Lentisphaerae* and in the healthy group was identified the phylum *Tenericutes*.

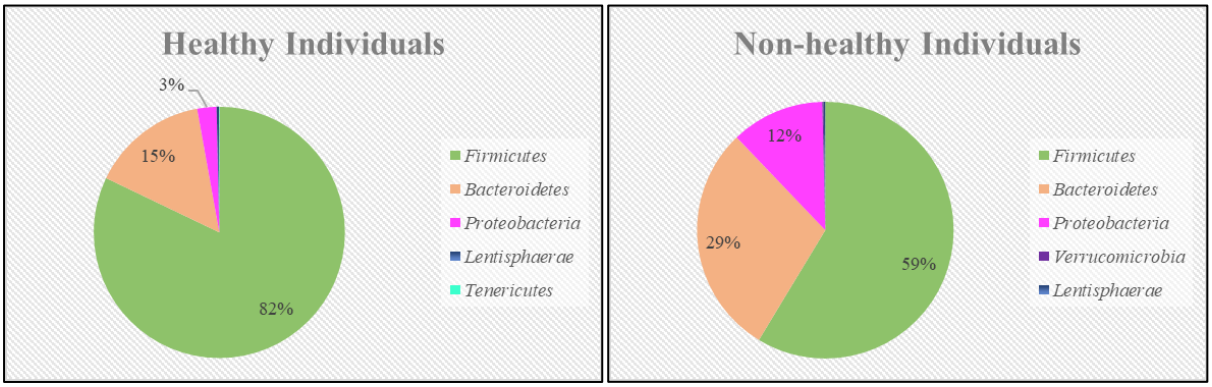


Figure 4.3.6. The five most abundant phyla in healthy and non-healthy faecal samples. Data provided by the EPI2ME platform. Percentage values less than 1% were not included in the analysis.

The analysis of the abundance of the bacterial genera identified in the non-healthy groups showed that the members of the *Bacteroides* genus were more abundant reaching 79 743 cumulative reads followed by the genus *Acidaminococcus* and *Oscillibacter* with 22 427 and 19 885 cumulative reads, The genus *Kineothrix* of the *Lachnospiraceae* family and the genus *Klebsiella* of the *Enterobacteriaceae* family were only observed in the non-

healthy group reaching 3 593 and 3126 cumulative reads, respectively (**Figure 4.3.7.A**). In the healthy group, the genus *Prevotella* showed to be the most abundant (43 111 cumulative reads), followed by the genus *Oscillibacter* (27 955 cumulative reads). The genus *Bacteroides* and *Dialister* also reached a high number of reads (11 400 and 19 992 cumulative reads, respectively). The genus *Lactobacillus* and *Streptococcus* were only enriched in the healthy group achieving 5 481 and 5 477 cumulative reads, respectively (**Figure 4.3.7.B**).

At the species level, in the non-healthy group, *B. vulgatus* (*P. vulgatus*) reached the highest cumulative reads (35 804) followed by *A. intestini* and *O. valericigenes* that reached 22 274 and 19 555 cumulative reads, respectively (**Figure 4.3.8.A**). *Bacteroides faecis* also reached a significant number of cumulative reads, 12 069. The unique species in this group the *K. alysoides* and *K. pneumoniae* reached quite close cumulative reads, 3 593 and 2 888, respectively (**Figure 4.3.8.A**).

In the healthy elderly *Prevotella copri* was the most enriched species (39 007 cumulative reads) followed by *O. valericigenes* with 27 317 cumulative reads. *Dialister invisus* was the third and most abundant specie, reaching 19 971 cumulative reads (**Figure 4.3.8.B**). The unique species in the healthy group, *Ruminococcus albus* and *L. mucosae* reached 5 679 and 4 478 cumulative reads, respectively. The other two unique species *Roseburia intestinalis* and *R. inulinivorans* reached 3 716 and 3 172 cumulative reads, respectively (**Figure 4.3.8.B**). Interestingly the common species in both groups, the *O. valericigenes* was slightly higher in the healthy group (**Figure 4.3.8. A, B**).

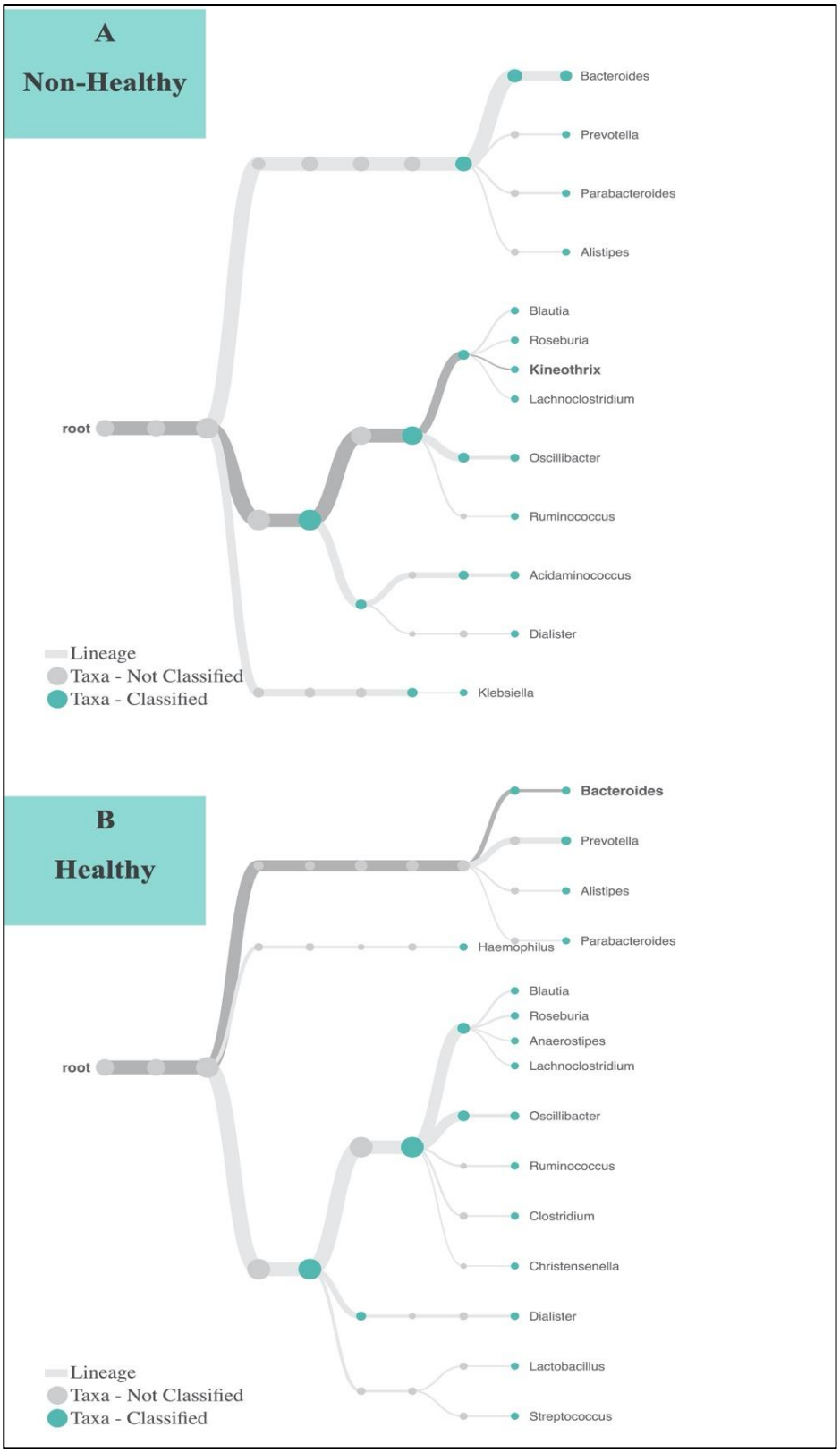


Figure 4.3.7. Phylogenetic representation of the genera identified in A) non-healthy and B) healthy faecal samples. Relative abundance is represented by the thickness of each branch. Minimum abundance cut off 0.5%. Data obtained with the EPI2ME platform.

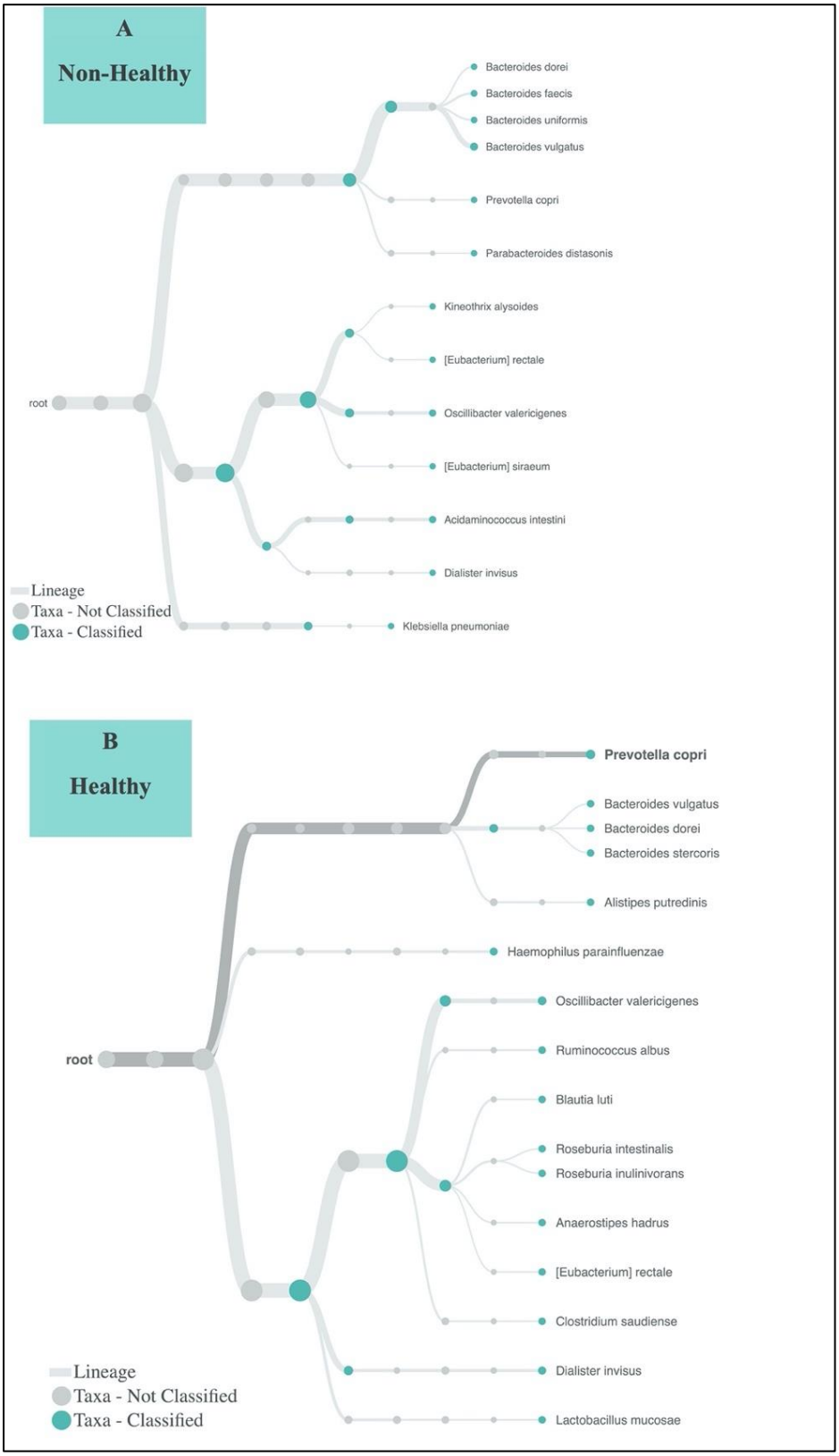


Figure 4.3.8. Phylogenetic representation of the species identified in A) non-healthy and B) healthy faecal samples. Relative abundance is represented by the thickness of each branch. Minimum abundance cut off 0.5%. Data obtained with the EPI2ME platform.

4.4. Representativeness of the Recovered Bacterial Isolates including *Bacteroides* spp. from the Faecal Samples in the Microbiome

The results obtained from the isolation and identification of *Bacteroides* spp. from each sample with their representativeness in the intestinal microbiome are summarized in **Table 4.4.1**. Interesting several bacterial isolates were not identified in the microbiome but it is assumed that they are included in their respective phylum, such as for *Proteobacteria* or *Enterobacteriaceae* family, namely *E. coli*, *Salmonella choleraesuis*, *Providencia alcalifaciens* and *Shigella* spp. Regarding the recovered isolates of the genus *Bacteroides* is evident that even those with very low percentages in the microbiome were possible to recover in culture, such as *B. koreensis* (0.002%) and *B. ovatus* (0.02 %) (**Table 4.4.1.**).

Table 4.4.1. Cultured bacterial isolates from each faecal sample and their observed percentages in the microbiome.

Sample	Identified Isolate	Microbiome (%)
S1	*Escherichia coli*	0.002
	Bacteroides koreensis	0.002
	Parabacteroides distasonis	0.27
MHA	*Escherichia coli*	*
MHB	*Escherichia coli*	*
	Bacteroides fragilis	0.57
	Dysgomonas spp.	0.14
	Phocaeicola dorei	0.30
MNA	*Escherichia coli*	0.009
	Phocaeicola dorei	0.51
	Bacteroides caccae	0.30
MNB	*Klebsiella pneumoniae*	0.36
	Salmonella choleraesuis	*
FHA	*Bacteroides* spp.	2
	Bacteroides ovatus	0.06
	Providencia alcalifaciens	*

Sample	Identified Isolate	Microbiome (%)
FHB	*Parabacteroides* spp.	0.21
	Bacteroides ovatus	0.02
	Bacteroides caccae	0.09
FHC	*Moellerella wisconsensis*	*
FHD	*Shigella* spp.	*
FHF	*Shigella* spp.	*
FNA	*Bacteroides ovatus*	0.05
FNB	*Bacteroides clarus*	0.19
	Phocaeicola dorei	1.3
	Bacteroides spp.	12

*- bacterial species not identified in the microbiome. The percentage value of the identified isolate on the microbiome was provided by the EPI2ME platform taking into account the total number of reads in the sample and the number of reads for the identified species.

5. Discussion

Ageing is a deteriorating and inevitable process that researchers around the world are trying to understand and counteract. Several factors are already described as triggering a typical ageing process, those triggered in each person, leading to the ageing of all systems and organs (Di Micco, R. *et al.*, 2021; Rossiello, F. *et al.*, 2022; Schumacher, B. *et al.*, 2021). However, nowadays, the main objective is to achieve successful ageing, an ageing process associated with minimal physiological and psychological impact. Thus, studying the associative factors between disease patterns and ageing has become an important approach to clarifying events that can be avoided by limiting pathological ageing. One of the most recently identified aspects was associated with the gut microbiota (Kong, F. et al., 2019; Kundu, P. *et al.*, 2017). The gut microbiota as a homeostatic microbial community is influenced by both the ageing process and disease, but its power to influence the host is also being clarified, and as such, trying to understand its role in the ageing process and disease could be a contribution to determine the path to successful ageing.

Establishing what is understood as a "healthy" gut microbiota composition constitutes a challenge. The gut microbiota is a volatile community influenced by intrinsic and environmental factors resulting in a unique microbial group for each person, and that fact tangles this establishment. Nevertheless, some general modulations seem to occur alongside the ageing process in the gut microbiota. Usually, there is a decrease in microbial diversity and an increase in the intestinal microbial abundance in the elderly in comparison with adulthood (Bana, B. & Cabreiro, F., 2019).

The main phyla found in the adult gut microbiota do not differ much from the ones found in the microbiota of old adults. Usually, major changes are observed in phyla proportions and species diversity (Rinninella, E. et al., 2019). Most studies evidenced that the phyla *Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia* and *Tenericutes* are the main five phyla represented in adulthood (Rinninella, E. *et al.*, 2019).

Despite the generalization, the majority of studies report differences between individuals of both genders (Haro, C. *et al.*, 2016; Valeri, F. & Endres, K., 2021; Yoon, K. & Kim, N., 2021). Accordingly, in the current study, female and male faecal samples showed different patterns at the phylum level. Both groups showed the four main phyla in common, *Firmicutes, Bacteroidetes, Proteobacteria* and *Lentisphaerae*, however, the

fifth main phylum in males was *Fusobacteria* while in the female group was *Tenericutes*. The proportion of phyla was also different between the two gender groups. Namely, the value of the *Firmicutes/Bacteroidetes* ratio is higher in the female samples. Regarding *Proteobacteria*, the proportion was higher in the male group with 15% to 4%. Thus, in the present study variations at the level of the presence of the different phylum between genders in the elderly were not noticed, instead, variations at the abundance level of these same phyla were observed. Such findings are in line with the ones reported by Valeri *et al*., (2021).

At the genus level, there are three predominant genera common to both gender groups, *Prevotella, Oscillibacter*, and *Bacteroides*, but the shared genera are in distinct amounts in each group, *Bacteroides* genus is at higher levels in the male group, while the genus *Prevotella* is at lower amounts in comparison with the female group. Although the differences in the bacterial patterns were not observed between healthy and non-healthy males, a more individual pattern was observed. In contrast with the current findings, Takagi *et al.,* (2019) reported higher levels of *Prevotella* in Japanese healthy elderly men. The other most prevalent genera that were different between the two genders were the genus *Dialister* and *Haemophilus* in females, while males showed the genus *Acidaminococcus* and *Parabacteroides*. Regarding the species pattern, it is important to highlight the diversity of *Bacteroides* species in the male group. In general, most of the bacterial species were identified in both genders. Nevertheless, amounts in the total compositions differ widely. *O. valericigene*s, the most abundant specie in the female group, is only the fourth most abundant in the male group, in the opposite position is *B. vulgatus,* which is the most abundant specie in the male group and its abundance is much lower in the female group. Moreover, the male group presents species, such as *Bacteroides faecis, B. coprocola, B. dorei, Parabacteroides distasonis, Paraprevotella clara, Acidaminococcus intestine* and *Klebsiella pneumoniae*, none of them represented in the female group. On the other hand, *Haemophilus parainfluenzae* and *Dialister invisus* are species mostly present in the female samples. This data suggest that gender might influence gut microbiota composition in elderly individuals. Despite the limited studies involving gender differences in gut microbiota within older individuals the current results provided in old adults are in accordance with the results reported in Yoon *et al.,* (2021).

As several factors can influence the gut microbiota, searching for patterns within individuals with the same age, gender and state of health might contribute to the

understanding of the gut microbiota dynamics. In the present study, the bacterial patterns in three healthy samples, all female participants with ages between 71 and 72 years, were examined. At the phylum level, the three samples showed similar profiles, with two samples (FHD and FHF) being more alike. Regarding genus and species, these two samples showed higher diversity in comparison with the third sample (FHA). *B. vulgatus* (*P. vulgatus*), *P. copri*, *R. inulinivorans* and *O. valericigenes* were the common species in the three samples, however, their abundances vary between the samples. This revealed a few parallels in the microbiota of individuals of the same age, gender, and state of health, however, it is not possible to establish a pattern in these samples. Nevertheless, the comparing of these healthy samples with another one, within the same age and gender, but in the non-healthy group, the gut microbiota composition widely varies. This data evidences that similar health status individuals can show different gut bacterial compositions and proportions in their gut, reinforcing the idea that the gut microbiota is unique to each person and can be affected by more than age, gender and health status (Al Bander, Z. et al., 2020; Gupta, V. K. et al., 2017; Rinninella, E. et al., 2019).

Diverse patterns of disease are now associated with alterations in the gut microbiota composition (Buford, T. W., 2017; Gemikonakli, G. et al., 2021; Leite, G. et al., 2021; Nagpal, R. et al., 2018; Rinninella, E. et al., 2019). Thus, trying to understand which microbial composition is associated with healthy and non-healthy individuals might be a way of achieving an idea of a "healthy" microbiota.

Usually, gut microbiota profiles in older individuals showed a low *Firmicutes/Bacteroidetes* ratio with a depletion in some genera like *Bacteroides*, *Lactobacillus* and *Bifidobacterium* and high levels of the *Escherichia coli* strains (Kong, F. et al., 2019; Leite, G. et al., 2021; Mariat, D. et al., 2009).

In the current study, individuals between ages 60 and 90 years of age showed high proportions of *Firmicutes*, with all healthy samples showing values higher than 70%, and 3 of them with a value higher than 85%. Regarding the non-healthy individuals, S1 showed a value of *Firmicutes* of 86%, FNA and FNB showed values of 73% and 78% respectively, and finally, MNA and MNB showed the lowest values, with 58% and 23% respectively. MNB was the only sample in which *Bacteroidetes* proportion surpassed the *Firmicutes*. This data however is not aligned with Kundu *et al.*, (2017) who summarized in their review that elderly patients usually possess a higher proportion of *Bacteroidetes* when compared to *Firmicutes*. Accordingly, the non-healthy individuals are the ones with

the lower *Firmicutes/Bacteroidetes* ratio, except S1, which values are similar to the healthy samples.

In this study, the three main phyla represented in all samples were *Firmicutes, Bacteroidetes* and *Proteobacteria,* in line with the work of Odamaki *et al.,* (2016) and Nagpal *et al.,* (2018) that reported these three phyla as predominant in the elderly, with *Proteobacteria* relative abundance increasing with age. Noteworthy, recent studies by Wilmasnki *et al.,* (2021) and Leite *et al.,* (2021) demonstrated that the relative abundance of the phylum *Bacteroidetes* tends to decrease in older individuals both in faecal and duodenal microbiota. Moreover, all healthy samples showed the phylum *Lentisphaerae* among the five main phyla except MHB. This phylum is also in the five main phyla in two of the non-healthy samples (MNA and FNA). Three of the non-healthy samples showed *Actinobacteria* in the five main phyla.

Overall, healthy individuals showed higher levels of *Firmicutes* and lower levels of *Bacteroidetes* and *Proteobacteria* in comparison with the non-healthy group. Wu *et al.,* (2021) and Du, Y *et al.,* (2020) demonstrated, respectively, that Alzheimer's, and depression patients usually have a higher proportion of *Bacteroidetes* than healthy controls, however, for other diseases is described the opposite, higher levels of *Bacteroidetes* are observed in the patients that suffer from obesity, Parkinson's, and colorectal cancer (Liu, R. *et al.,* 2017; Rinninella, E. et al., 2019; Unger, M. M. et al., 2016). High levels of *Proteobacteria* were associated with colorectal cancer by Lu *et al.,* (2017).

At the genus and species level, non-healthy samples displayed a higher diversity in comparison with the healthy group, and the two groups showed very different profiles with only three species in common, namely *B. vulgatus, O. valericigenes* and *P. copri*. This data aligns with several studies in which the elderly revealed different gut microbiota composition in comparison with healthy controls (Buford, T. W., 2017; Gemikonakli, G. et al., 2021; Leite, G. et al., 2021; Nagpal, R. et al., 2018; Rinninella, E. et al., 2019). This data is still controversial since in several studies, ill patients usually show lower diversity in comparison with the healthy controls (Guan, Q., 2019; Liu, R. et al., 2017; Wu, S. et al., 2021).

Bacteroides is a genus of high relevance in the intestinal tract. Some studies have revealed alterations in this genus levels with ageing (Ghosh, T. S. et al., 2022; Li, Z. et al., 2018;

Saji, N. et al., 2019; Yoshida, N. et al., 2018). In the current study, the identification of the *Bacteroides* spp. and their respective counts in faecal samples of elderly, both with a healthier status and a non-healthy status, was determined. The isolation of *Bacteroides* in BVSA revealed a higher number of *Bacteroides* CFUs/g of faeces in the non-healthy group, with a higher diversity of *Bacteroides* spp. in the healthy group. Moreover, the microbiome analysis also revealed a higher level of the genus *Bacteroides* in the non-healthy group, however, the group that showed higher diversity was the non-healthy.

Noteworthy, all isolates identified by the culture-dependent method, when confronted with the culture-independent method results, were in small amounts or not present at all. These might be explained by the fact that not all sequences per sample were classified with the EPI2ME software, out of a total of 1078743 reads, 14586 (around 1%) were not classified. Moreover, *E coli* representation is not elevated when looking into the microbiome analysis, but the *E. coli* isolates were 29% of the total number of isolates recovered. These data are unaligned with that reported by Kong *et al.*, (2019) who demonstrated high quantities of *E. coli* in the elderly using the microbiome data of a long-living Chinese cohort. These isolates revealed multi-resistance to antibiotics, specifically clindamycin and colistin, which is in line with some works that showed that bacteria from the gut usually possess resistance to antimicrobials (Lamberte, L. E. & van Schaik, W., 2022; Tavella, T. *et al.*, 2021).

6. Conclusions

In this study, the bacteriome analysis of twelve faecal samples of old adults was performed using an Oxford Nanopore device and a culture-dependent approach was used to evaluate the culturable *Bacteroides* spp. Seven old adults showed a healthy status and five were included in the non-healthy group.

Regarding the individual pattern for the main phyla, it was observed that the three main phyla included *Firmicutes, Bacteroidetes* and *Proteobacteria*. The phylum *Firmicutes* dominated in all samples, except one for which the phylum *Bacteroidetes* prevailed. The results evidenced that *O. valericigenes* was the bacterial specie most abundant across all samples.

The phylum *Proteobacteria* was more abundant in male samples. Regarding the bacterial species according to gender, it was observed that *A. intestini, P. distasonis*, and *K. pneumoniae* were only identified in the male group, whereas *H. parainfluenzae* and *D. invisus* were only observed in the female group.

Concerning the health status of the participants involved in the current study, the results evidenced that the fourth and fifth most abundant phyla were slightly different between these two groups, namely, in the non-healthy group the fourth phylum was *Verrucomicrobia* and the fifth was *Lentisphaerae,* in contrast in the healthy group the fourth most abundant phylum was *Lentisphaerae* and the fifth was *Tenericutes*. Regarding bacterial species, it was observed that *B. vulgatus* (*P. vulgatus*) was more abundant in the non-healthy group, whereas *P. copri* was more abundant in the healthy group.

The results of the culture-dependent approach to analyse the culturable *Bacteroides* spp. population evidenced that even those species with very low percentages in the microbiome were recovered in culture, such as *B. koreensis* and *B. ovatus*.

Interesting the culture-dependent approach also allowed us to observe that some species of the phylum *Proteobacteria* and *Enterobacteriaceae* family, which were recovered in culture were not identified in the microbiome analysis. Such finding suggests that the accuracy of the reads was not sufficient to correctly identify the species.

This study was performed as a preliminary approach to the project “ALgarve Fit Aging Score (ALFA Score): a new integrated tool to improve quality of life and healthy ageing,

therefore the number of samples is limited which restrains the current analysis. Nevertheless, it was possible to observe the main differences in intestinal bacteriome of the enrolled old adults, which is promising for the project in order to obtain a gut microbiome profile of elderly from the Algarve region.

7. Future Perspectives

The advance in sequencing techniques and technologies allows more and more data to be processed. The utilization of new metagenomic methods for microbiota research have been resulting in a large amount of data. However, finding a line of causation between gut microbiota composition and state of health remains a challenge. More studies are needed, especially long-term studies that allow monitoring the fluctuations in the microbial community in certain life stages.

Following the project with the appropriate number of samples the required parameters, such as the α and β- diversity in each group will be determined.

Another approach that will improve the knowledge about the impact of the gut microbiota on the ageing process is the examination of the intestinal metabolome. For this, in future work, the tracing of the metabolic profile of each sample will be crucial to properly evaluate the functionality of the intestinal microbiome.

References

Al Bander, Z., Nitert, M.D., Mousa, A., & Naderpoor, N. (2020). The gut microbiota and inflammation: An overview. *Int J Environ Res Public Health, 17*(20). doi:10.3390/ijerph17207618

Aleksandrova, K., Romero-Mosquera, B., & Hernandez, V. (2017). Diet, gut microbiome and epigenetics: Emerging links with inflammatory bowel diseases and prospects for management and prevention. *Nutrients, 9*(9). doi:10.3390/nu9090962

Alexopoulos, G.S. (2005). Depression in the elderly. *The Lancet, 365*(9475), 1961-1970. doi:https://doi.org/10.1016/S0140-6736(05)66665-2

Almeida, O.P. (2014). Prevention of depression in older age. *Maturitas, 79*(2), 136-141. doi:10.1016/j.maturitas.2014.03.005

An, R., Wilms, E., Masclee, A.a.M., Smidt, H., Zoetendal, E.G., & Jonkers, D. (2018). Age-dependent changes in gi physiology and microbiota: Time to reconsider? *Gut, 67*(12), 2213-2222. doi:10.1136/gutjnl-2017-315542

Ananthakrishnan, A.N., Nguyen, G.C., & Bernstein, C.N. (2021). Aga clinical practice update on management of inflammatory bowel disease in elderly patients: Expert review. *Gastroenterology, 160*(1), 445-451. doi:10.1053/j.gastro.2020.08.060

Anderson, K.M., Ferranti, E.P., Alagha, E.C., Mykityshyn, E., French, C.E., & Reilly, C.M. (2022). The heart and gut relationship: A systematic review of the evaluation of the microbiome and trimethylamine-n-oxide (tmao) in heart failure. *Heart Fail Rev*. doi:10.1007/s10741-022-10254-6

Arvelo, F., Sojo, F., & Cotte, C. (2015). Biology of colorectal cancer. *Ecancermedicalscience, 9*, 520. doi:10.3332/ecancer.2015.520

Bacic, M.K., & Smith, C.J. (2008). Laboratory maintenance and cultivation of *bacteroides* species. *Curr Protoc Microbiol, Chapter 13*, Unit 13C.11. doi:10.1002/9780471729259.mc13c01s9

Bakir, M.A., Sakamoto, M., Kitahara, M., Matsumoto, M., & Benno, Y. (2006). *Bacteroides dorei* sp. Nov., isolated from human faeces. *Int J Syst Evol Microbiol, 56*(Pt 7), 1639-1643. doi:10.1099/ijs.0.64257-0

Bamola, V.D., Kapardar, R., Lal, B., Sharma, A., & Chaudhry, R. (2022). A metagenomic assessment of gut microbiota in indian colon cancer patients. *J Cancer Res Ther, 18*(1), 96-102. doi:10.4103/0973-1482.341139

Bana, B., & Cabreiro, F. (2019). The microbiome and aging. *Annu Rev Genet, 53*, 239-261. doi:10.1146/annurev-genet-112618-043650

Bonnet, M., Buc, E., Sauvanet, P., Darcha, C., Dubois, D., Pereira, B., Déchelotte, P., Bonnet, R., Pezet, D., & Darfeuille-Michaud, A. (2014). Colonization of the human gut by *E. coli* and colorectal cancer risk. *Clin Cancer Res, 20*(4), 859-867. doi:10.1158/1078-0432.Ccr-13-1343

Brenner, H., Kloor, M., & Pox, C.P. (2014). Colorectal cancer. *The Lancet, 383*(9927), 1490-1502. doi:https://doi.org/10.1016/S0140-6736(13)61649-9

Buford, T.W. (2017). (dis)trust your gut: The gut microbiome in age-related inflammation, health, and disease. *Microbiome, 5*(1), 80. doi:10.1186/s40168-017-0296-0

Calvani, R., Picca, A., Cesari, M., Tosato, M., Marini, F., Manes-Gravina, E., Bernabei, R., Landi, F., & Marzetti, E. (2018). Biomarkers for sarcopenia: Reductionism vs. Complexity. *Curr Protein Pept Sci, 19*(7), 639-642. doi:10.2174/1389203718666170516115422

Cesari, M., Calvani, R., & Marzetti, E. (2017). Frailty in older persons. *Clin Geriatr Med, 33*(3), 293-303. doi:10.1016/j.cger.2017.02.002

Chioncel, O., & Ambrosy, A.P. (2019). Trimethylamine n-oxide and risk of heart failure progression: Marker or mediator of disease. *Eur J Heart Fail, 21*(7), 887-890. doi:10.1002/ejhf.1409

Chung, H.Y., Kim, D.H., Lee, E.K., Chung, K.W., Chung, S., Lee, B., Seo, A.Y., Chung, J.H., Jung, Y.S., Im, E., Lee, J., Kim, N.D., Choi, Y.J., Im, D.S., & Yu, B.P. (2019). Redefining chronic inflammation in aging and age-related diseases: Proposal of the senoinflammation concept. *Aging Dis, 10*(2), 367-382. doi:10.14336/ad.2018.0324

Coker, O.O., Liu, C., Wu, W.K.K., Wong, S.H., Jia, W., Sung, J.J.Y., & Yu, J. (2022). Altered gut metabolites and microbiota interactions are implicated in colorectal carcinogenesis and can be non-invasive diagnostic biomarkers. *Microbiome, 10*(1), 35. doi:10.1186/s40168-021-01208-5

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., Mcgarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., & Tiedje, J.M. (2013). Ribosomal database project: Data and tools for high throughput rrna analysis. *Nucleic Acids Research, 42*(D1), D633-D642. doi:10.1093/nar/gkt1244 %J Nucleic Acids Research

Cruz-Jentoft, A.J., Bahat, G., Bauer, J., Boirie, Y., Bruyère, O., Cederholm, T., Cooper, C., Landi, F., Rolland, Y., Sayer, A.A., Schneider, S.M., Sieber, C.C., Topinkova, E., Vandewoude, M., Visser, M., & Zamboni, M. (2019). Sarcopenia: Revised european consensus on definition and diagnosis. *Age Ageing, 48*(1), 16-31. doi:10.1093/ageing/afy169

Cui, X., Ye, L., Li, J., Jin, L., Wang, W., Li, S., Bao, M., Wu, S., Li, L., Geng, B., Zhou, X., Zhang, J., & Cai, J. (2018). Metagenomic and metabolomic analyses unveil dysbiosis of gut microbiota in chronic heart failure patients. *Sci Rep, 8*(1), 635. doi:10.1038/s41598-017-18756-2

Cunningham, A.L., Stephens, J.W., & Harris, D.A. (2021). Gut microbiota influence in type 2 diabetes mellitus (t2dm). *Gut Pathogens, 13*(1), 50. doi:10.1186/s13099-021-00446-0

Czajkowska, A., Kaźmierczak-Siedlecka, K., Jamioł-Milc, D., Gutowska, I., & Skonieczna-Żydecka, K. (2020). Gut microbiota and its metabolic potential. *Eur Rev Med Pharmacol Sci, 24*(24), 12971-12977. doi:10.26355/eurrev_202012_24201

Dejong, E.N., Surette, M.G., & Bowdish, D.M.E. (2020). The gut microbiota and unhealthy aging: Disentangling cause from consequence. *Cell Host Microbe, 28*(2), 180-189. doi:https://doi.org/10.1016/j.chom.2020.07.013

Di Micco, R., Krizhanovsky, V., Baker, D., & D'adda Di Fagagna, F. (2021). Cellular senescence in ageing: From mechanisms to therapeutic opportunities. *Nature Reviews Molecular Cell Biology, 22*(2), 75-95. doi:10.1038/s41580-020-00314-w

Dieterich, W., Schink, M., & Zopf, Y. (2018). Microbiota in the gastrointestinal tract. *Med Sci (Basel), 6*(4). doi:10.3390/medsci6040116

Dominguez-Bello, M.G., Godoy-Vitorino, F., Knight, R., & Blaser, M.J. (2019). Role of the microbiome in human development. *Gut, 68*(6), 1108. doi:10.1136/gutjnl-2018-317503

Dong, T.S., Guan, M., Mayer, E.A., Stains, J., Liu, C., Vora, P., Jacobs, J.P., Lagishetty, V., Chang, L., Barry, R.L., & Gupta, A. (2022). Obesity is associated with a distinct brain-gut microbiome signature that connects prevotella and bacteroides to the brain's reward center. *Gut Microbes, 14*(1), 2051999. doi:10.1080/19490976.2022.2051999

Dong, Z., Zheng, S., Shen, Z., Luo, Y., & Hai, X. (2021). Trimethylamine n-oxide is associated with heart failure risk in patients with preserved ejection fraction. *Lab Med, 52*(4), 346-351. doi:10.1093/labmed/lmaa075

Du, Y., Gao, X.R., Peng, L., & Ge, J.F. (2020). Crosstalk between the microbiota-gut-brain axis and depression. *Heliyon, 6*(6), e04097. doi:10.1016/j.heliyon.2020.e04097

Dyson, P.A. (2010). The therapeutics of lifestyle management on obesity. *Diabetes Obes Metab, 12*(11), 941-946. doi:10.1111/j.1463-1326.2010.01256.x

Ejtahed, H.S., Angoorani, P., Soroush, A.R., Hasani-Ranjbar, S., Siadat, S.D., & Larijani, B. (2020). Gut microbiota-derived metabolites in obesity: A systematic review. *Biosci Microbiota Food Health, 39*(3), 65-76. doi:10.12938/bmfh.2019-026

European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint Tables for Interpretation of MICs and Zone Diameters. Available online: http://www.eucast.org (accessed on July 2022)

Ferrucci, L., & Fabbri, E. (2018). Inflammageing: Chronic inflammation in ageing, cardiovascular disease, and frailty. *Nat Rev Cardiol, 15*(9), 505-522. doi:10.1038/s41569-018-0064-2

Finlay, B.B., Pettersson, S., Melby, M.K., & Bosch, T.C.G. (2019). The microbiome mediates environmental effects on aging. *Bioessays, 41*(10), e1800257. doi:10.1002/bies.201800257

Gaio, V., Antunes, L., Namorado, S., Barreto, M., Gil, A., Kyslaya, I., Rodrigues, A.P., Santos, A., Bøhler, L., Castilho, E., Vargas, P., Do Carmo, I., Nunes, B., & Dias, C.M. (2018). Prevalence of overweight and obesity in portugal: Results from the first portuguese health examination survey (insef 2015). *Obes Res Clin Pract, 12*(1), 40-50. doi:10.1016/j.orcp.2017.08.002

Gao, Z., Guo, B., Gao, R., Zhu, Q., & Qin, H. (2015). Microbiota disbiosis is associated with colorectal cancer. *Front Microbiol, 6*, 20. doi:10.3389/fmicb.2015.00020

Garcia-Gutierrez, E., Narbad, A., & Rodríguez, J.M. (2020). Autism spectrum disorder associated with gut microbiota at immune, metabolomic, and neuroactive level. *Front Neurosci, 14*, 578666. doi:10.3389/fnins.2020.578666

García-López, M., Meier-Kolthoff, J.P., Tindall, B.J., Gronow, S., Woyke, T., Kyrpides, N.C., Hahnke, R.L., & Göker, M. (2019). Analysis of 1,000 type-strain genomes improves taxonomic classification of bacteroidetes. *Front Microbiol, 10*, 2083. doi:10.3389/fmicb.2019.02083

Gemikonakli, G., Mach, J., & Hilmer, S.N. (2021). Interactions between the aging gut microbiome and common geriatric giants: Polypharmacy, frailty, and dementia. *J Gerontol A Biol Sci Med Sci, 76*(6), 1019-1028. doi:10.1093/gerona/glaa047

Ghosh, T.S., Shanahan, F., & O'toole, P.W. (2022). The gut microbiome as a modulator of healthy ageing. *Nature Reviews Gastroenterology & Hepatology, 19*(9), 565-584. doi:10.1038/s41575-022-00605-x

Guan, Q. (2019). A comprehensive review and update on the pathogenesis of inflammatory bowel disease. *J Immunol Res, 2019*, 7247238. doi:10.1155/2019/7247238

Gupta, V.K., Paul, S., & Dutta, C. (2017). Geography, ethnicity or subsistence-specific variations in human microbiome composition and diversity. *8*. doi:10.3389/fmicb.2017.01162

Haran, J.P., & Mccormick, B.A. (2021). Aging, frailty, and the microbiome-how dysbiosis influences human aging and disease. *Gastroenterology, 160*(2), 507-523. doi:10.1053/j.gastro.2020.09.060

Haro, C., Rangel-Zúñiga, O.A., Alcalá-Díaz, J.F., Gómez-Delgado, F., Pérez-Martínez, P., Delgado-Lista, J., Quintana-Navarro, G.M., Landa, B.B., Navas-Cortés, J.A., Tena-Sempere, M., Clemente, J.C., López-Miranda, J., Pérez-Jiménez, F., & Camargo, A. (2016). Intestinal microbiota is influenced by gender and body mass index. *PLOS ONE, 11*(5), e0154090. doi:10.1371/journal.pone.0154090

Heianza, Y., Ma, W., Didonato, J.A., Sun, Q., Rimm, E.B., Hu, F.B., Rexrode, K.M., Manson, J.E., & Qi, L. (2020). Long-term changes in gut microbial metabolite trimethylamine n-oxide and coronary heart disease risk. *J Am Coll Cardiol, 75*(7), 763-772. doi:10.1016/j.jacc.2019.11.060

Horvath, S. (2013). DNA methylation age of human tissues and cell types. *Genome Biology, 14*(10), 3156. doi:10.1186/gb-2013-14-10-r115

Howell, K.J., Kraiczy, J., Nayak, K.M., Gasparetto, M., Ross, A., Lee, C., Mak, T.N., Koo, B.K., Kumar, N., Lawley, T., Sinha, A., Rosenstiel, P., Heuschkel, R., Stegle, O., & Zilbauer, M. (2018). DNA methylation and transcription patterns in intestinal epithelial cells from pediatric patients with inflammatory bowel diseases differentiate disease subtypes and associate with outcome. *Gastroenterology, 154*(3), 585-598. doi:10.1053/j.gastro.2017.10.007

Iftekhar, A., Berger, H., Bouznad, N., Heuberger, J., Boccellato, F., Dobrindt, U., Hermeking, H., Sigal, M., & Meyer, T.F. (2021). Genomic aberrations after short-term exposure to colibactin-producing *E. coli* transform primary colon epithelial cells. *Nat Commun, 12*(1), 1003. doi:10.1038/s41467-021-21162-y

Israr, M.Z., Bernieh, D., Salzano, A., Cassambai, S., Yazaki, Y., Heaney, L.M., Jones, D.J.L., Ng, L.L., & Suzuki, T. (2021). Association of gut-related metabolites with outcome in acute heart failure. *Am Heart J, 234*, 71-80. doi:10.1016/j.ahj.2021.01.006

Jackson, M.A., Jeffery, I.B., Beaumont, M., Bell, J.T., Clark, A.G., Ley, R.E., O'toole, P.W., Spector, T.D., & Steves, C.J. (2016). Signatures of early frailty in the gut microbiota. *Genome Medicine, 8*(1), 8. doi:10.1186/s13073-016-0262-7

Kato, I., Boleij, A., Kortman, G.A., Roelofs, R., Djuric, Z., Severson, R.K., & Tjalsma, H. (2013). Partial associations of dietary iron, smoking and intestinal bacteria with colorectal cancer risk. *Nutr Cancer, 65*(2), 169-177. doi:10.1080/01635581.2013.748922

Kim, M.S., Kim, Y., Choi, H., Kim, W., Park, S., Lee, D., Kim, D.K., Kim, H.J., Choi, H., Hyun, D.W., Lee, J.Y., Choi, E.Y., Lee, D.S., Bae, J.W., & Mook-Jung, I. (2020). Transfer of a healthy microbiota reduces amyloid and tau pathology in an alzheimer's disease animal model. *Gut, 69*(2), 283-294. doi:10.1136/gutjnl-2018-317431

Kong, F., Deng, F., Li, Y., & Zhao, J. (2019). Identification of gut microbiome signatures associated with longevity provides a promising modulation target for healthy aging. *Gut Microbes, 10*(2), 210-215. doi:10.1080/19490976.2018.1494102

Korpela, K., Salonen, A., Saxen, H., Nikkonen, A., Peltola, V., Jaakkola, T., De Vos, W., & Kolho, K.L. (2020). Antibiotics in early life associate with specific gut microbiota signatures in a prospective longitudinal infant cohort. *Pediatr Res, 88*(3), 438-443. doi:10.1038/s41390-020-0761-5

Kundu, P., Blacher, E., Elinav, E., & Pettersson, S. (2017). Our gut microbiome: The evolving inner self. *Cell, 171*(7), 1481-1493. doi:10.1016/j.cell.2017.11.024

Lakshminarayanan, B., Stanton, C., O'toole, P.W., & Ross, R.P. (2014). Compositional dynamics of the human intestinal microbiota with aging: Implications for health. *J Nutr Health Aging, 18*(9), 773-786. doi:10.1007/s12603-014-0549-6

Lamberte, L.E., & Van Schaik, W. (2022). Antibiotic resistance in the commensal human gut microbiota. *Curr Opin Microbiol, 68*, 102150. doi:https://doi.org/10.1016/j.mib.2022.102150

Landi, F., Calvani, R., Cesari, M., Tosato, M., Martone, A.M., Ortolani, E., Savera, G., Salini, S., Sisto, A., Picca, A., & Marzetti, E. (2018). Sarcopenia: An overview on current definitions, diagnosis and treatment. *Curr Protein Pept Sci, 19*(7), 633-638. doi:10.2174/1389203718666170607113459

Leite, G., Pimentel, M., Barlow, G.M., Chang, C., Hosseini, A., Wang, J., Parodi, G., Sedighi, R., Rezaie, A., & Mathur, R. (2021). Age and the aging process significantly alter the small bowel microbiome. *Cell Reports, 36*(13), 109765. doi:https://doi.org/10.1016/j.celrep.2021.109765

Leroith, D., Biessels, G.J., Braithwaite, S.S., Casanueva, F.F., Draznin, B., Halter, J.B., Hirsch, I.B., Mcdonnell, M.E., Molitch, M.E., Murad, M.H., & Sinclair, A.J. (2019). Treatment of diabetes in older adults: An endocrine society* clinical practice guideline. *J Clin Endocrinol Metab, 104*(5), 1520-1574. doi:10.1210/jc.2019-00198

Levine, M.E., Lu, A.T., Quach, A., Chen, B.H., Assimes, T.L., Bandinelli, S., Hou, L., Baccarelli, A.A., Stewart, J.D., Li, Y., Whitsel, E.A., Wilson, J.G., Reiner, A.P., Aviv, A., Lohman, K., Liu, Y., Ferrucci, L., & Horvath, S. (2018). An epigenetic biomarker of aging for lifespan and healthspan. *Aging (Albany NY), 10*(4), 573-591. doi:10.18632/aging.101414

Li, Z., Quan, G., Jiang, X., Yang, Y., Ding, X., Zhang, D., Wang, X., Hardwidge, P.R., Ren, W., & Zhu, G. (2018). Effects of metabolites derived from gut microbiota and hosts on pathogens. *Front Cell Infect Microbiol, 8*, 314. doi:10.3389/fcimb.2018.00314

Lin, D., Peters, B.A., Friedlander, C., Freiman, H.J., Goedert, J.J., Sinha, R., Miller, G., Bernstein, M.A., Hayes, R.B., & Ahn, J. (2018). Association of dietary fibre intake and gut microbiota in adults. *Br J Nutr, 120*(9), 1014-1022. doi:10.1017/s0007114518002465

Liu, R., Hong, J., Xu, X., Feng, Q., Zhang, D., Gu, Y., Shi, J., Zhao, S., Liu, W., Wang, X., Xia, H., Liu, Z., Cui, B., Liang, P., Xi, L., Jin, J., Ying, X., Wang, X., Zhao, X., Li, W., Jia, H., Lan, Z., Li, F., Wang, R., Sun, Y., Yang, M., Shen, Y., Jie, Z., Li, J., Chen, X., Zhong, H., Xie, H., Zhang, Y., Gu, W., Deng, X., Shen, B., Xu, X., Yang, H., Xu, G., Bi, Y., Lai, S., Wang, J., Qi, L., Madsen, L., Wang, J., Ning, G., Kristiansen, K., & Wang, W. (2017). Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention. *Nat Med, 23*(7), 859-868. doi:10.1038/nm.4358

Lu, R., Bosland, M., Xia, Y., Zhang, Y.G., Kato, I., & Sun, J. (2017). Presence of salmonella avra in colorectal tumor and its precursor lesions in mouse intestine and human specimens. *Oncotarget, 8*(33), 55104-55115. doi:10.18632/oncotarget.19052

Mallappallil, M., Friedman, E.A., Delano, B.G., Mcfarlane, S.I., & Salifu, M.O. (2014). Chronic kidney disease in the elderly: Evaluation and management. *Clin Pract (Lond), 11*(5), 525-535. doi:10.2217/cpr.14.46

Margiotta, E., Miragoli, F., Callegari, M.L., Vettoretti, S., Caldiroli, L., Meneghini, M., Zanoni, F., & Messa, P. (2020). Gut microbiota composition and frailty in elderly patients with chronic kidney disease. *PLOS ONE, 15*(4), e0228530. doi:10.1371/journal.pone.0228530

Mariat, D., Firmesse, O., Levenez, F., Guimarăes, V.D., Sokol, H., Doré, J., Corthier, G., & Furet, J.P. (2009). The *Firmicutes/Bacteroidetes* ratio of the human microbiota changes with age. *BMC Microbiology, 9*(1), 123. doi:10.1186/1471-2180-9-123

Martinez-Guryn, K., Leone, V., & Chang, E.B. (2019). Regional diversity of the gastrointestinal microbiome. *Cell Host Microbe, 26*(3), 314-324. doi:10.1016/j.chom.2019.08.011

Marzetti, E., Calvani, R., Tosato, M., Cesari, M., Di Bari, M., Cherubini, A., Collamati, A., D'angelo, E., Pahor, M., Bernabei, R., & Landi, F. (2017). Sarcopenia: An overview. *Aging Clin Exp Res, 29*(1), 11-17. doi:10.1007/s40520-016-0704-5

Meseeha, M., & Attia, M. (2022). Colon polyps. In *Statpearls*. Treasure Island (FL): StatPearls Publishing Copyright © 2022, StatPearls Publishing LLC.

Milani, C., Ticinesi, A., Gerritsen, J., Nouvenne, A., Lugli, G.A., Mancabelli, L., Turroni, F., Duranti, S., Mangifesta, M., Viappiani, A., Ferrario, C., Maggio, M., Lauretani, F., De Vos, W., Van Sinderen, D., Meschi, T., & Ventura, M. (2016). Gut microbiota composition and clostridium difficile infection in hospitalized elderly individuals: A metagenomic study. *Sci Rep, 6*, 25945. doi:10.1038/srep25945

Mira-Pascual, L., Cabrera-Rubio, R., Ocon, S., Costales, P., Parra, A., Suarez, A., Moris, F., Rodrigo, L., Mira, A., & Collado, M.C. (2015). Microbial mucosal colonic shifts associated with the development of colorectal cancer reveal the presence of different bacterial and archaeal biomarkers. *J Gastroenterol, 50*(2), 167-179. doi:10.1007/s00535-014-0963-x

Nagpal, R., Mainali, R., Ahmadi, S., Wang, S., Singh, R., Kavanagh, K., Kitzman, D.W., Kushugulova, A., Marotta, F., & Yadav, H. (2018). Gut microbiome and aging: Physiological and mechanistic insights. *Nutr Healthy Aging, 4*(4), 267-285. doi:10.3233/nha-170030

Odamaki, T., Kato, K., Sugahara, H., Hashikura, N., Takahashi, S., Xiao, J.-Z., Abe, F., & Osawa, R. (2016). Age-related changes in gut microbiota composition from newborn to centenarian: A cross-sectional study. *BMC Microbiology, 16*(1), 90. doi:10.1186/s12866-016-0708-5

Peery, R.C., Pammi, M., Claud, E., & Shen, L. (2021). Epigenome - a mediator for host-microbiome crosstalk. *Semin Perinatol, 45*(6), 151455. doi:10.1016/j.semperi.2021.151455

Peng, L., Li, Z.R., Green, R.S., Holzman, I.R., & Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of amp-activated protein kinase in caco-2 cell monolayers. *J Nutr, 139*(9), 1619-1625. doi:10.3945/jn.109.104638

Pitcher, D.G., Saunders, N.A., & Owen, R.J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *8*(4), 151-156. doi:10.1111/j.1472-765X.1989.tb00262.x

Ramirez, J., Guarner, F., Bustos Fernandez, L., Maruy, A., Sdepanian, V.L., & Cohen, H. (2020). Antibiotics as major disruptors of gut microbiota. *Front Cell Infect Microbiol, 10*, 572912. doi:10.3389/fcimb.2020.572912

Rashidi, A., Ebadi, M., Rehman, T.U., Elhusseini, H., Nalluri, H., Kaiser, T., Holtan, S.G., Khoruts, A., Weisdorf, D.J., & Staley, C. (2021). Gut microbiota response to antibiotics is personalized and depends on baseline microbiota. *Microbiome, 9*(1), 211. doi:10.1186/s40168-021-01170-2

Ravi, A., Halstead, F.D., Bamford, A., Casey, A., Thomson, N.M., Van Schaik, W., Snelson, C., Goulden, R., Foster-Nyarko, E., Savva, G.M., Whitehouse, T., Pallen, M.J., & Oppenheim, B.A. (2019). Loss of microbial diversity and

pathogen domination of the gut microbiota in critically ill patients. *Microb Genom, 5*(9). doi:10.1099/mgen.0.000293

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G.a.D., Gasbarrini, A., & Mele, M.C. (2019). What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases. *Microorganisms, 7*(1). doi:10.3390/microorganisms7010014

Romanenko, M., Kholin, V., Koliada, A., & Vaiserman, A. (2021). Nutrition, gut microbiota, and alzheimer's disease. *Front Psychiatry, 12*, 712673. doi:10.3389/fpsyt.2021.712673

Roquette, R., Painho, M., & Nunes, B. (2019). Geographical patterns of the incidence and mortality of colorectal cancer in mainland portugal municipalities (2007–2011). *BMC Cancer, 19*(1), 512. doi:10.1186/s12885-019-5719-9

Rossiello, F., Jurk, D., Passos, J.F., & D'adda Di Fagagna, F. (2022). Telomere dysfunction in ageing and age-related diseases. *Nature Cell Biology, 24*(2), 135-147. doi:10.1038/s41556-022-00842-x

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., & Tuohy, K. (2018). Gut microbiota functions: Metabolism of nutrients and other food components. *Eur J Nutr, 57*(1), 1-24. doi:10.1007/s00394-017-1445-8

Ryan, E., Mcnicholas, D., Creavin, B., Kelly, M.E., Walsh, T., & Beddy, D. (2019). Sarcopenia and inflammatory bowel disease: A systematic review. *Inflamm Bowel Dis, 25*(1), 67-73. doi:10.1093/ibd/izy212

Saji, N., Niida, S., Murotani, K., Hisada, T., Tsuduki, T., Sugimoto, T., Kimura, A., Toba, K., & Sakurai, T. (2019). Analysis of the relationship between the gut microbiome and dementia: A cross-sectional study conducted in japan. *Scientific Reports, 9*(1), 1008. doi:10.1038/s41598-018-38218-7

Sakamoto, M., Sakurai, N., Tanno, H., Iino, T., Ohkuma, M., & Endo, A. (2022). Genome-based, phenotypic and chemotaxonomic classification of faecalibacterium strains: Proposal of three novel species faecalibacterium duncaniae sp. Nov., faecalibacterium hattorii sp. Nov. And faecalibacterium gallinarum sp. Nov. *Int J Syst Evol Microbiol, 72*(4). doi:10.1099/ijsem.0.005379

Santacroce, L., Man, A., Charitos, I.A., Haxhirexha, K., & Topi, S. (2021). Current knowledge about the connection between health status and gut microbiota from birth to elderly. A narrative review. *Front Biosci (Landmark Ed), 26*(6), 135-148. doi:10.52586/4930

Santoni, M., Miccini, F., & Battelli, N. (2021). Gut microbiota, immunity and pain. *Immunology Letters, 229*, 44-47. doi:https://doi.org/10.1016/j.imlet.2020.11.010

Schumacher, B., Pothof, J., Vijg, J., & Hoeijmakers, J.H.J. (2021). The central role of DNA damage in the ageing process. *Nature, 592*(7856), 695-703. doi:10.1038/s41586-021-03307-7

Senghor, B., Sokhna, C., Ruimy, R., & Lagier, J.-C. (2018). Gut microbiota diversity according to dietary habits and geographical provenance. *Human Microbiome Journal, 7-8*, 1-9. doi:https://doi.org/10.1016/j.humic.2018.01.001

Shafiee, G., Keshtkar, A., Soltani, A., Ahadi, Z., Larijani, B., & Heshmat, R. (2017). Prevalence of sarcopenia in the world: A systematic review and meta- analysis of general population studies. *J Diabetes Metab Disord, 16*, 21. doi:10.1186/s40200-017-0302-x

Shen, X.J., Rawls, J.F., Randall, T., Burcal, L., Mpande, C.N., Jenkins, N., Jovov, B., Abdo, Z., Sandler, R.S., & Keku, T.O. (2010). Molecular characterization of mucosal adherent bacteria and associations with colorectal adenomas. *Gut Microbes, 1*(3), 138-147. doi:10.4161/gmic.1.3.12360

Shintouo, C.M., Mets, T., Beckwee, D., Bautmans, I., Ghogomu, S.M., Souopgui, J., Leemans, L., Meriki, H.D., & Njemini, R. (2020). Is inflammageing influenced by the microbiota in the aged gut? A systematic review. *Exp Gerontol, 141*, 111079. doi:10.1016/j.exger.2020.111079

Stachura, A., Gierszon, P., Paziewska, M., & Łagowska-Batyra, A. (2020). Biological signs of aging in the elderly. *Acta Balneologica, 62*, 166-170. doi:10.36740/ABal202003106

Suzuki, T., Yazaki, Y., Voors, A.A., Jones, D.J.L., Chan, D.C.S., Anker, S.D., Cleland, J.G., Dickstein, K., Filippatos, G., Hillege, H.L., Lang, C.C., Ponikowski, P., Samani, N.J., Van Veldhuisen, D.J., Zannad, F., Zwinderman, A.H., Metra, M., & Ng, L.L. (2019). Association with outcomes and response to treatment of trimethylamine n-oxide in heart failure: Results from biostat-chf. *Eur J Heart Fail, 21*(7), 877-886. doi:10.1002/ejhf.1338

Takagi, T., Naito, Y., Inoue, R., Kashiwagi, S., Uchiyama, K., Mizushima, K., Tsuchiya, S., Dohi, O., Yoshida, N., Kamada, K., Ishikawa, T., Handa, O., Konishi, H., Okuda, K., Tsujimoto, Y., Ohnogi, H., & Itoh, Y. (2019). Differences in gut microbiota associated with age, sex, and stool consistency in healthy japanese subjects. *J Gastroenterol, 54*(1), 53-63. doi:10.1007/s00535-018-1488-5

Tan, H., Zhai, Q., & Chen, W. (2019). Investigations of *Bacteroides* spp. Towards next-generation probiotics. *Food Res Int, 116*, 637-644. doi:10.1016/j.foodres.2018.08.088

Tang, W.H., & Hazen, S.L. (2017). The gut microbiome and its role in cardiovascular diseases. *Circulation, 135*(11), 1008-1010. doi:10.1161/circulationaha.116.024251

Tavella, T., Turroni, S., Brigidi, P., Candela, M., & Rampelli, S. (2021). The human gut resistome up to extreme longevity. *6*(5), e00691-00621. doi:doi:10.1128/mSphere.00691-21

Thomson, C., Garcia, A.L., & Edwards, C.A. (2021). Interactions between dietary fibre and the gut microbiota. *Proceedings of the Nutrition Society, 80*(4), 398-408. doi:10.1017/S0029665121002834

Thursby, E., & Juge, N. (2017). Introduction to the human gut microbiota. *Biochem J, 474*(11), 1823-1836. doi:10.1042/bcj20160510

Ticinesi, A., Milani, C., Lauretani, F., Nouvenne, A., Mancabelli, L., Lugli, G.A., Turroni, F., Duranti, S., Mangifesta, M., Viappiani, A., Ferrario, C., Maggio, M., Ventura, M., & Meschi, T. (2017). Gut microbiota composition is associated with polypharmacy in elderly hospitalized patients. *Sci Rep, 7*(1), 11102. doi:10.1038/s41598-017-10734-y

Trøseid, M., Ueland, T., Hov, J.R., Svardal, A., Gregersen, I., Dahl, C.P., Aakhus, S., Gude, E., Bjørndal, B., Halvorsen, B., Karlsen, T.H., Aukrust, P., Gullestad, L., Berge, R.K., & Yndestad, A. (2015). Microbiota-dependent metabolite trimethylamine-n-oxide is associated with disease severity and survival of patients with chronic heart failure. *J Intern Med, 277*(6), 717-726. doi:10.1111/joim.12328

Umirah, F., Neoh, C.F., Ramasamy, K., & Lim, S.M. (2021). Differential gut microbiota composition between type 2 diabetes mellitus patients and healthy controls: A systematic review. *Diabetes Res Clin Pract, 173*, 108689. doi:10.1016/j.diabres.2021.108689

Unger, M.M., Spiegel, J., Dillmann, K.U., Grundmann, D., Philippeit, H., Bürmann, J., Faßbender, K., Schwiertz, A., & Schäfer, K.H. (2016). Short chain fatty acids and gut microbiota differ between patients with parkinson's disease and age-matched

controls. *Parkinsonism Relat Disord, 32*, 66-72. doi:10.1016/j.parkreldis.2016.08.019

Valeri, F., & Endres, K. (2021). How biological sex of the host shapes its gut microbiota. *Frontiers in Neuroendocrinology, 61*, 100912. doi:https://doi.org/10.1016/j.yfrne.2021.100912

Valles-Colomer, M., Falony, G., Darzi, Y., Tigchelaar, E.F., Wang, J., Tito, R.Y., Schiweck, C., Kurilshikov, A., Joossens, M., Wijmenga, C., Claes, S., Van Oudenhove, L., Zhernakova, A., Vieira-Silva, S., & Raes, J. (2019). The neuroactive potential of the human gut microbiota in quality of life and depression. *Nature Microbiology, 4*(4), 623-632. doi:10.1038/s41564-018-0337-x

Van Tongeren, S.P., Slaets, J.P., Harmsen, H.J., & Welling, G.W. (2005). Fecal microbiota composition and frailty. *Appl Environ Microbiol, 71*(10), 6438-6442. doi:10.1128/aem.71.10.6438-6442.2005

Vital, M., Howe, A.C., & Tiedje, J.M. (2014). Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data. *5*(2), e00889-00814. doi:doi:10.1128/mBio.00889-14

Vogt, N.M., Kerby, R.L., Dill-Mcfarland, K.A., Harding, S.J., Merluzzi, A.P., Johnson, S.C., Carlsson, C.M., Asthana, S., Zetterberg, H., Blennow, K., Bendlin, B.B., & Rey, F.E. (2017). Gut microbiome alterations in alzheimer's disease. *Scientific Reports, 7*(1), 13537. doi:10.1038/s41598-017-13601-y

Vuotto, C., Battistini, L., Caltagirone, C., & Borsellino, G. (2020). Gut microbiota and disorders of the central nervous system. *The Neuroscientist, 26*(5-6), 487-502. doi:10.1177/1073858420918826

Wang, T., Cai, G., Qiu, Y., Fei, N., Zhang, M., Pang, X., Jia, W., Cai, S., & Zhao, L. (2012). Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *Isme j, 6*(2), 320-329. doi:10.1038/ismej.2011.109

Wei, H., Zhao, M., Huang, M., Li, C., Gao, J., Yu, T., Zhang, Q., Shen, X., Ji, L., Ni, L., Zhao, C., Wang, Z., Dong, E., Zheng, L., & Wang, D.W. (2022). Fmo3-tmao axis modulates the clinical outcome in chronic heart-failure patients with reduced ejection fraction: Evidence from an asian population. *Front Med, 16*(2), 295-305. doi:10.1007/s11684-021-0857-2

Wexler, H.M. (2007). Bacteroides: The good, the bad, and the nitty-gritty. *Clinical microbiology reviews, 20*(4), 593-621. doi:10.1128/cmr.00008-07

World Health Organization, 2017. Mental health of older adults. https://www.who.int/news-room/fact-sheets/detail/mental-health-of-older-adults. (Accessed on 25th August 2022)

World Health Organization. (2021, June 9th). Obesity. Retrieved from https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (Accessed on 25th August 2022)

World Health Organization. (2021, June 11th). Cardiovascular diseases (CVDs). Retrieved from https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (Accessed on 25th August 2022)

World Health Organization. (2021, November 10th). Diabetes. Retrieved from https://www.who.int/news-room/fact-sheets/detail/diabetes (Accessed on 25th August 2022)

World Health Organization. (2021, September 13th). Depression. Retrieved from https://www.who.int/news-room/fact-sheets/detail/depression/mental-health-of-older-adults. (Accessed on 25th August 2022)

Wilmanski, T., Diener, C., Rappaport, N., Patwardhan, S., Wiedrick, J., Lapidus, J., Earls, J.C., Zimmer, A., Glusman, G., Robinson, M., Yurkovich, J.T., Kado, D.M., Cauley, J.A., Zmuda, J., Lane, N.E., Magis, A.T., Lovejoy, J.C., Hood, L., Gibbons, S.M., Orwoll, E.S., & Price, N.D. (2021). Gut microbiome pattern reflects healthy ageing and predicts survival in humans. *Nat Metab, 3*(2), 274-286. doi:10.1038/s42255-021-00348-0

Witkowski, M., Weeks, T.L., & Hazen, S.L. (2020). Gut microbiota and cardiovascular disease. *127*(4), 553-570. doi:doi:10.1161/CIRCRESAHA.120.316242

Woo, V., & Alenghat, T. (2022). Epigenetic regulation by gut microbiota. *Gut Microbes, 14*(1), 2022407. doi:10.1080/19490976.2021.2022407

Wu, S., Liu, X., Jiang, R., Yan, X., & Ling, Z. (2021). Roles and mechanisms of gut microbiota in patients with alzheimer's disease. *Front Aging Neurosci, 13*, 650047. doi:10.3389/fnagi.2021.650047

Xiao, L., Liu, Q., Luo, M., & Xiong, L. (2021). Gut microbiota-derived metabolites in irritable bowel syndrome. *Front Cell Infect Microbiol, 11*, 729346. doi:10.3389/fcimb.2021.729346

Xu, C., Zhu, H., & Qiu, P. (2019). Aging progression of human gut microbiota. *BMC Microbiology, 19*(1), 236. doi:10.1186/s12866-019-1616-2

Yang, L., Bajinka, O., Jarju, P.O., Tan, Y., Taal, A.M., & Ozdemir, G. (2021). The varying effects of antibiotics on gut microbiota. *AMB Express, 11*(1), 116. doi:10.1186/s13568-021-01274-w

Yoo, J.Y., Groer, M., Dutra, S.V.O., Sarkar, A., & Mcskimming, D.I. (2020). Gut microbiota and immune system interactions. *Microorganisms, 8*(10). doi:10.3390/microorganisms8101587

Yoon, K., & Kim, N. (2021). Roles of sex hormones and gender in the gut microbiota. *Journal of neurogastroenterology and motility, 27*(3), 314-325. doi:10.5056/jnm20208

Yoshida, N., Emoto, T., Yamashita, T., Watanabe, H., Hayashi, T., Tabata, T., Hoshi, N., Hatano, N., Ozawa, G., Sasaki, N., Mizoguchi, T., Amin, H.Z., Hirota, Y., Ogawa, W., Yamada, T., & Hirata, K.I. (2018). *Bacteroides vulgatus* and *Bacteroides dorei* reduce gut microbial lipopolysaccharide production and inhibit atherosclerosis. *Circulation, 138*(22), 2486-2498. doi:10.1161/circulationaha.118.033714

Yoshida, N., Yamashita, T., Osone, T., Hosooka, T., Shinohara, M., Kitahama, S., Sasaki, K., Sasaki, D., Yoneshiro, T., Suzuki, T., Emoto, T., Saito, Y., Ozawa, G., Hirota, Y., Kitaura, Y., Shimomura, Y., Okamatsu-Ogura, Y., Saito, M., Kondo, A., Kajimura, S., Inagaki, T., Ogawa, W., Yamada, T., & Hirata, K.I. (2021). *Bacteroides* spp. Promotes branched-chain amino acid catabolism in brown fat and inhibits obesity. *iScience, 24*(11), 103342. doi:10.1016/j.isci.2021.103342

Zafar, H., & Saier, M.H., Jr. (2021). Gut bacteroides species in health and disease. *Gut Microbes, 13*(1), 1-20. doi:10.1080/19490976.2020.1848158

Zapata, H.J., & Quagliarello, V.J. (2015). The microbiota and microbiome in aging: Potential implications in health and age-related diseases. *J Am Geriatr Soc, 63*(4), 776-781. doi:10.1111/jgs.13310

Zheng, S.Y., Li, H.X., Xu, R.C., Miao, W.T., Dai, M.Y., Ding, S.T., & Liu, H.D. (2021). Potential roles of gut microbiota and microbial metabolites in parkinson's disease. *Ageing Res Rev, 69*, 101347. doi:10.1016/j.arr.2021.101347

Zhou, X., Jin, M., Liu, L., Yu, Z., Lu, X., & Zhang, H. (2020). Trimethylamine n-oxide and cardiovascular outcomes in patients with chronic heart failure after myocardial infarction. *ESC Heart Fail, 7*(1), 188-193. doi:10.1002/ehf2.12552

Appendix 1

Table A1. Similarity scores obtained with NCBI and RDP databases for the identification of each isolate.

Isolate	NCBI		RDP
	Query Cover (%)[a]	Percent Identity (%)[b]	S_ab score[c]
S1 GBL1 (*Escherichia coli*)	99 99 99 99 99	99.51 99.51 99.51 99.51 99.51	0.973 0.973 0.973 0.973 0.973
S1 GBL2 (*Escherichia fergusonii*)	98 98 98 98 98	99.9 99.8 99.8 99.8 99.8	0.99 0.987 0.987 0.987 0.986
S1 GYR1 (*Bacteroides koreensis)*	99 99 99 99 99	100 100 100 100 100	0.999 0.999 0.999 0.999 0.999
S1 MYR1 (*Parabacteroides distasonis*)	99 99 99 99 99	99.63 99.5 99.5 99.5 99.5	0.979 0.978 0.978 0.978 0.978
S1 MBR1 (*Escherichia coli*)	99 99 99 99 99	98.94 98.94 98.94 98.94 98.94	0.944 0.944 0.941 0.941 0.94
S1 PBR1 (*Escherichia coli*)	100 100 100 100 100	97.15 97.15 97.15 97.15 97.15	0.873 0.872 0.872 0.872 0.872
MHA GEHL (*Escherichia coli*)	96 96 96 97 96	98.43 98.43 98.43 98.35 98.26	0.864 0.863 0.862 0.861 0.861

Isolate	NCBI		RDP
	Query Cover (%)[a]	Percent Identity (%)[b]	S_ab score[c]
MHA GCEL (*Escherichia coli*)	98 98 98 99 98	96.61 96.61 96.61 96.41 96.41	0.957 0.954 0.953 0.953 0.952
MHB GCL (*Escherichia coli*)	99 99 99 99 99	99.82 99.81 99.81 99.81 99.81	0.984 0.984 0.983 0.982 0.982
MHB PEB1 (*Dysgonomonas* spp.)	99 99 99 99 99	100 100 100 100 100	0.997 0.997 0.997 0.997 0.997
MHB PEB2 (*Bacteroides fragilis*)	100 100 100 100 100	99.78 99.78 99.78 99.78 99.78	0.987 0.987 0.987 0.985 0.985
MHB PEB4 (*Phocaeicola dorei*)	100 100 100 100 100	99.65 99.65 99.65 99.65 99.65	0.98 0.98 0.98 0.98 0.98
MHB MEB3 (*Phocaeicola* spp.)	94 94 94 94 94	98 97.99 97.99 97.99 97.99	0.878 0.878 0.878 0.878 0.878
MNA MCL1 (*Escherichia coli*)	100 100 100 100 100	99.89 99.89 99.89 99.89 99.89	0.994 0.989 0.988 0.987 0.987
MNA MCL2 (*Shigella flexneri*)	100 100 100 100 100	99.35 99.35 99.35 99.35 99.35	0.976 0.972 0.97 0.969 0.969

Isolate	NCBI		RDP
	Query Cover (%)[a]	Percent Identity (%)[b]	S_ab score[c]
MNA PEB1 (*Escherichia* spp.)	99 99 99 98 98	99,8 99,8 99,8 100 99,9	0,989 0,987 0,987 0,987 0,987
MNA PEB3 (*Phocaeicola dorei*)	100 100 100 100 100	99.37 99.37 99.37 99.37 99.37	0.97 0.966 0.966 0.966 0.966
MNA MEB3 (*Bacteroides caccae*)	100 100 100 100 100	99.22 99.08 99.08 99.08 99.08	0.965 0.961 0.961 0.958 0.958
MNB GEL (*Klebsiella pneumoniae*)	100 100 100 100 100	99.89 99.77 99.77 99.77 99.77	0.991 0.991 0.991 0.991 0.991
FHA HEHP1 (*Bacteroides* spp.)	99 99 91 91 91	97.04 97.04 99.6 99.6 99.6	0.911 0.911 0.911 0.911 0.911
FHA HEHC2 (*Bacteroides ovatus*)	100 100 100 100 100	99.87 99.87 99.87 99.87 99.87	0.993 0.993 0.993 0.993 0.993
FHB2 (*Parabacteroides* spp.)	100 100 100 100 100	98.22 98.22 98.06 98.06 98.06	0.926 0.925 0.915 0.915 0.913
FHB5 (*Bacteroides ovatus*)	94 94 94 94 94	97.35 97.35 97.35 97.35 97.35	0.807 0.807 0.807 0.807 0.805

Isolate	NCBI		RDP
	Query Cover (%)[a]	Percent Identity (%)[b]	S_ab score[c]
FHB6 (*Bacteroides caccae*)	100 100 100 100 100	100 100 100 100 100	1 1 1 1 1
FHD PEB3 (*Phocaeicola* spp.)	100 100 90 100 100	92.14 92.14 94.7 91.92 91.92	0.708 0.708 0.695 0.695 0.695
FNA GEL (*Bacteroides ovatus*)	100 100 100 100 100	99.34 99.34 99.34 99.34 99.34	0.963 0.963 0.961 0.961 0.961
FNB PEB4 (*Bacteroides clarus*)	79 79 79 79 79	99.05 99.05 98.81 98.81 98.57	0.8 0.791 0.781 0.781 0.779
FNB MEB4 (*Phocaeicola dorei*)	98 98 98 98 98	99.61 99.61 99.61 99.61 99.61	0.967 0.966 0.966 0.966 0.964
FNB MEBH1 (*Bacteroides* spp.)	65 65 65 65 65	91.19 91.19 91.19 91.19 90.83	0.439 0.43 0.424 0.421 0.421

[a] Query Cover - the percentage of the query sequence (your specimen) that overlaps the reference sequence.
[b] Percent Identity - a number that describes how similar the query sequence is to the target sequence (how many characters in each sequence are identical).
[c] S_ab score - the number of (unique) 7-base oligomers shared between your sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences.

Table A2. Inhibition zone diameters for each tested bacterial isolate by Kirby-Bauer test.

Isolate	Replicates	CD (mm)	MXF (mm)	IMI (mm)
S1 GBL1	A1	0	28	25
	A2	0	30	25
	B1	0	30	26
	B2	0	31	25
S1 GBL2	A1	0	28	26
	A2	0	26	24
	B1	0	26	23
	B2	0	26	25
MHA GCEL	A1	0	29	27
	A2	0	30	28
	B1	0	30	28
	B2	0	28.5	25
MHA GEHL	A1	0	27	25
	A2	0	7	14
	B1	0	27	23
	B2	0	29	27

Isolate	Mean ± Standard Deviation		
	CD	MXF	IMI
S1 GBL1	0	29.8 ± 1.3	25.3 ± 0.5
S1 GBL2	0	26.5 ± 1.0	24.5 ± 1.3
MHA GCEL	0	29.4 ± 0.8	27.0 ± 1.4
MHA GEHL	0	22.5 ± 10.4	22.3 ± 5.7

Replicates A and B – biological replicas. Replicates 1 and 2 – technical replicas. The mean value of the double replicates inhibition zones was used establish the breakpoints according to EUCAST (2022).
CD – clindamycin. MXF – moxifloxacin. IMI – imipenem.

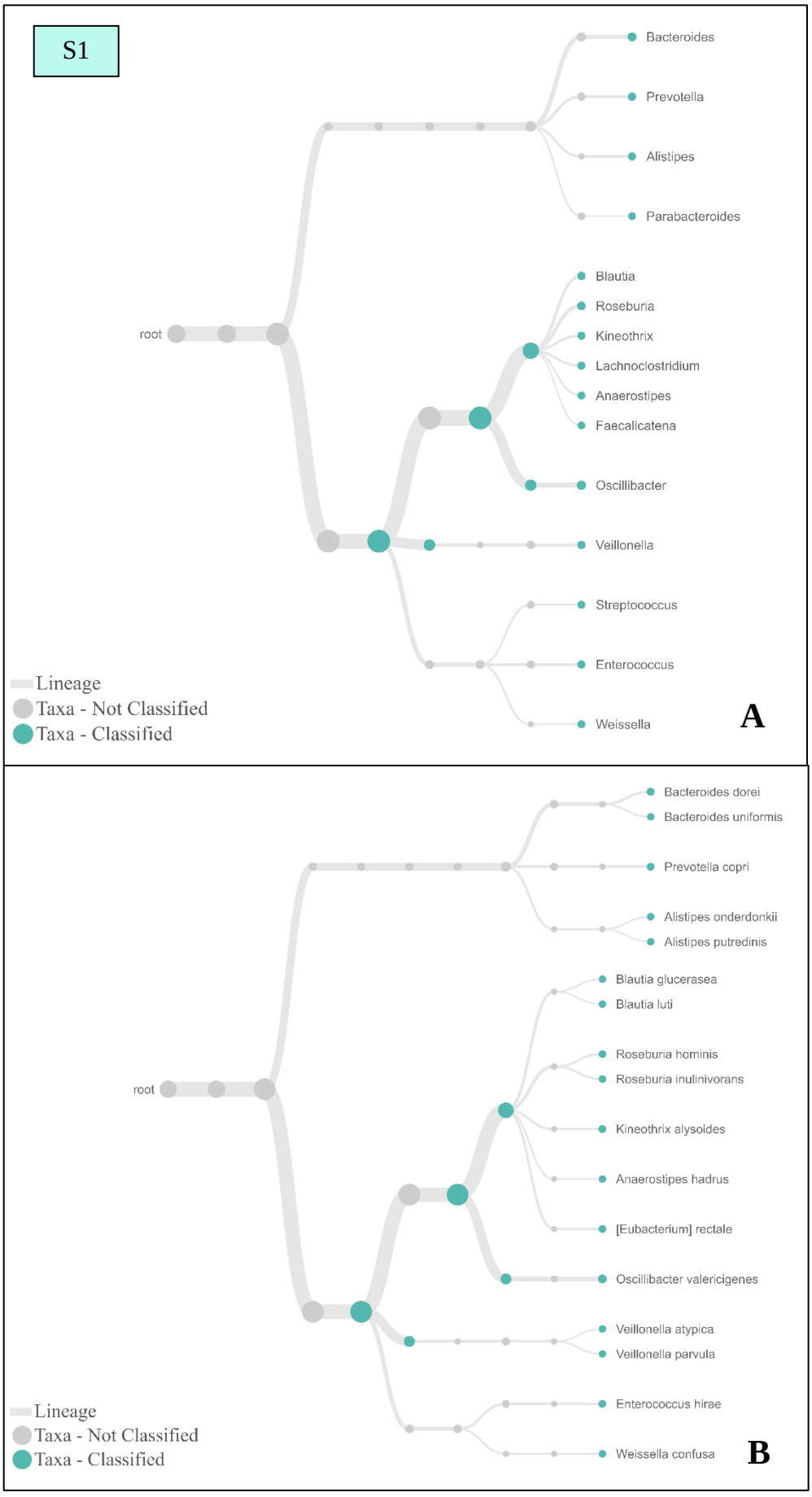


Figure A1. Phylogenetic representation of the genera (A) and species (B) found in all samples. Relative abundance is represented by the thickness of each branch. Data collected with the EPI2ME software.

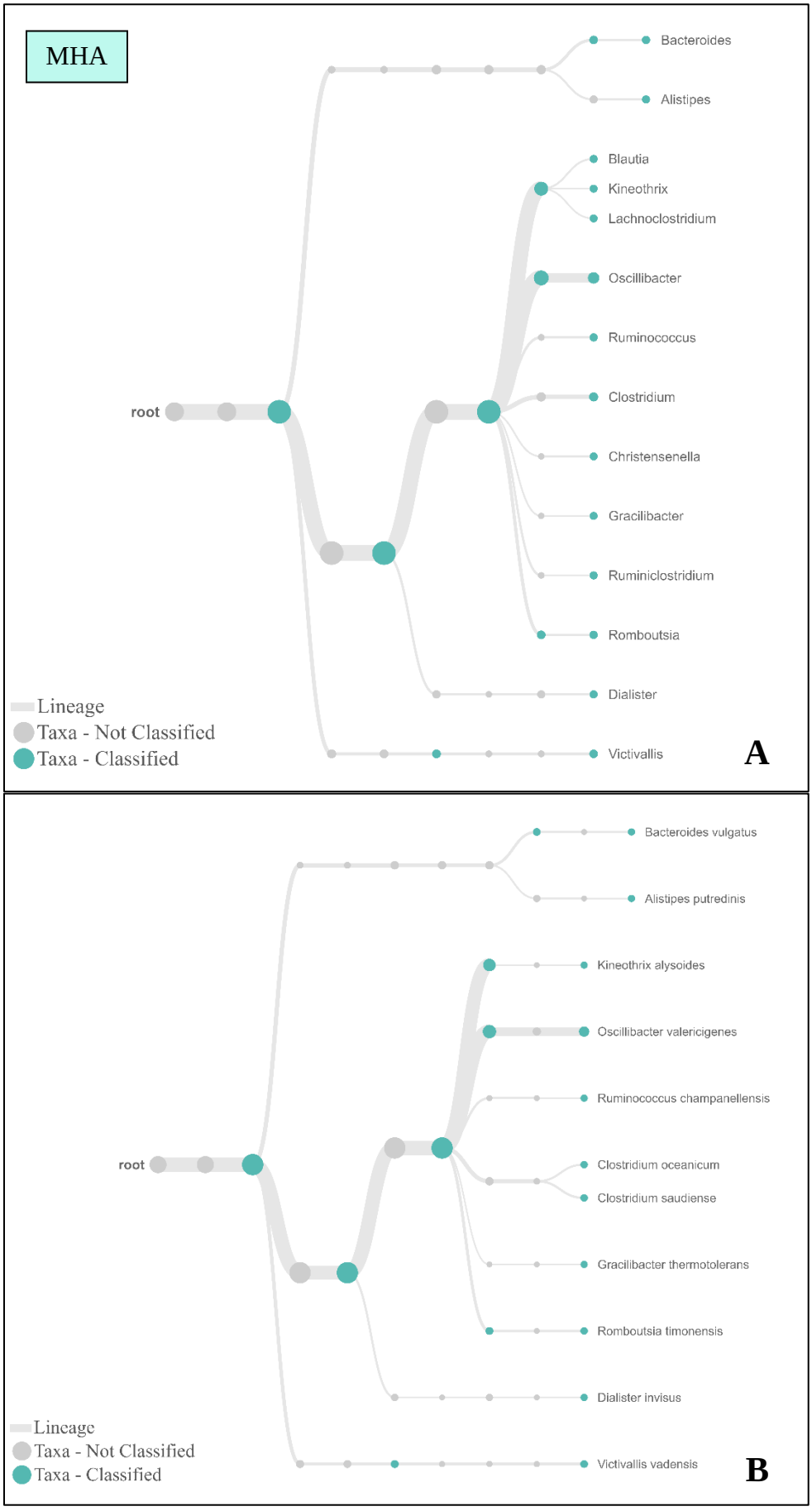


Figure A1. (continued)

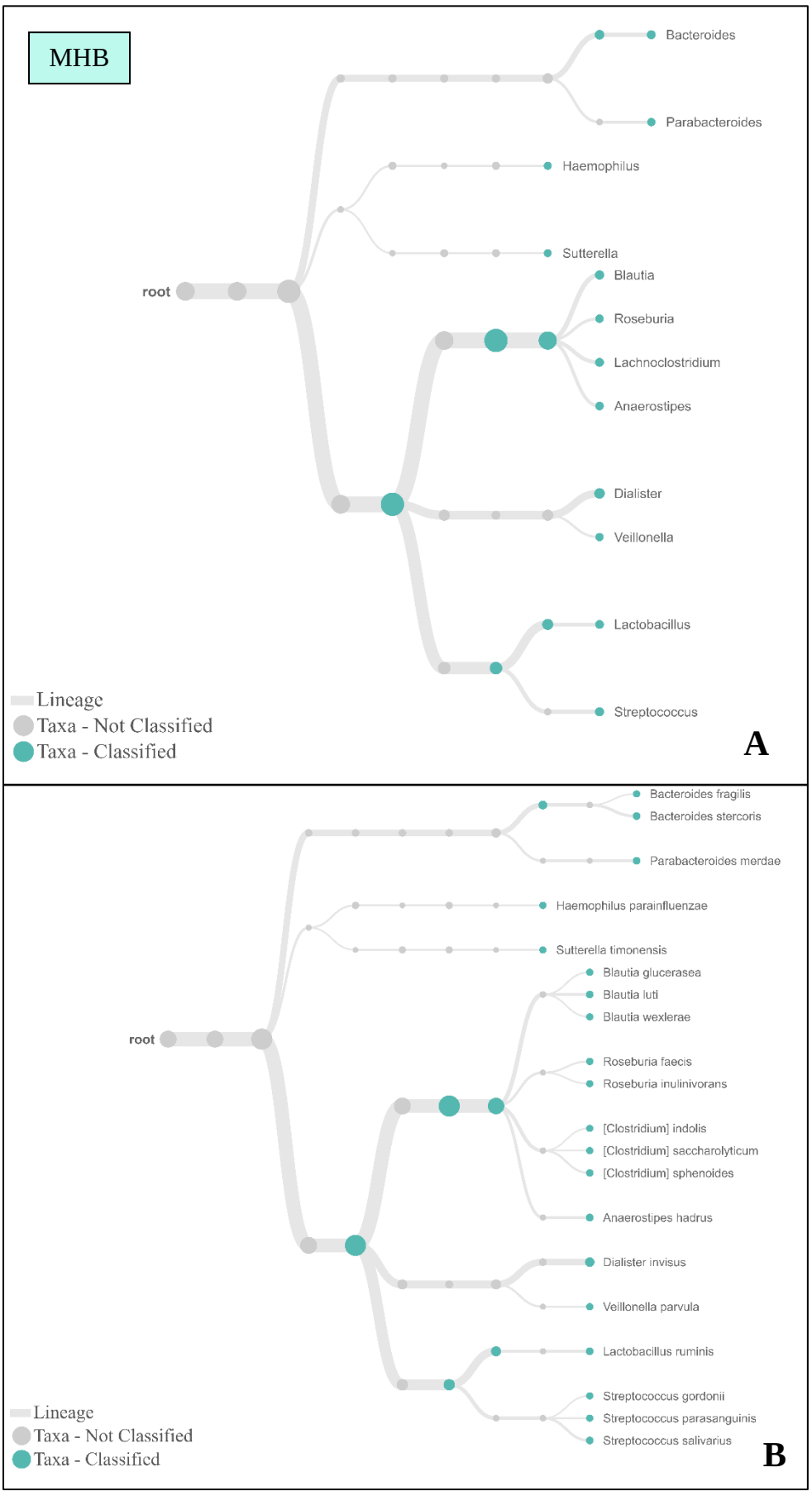


Figure A1. (continued)

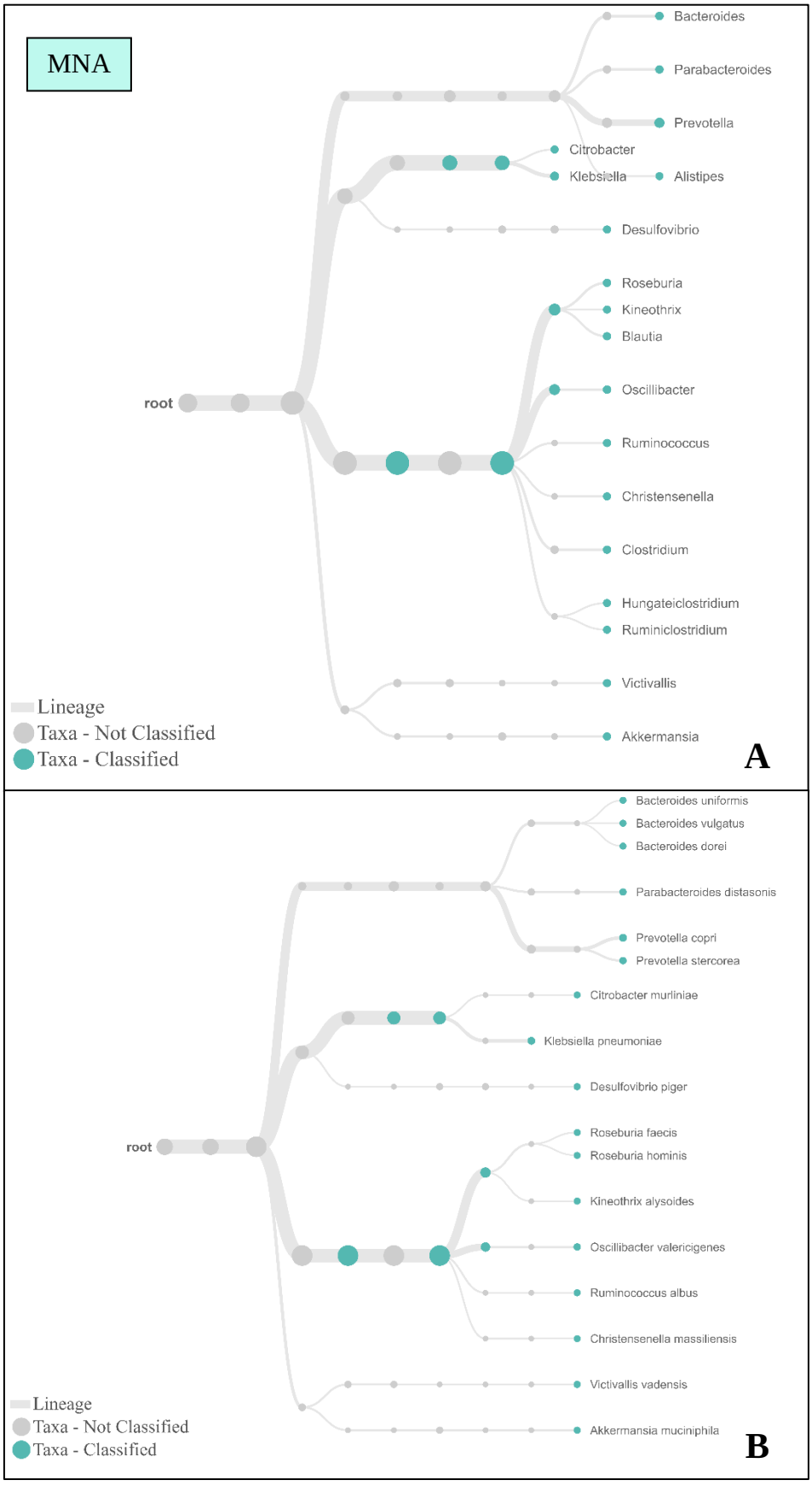


Figure A1. (continued)

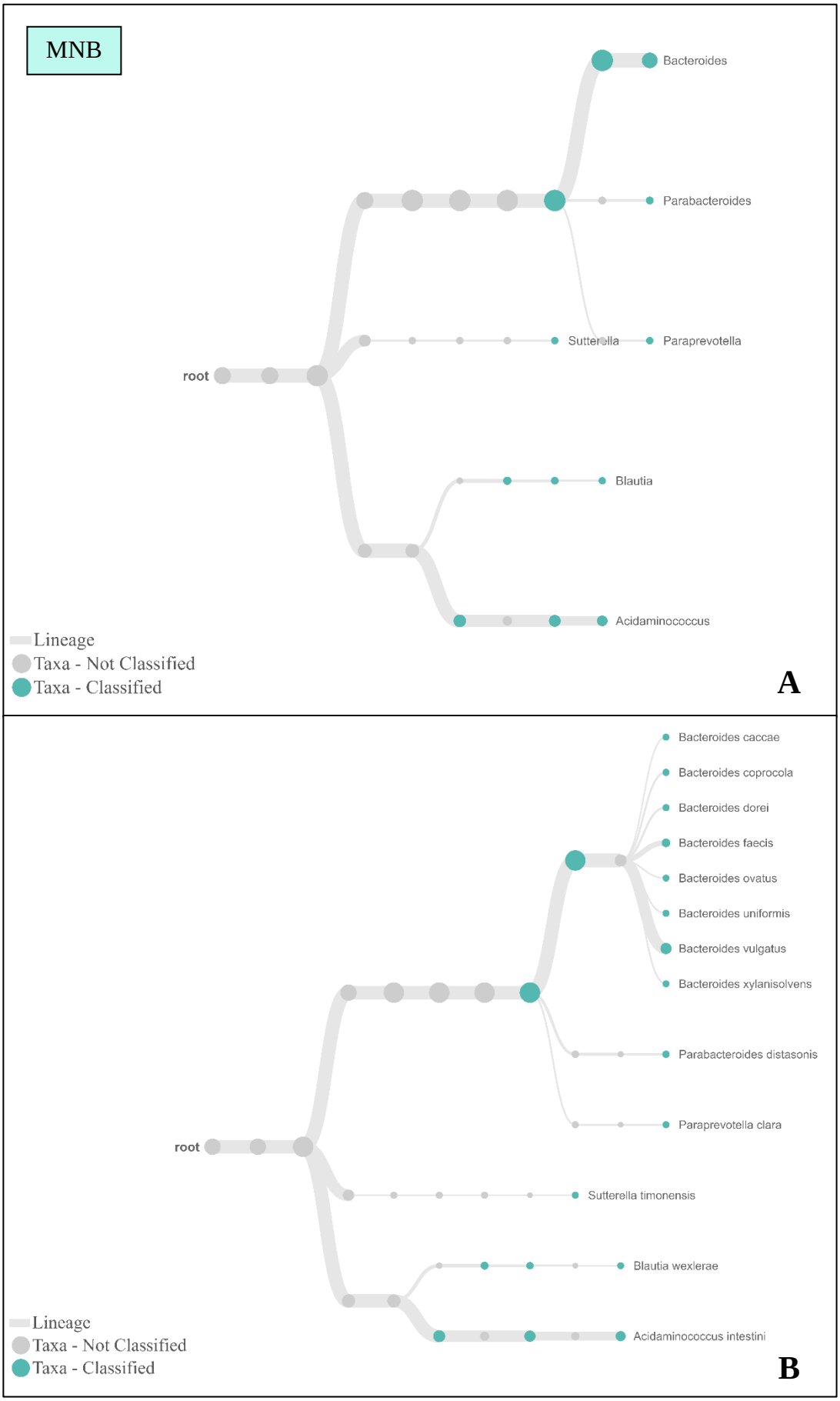


Figure A1. (continued)

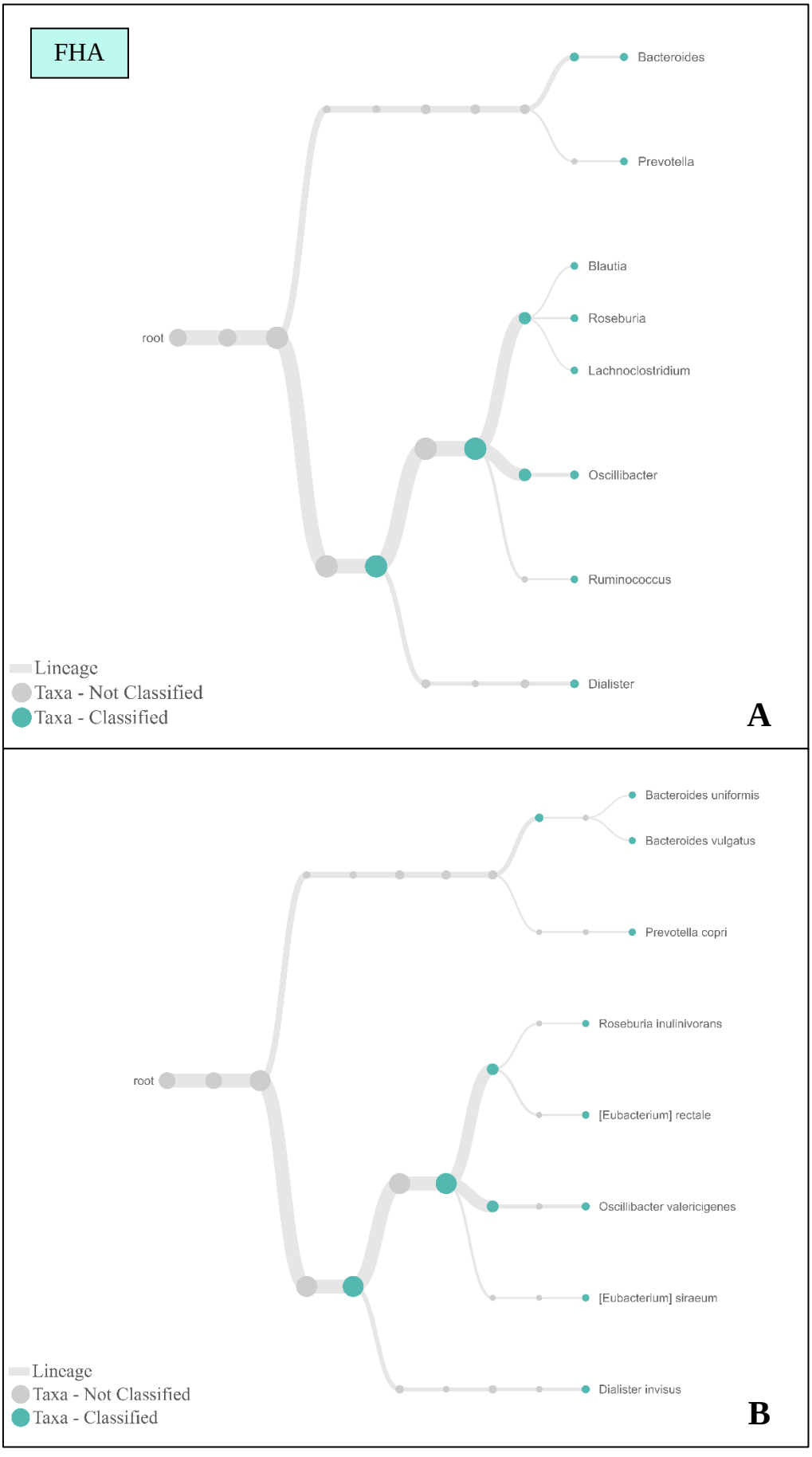


Figure A1. (continued)

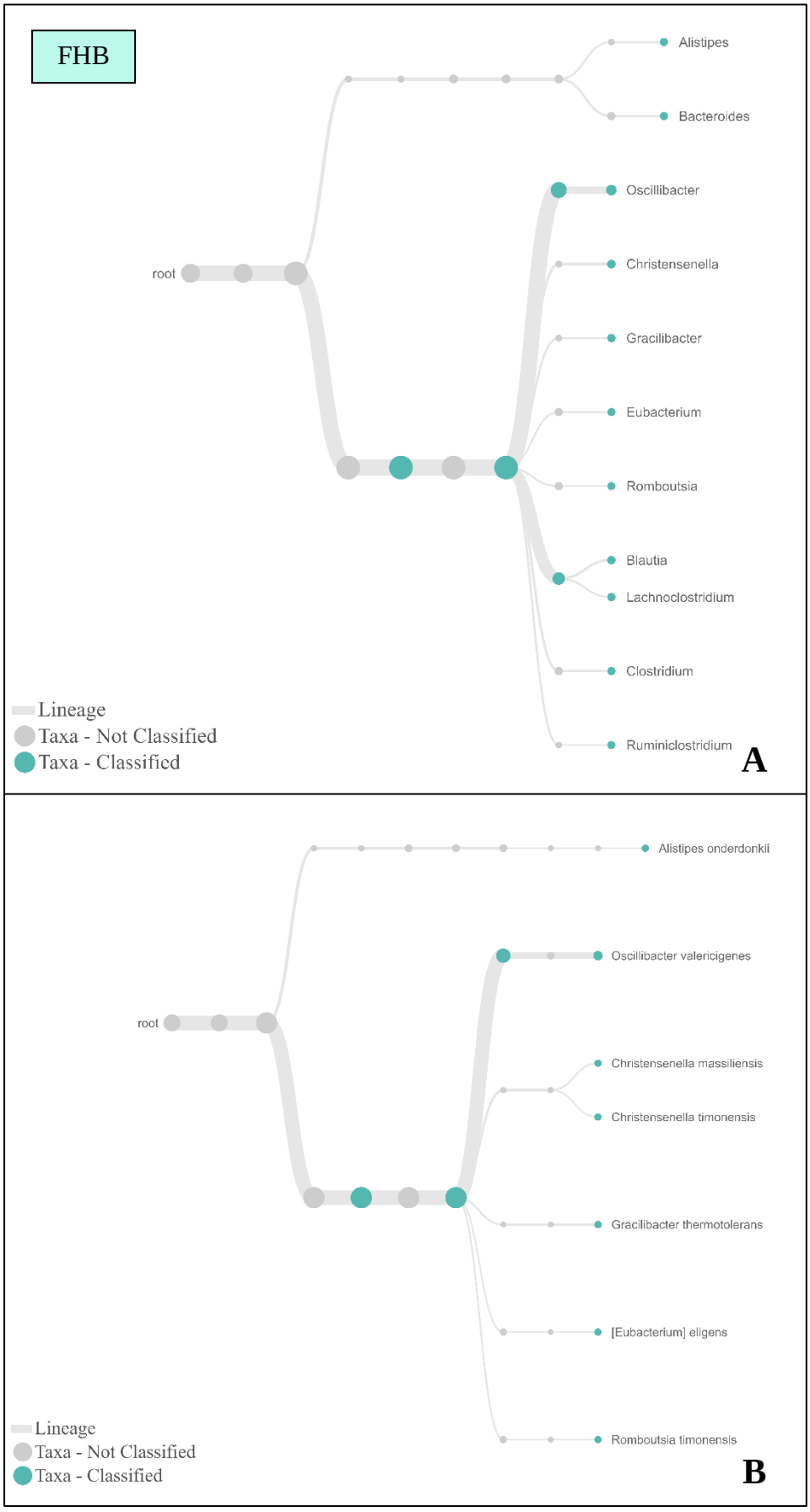


Figure A1. (continued)

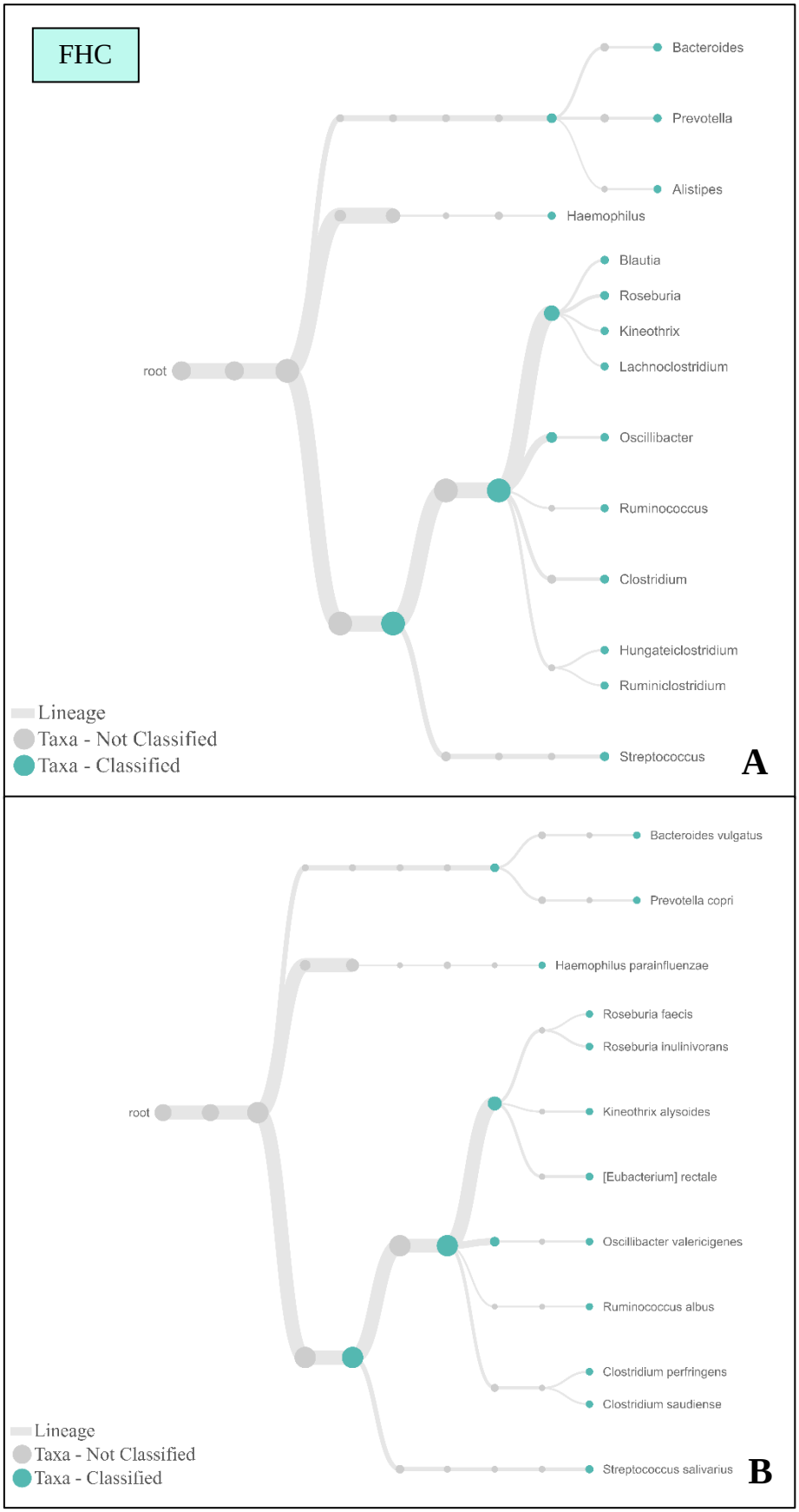


Figure A1. (continued)

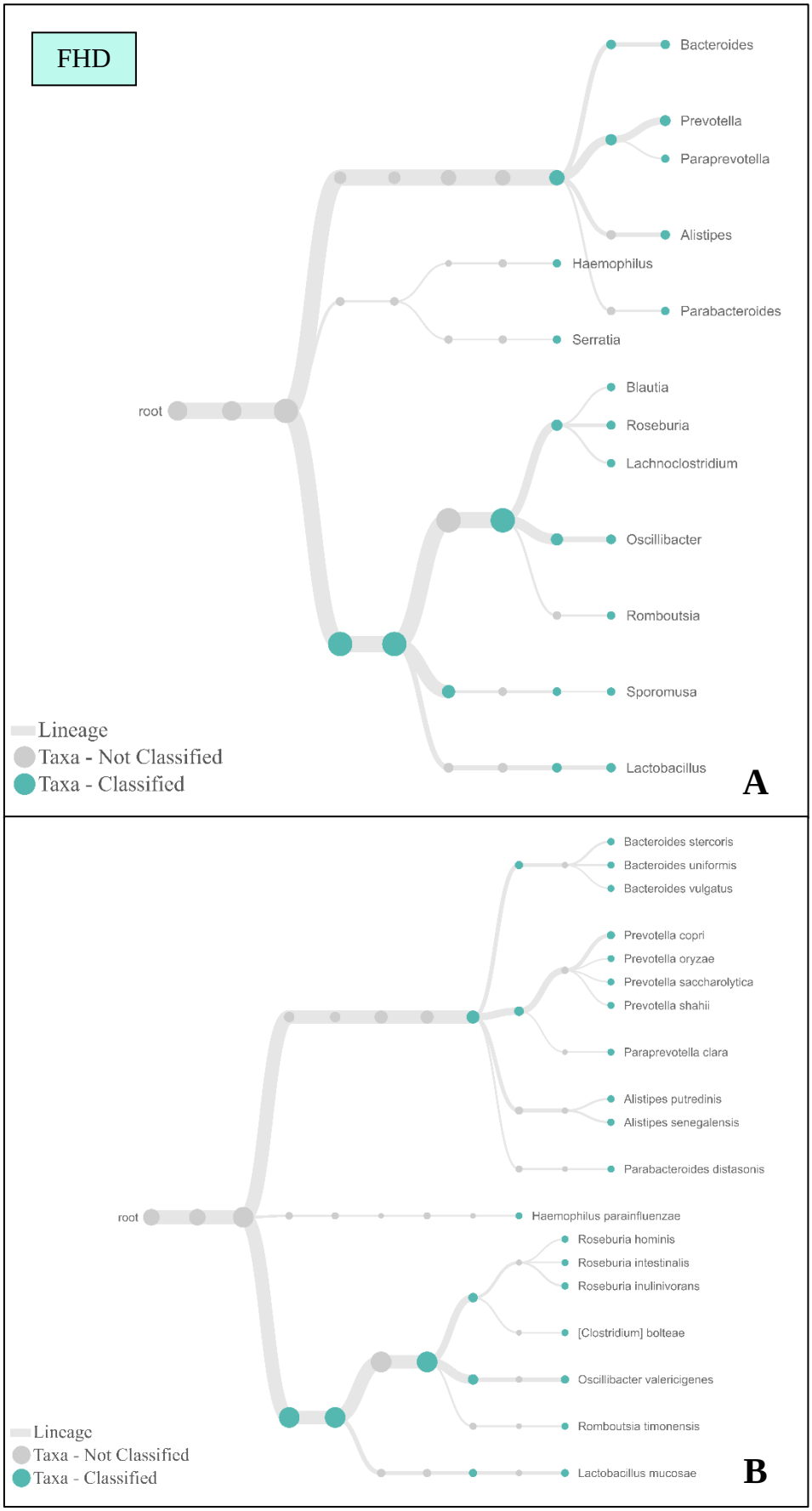


Figure A1. (continued)

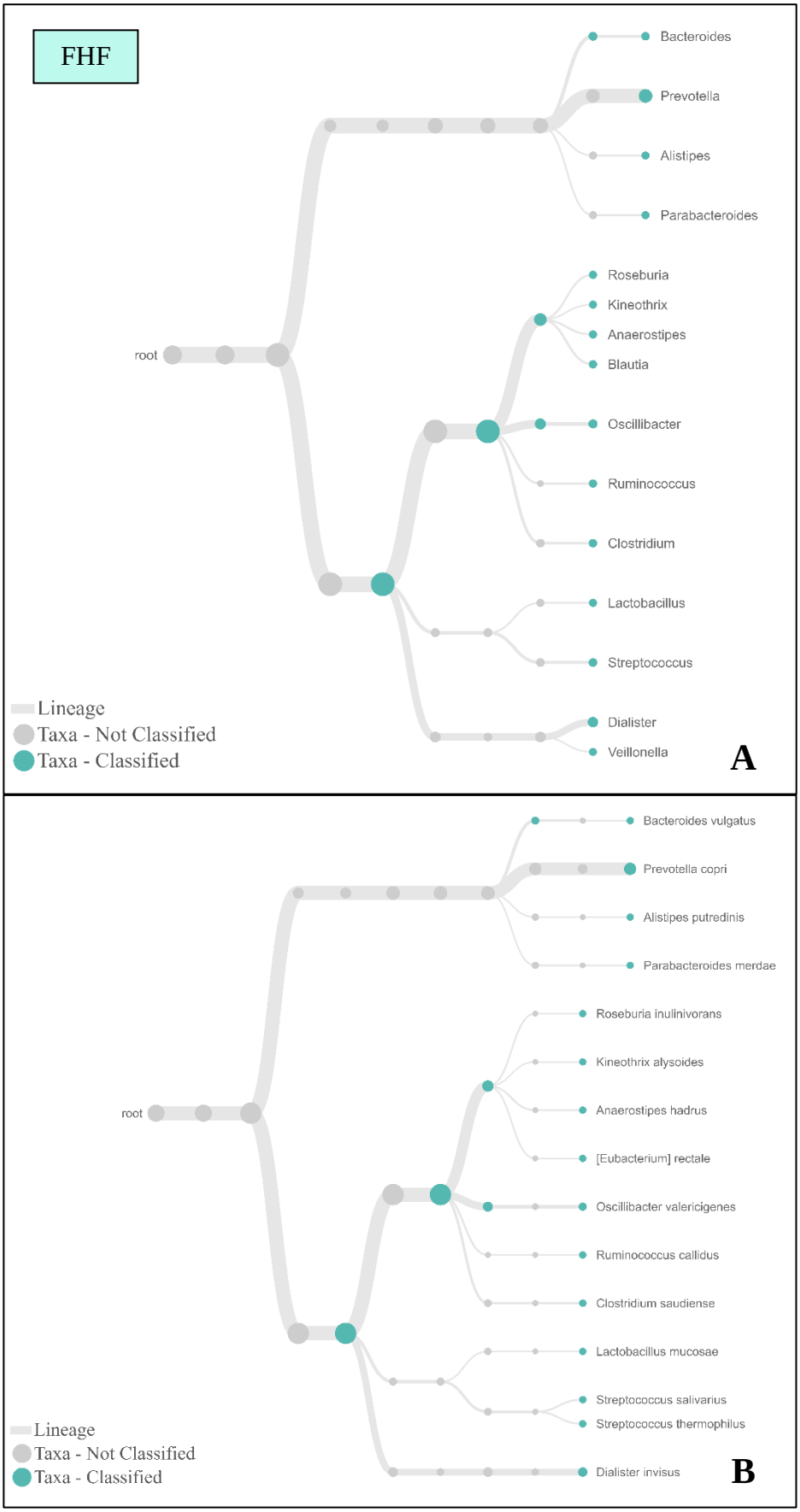


Figure A1. (continued)

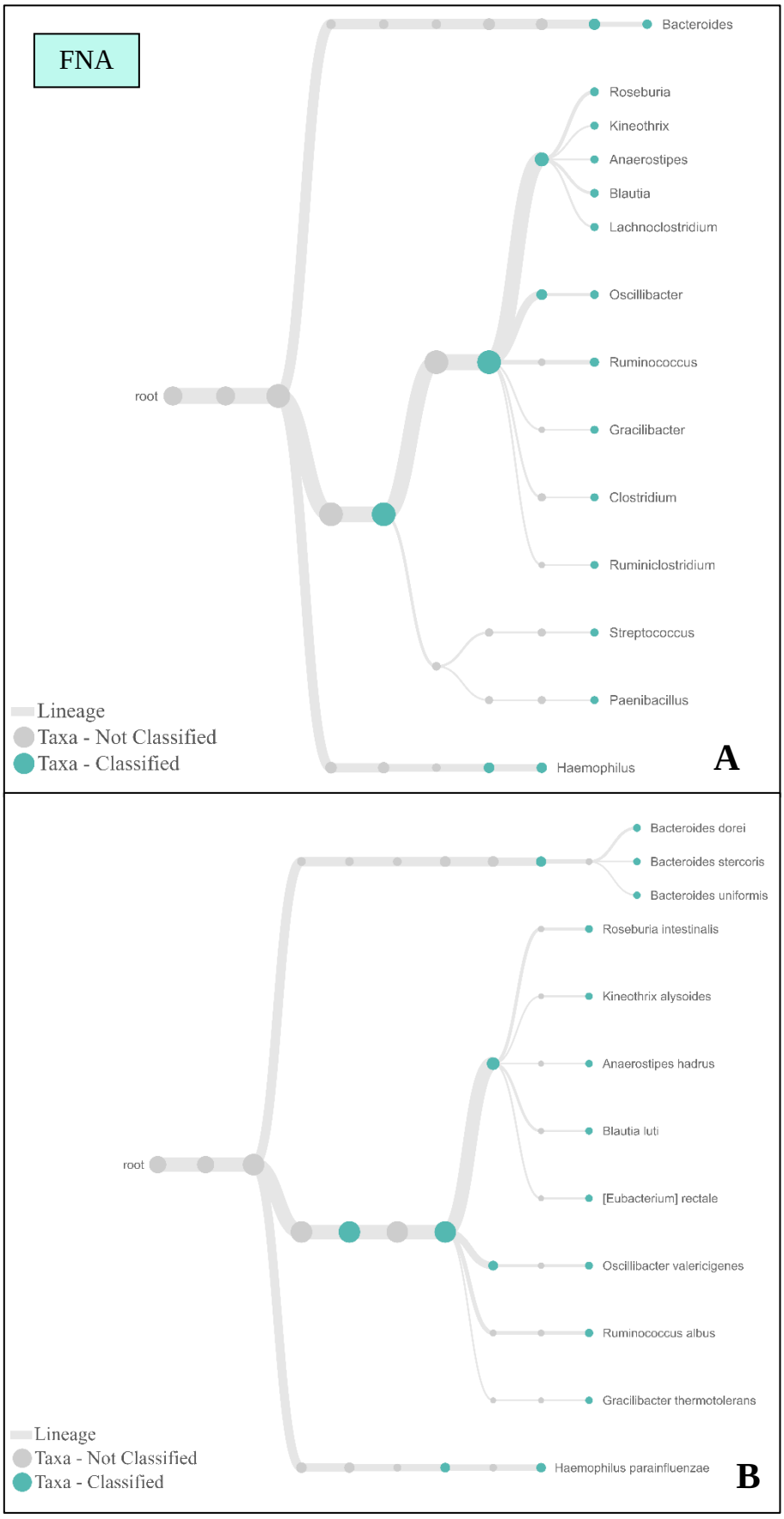


Figure A1. (continued)

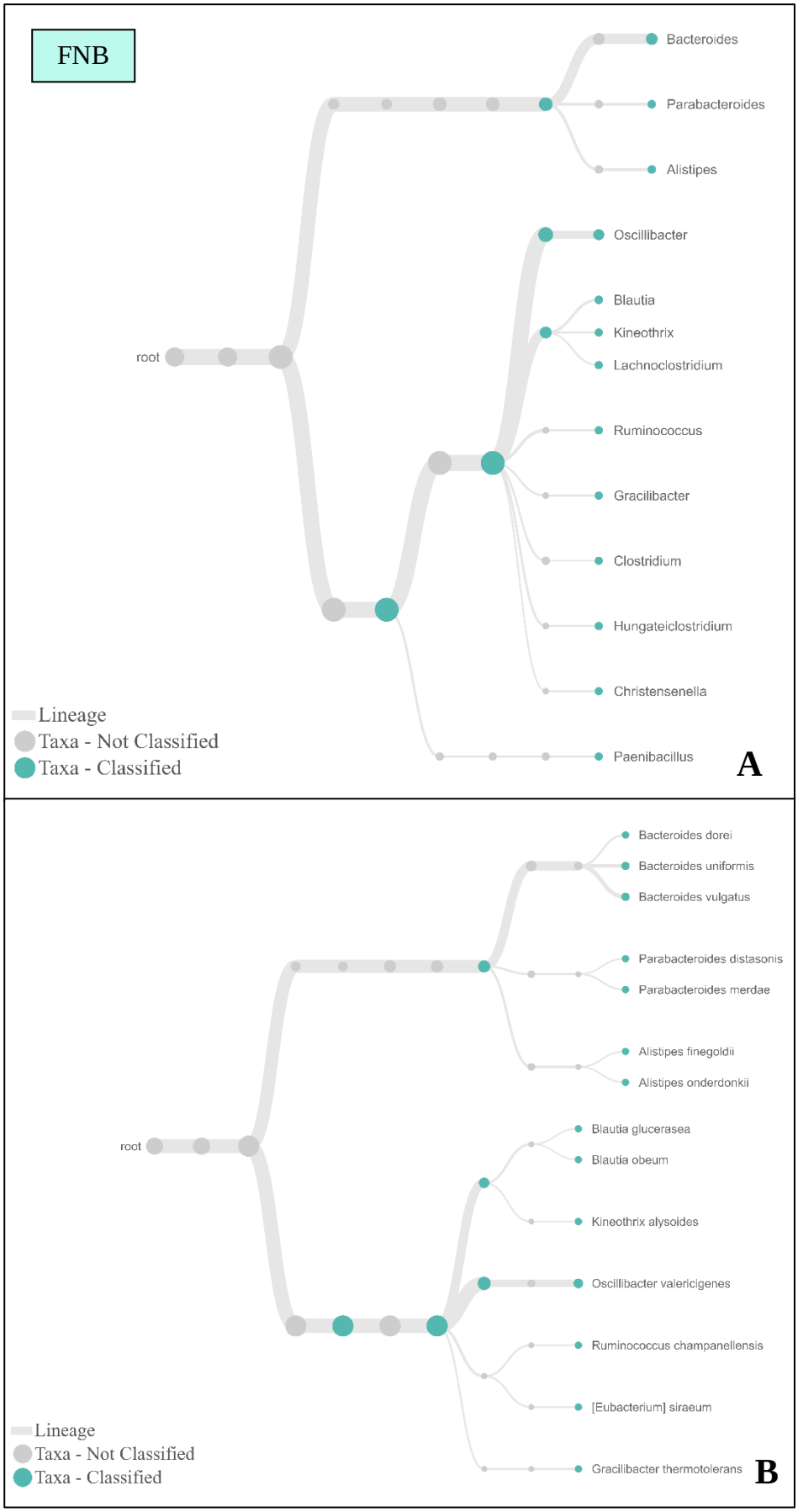


Figure A1. (continued)