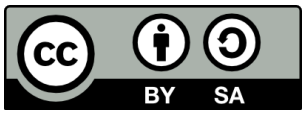
Universitat de Girona

MICROBIOTA AS INDICATOR OF INTESTINAL HEALTH AND MODULATION STRATEGIES

Lia Oliver Galindo



Universitat de Girona

DOCTORAL THESIS

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Lia Oliver Galindo

2024

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Microbiota as indicator of intestinal health and modulation strategies

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Doctoral program in Molecular Biology, Biomedicine and Health

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PhD thesis submitted in fulfilment of the requirements to obtain the doctoral degree from the University of Girona

Certificate of thesis direction

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CERTIFY:

That this doctoral thesis entitled "Microbiota as indicator of intestinal health and modulation strategies", that Lia Oliver Galindo submitted to obtain the doctoral degree from Universitat de Girona, has been completed under their supervision.

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AGRAÏMENTS

Diuen que si vols anar de pressa vagis sol, però que si vols arribar lluny vagis acompanyat, i en el meu camí he tingut a grans companys de viatge sense els quals tot això no hauria estat possible i que m'agradaria reconèixer aquí.

Començant per en **Jesús**, un dels principals "culpables" que hi hagi aquesta tesi i el codirector. Recordo com si fos ara la teva xerrada el desembre del 2020 quan em vas acabar de convèncer per fer la tesi i em vas dir que m'ajudaries a portar-ho a terme. I ho has complert. Gràcies pel teu suport i orientació, i per compartir els teus coneixements amb entusiasme. A en **Xevi**, l'altre codirector de la tesi. La teva visió mèdica ha estat fonamental per al desenvolupament de la tesi i la meva carrera professional a GoodGut. Agraeixo de tot cor la teva dedicació i el temps que sempre has trobat per a mi malgrat la teva ajustada agenda.

Aquesta tesi no hi seria si no fos per la **Mariona,** la "jefa". La teva confiança en el meu potencial i el teu suport incondicional m'han animat a superar-me i afrontar nous reptes, incloent-hi l'aventura de realitzar aquesta tesi doctoral. Hi ha moments que encara em pregunto com he arribat fins aquí, si jo mai he volgut fer una tesi! La teva perseverança és inspiradora i gràcies a tu, he arribat a llocs que mai hauria imaginat. GRÀCIES per tot sempre!

A l'aventura de GoodGut, agraeixo la companyia dels millors companys de viatge possibles, formant un autèntic *dream team*. En **Joanet**, el meu primer company, hem compartit mil històries, cançons Disney, hem parlat de viatges i ets el meu bessó de mà. Amb la **Marta**, hem creat una connexió única compartint rialles i llàgrimes en tots els moments. Amb la **Sara** ens coneixíem de la uni i he vist en tu una enorme evolució com a persona i professional i m'has donat suport sempre en tot. A la **Rosor** li agraeixo sortir de l'àmbit científic i compartir la seva saviesa sobre diners, recursos humans i la vida en general. A en **Dani**, per ser tant bo i recomanar-me sèries, monòlegs i *podcasts* de riure, gràcies a tu he rigut molt i això ha sigut un gran regal. I a tota la resta de persones que formen GoodGut, **Mireia**, **Sandra**, **Gemma**, **JuanVi**, **Marcel**, **Paula** i **Marc**, us estic molt agraïda per fer de GoodGut un lloc encara millor.

A l'**Anna Bahí**, en part estic a GoodGut per tu, perquè ens coneixíem d'abans i vas compartir a LinkedIn que buscaven algú, i aquí estic quasi vuit anys després. A l'**Aleix**, per ser primer company de laboratori i després ajudar-nos a trobar participants i mostres d'estudis. Per realitzar aquesta tesi, he rebut el suport de professionals mèdics excepcionals

com **Esther Fort**, **David Busquets**, **Laura Pardo** i **Sara Oduber**. Agraeixo la vostra amabilitat i dedicació. També vull expressar el meu agraïment als meus professors d'estadística, en **Marc Saez** i en **Martí Casals**, per ajudar-me amb les anàlisis estadístiques.

No puc oblidar que aquesta tesi s'ha escrit en el meu pitjor moment personal, i ara és el moment d'agrair a aquelles persones que m'han ajudat i acompanyat en aquest tràngol. Començant per la **Susanna**, ex veïna que malauradament vas haver de viure la mateixa experiència fa uns anys. Les teves trucades i visites sempre m'han reconfortat. A la **Marta** per la seva confiança i suport constant i sorprendre'm amb un detall post operació. A en **Yuri** i la **Laura** per estar pendents de mi i donar-nos suport mútuament. A l'**Eva**, agraeixo les teves trucades i la teva capacitat per distreure'm en els moments difícils. A la **Judith**, per fer temps per visitar-me i trucar-me. A la **Juani** i en **Juan** per cuidar-me i cuidar-nos tant com si fóssim de la vostra família, ens heu facilitat molt la vida en un moment molt complicat i això no ho oblidarem.

A la **Marina**, que tot i també estar passant per un procés molt difícil personal, sempre has trobat temps i forces per estar pendent de mi. A la **tieta Tere**, per ser la meva infermera sempre present des de la distància, m'he sentit molt acompanyada i cuidada per tu. A l'**àvia Montse**, perquè encara que diguis que ara no ens pots ajudar, ens has ajudat i tenir-te ha sigut imprescindible. A la **iaia Mercè**, per acollir-nos a casa teva i cuidar-nos tant, els teus menjars han sigut clau per la meva recuperació. A l'**Aniol** per ser-hi en els moments més difícils, sorprendrem amb detalls com una perruca de fantasia i ajudar-me sempre amb tot el que necessito. A la **Mama** i el **Papa,** a qui estic eternament agraïda per ser-hi sempre, per mimar-me, per cuidar-me, animar-me i tots els verbs que impliquen amor. Gràcies a vosaltres jo soc qui soc, i sense una base tan sòlida, mai m'hauria embarcat en aquesta aventura, i menys encara hagués arribat al final!

Al meu company de vida, **Oriol**, t'estic infinitament agraïda per tot el que fas, però no tinc prou paraules per descriure-ho. Gràcies per aportar-me tranquil·litat, acompanyar-me magníficament en tots els moments, per celebrar les petites i grans victòries i per creure en mi sempre.

I també em vull agrair a mi mateixa tot l'esforç fet. Aquesta tesi s'ha escrit en el pitjor moment personal, però ha sigut també un refugi. Quan el meu cap tenia ganes de pensar, jo li he dit: vols pensar? Doncs pensem, però pensaràs en el que jo et digui. I així hem escrit aquesta tesi. Així que em felicito per haver fet la tesi, per ser tan treballadora i valenta. Perquè si he pogut amb això en aquest moment tan complicat, sempre recordi que puc amb qualsevol cosa.

LIST OF PUBLICATIONS

Some results of this Doctoral Thesis have been published or submitted in scientific journals included in the Journal Citation Report of the Institute of Scientific Information. Peer reviewed publication obtained from this thesis are listed below:

1. Oliver L, Ramió-Pujol S, Amoedo J, Malagón M, Serrano M, Bahí A, Lluansí A, Torrealba L, Busquets D, Pardo L, Serra-Pagès M, Aldeguer X, Garcia-Gil J. **A novel grape-derived prebiotic selectively enhances abundance and metabolic activity of butyrate-producing bacteria in faecal samples.** Frontiers in Microbiology. Volume 12-2021. https://doi.org/10.3389/fmicb.2021.639948
   Frontiers in Microbiology Q1; IF_{2021}= 6.064
2. Oliver L, Ramió-Pujol S, Malagón M, Sánchez-Vizcaino M, Sánchez-Algans R, Lacosta E, Saéz-Zafra M, Serra-Pagès M, Aldeguer X, Garcia-Gil J, Oduber S. **Categorizing and characterizing intestinal dysbiosis: evaluating stool microbial test clinical utility.** Submitted to *Microbiome* on April 10th.
3. Oliver L, Fort E, Ramió-Pujol S, Malagón M, Bahí A, Puig C, Llirós M, Serra-Pagès M, Garcia-Gil J, Aldeguer X. **Increased abundance of gut microbiota with enzymatic activity in patients with exocrine pancreatic insufficiency.** Submitted to *Frontiers in Microbiology* on February 12th.

LIST OF ABBREVIATIONS

AKK	*Akkermansia muciniphila*
B46	best BLAST match *Subdolinogranulum variabile*
BAC	Bacteroidetes
BAs	Bile acids
BMI	Body mass index
CAN	*Candida albicans*
CD	Crohn's disease
CDI	*Clostridioides difficile* infection
CFS	Cell-free supernatant
CLA	Conjugated linoleic acid
CLO	*Clostridium* cluster I
CLR	Centred logratio transformation
CP	Chronic pancreatitis
CRC	Colorectal cancer
ECO	*Escherichia coli*
ENB	*Enterobacteriaceae* group
ENT	*Enterococcus* sp.
EPS	Exopolysaccharides
EUB	Eubacteria
FAE	*Faecalibacterium prausnitzii*
FIR	Firmicutes
FMT	Faecal microbial transplantation
FOS	Fructooligosaccharides
GAM	Gamma-proteobacteria
GI	Gastrointestinal
GLM	Generalized linear model
GOS	Galactooligosaccharides
GSPE	Grape seed extract
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IECs	Intestine epithelial cells

IESCs	Intestine epithelial stem cells
IFN	Interferon
IL	Interleukin
LAC	*Lactobacillus* sp.
LTAs	Lipoteichoic acids
MOS	Maltooligosaccharides
MSM	*Methanobrevibacter smithii*
NAFLD	Non-alcoholic fatty liver disease
NODs	Nucleotide-binding oligomerization domain
NSAIDs	Non-steroidal anti-inflammatory drugs
OR	Odds ratio
PEI	Pancreatic exocrine insufficiency
PERT	Pancreatic enzyme replacement therapy
PHGI	*Faecalibacterium prausnitzii* phylogroup I
PHGII	*Faecalibacterium prausnitzii* phylogroup II
PPIs	Proton pump inhibitors
PUFA	Polyunsaturated fatty acids
ROS	*Roseburia* sp.
RUM	*Ruminococcus* spp.
SCFAs	Short-chain fatty acids
T2DM	Type 2 diabetes mellitus
TAs	Teichoic acids
TLRs	Toll-like receptors
TMAO	Trimethylamine-N-oxide
TNF	Tumour necrosis factor
UC	Ulcerative colitis
WTAs	Wall teichoic acids
XOS	Xylooligosaccharides
XIV	*Clostridium* cluster XI

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SUMMARY

Intestinal microbiota encompasses a diverse community of microorganisms, comprising bacteria, viruses, fungi, and archaea, residing in the gastrointestinal tract. These microorganisms play a pivotal role in fundamental physiological functions, such as digestion, nutrient absorption, and immune system modulation, contributing significantly to overall gut health.

Conversely, dysbiosis denotes an imbalance or disruption in the composition and function of the intestinal microbiota. This disturbance may result in a shift in the microbial community, potentially fostering the overgrowth of harmful microorganisms while compromising the beneficial ones. Dysbiosis is intricately linked to various health conditions, implicated in the development or aggravation of gastrointestinal disorders and systemic diseases, including inflammatory bowel disease, irritable bowel syndrome, colorectal cancer, metabolic diseases, and chronic pancreatitis among others.

Throughout an individual's lifespan, the gut microbiota is shaped by a multitude of factors, ranging from birth, early life experiences, and breastfeeding to genetics, environmental exposures, diet, medications, and lifestyle habits. These elements collectively mould the diversity and composition of the microbial community in the gastrointestinal tract. Additionally, various modulation strategies, such as probiotics, prebiotics, synbiotics, postbiotics, and faecal microbiota transplant, offer interventions to restore or maintain a balanced microbial environment.

The primary focus of this thesis is to explore the intricate role of intestinal microbiota, including dysbiosis, in diverse symptoms and digestive disorders, while assessing the modulating potential of a novel grape-derived prebiotic.

To accomplish this objective, the clinical utility of an intestinal microbiota analysis test was examined through the analysis of different microbial markers in stool samples. This initial study assessed the test's robustness concerning sex and age and correlated alterations in microbial markers with digestive symptoms or conditions. Moreover, dysbiosis was categorized and characterized for the first time using a previously described index comprising two markers, *F. prausnitzii* and *E. coli*, distinguishing between transient and pathological dysbiosis.

Subsequently, the *in vitro* efficacy of a new grape-derived prebiotic was evaluated in stool samples from both control subjects and patients with inflammatory bowel diseases and irritable bowel syndrome. This prebiotic demonstrated significant efficacy in increasing species producing short-chain fatty acids, predominantly from *Roseburia* sp. and *F. prausnitzii*. Notably, the production of short-chain fatty acids saw significant increases, with acetate, butyrate, and propionate concentrations exhibiting marked improvements compared to control samples.

Finally, an analysis of the intestinal microbiota in patients with chronic pancreatitis and developed exocrine pancreatic insufficiency was conducted to identify potential alterations compared to control subjects. These patients, characterised by a lack of pancreatic enzyme production (amylase, protease, and lipase), exhibited an increase in microbial species with enzymatic activity, particularly lipase, compared to controls subjects. This suggests a possible compensatory effect of the intestinal microbiota in response to the enzyme deficiency.

The results obtained in this thesis validate the clinical utility of an intestinal microbiota test in stool, reveal the prebiotic's efficacy in improving short-chain fatty acid-producing species, and highlight a tailored gut dysbiosis in individuals with abnormal pancreatic conditions. These findings underscore the need to continue researching the complex relationship between gut microbiota and human health. Understanding and manipulating these influences and modulation strategies can optimize gut microbiota composition, promoting overall health and potentially preventing or alleviating various gastrointestinal and systemic conditions associated with dysbiosis.

RESUM

La microbiota intestinal engloba una comunitat diversa de microorganismes, que inclou bacteris, virus, fongs i arquees, que resideixen al tracte gastrointestinal. Aquests microorganismes tenen un paper fonamental en les funcions fisiològiques fonamentals, com ara la digestió, l'absorció de nutrients i la modulació del sistema immunitari, contribuint significativament a la salut intestinal general.

Per contra, la disbiosi denota un desequilibri o una interrupció en la composició i funció de la microbiota intestinal. Aquesta pertorbació pot provocar un canvi en la comunitat microbiana, fomentant el creixement excessiu de microorganismes potencialment nocius alhora que compromet els beneficiosos. La disbiosi està íntimament relacionada amb diverses condicions de salut, implicades en el desenvolupament o l'agreujament de trastorns gastrointestinals i malalties sistèmiques, com ara la malaltia inflamatòria intestinal, la síndrome de l'intestí irritable, el càncer colorectal, les malalties metabòliques i la pancreatitis crònica, entre d'altres.

Al llarg de la vida d'un individu, la microbiota intestinal està configurada per multitud de factors, que van des del naixement, les primeres experiències de la vida i la lactància materna fins a la genètica, les exposicions ambientals, la dieta, els medicaments i els hàbits de vida. Aquests elements modelen col·lectivament la diversitat i la composició de la comunitat microbiana del tracte gastrointestinal. A més, diverses estratègies de modulació, com ara probiòtics, prebiòtics, simbiòtics, post biòtics i trasplantament de microbiota fecal, ofereixen intervencions per restaurar o mantenir un ambient microbià equilibrat.

L'objectiu principal d'aquesta tesi és explorar el complex paper de la microbiota intestinal, inclosa la disbiosi, en diversos símptomes i trastorns digestius, alhora que s'avalua el potencial modulador d'un nou prebiòtic derivat del raïm.

Per aconseguir aquest objectiu, es va examinar la utilitat clínica d'una prova d'anàlisi de microbiota intestinal mitjançant l'anàlisi de diferents marcadors microbians en mostres de femta. Aquest estudi inicial va avaluar la robustesa de la prova pel que fa al sexe i l'edat i les alteracions correlacionades en els marcadors microbians amb símptomes o condicions digestives. A més, la disbiosi es va categoritzar i caracteritzar per primera vegada mitjançant un índex prèviament definit que consta de dos marcadors, *F. prausnitzii* i *E. coli*, distingint entre disbiosi transitòria i patològica.

Posteriorment, es va avaluar l'eficàcia *in vitro* d'un nou prebiòtic derivat del raïm en mostres de femta tant de subjectes control com de pacients amb malalties inflamatòries intestinals i síndrome d'intestí irritable. Aquest prebiòtic va demostrar una eficàcia significativa en l'augment d'espècies productores d'àcids grassos de cadena curta, principalment de *Roseburia* sp. i *F. prausnitzii*. En concret, la producció d'àcids grassos de cadena curta va experimentar augments significatius, amb les concentracions d'acetat, butirat i propionat que van mostrar millores notables en comparació amb les mostres de control.

Finalment, es va realitzar una anàlisi de la microbiota intestinal en pacients amb pancreatitis crònica i insuficiència pancreàtica exocrina desenvolupada per identificar possibles alteracions en comparació amb els subjectes control. Aquests pacients, caracteritzats per una manca de producció d'enzims pancreàtics (amilasa, proteasa i lipasa), van mostrar un augment d'espècies microbianes amb activitat enzimàtica, especialment lipasa, en comparació amb els subjectes controls. Això suggereix un possible efecte compensatori de la microbiota intestinal en resposta a la deficiència enzimàtica.

Els resultats obtinguts en aquesta tesi validen la utilitat clínica d'una prova de microbiota intestinal en femta, revelen l'eficàcia del prebiòtic per millorar les espècies productores d'àcids grassos de cadena curta i posen de manifest una disbiosi intestinal a mida en individus amb condicions pancreàtiques anormals. Aquestes troballes subratllen la necessitat de continuar investigant la complexa relació entre la microbiota intestinal i la salut humana. Comprendre i manipular aquestes influències i estratègies de modulació pot optimitzar la composició de la microbiota intestinal, promoure la salut general i prevenir o alleujar diverses condicions gastrointestinals i sistèmiques associades a la disbiosi.

RESUMEN

La microbiota intestinal engloba a una comunidad diversa de microorganismos, que incluye bacterias, virus, hongos y arqueas, que residen en el tracto gastrointestinal. Estos microorganismos desempeñan un papel fundamental en las funciones fisiológicas fundamentales, como la digestión, la absorción de nutrientes y la modulación del sistema inmunitario, contribuyendo significativamente a la salud intestinal general.

Por el contrario, la disbiosis denota un desequilibrio o interrupción en la composición y función de la microbiota intestinal. Esta perturbación puede provocar un cambio en la comunidad microbiana, fomentando el crecimiento excesivo de microorganismos potencialmente nocivos a la vez que compromete a los beneficiosos. La disbiosis está íntimamente relacionada con diversas condiciones de salud, implicadas en el desarrollo o agravamiento de trastornos gastrointestinales y enfermedades sistémicas, como la enfermedad inflamatoria intestinal, el síndrome del intestino irritable, el cáncer colorrectal, las enfermedades metabólicas y la pancreatitis crónica, entre otros.

A lo largo de la vida de un individuo, la microbiota intestinal está configurada por multitud de factores, que van desde el nacimiento, las primeras experiencias de la vida y la lactancia materna hasta la genética, las exposiciones ambientales, la dieta, los medicamentos y los hábitos de vida. Estos elementos moldean colectivamente la diversidad y la composición de la comunidad microbiana del trato gastrointestinal. Además, diversas estrategias de modulación, como probióticos, prebióticos, simbióticos, post bióticos y trasplante de microbiota fecal, ofrecen intervenciones para restaurar o mantener un ambiente microbiano equilibrado.

El objetivo principal de esta tesis es explorar el complejo papel de la microbiota intestinal, incluida la disbiosis, en varios síntomas y trastornos digestivos, al tiempo que se evalúa el potencial modulador de un nuevo prebiótico derivado de la uva.

Para conseguir este objetivo, se examinó la utilidad clínica de una prueba de análisis de microbiota intestinal mediante el análisis de diferentes marcadores microbianos en muestras de heces. Este estudio inicial evaluó la robustez de la prueba en lo que se refiere al sexo y la edad y las alteraciones correlacionadas en los marcadores microbianos con síntomas o condiciones digestivas. Además, la disbiosis se categorizó y caracterizó por primera vez mediante un índice previamente descrito que consta de dos marcadores, *F. prausnitzii* y *E. coli*, distinguiendo entre disbiosis transitoria y patologica.

Posteriormente, se evaluó la eficacia in vitro de un nuevo prebiótico derivado de la uva en muestras de heces tanto de sujetos control como de pacientes con enfermedades inflamatorias intestinales y síndrome de intestino irritable. Este prebiótico demostró una eficacia significativa en el aumento de especies productoras de ácidos grasos de cadena corta, principalmente de *Roseburia* sp. y *F. prausnitzii*. En concreto, la producción de ácidos grasos de cadena corta experimentó aumentos significativos, con las concentraciones de acetato, butirato y propionato que mostraron mejoras notables en comparación con las muestras de control.

Por último, se realizó un análisis de la microbiota intestinal en pacientes con pancreatitis crónica e insuficiencia pancreática exocrina desarrollada para identificar posibles alteraciones en comparación con los sujetos control. Estos pacientes, caracterizados por una carencia de producción de enzimas pancreáticas (amilasa, proteasa y lipasa), mostraron un aumento de especies microbianas con actividad enzimática, especialmente lipasa, en comparación con los sujetos controles. Esto sugiere un posible efecto compensatorio de la microbiota intestinal en respuesta a la deficiencia enzimática.

Los resultados obtenidos en esta tesis validan la utilidad clínica de una prueba de microbiota intestinal en heces, revelan la eficacia del prebiótico para mejorar las especies productoras de ácidos grasos de cadena corta y ponen de manifiesto una disbiosis intestinal a medida en individuos con condiciones pancreáticas anormales. Estos hallazgos subrayan la necesidad de seguir investigando la compleja relación entre la microbiota intestinal y la salud humana. Comprender y manipular estas influencias y estrategias de modulación puede optimizar la composición de la microbiota intestinal, promover la salud general y prevenir o aliviar diversas condiciones gastrointestinales y sistémicas asociadas a la disbiosis.

1

Introduction

1 Human microbiome

The human microbiota encompasses a diverse collection of microorganisms, including Eukaryotes, Archaea, Bacteria, and viruses, residing in different body habitats such as the skin, oral cavity, respiratory tract, gastrointestinal tract, urinary tract, and reproductive tract (1). These communities engage in a range of interactions, including commensalism, mutualism, and pathogenicity. They form a complex and discrete ecosystem that adapts to the environmental conditions of each niche (2). The term "human microbiome" refers to the genomic content of the microbiota at a specific body site. The human microbiota, primarily located in the gastrointestinal tract, is crucial in maintaining human health and homeostasis by influencing nutrient metabolism, immune system development, and defence against pathogenic invaders (3).

1.1 Structure and diversity of gut microbiota

The human gastrointestinal (GI) tract harbours a highly diverse and abundant microbial community known as the gut microbiota. This intricate ecosystem comprises over 10^{14} microorganisms, consisting of hundreds to thousands of different microbial species, which exhibit variations in composition, abundance, and diversity along the GI tract (4,5).

The gastrointestinal (GI) tract harbours a symbiotic relationship between the human host and the gut microbiota, which has undergone co-evolution. The microbiota populations have adapted to the various physiological conditions encountered along the GI tract, including changes in pH, oxygen levels, and nutrient availability (Figure 1). Consequently, anaerobic and facultative anaerobic bacteria tend to predominate in specific compartments of the organs (5). The majority of the human microbiota resides in the large intestine, primarily consisting of obligate anaerobes, surpassing the number of human cells by an order of magnitude (6). The duration of transit in the small intestine influences bacterial adherence and colonization. As oxygen levels are higher in the small intestine, the bacterial species inhabiting this environment are primarily facultative anaerobes capable of tolerating lower pH levels, such as the *Lactobacillaceae* family (5). In contrast, the colon exhibits a limited availability of easily digestible carbon sources, requiring bacteria capable of breaking down "resistant" polysaccharides, thereby promoting the growth of fermentative anaerobes (5).

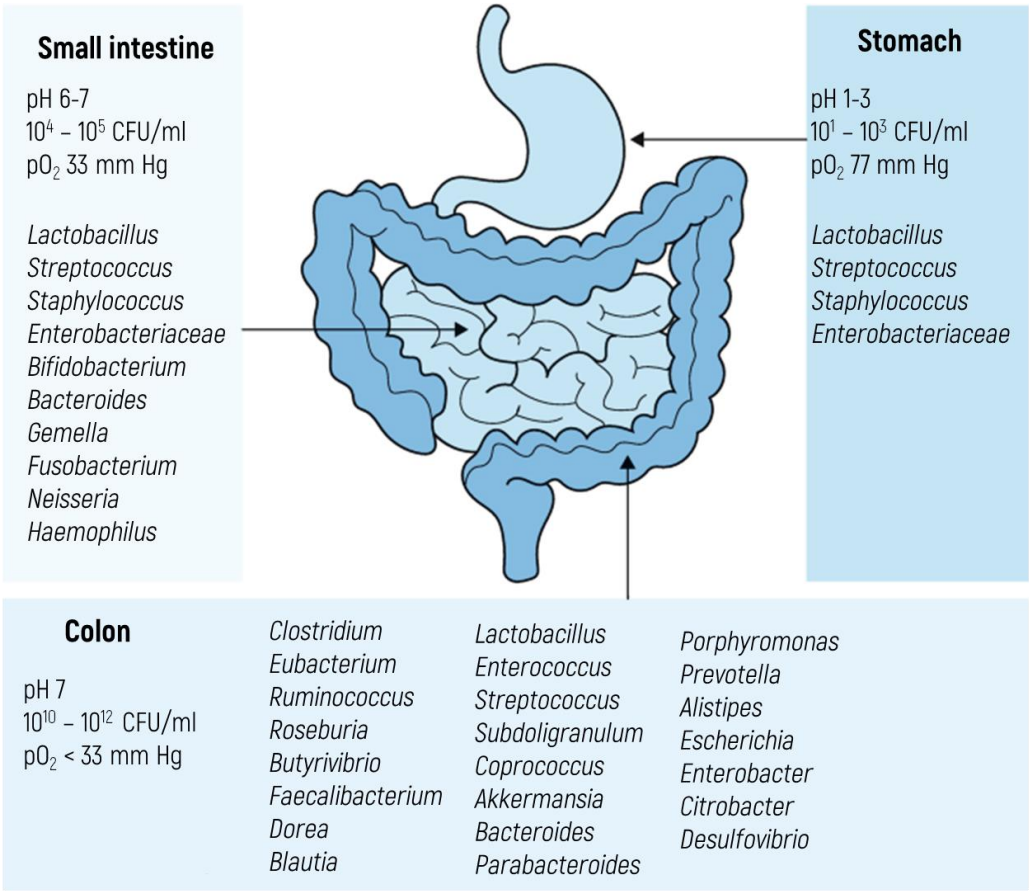

Figure 1. Distribution and abundance of bacteria in the human GI tract.

All parts of the gastrointestinal tract are covered by mucus. The mucus is mainly composed of water, and highly glycosylated gel-forming mucins, which are produced and continuously renewed by goblet cells (7). The mucus layer in the colon is much thicker than in the small intestine and is composed of outer and inner layers (7,8). The thickness, as well as the microbial composition, is different between these two layers (5,7). Also, the thickness of these layers is very variable and influenced by the bacteria present.

The composition of the intestinal microbiota varies between the luminal and mucosal compartments, with Bacteroidetes and Firmicutes phyla accounting for over 90% of the microbial population, while other less predominant phyla, such as Proteobacteria, Actinobacteria, Fusobacteria, fungi, and viruses, compose the remaining populations (4,9).

Firmicutes, the largest phylum in the gut microbiota, comprises numerous genera, with *Clostridium* and *Lactobacillus* being the most abundant. Firmicutes bacteria are gram-positive possessing efficient capabilities for energy extraction from the host diet (10). Bacteroidetes, the second most prevalent phylum, encompasses three classes of gram-negative, non-spore-forming, rod-shaped bacteria: *Flavobacteria*, *Sphingobacteria*, and the extensively studied *Bacteroides*. The genus

Bacteroides plays a vital role in metabolic processes within the human host (11). Proteobacteria, another phylum present in the gut microbiota, consists of five classes of gram-negative bacteria, classified primarily based on trophic status. Gamma-proteobacteria is the most diverse class and includes human pathogens like *Vibrio cholerae* and enteric bacteria such as *Escherichia coli* (12). Actinobacteria, a gram-positive phylum, contains clinically relevant genera such as *Bifidobacterium* and Actinomyces (13).

While the core phyla remain consistent among individuals, substantial inter- and intra-individual variation in microbial diversity has been observed (4,14).

1.2 Acquisition and stability

The development of the intestinal microbiota system primarily occurs within the initial year of life. The colonization process of the gut microbiota initiates even during pregnancy, which challenges the conventional notion of a sterile foetal environment. Recent research has provided evidence that microbial communities resembling those in the oral cavity can exist in the placenta and amniotic fluid, indicating the potential for in-utero colonization (15,16). Nevertheless, the primary acquisition of the initial gut microbiota primarily takes place during and after birth, with factors such as the mode of delivery and early feeding patterns exerting influence (17).

Vaginally born infants acquire their first gut microbiota from the maternal vaginal and faecal microbiota, leading to the establishment of their initial intestinal microbiota in the early months of life. The vagina-harboured genera such as *Bacteroides*, *Bifidobacterium*, *Escherichia*, *Shigella*, and *Parabacteroides* are among the first colonisers. In contrast, infants delivered by caesarean section acquire their first colonisers from the mother's skin, with microbes such as *Enterobacter hormaechei*, *Haemophilus parainfluenzae*, *Enterococcus faecalis*, and *Streptococcus australis*. Additionally, environmental microbes in the surrounding environment are another significant source of the infants' intestinal microbes (17,18).

The intestinal microbiota exhibits considerable fluctuations throughout infancy before developing a more stable microbiota in adulthood. These fluctuations primarily occur during the first year of life due to various environmental exposures, including exposure to the microbiota of family members and dietary influences. Factors such as feeding patterns (breastfeeding or formula-feeding), the introduction of solid food, and antibiotic treatment significantly influence the gut microbiota, leading to changes in its composition based on dietary and health conditions (19,20).

Breastfeeding plays a crucial role in promoting the growth of beneficial bacteria, particularly *Bifidobacteria* and *Lactobacilli*, thus shaping the early composition of the gut microbiota. In

contrast, formula-fed infants exhibit a distinct microbial profile characterised by a predominance of *Bacteroides* and *Bifidobacteria*, along with the presence of *Escherichia coli* and *Staphylococci* (17,21). Furthermore, introducing solid food increases the abundance of Bacteroidetes, which may enhance the capacity for short-chain fatty acids (SCFAs) synthesis, carbohydrate utilisation, and vitamin biosynthesis (22). Adequate nutrition is also a critical factor in shaping the development of the intestinal microecosystem. A sufficient nutritional supply is necessary for colonising the gut microbiota, as malnutrition can lead to an imbalanced microecosystem (23).

By the end of the third year after birth, children's intestinal microbiota gradually matures, resulting in a more stable and adult-like microecosystem structure. Therefore, the first three years of life represent the most critical period for dietary interventions to improve child growth and development. This is the period when the intestinal microbiota, a vital asset for health and neurodevelopment, is established, and its alteration during this period has the potential to affect host health and development (19,20) profoundly. This stable microecosystem persists throughout life, although it can be altered by factors such as pathogen infection, antibiotic abuse, and long-term changes in diet and lifestyle. Maintaining the stability of the intestinal microecosystem is beneficial for the host's health, as it contributes to the production of important metabolites, the maturation of the host immune system, and protection against pathogen infection. Conversely, disruptions and abnormalities in the gut microbiota can increase the risk of various diseases (19).

1.3 Gut microbiota functionality

The intestinal microbiota has co-evolved with human hosts to establish a state of mutualistic symbiosis, in which humans provide nutrition and a physiological environment. In contrast, the microbiota fulfils a broad range of essential functions that promote host health.

- Nutrient Metabolism and Digestion

The intestinal microbiota plays a crucial role in nutrient metabolism and digestion processes. It includes microbial species with enzymes capable of breaking down complex carbohydrates, such as dietary fibres, resistant to human digestive enzymes. These microbes perform fermentation, producing vitamins and SCFAs such as acetate, propionate, and butyrate. SCFAs serve as an energy source for the host and contribute to the colon's overall health. Furthermore, gut bacteria metabolise bile acids, amino acids, and vitamins, helping extract nutrients from ingested food (24,25) (see section 1.3 for further information).

- Development and function of the immune system

The intestinal microbiota plays a crucial role in the development and function of the host's immune system. Commensal microorganisms stimulate immune cell maturation and promote immune tolerance, leading to host-microbiota symbiosis. Microbes within the gut produce metabolites that modulate immune responses, regulate inflammation, and maintain intestinal barrier integrity. Dysbiosis, an imbalance in the gut microbial composition, has been linked to immune-related disorders, including allergies, inflammatory bowel disease (IBD), and autoimmune conditions (26,27).

Exposure to commensal microbes during early life is essential for proper immune training, leading to the expansion of regulatory T cells rather than inflammatory effectors (27). Perturbed microbiota-mediated immunity training in early years has been associated with increased susceptibility to infection and disease. Studies on germ-free mice have revealed the importance of the microbiota in immune system development, as germ-free mice display impaired immune function (27). The distribution of immune cells in the intestine varies, with the small intestine focusing on tolerance towards food antigens and defence against pathogens. In contrast, the large intestine focuses on homeostasis with the commensal microbiota. Some bacteria have immunomodulatory abilities that promote tolerance to the microbiota.

The microbiota also influences the mucosal glial cell network and intestinal barrier function. It contributes to the postnatal development and continuous homeostasis of the glial cell network and affects mucus renewal and intestinal barrier function. The host and bacteria have mutually adapted to each other, contributing to a remarkable symbiosis. However, the mechanisms by which the immune system distinguishes between pathogens and commensal bacteria are not fully understood. Microbial metabolites have been implicated in the maintenance of immune homeostasis. Early colonisation of the intestinal microbiota during a critical window appears crucial in establishing a healthy relationship and maintaining homeostasis (28).

- Protection against pathogens

The intestinal microbiota is crucial in providing barrier effects and protecting the gut from colonisation and invasion by potentially pathogenic microorganisms and toxins. Commensal bacteria compete for resources, acting as a physical barrier against pathogenic invasion and controlling overgrowth. Furthermore, they produce antimicrobial substances that inhibit the growth of similar species. Additionally, the production of antimicrobial peptides and SCFAs reinforces the integrity of the gut epithelial layer by adjusting the local pH and inhibiting the growth of potential pathogens, preventing the translocation of harmful bacteria into the bloodstream.

Finally, gut bacteria can directly interact with pathogens, impeding their adhesion and colonisation in the intestinal mucosa (25,29).

- Metabolic regulation and energy homeostasis

Increasing evidence suggests that the gut microbiota is implicated in metabolic regulation and energy homeostasis. The composition of the gut microbiota has been associated with the development of metabolic disorders, including obesity and type 2 diabetes. Microbial metabolites, such as SCFAs, have been shown to influence host metabolism, appetite regulation, and insulin sensitivity. Furthermore, gut bacteria participate in the enterohepatic circulation of bile acids, which play a role in lipid metabolism and energy utilisation (24).

- Neurological and mental health

The gut-brain axis, a bidirectional communication system between the gut microbiota and the central nervous system, has gained attention recently. Gut microbes produce neurotransmitters and neuroactive compounds that can influence brain function and behaviour. Additionally, alterations in gut microbiota composition can impact stress responses and cognitive function. Signals from the brain also affect the gut microbiota, and stress and inflammatory mediators can alter the interaction between the gut mucosa and microbiota. The involvement of neural pathways and enteroendocrine cells, which release hormones and react to microbial presence, play essential roles in microbiota-gut-brain signalling. Inflammation can disrupt microbiota-gut-brain communication through increased gut permeability. This axis is implicated in neurological disorders like autism spectrum disorder and depression (30,31).

- Drug metabolism

The intestinal microbiota contributes to the metabolism and biotransformation of various drugs and xenobiotics. Some microbial enzymes can modify the chemical structure of drugs, affecting their efficacy and toxicity. Moreover, interactions between gut bacteria and medications can impact drug absorption, distribution, and elimination, potentially influencing therapeutic outcomes. Understanding the interplay between gut microbiota and drug metabolism is crucial for personalised medicine and optimising drug therapies (32).

- Intestinal barrier function

Maintaining the integrity of the intestinal barrier is crucial for preventing the entry of harmful substances into the systemic circulation. The gut microbiota plays a vital role in strengthening the intestinal barrier by promoting mucus production, enhancing tight junction proteins, and preventing the colonisation of pathogenic bacteria. Disruption of the gut microbiota composition

can compromise the integrity of the intestinal barrier, leading to increased permeability and the potential for inflammatory responses (33,34).

1.4 Microbial metabolites

A crucial contribution of the gut microbiota to host physiology lies in producing diverse metabolites and other small molecules. These metabolites can be absorbed across the host gut, found in host circulation, and potentially impact human health directly or indirectly (35). Gut microbial metabolites encompass various classes of compounds which can be differentiated into three main groups (Figure 2) (36):

1. Metabolites produced by the gut microbiota from dietary compounds.

- Short Chain Fatty Acids (SCFAs)

SCFAs are fatty acids composed of fewer than six carbon atoms. They are primarily produced through the colon's bacterial fermentation of dietary fibres. The main SCFAs include acetate, propionate, and butyrate. Anaerobic bacteria in the colon mediate the conversion of starch into SCFAs through specific enzymes, making them resistant to digestion and absorption in the small intestine. Acetate, a major SCFA, is produced by various enteric bacteria such as *Akkermansia muciniphila*, *Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., *Ruminococcus* spp., *Clostridium* spp., and *Streptococcus* spp. from pyruvate using acetyl-CoA. Propionate, on the other hand, is synthesised through three distinct pathways: the succinate, acrylate, and propanediol pathways, which involve specific microbes, including *Bacteroides* spp., *Dialister* spp., *Veillonella* spp., *Roseburia inulinivorans*, and *Ruminococcus obeum*. Butyrate synthesis involves two pathways and requires species such as *Coprococcus comes*, *Anaerostipes* spp., *Faecalibacterium prausnitzii*, and *Roseburia* spp (36).

SCFAs are important in maintaining health and can influence the onset of various diseases. Although SCFA production and absorption primarily occur within the gut, systemic circulation underscores their significance in maintaining intestinal homeostasis and regulating multiple physiological processes in the host. These processes include energy expenditure, adipocyte metabolism, and immunological homeostasis, which has implications for allergies, colitis, type 2 diabetes, cirrhosis, and other conditions (35–38).

- Trimethylamine-N-oxide (TMAO)

TMAO is a metabolite generated through gut microbial metabolism after ingesting foods rich in phosphatidylcholine, choline, and carnitine. Enzymes encoded by the gut microbiota facilitate the conversion of these dietary components into trimethylamine (TMA). Subsequently, TMA is absorbed from the gut lumen into the portal vein and undergoes further metabolism in the liver,

producing TMAO. Several human gut bacteria belonging to phyla, such as Firmicutes, Actinobacteria, and Proteobacteria, can produce TMAO. In contrast, no members of the Bacteroidetes phylum have been found to possess this ability (35).

Elevated TMAO levels have been linked to atherosclerosis, cardiovascular disease, and renal dysfunction. TMAO promotes the formation of macrophage foam cells, augments platelet hyperreactivity, and induces endothelial dysfunction, thereby contributing to the development of cardiovascular pathologies (35).

2. Metabolites produced by the host and modified by the gut microbiota.

- Bile Acids (BAs)

BAs, components of bile, are produced by hepatocytes through the oxidation of cholesterol in the liver. They act as detergents and support the digestion of lipids and cholesterol homeostasis in the intestine. In humans, BAs are classified into two groups: primary and secondary. Primary BAs consist of cholic acid and chenodeoxycholic acid. The liver secretes approximately 200-600 mg BAS daily in humans, which is stored in the gallbladder and released into the duodenum after meals. Most BAs are reabsorbed in the terminal ileum and re-enter the liver via the enterohepatic circulation. However, about 15% of conjugated BAs are not reabsorbed in the terminal ileum and reach the colon, where they undergo deconjugation and biotransformation into secondary BAs through microbial activity. This process is facilitated by the enzyme bile salt hydrolase, produced by certain gut bacteria, including *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium* spp., and *Bacteroides* spp. Secondary BAs possess distinct physiological properties. BAs also act as signalling molecules, modulating host metabolic pathways and immune responses by activating nuclear receptors. Dysregulation of bile acid metabolism has been associated with liver diseases, colorectal cancer, and metabolic disorders (36,37).

- Indoles

Indoles are aromatic compounds derived from the metabolism of dietary tryptophan by gut bacteria. Microbial metabolism of tryptophan results in the production of several catabolites, including indole, tryptamine, indole ethanol, indolepropionic acid, indoleacetic acid, indoleacetic acid, skatole, indole aldehyde, and indole acrylic acid. These metabolites affect host health, including anti-inflammatory and antioxidant properties. Indoles can influence host immune responses and gut barrier function and even affect neurological processes, as tryptophan is a necessary precursor to produce serotonin. Notably, *Lactobacillus* species play a pivotal role in this metabolic pathway. Altered levels of indoles have been implicated in conditions such as IBD, autism spectrum disorders, and mood disorders (35,37).

3. Metabolites synthesised *de novo* by gut microbiota.

- Polyamines

Polyamines are cationic aliphatic amines that are multifunctional and ubiquitous, present in eukaryotic and prokaryotic organisms. Owing to their ionic characteristics, they interact with nucleic acids, ATP, acidic phospholipids, and specific types of proteins. Putrescine, spermidine, and spermine are the three major polyamines produced by mammalian cells. Polyamines are crucial in many fundamental biological functions, such as gene regulation, stress resistance, cell growth, survival, proliferation, and differentiation in health and disease. Various biosynthetic and salvage mechanisms tightly regulate intracellular polyamine levels. Gut microbiota is the primary contributor to polyamine production in the intestine. Gut bacteria, such as *Enterococcus faecalis*, *Campylobacter jejuni*, *Bifidobacterium* spp., and *Escherichia coli*, contribute significantly to polyamine synthesis through the decarboxylation of amino acids. Dysregulated polyamine metabolism has been associated with colorectal cancer, IBD, and intestinal inflammation (36).

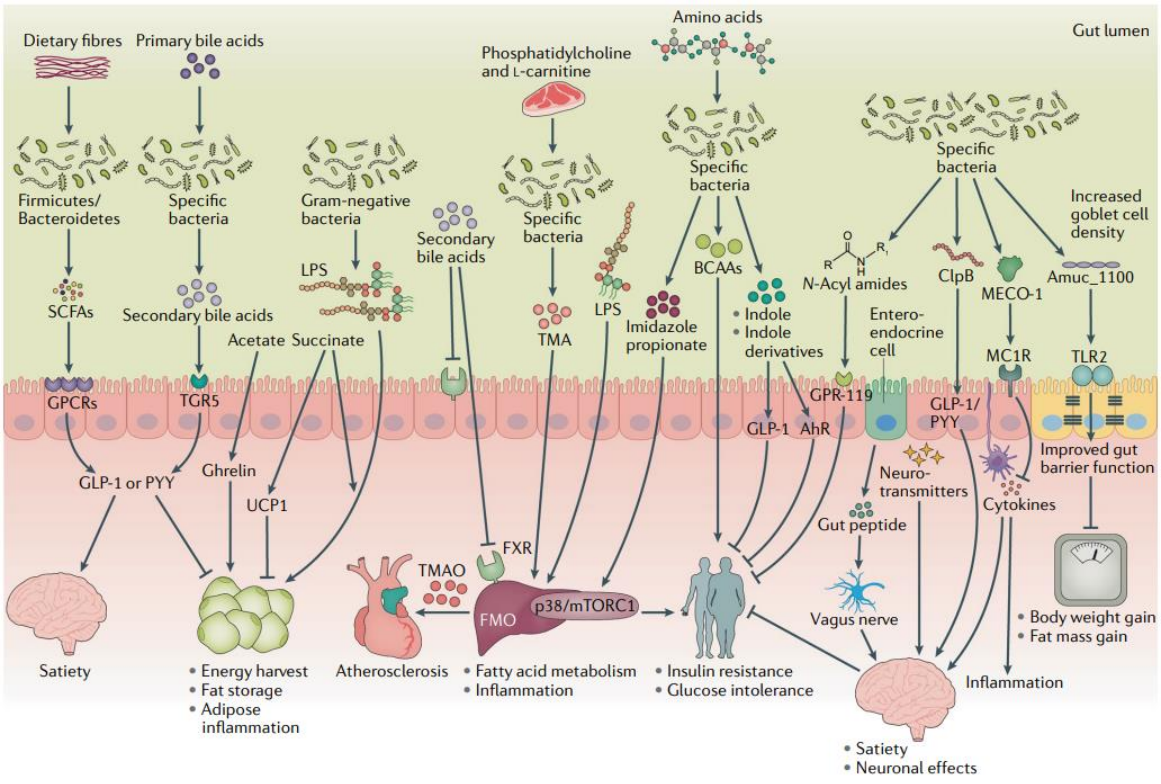

Figure 2. Overview of microbial metabolites and their effects and functions in the host (38).

1.5 Intestinal homeostasis by gut microbiota

The intestinal microbiota serves various beneficial developmental, metabolic, and protective functions within the host and is also implicated in maintaining intestinal homeostasis. The intestinal epithelium consists of a monocellular layer of intestine epithelial cells (IECs) arranged into specialised villi and crypts, known as the crypts of Lieberkühn. The IEC layer is continuously replenished through the division and differentiation of pluripotent intestinal epithelial stem cells (IESCs) residing at the base of the crypts and give rise to different subsets of functional IECs. Intestinal homeostasis is achieved when the rate of IESC proliferation equals the rate of programmed cell death, either through apoptosis, thereby maintaining a steady cell population (39,40).

Intestinal homeostasis refers to the balanced and stable state within the GI tract essential for optimal functioning. It involves maintaining a harmonious relationship between the intestinal epithelium, gut microbiota, immune system, and other factors to ensure proper digestion, nutrient absorption, and immune responses while preventing excessive inflammation, infection, and tissue damage. Intestinal homeostasis encompasses various processes: nutrient metabolism, immune regulation, epithelial barrier integrity maintenance, and modulation of gut motility. Disruptions of intestinal homeostasis, such as dysbiosis or impaired barrier function, can lead to gastrointestinal disorders and impact overall health (41,42).

Various signals from the gut microbiota and host immune cells regulate mucosal barrier function. IECs express pattern recognition receptors, including Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain-containing proteins (NODs), to sense bacterial components directly. The production of antimicrobial molecules by IECs is controlled by TLR4/MyD88 signalling and NOD2 signalling, which are driven by gut microorganisms. Metabolites from gut bacteria also directly enhance the mucosal barrier function of IECs. Butyrate, one of the SCFAs produced by gut bacteria, upregulates mucus secretion from goblet cells. Recent evidence has revealed that indole, a metabolite of dietary tryptophan from commensal bacteria, enhances the expression of cell junction-associated molecules such as occludins and claudins in IECs. The mucosal barrier function of IECs is further enhanced by cytokines from immune cells activated by gut commensal or pathogenic bacteria. In cases of mucosal injury, interleukin-6 derived from intraepithelial lymphocytes promotes intestinal epithelial cell proliferation and contributes to mucosal healing.

Moreover, activated macrophages, differentiated from monocytes recruited to the mucosal wound site, stimulate the colonic epithelial progenitor niche through direct cell-cell contact, thereby promoting epithelial regeneration and aiding in the restoration of the mucosal barrier. Conversely,

specific pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α and interferon (IFN), inhibit epithelial cell proliferation by suppressing ß-catenin/T cell factor signalling. Therefore, maintaining mucosal barrier function in IECs relies on the intestinal microbiota and cytokines derived from immune cells (43–46).

1.6 Intestinal dysbiosis

Under normal conditions, the mechanisms discussed thus far maintain intestinal homeostasis and promote the health of the human host. However, the disruption of such mechanisms can lead to severe pathological consequences. Intestinal dysbiosis refers to an imbalance or disruption in the normal composition of microbial communities, specifically bacteria, that inhabit the GI tract (47).

Instead, dysbiosis often results in a loss of host tolerance mechanisms and gives rise to non-specific chronic inflammation. Dysbiosis is characterised by the expansion of pathobionts, depletion of commensals, and reduced microbial diversity (Figure 3). These three deviations often coexist. Each feature will be discussed separately and considered with associated immunological and clinical consequences.

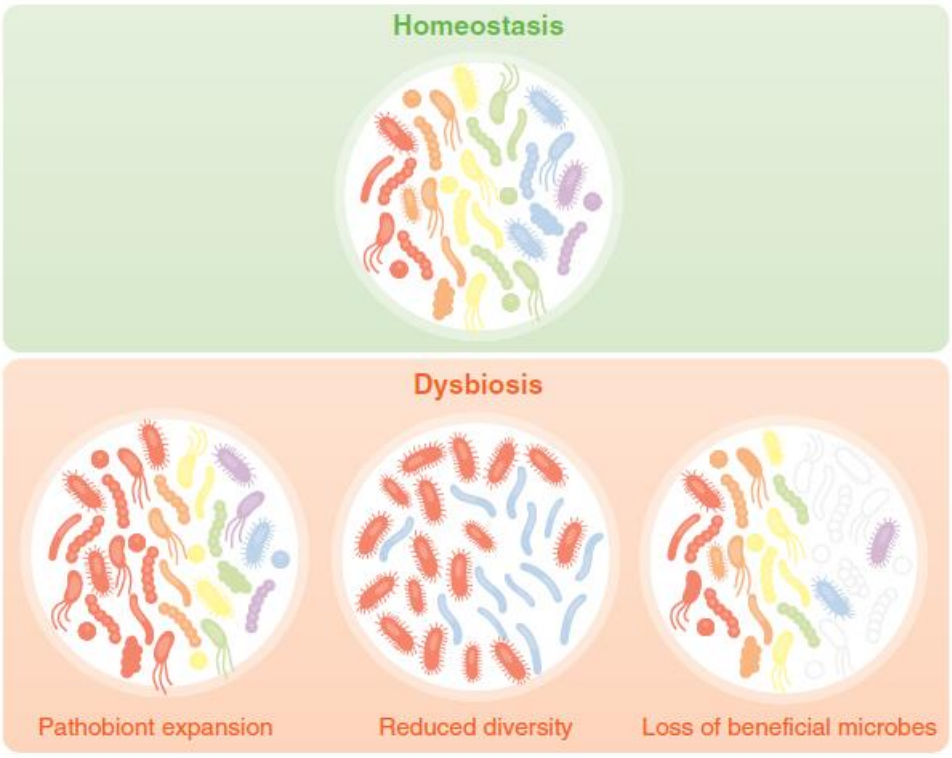

Figure 3. A loss of beneficial microbes, expansion of pathobionts, and loss of diversity are events that encompass dysbiosis (48).

Several members of the intestinal microbiota have been categorised as beneficial bacteria or symbionts due to their role in promoting host-microbiota homeostasis through diverse mechanisms that mitigate the host's inflammatory response. Depletion of these beneficial bacteria

can disrupt intestinal homeostasis and potentially lead to a state of dysbiosis. Among these beneficial bacteria, *Faecalibacterium prausnitzii* is one of the most prevalent commensal organisms in the healthy adult intestinal tract (49–51). *F. prausnitzii* exhibits significant anti-inflammatory activities, characterised by a reduction in the production of pro-inflammatory cytokines such as interleukin (IL)-8, IL-12, and IFN-γ, and an increase in the production of the anti-inflammatory cytokine IL-10. Notably, depletion of *F. prausnitzii* has been observed in patients with IBD, and its abundance has been associated with the degree of disease severity and the risk of relapse (52,53).

As previously discussed, the abundance of pathobionts in the intestinal microbiota is typically controlled through host defence mechanisms and competition for microbiota niches. However, under certain circumstances, the overgrowth of pathobionts can occur, contributing to disease pathology. The most frequently reported case of pathobiont expansion is observed in the Enterobacteriaceae family, particularly *E. coli* and *Shigella* species. The parallel increase in pathobiont expansion and translocation causes heightened host-microbial basolateral interactions, chronic activation of IEC signalling, and activation of innate immune cells. Subsequent expression of pro-inflammatory cytokines recruits and activates T cells, initiating a robust immune response. The expression of IFN-γ and TNF-α leads to extensive recruitment of immune cells, apoptosis of IECs, and a loss of tight junctional complexes. These events contribute to the breakdown of intestinal homeostasis and form a positive feedback loop due to the destruction of epithelial barrier function and the influx of microbiota-associated products that stimulate TLR receptors on the basolateral IEC membrane and dendritic cells in the lamina propria. In response to activation, these immune cells secrete significant quantities of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, stimulating effector T cell populations and inducing substantial immune responses against the intestinal microbiota. It has been suggested that such immune responses fall into a positive feedback loop due to a consequent breakdown of the epithelial barrier mediated by TNF-α and IFN-γ, leading to increased translocation of resident microflora and associated products, further exacerbating the immune response (54–57).

A depletion in intestinal microbiota diversity is the third defining feature of intestinal dysbiosis. De Filippo's study uncovered a distinct range of microbial diversity regarding Firmicutes/Bacteroidetes ratio in children from different geographical regions, highlighting the impact of diet on gut microbiota composition. This results emphasized the importance of environmental and dietary factors in shaping the microbiome and contributing to potential disruptions in gut health (58).

Also, as previously discussed, regulatory T cells are crucial in maintaining host-microbiota homeostasis. Gnotobiological studies have shown that multiple species of Clostridia contribute to

the activation of regulatory T cells. However, this stimulation is abolished when the gut is colonised with a single species of Clostridia, indicating the functional importance of less abundant commensal microbes. Similar to the depletion of symbionts and the expansion of pathobionts, a reduction in the diversity of the intestinal microbiota has been observed in patients with IBD compared to healthy individuals, thus highlighting the functional decline associated with a microbiota that lacks diversity (51,56).

Intestinal dysbiosis has been associated with a range of health issues, including GI disorders such as irritable bowel syndrome (IBS), IBD, and colorectal cancer (CRC). Furthermore, it has been linked to metabolic disorders, autoimmune conditions, allergies, and even mental health disorders (for further information, refer to section 2) (54). Ongoing research in this field continues to explore the causal or consequential role of dysbiosis in these conditions. In contrast, the precise mechanisms by which dysbiosis contributes to these conditions are not fully understood.

Treatment approaches for intestinal dysbiosis typically involve modulation strategies to restore a healthy balance of gut bacteria (for further information, refer to section 3.2) (59).

1.7 Diagnostics

The regulation of the gut microbiome has emerged as a promising avenue in the therapeutic management of chronic diseases (60). Consequently, this has led to a surge of interest in developing microbial stool tests that can accurately assess gut dysbiosis and provide insights into its implications for human health.

In recent years, extensive research has been dedicated to characterising the diversity and functional capacity of the gut microbiota, leading to the development of numerous microbial stool tests for dysbiosis assessment (60). Challenges associated with applying microbial stool tests in regular clinical practice have limited their widespread use. Factors such as the substantial volume of data generated from microbiome tests, the considerable interindividual variation in gut microbial composition, and the lack of disease condition-specific microbial profiles have hindered the seamless integration of these tests into routine clinical workflows (61,62).

Efforts are being made to overcome these challenges which aim to enhance the practical applicability of gut microbiome information, ultimately facilitating its effective utilization in clinical decision-making and personalised treatment approaches (63,64).

Nevertheless, the definition of a "healthy" gut microbiome remains a challenge, rendering the accurate determination of dysbiosis even more complex. The gut microbiota composition is highly individualised and influenced by various factors, including genetics, diet, lifestyle, and

environmental exposures. Considerable interindividual variation in microbial diversity and abundance makes it difficult to establish a clear definition of a "normal" or "healthy" gut microbiome (61,62,65). Consequently, identifying dysbiosis becomes subjective since it represents a deviation from an undefined healthy state (63)

Today analysis of the gut microbiome is based on microbe isolation through culture-dependent characterization and DNA extraction through culture-independent characterization. Culture-based tests are inaccurate as they favour aerobic bacteria, most of which are pathogens, while missing anaerobic bacteria, which include most gut commensals. To overcome that limitation, emerging technologies such as culturomics (high-throughput cell culture of bacteria) are being developed to isolate anaerobic microbes from the human gut (66). The second major type of microbiome testing is molecular-based stool tests using DNA based methods, such as next generation sequencing of 16S ribosomal RNA genes, whole genome shotgun sequencing or qPCR analysis (67).

Some potential applications of microbiome tests that are emerging, being some already ready to use as routine practice in the clinic yet include the use of specific faecal microbial signatures to predict which patients will respond better to interventions. For instance, some research has shown that the gut microbiome might help discriminate between responders and non-responders to dietary interventions in patients with irritable bowel syndrome or obesity (68). Other studies have shown the potential of faecal microbial signatures to predict the response to biological treatment in patients with inflammatory bowel diseases (69) or for colorectal cancer screening (70).

As our understanding of the complex relationship between microbiota and various diseases, including endocrine and neurological disorders, continues to evolve, the notion of employing generic microbiota analysis as a predictive tool for disease risk assessment and prevention, akin to routine blood tests, emerges as a potent concept within the ambit of public health. This approach underscores the potential for leveraging healthy habits to modulate microbiota composition and mitigate disease risks. However, it is imperative to acknowledge the substantial groundwork that remains to be covered in terms of defining normative values, establishing temporal dynamics, and unequivocally demonstrating causal relationships with these multifaceted diseases. Presently, the majority evidence primarily pertains to gastrointestinal disorders, underscoring the need for further research to unravel the broader implications across diverse health domains.

2 Intestinal microbiota in dysbiosis-associated diseases

2.1 Inflammatory bowel diseases

IBD are chronic inflammatory disorders of the gastrointestinal tract that encompass two major forms Crohn's disease (CD) and ulcerative colitis (UC). The aetiology of IBD remains incompletely understood, but emerging evidence suggests a crucial involvement of the gut microbiota. Dysbiosis, characterised by alterations in the composition, diversity, and function of the gut microbial community, has been implicated as a key factor contributing to IBD development and progression. Compared to healthy individuals, IBD patients often exhibit a decrease in microbial diversity and a shift in the relative abundance of specific bacterial taxa. For instance, reductions in the phylum Firmicutes and the family *Lachnospiraceae* have been consistently observed in CD and UC patients. Conversely, there is an increase in the relative abundance of Proteobacteria, particularly Enterobacteriaceae, in the inflamed gut mucosa of IBD patients (57,71).

Furthermore, studies have identified alterations at the species level, such as a decreased abundance of *F. prausnitzii,* a commensal bacterium with anti-inflammatory properties, in individuals with CD. Similarly, reduced levels of *Bacteroides* species, known for their immunomodulatory effects, have been observed in UC patients. These dysregulated microbial communities contribute to an imbalanced gut ecosystem, promoting chronic inflammation and disease progression in IBD (72–74). The disruption of microbial diversity, characterised by decreased overall species richness and evenness, is a hallmark of dysbiosis in IBD. Reduced diversity has been associated with increased disease severity and poorer clinical outcomes. The loss of microbial diversity can impair important ecosystem functions in the gut, including producing SCFAs, maintaining the intestinal barrier, and regulating immune homeostasis (75).

Several factors contribute to decreased microbial diversity in IBD. Prolonged inflammation and the associated mucosal damage create an unfavourable environment for commensal bacteria, leading to depletion. Antibiotics, a common therapeutic approach in IBD management, can further disrupt microbial diversity by indiscriminately targeting beneficial and pathogenic microbes. Alongside alterations in microbial composition and diversity, dysbiosis in IBD is accompanied by functional changes within the gut microbiota. The imbalanced microbial community exhibits altered metabolic activities, resulting in the aberrant production of microbial metabolites that can impact host physiology and immune responses (76).

Furthermore, dysbiosis-associated microbial dysfunctions can lead to the generation of pro-inflammatory metabolites. For example, increased hydrogen sulphide production by sulphate-

reducing bacteria has been implicated in IBD pathogenesis, which promotes inflammation and disrupts epithelial integrity, exacerbating intestinal inflammation in IBD (Figure 4) (57,77).

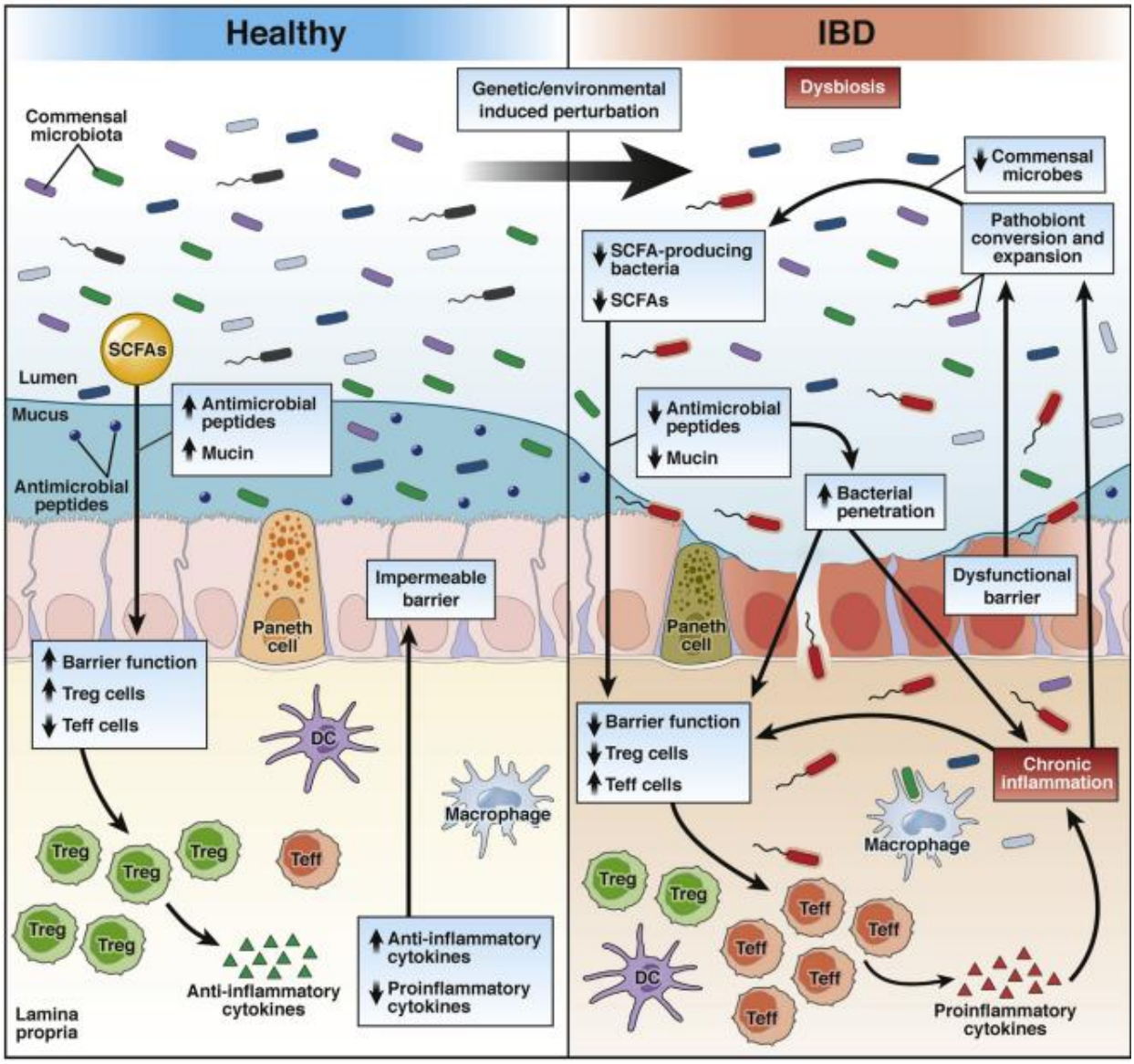

Figure 4. Factors influencing dysbiotic and chronic inflammatory state in IBD compared to healthy individuals (76).

2.2 Irritable bowel syndrome

IBS is a functional gastrointestinal disorder characterised by chronic abdominal pain or discomfort, bloating, and altered bowel habits. The aetiology of IBS is multifactorial and complex, with emerging evidence implicating dysbiosis, an imbalance in the gut microbiota, as a potential contributor to its pathogenesis (78).

Emerging evidence suggests that individuals with IBS exhibit alterations in the composition of their gut microbiota compared to healthy individuals. While the specific microbial profiles

associated with IBS may vary, studies have consistently reported changes in the relative abundance of certain bacterial taxa. For instance, reductions in the abundance of *Bifidobacteria*, *Lactobacilli*, and *F. prausnitzii* have been observed in individuals with IBS. Conversely, there may be an increase in potentially pathogenic bacteria such as Enterobacteriaceae. Dysbiosis in IBS extends beyond alterations in microbial composition and diversity and involves disruptions in microbial function. The dysbiotic microbiota in individuals with IBS may exhibit impaired metabolic activities and perturbations in producing microbial metabolites. These alterations can profoundly affect host physiology and contribute to IBS symptomatology (79–81).

Furthermore, dysbiosis in IBS can alter the gut microbiota's production of neuroactive compounds and neurotransmitters. These molecules can influence the gut-brain axis and impact GI motility, visceral sensitivity, and pain perception, central features of IBS symptomatology (82).

2.3 Colorectal cancer

CRC ranks among the most prevalent cancer types affecting both men and women. Most cases are classified as sporadic cancers (85-95%), likely influenced by various environmental factors. Conversely, some cases can be attributed to hereditary factors or specific predisposing conditions, such as IBD.

The involvement of gut microbiota in the development of CRC has been noted for some time. Gut microbiota can promote the development and progression of CRC by different processes, including the induction of a chronic inflammatory state or immune response, altering stem cell dynamics, the biosynthesis of toxic and genotoxic metabolites, and affecting the host metabolism (83).

Notably, CRC patients exhibit a decrease in community diversity and an important reduction in beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*. Conversely, there is an increase in potentially pathogenic bacteria, including *Fusobacterium nucleatum*, *Bacteroides fragilis*, *Porphyromonas* and *Escherichia coli* (84–86). This dysbiotic microbiota can produce metabolites that contribute to tumour development and progression. For instance, TMAO, a microbial metabolite, has been associated with CRC development and has pro-tumorigenic effects, including promoting cancer cell proliferation and inhibiting apoptosis. Furthermore, dysbiosis can lead to an increase in the production of secondary BAs, which can exhibit cytotoxic and genotoxic properties. These BAs can damage DNA, disrupt cellular processes, and contribute to CRC initiation and progression (83,85).

2.4 Metabolic diseases

Metabolic diseases, including obesity, type 2 diabetes mellitus (T2DM), and obesity-associated non-alcoholic fatty liver disease (NAFLD), have reached epidemic proportions worldwide (87).

Notable alterations in the composition of the gut microbiota often accompany metabolic diseases. Studies have demonstrated specific changes in microbial taxa associated with metabolic dysfunction. The dysbiotic signatures in the gut microbiota associated with metabolic disease phenotypes include an increased ratio of Firmicutes to Bacteroidetes at the phylum level, particularly within the butyrate-producing groups such as the genera *Eubacterium*, *Roseburia*, and *Faecalibacterium*. Furthermore, there is an expansion of Proteobacteria and a reduced abundance of *Akkermansia*. Insulin-resistant phenotypes also exhibit a pronounced proliferation of *Prevotella copri* and *Bacteroides vulgatus*, which elevate circulating levels of branched-chain amino acids. Additionally, an obese microbiota is characterised by augmented serum glutamate levels due to the reduced abundance of *Bacteroides thetaiotaomicron*, which is responsible for glutamate conversion. Moreover, the gut microbiota associated with advanced fibrosis in NAFLD is characterised by an increased abundance of Proteobacteria and *Escherichia coli*, accompanied by a reduction in the population of Firmicutes (88,89).

SCFAs and succinate might prevent obesity by increasing energy expenditure, enhancing the production of anorexic hormones, and improving appetite regulation. These SCFAs play a crucial role in maintaining gut homeostasis, as well as in adipose tissue and liver substrate metabolism and function. Through these mechanisms, SCFAs can potentially prevent the progression of T2DM and NAFLD (87,90).

2.5 Chronic pancreatitis

Chronic pancreatitis (CP) is a fibro-inflammatory syndrome of the pancreas in individuals with genetic, environmental, and other risk factors who develop persistent pathologic responses to parenchymal injury or stress. Persistent inflammatory episodes lead to fibrotic tissue replacement, resulting in exocrine and endocrine pancreatic insufficiency (91).

Studies on intestinal microbiota and pancreatic diseases are recent and scarce, but the exocrine pancreas represents one of the most important host factors regulating gut microbiota composition. These recent studies have shown that CP patients show reductions in the abundance of Bacteroidetes, Actinobacteria, and Firmicutes such as *F. prausnitzii*, *Ruminococcus bromii* and *Prevotella*, and an increase in Proteobacteria phylum (92,93).

3 Intestinal microbiota modulation

3.1 Factors influencing intestinal microbiota

3.1.1 Birth and nursing

The mode of delivery, whether vaginal or caesarean section, profoundly impacts the initial colonisation of the infant's gut microbiota. Vaginally born infants acquire their first microbial exposure from the birth canal, rich in maternal vaginal and faecal bacteria. These microbes contribute to the early colonisation of the infant's gut. In contrast, infants delivered by caesarean section are exposed to a different microbial environment, primarily influenced by the skin and hospital surroundings. This results in a distinct microbial composition, often characterised by a higher abundance of opportunistic pathogens and reduced diversity compared to vaginally delivered infants. The differences in the gut microbiota composition associated with birth mode may affect the infant's health. Studies have shown that infants born via caesarean section are more likely to develop certain conditions, including allergies, asthma, obesity, and autoimmune diseases. The altered microbial colonisation pattern resulting from caesarean delivery may disrupt the development of resilient and diverse microbiota, impacting immune system maturation and increasing disease susceptibility (94–96).

In addition, feeding mode significantly shapes gut microbiota structure. Breast milk is a vital source of nutrients for infants and an essential contributor to the development of healthy gut microbiota. Breast milk contains a diverse array of bioactive compounds, including human milk oligosaccharides, which serve as prebiotics which selectively promote the growth of specific beneficial bacteria and SCFAs production. Breastfeeding has been consistently linked to a more favourable microbiota composition in infants. Breastfed infants display higher microbial diversity, increased abundance of beneficial bacteria, and lower levels of potentially harmful microbes than formula-fed infants. The unique composition of breast milk, including immune-modulating factors and antimicrobial peptides, further contributes to the establishment of a resilient gut microbiota and helps protect against infections and inflammatory diseases. It is worth noting that the duration of breastfeeding also plays a role in microbiota development. Beyond six months, prolonged breastfeeding has been associated with a more pronounced and lasting effect on the gut microbiota composition, promoting a diverse and stable microbial community (94,97).

3.1.2 Genetics

The human genome exhibits significant genetic variation playing a substantial role in shaping the composition and function of the intestinal microbiota. Several studies have identified associations between specific host genetic variants and alterations in microbial community structure. Genome-wide association studies have identified specific genetic loci associated with microbiota-related traits. These studies have revealed associations between host genetic variations, the abundance of specific bacterial taxa, and the production of microbial metabolites. Genetic variations can influence host-microbiota interactions, impacting the intestinal microbiota's composition and function. The host immune system plays a critical role in shaping the microbiota, and genetic variations in immune-related genes can affect host immune responses to microbial colonisation, establishment, and composition (98,99). In addition, host genetic factors can shape the overall structure of the microbial community in the intestine. Studies comparing the gut microbiota composition between monozygotic (genetically identical) and dizygotic (non-identical) twins have provided insights into the heritability of the microbiota. Monozygotic twins have been found to exhibit higher similarity in gut microbial composition compared to dizygotic twins, suggesting a role for host genetics in shaping the microbiota (100).

3.1.3 Environment

Environmental factors, such as geography, air pollution, altitude, temperature, humidity, or the presence of household pets, can significantly impact the prevalence and abundance of specific microbial taxa. Microorganisms in various environmental conditions have adapted to their surroundings, influencing their capacity to colonise and thrive within the human gut.

For instance, rural communities may experience greater exposure to soil-associated bacteria, while urban populations might have higher exposure to environmental pollutants or urban-specific microbes. In urban regions, pollutants, and chemicals, including particulate matter, heavy metals, pesticides, and industrial chemicals, can enter the body through inhalation, ingestion, or dermal absorption, potentially disrupting the gut microbial ecosystem. Numerous studies have demonstrated associations between exposure to air pollution and alterations in the gut microbiota. Airborne pollutants can directly interact with the gut microbiota by promoting the growth of certain pathogenic bacteria or reducing the abundance of beneficial bacteria. Such disruptions in the microbial balance can contribute to dysbiosis and increase susceptibility to diseases. Additionally, exposure to chemicals such as pesticides or industrial pollutants may directly affect the gut microbiota, disrupting microbial communities, impairing microbial metabolism, and influencing the production of microbial metabolites crucial for host health (101,102).

Furthermore, Tibetans living at high altitudes (4800 m) have exhibited an enriched gut microbiota containing butyrate-producing bacteria as an adaptive response to hard environments. SCFAs produced by bacteria such as *Clostridium*, *Desulfovibrio*, *Bacteroides*, *Lactobacillus*, and *Prevotella* can assist in reducing blood pressure and adapting to energy demands and pulmonary hypertension (103).

Moreover, studies have reported general microbial alterations associated with pet-keeping and specifically with dogs and cats. These alterations manifest as microbial richness, diversity, and composition changes when comparing individuals living with furry pets to those in pet-free homes (94).

3.1.4 Diet

Several studies have investigated the impact of diet on the microbiota structure and composition, which can undergo rapid changes in response to dietary modifications and contribute to both positive and adverse health effects (104,105).

In developed countries, populations tend to consume low-fibre diets. A comparison between children from rural Burkina Faso in Africa and Italy in Europe has revealed significant differences that can primarily be attributed to variations in dietary habits. African children, who consume a diet rich in plant-derived fibre, exhibited a significantly higher abundance of *Prevotella* and *Xylanibacter* in their microbiota. At the same time, their Firmicutes levels were depleted compared to the Italian children. Additionally, African children had significantly higher levels of SCFAs and lower levels of Enterobacteriaceae. In contrast, the Italian children consumed a diet low in dietary fibre and had elevated levels of Enterobacteriaceae, particularly *Shigella* and *Escherichia* (58).

In addition, the analysis of adult faecal microbiota in a North American cohort revealed that enterotypes were associated with long-term dietary habits. A diet rich in animal proteins and fats, typical of Western societies that have undergone nutritional transitions over the past 60 years, was found to favour the Bacteroides enterotype. On the other hand, the *Prevotella* enterotype was more prevalent in individuals consuming high-fibre diets rich in fruits and vegetables (106).

The quantity of diet can also influence the microbiota. Short-term carbohydrate restriction leads to a decrease in SCFA-producing bacteria and alterations in the composition of the microbiome, such as a decrease in *Blautia coccoides* and an increase in Bacteroides. Another example is the correlation between the abundance of *Akkermansia muciniphila* in faecal samples and improved metabolic outcomes following calorie restriction interventions in individuals with overweight or obesity (104).

3.1.5 Medication

Medication has recently emerged as one of the most influential determinants of gut microbiota composition and activity. Several classes of drugs can shape gut microbiota by directly targeting certain bacteria or causing imbalances in the microbial composition. The main medications affecting gut microbiota are:

- Antibiotics

Antibiotics are medications used to treat bacterial infections. Antibiotic treatment reduces the overall diversity of gut microbiota species, including the loss of some important taxa. This reduction in diversity leads to metabolic shifts, increases gut susceptibility to colonisation, and stimulates the development of bacterial antibiotic resistance. Post-antibiotic dysbiosis is commonly characterised by reduced diversity of the phyla Firmicutes and Bacteroidetes, often accompanied by an overgrowth of the family *Enterobacteriaceae*. This decrease in diversity and dysbiosis can be critical as the healthy microbiota provides colonisation resistance against invading pathogenic bacteria, while dysbiosis increases vulnerability to the post-antibiotic expansion of enteropathogenic strains like *Clostridioides difficile* (former *Clostridium difficile*) (107,108).

In children, restoration of microbial diversity following antibiotic treatment has been reported to take approximately one month, while in adults, the gut microbiota mainly was restored within 1.5 months, although several common species remained undetectable for more than 180 days (109).

- Proton pump inhibitors (PPIs)

PPIs are among the most used drugs worldwide to reduce stomach acid production and treat conditions like gastroesophageal reflux disease and stomach ulcers. Studies have shown that long-term PPI use is associated with profound changes in the gut microbiome, increasing susceptibility to *Clostridioides difficile*, *Salmonella* spp., *Shigella* spp., and *Campylobacter* spp. PPIs alter the gut microbiome through their direct effect on stomach acid. Stomach acid is vital in defending against bacterial influx during food ingestion and oral mucus contact. PPIs reduce stomach acidity, allowing more bacteria to survive this barrier (110–112).

- Metformin

Metformin is commonly used as the first-line drug for the medication of T2DM and exerts several actions within the gut. It increases intestinal glucose uptake and lactate production, elevates glucagon-like peptide one concentration, and affects the bile acid pool within the intestine. Metformin also induces changes in the gut microbiota. Significant differences observed with metformin intake include decreased abundances of Firmicutes and Bacteroidetes after four weeks

of treatment. The genus *Bacteroides* (phylum Bacteroidetes) and the genus *Faecalibacterium* (genus Firmicutes) show decreased abundance following metformin administration. Additionally, reductions in the genera *Clostridium*, *Roseburia*, and *Dorea* have been observed. *Pseudomonas aeruginosa*, characterised by its high biofilm-forming capacity, also shows decreased abundance with metformin treatment due to drug's antibacterial effects, which increase claudin-1 production and occluding protein abundance (113).

- Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs, such as Ibuprofen and Naproxen, are widely used for pain relief and inflammation reduction. Chronic NSAID use has been associated with alterations in the gut microbiota and an increased risk of gastrointestinal complications, including bleeding, inflammation, and ulceration in the stomach and small intestine. NSAIDs have been demonstrated to disrupt the intestinal barrier function, leading to bacterial translocation and the alteration of gut microbial balance. Notably, the effects of NSAIDs appear to vary depending on the type of NSAID ingested (114).

For example, treatment with aspirin induces shifts in the composition of the gut microbiota, affecting *Prevotella*, *Bacteroides*, *Ruminococcaceae*, and *Barnesiella*, while celecoxib and Ibuprofen increase the abundance of *Acidaminococcaceae* and *Enterobacteriaceae*. In particular, Ibuprofen promotes the enrichment of species belonging to *Propionibacteriaceae*, *Pseudomonadaceae*, *Puniceicoccaceae*, and *Rikenellaceae*, compared to non-users and naproxen users. These findings support the notion that drug administration can influence the composition of the microbiota, favouring taxa with metabolic capabilities for those specific drugs (115).

- Chemotherapy

Chemotherapy is a cancer treatment that can affect both cancer and healthy cells, including those in the gut. GI toxicity, in the form of mucositis, is a common adverse effect of chemotherapy, leading to symptoms such as diarrhoea, pain, and weight loss. Alongside the multiple host pro-inflammatory and apoptotic pathways activated by chemotherapy, the gut microbiota plays a crucial role in the pathogenesis of mucositis. Following chemotherapy treatment, the gut microbiota exhibits reduced diversity and richness, with a general increase in Proteobacteria and a decrease in the abundance of Firmicutes and Actinobacteria. Taxa are known to mitigate inflammation by modulating the NF-kβ pathway, and the production of SCFAs is depleted after chemotherapy (116,117).

- Immunosuppressive drugs

Medications utilised to suppress the immune system, including corticosteroids and certain drugs employed in organ transplantation, can influence the gut microbiota. As previously mentioned, the

microbiota plays a pivotal role in shaping the development and regulation of the immune system. In contrast, reciprocally, the immune system maintains control over the microbiota through the production and secretion of antimicrobial peptides and secretory IgA. The absence of such controls has been demonstrated to induce shifts in microbial composition, localisation, and activity (118).

While limited human studies exist to discern the effects of immunosuppressive drugs on the microbiota, one study conducted in liver transplantation patients revealed dysbiosis characterised by a decrease in the total bacterial mass and a decline in beneficial bacteria, such as *Bifidobacterium* spp., *F. prausnitzii*, and *Lactobacillus* spp., along with an increase in *Enterobacteriaceae* and *Enterococcus* spp. However, within 1-2 years after transplantation, the population of all bacterial species tended to return to normal levels (119).

3.1.6 Lifestyle

- Physical activity

Physical activity is widely recognised for its numerous health benefits, and emerging evidence suggests that it also influences the composition of the gut microbiota. Several studies have found a positive correlation between physical activity levels and microbial richness, indicating that individuals who exercise regularly tend to have a more diverse and abundant gut microbial community (120,121).

Exercise-induced changes in the gut microbiota are believed to occur through various mechanisms. Muscle contraction in response to physical exercise triggers a cascade of acute and chronic physiological changes in the body, many of which are associated with disease prevention and improved health. Blood flow suppression in the gastrointestinal system depends on the intensity of the exercise. Mild-to-moderate exercise can help preserve mucosal integrity and enhance intestinal motility, facilitating food movement through the gastrointestinal tract and promoting nutrient availability for gut microbes. Conversely, high-intensity exercise has been linked to epithelial injury, increased permeability, reduced gastric motility, and other imbalances. It is worth noting that even in a high-fat diet, exercise may reduce inflammatory infiltration and protect the morphology and integrity of the intestine. Additionally, exercise influences the production and circulation of certain gut hormones and neurotransmitters that regulate appetite, such as peptide YY and glucagon-like peptide 1, which can impact microbial composition and function (122).

Moreover, exercise-induced systemic immune function and inflammation alterations may indirectly affect the gut microbiota. Regular physical activity has been shown to reduce chronic

low-grade inflammation, which is associated with dysbiosis and an increased risk of various diseases. Changes in immune function and inflammatory markers due to exercise can modulate the gut environment, creating a more favourable niche for beneficial microbial populations. For instance, physically fit individuals exhibit a greater diversity within the Firmicutes phylum, including species such as *F. prausnitzii*. Furthermore, a microbiome enriched in butyrate-producing taxa, such as Clostridiales, *Roseburia*, *Akkermansia muciniphila*, Lachnospiraceae, and Erysipelotrichaeae, has been observed in physically active individuals, resulting in increased butyrate production, which is considered an indicator of gut health (120,122).

- Stress

Acute and chronic stress can significantly impact the communication between the gut and the brain, leading to alterations in the composition and function of the gut microbiota. Stress triggers the activation of the hypothalamic-pituitary-adrenal axis, releasing stress hormones, such as cortisol. These stress hormones can directly influence the gut environment, causing gut motility, intestinal permeability, and mucus secretion changes. As a result, bacteria can translocate across the intestinal mucosa, gaining direct access to immune cells and neuronal cells of the enteric nervous system (123).

Furthermore, stress can also indirectly impact the gut microbiota through alterations in behaviour and dietary patterns. Stress can lead to changes in eating behaviours, such as emotional eating or a preference for high-fat and high-sugar foods, which can impair the composition of the gut microbial community. Additionally, stress-related changes in sleep patterns, physical activity levels, and overall lifestyle can indirectly influence the diversity and function of the gut microbiota (124).

The alterations in gut microbiota composition due to stress can further influence bidirectional communication with the brain. The gut microbiota can produce various neuroactive compounds, including neurotransmitters, neuropeptides, and metabolites, which can modulate neural activity and neurotransmission. Dysbiosis of the gut microbiota induced by stress has been associated with mood disorders, such as anxiety and depression, as well as cognitive impairments (125). Studies have shown that alterations in the gut microbiota reduce microbial diversity, leading to a decrease in the relative abundance of *Bacteroides* spp. and *Lactobacillus* spp., while bacteria in the genus *Clostridium* increase, ultimately affecting behaviour mediated by the microbiota (126).

- Circadian rhythms

Circadian rhythms are intrinsic biological rhythms that regulate various physiological processes, including sleep-wake cycles, hormone secretion, and metabolism. Disruptions in the circadian rhythm, such as irregular sleep patterns or shift work, can profoundly affect the gut microbiota.

The gut microbiota exhibits circadian rhythms, characterised by fluctuations in microbial composition and function throughout the day. Disruptions in the host's circadian rhythm can lead to dysbiosis, microbial diversity, and activity alterations. Animal studies have demonstrated that circadian disruption, such as exposure to constant light or irregular feeding schedules, can induce changes in the composition of the gut microbiota, characterised by an overgrowth of potentially pathogenic bacteria and a reduction in beneficial bacteria (127).

Chronic circadian rhythm disruption has been associated with obesity, diabetes, and cardiometabolic disorders, which in turn have been linked to alterations in intestinal hyperpermeability. The effects of circadian rhythm-induced changes in microbiome function include the production of SCFAs, metabolites implicated in stress, obesity, insulin resistance, inflammation, and the gut-brain axis (128).

- Smoking

Smoking is widely recognised as a significant risk factor for various health conditions, encompassing cardiovascular disease, respiratory disorders, and cancer. Within the gastrointestinal tract, smoking exerts notable effects on multiple functions, including mucin production, alterations in tight junctions in the small intestine, and disruption of gut barrier function. Cigarette smoke comprises many toxic substances, such as polycyclic aromatic hydrocarbons, aldehydes, nitrosamines, and heavy metals inhaled into the lungs. These substances have the potential to reach the gastrointestinal tract, thereby initiating microbiota dysbiosis through diverse mechanisms, such as antimicrobial activity or modulation of the intestinal microenvironment (129).

Exposure to cigarette smoking induces a significant alteration in the composition of the gut microbiome. Individuals who smoke exhibit a distinct faecal microbiome composition compared to non-smokers. Among healthy smokers, the faecal microbiome has an elevated abundance of *Prevotella*, *Veillonella*, *Bacteroides*, *Acidaminococcus*, and *Oscillospira*. Moreover, smokers demonstrate a decreased abundance of Firmicutes, specifically a depletion of the *Lachnospira* genus (130).

- Alcohol consumption

Excessive alcohol consumption is linked to various health problems, including liver disease, GI disorders, and an elevated risk of certain cancers. Notably, alcohol consumption substantially alters the quality and quantity of gut microbiota, mucosal changes, and increased gut permeability, leading to endotoxemia. Specifically, the heightened gut permeability resulting from alcohol abuse leads to an increased concentration of lipopolysaccharides in the porta blood flow. These

lipopolysaccharides bind to TLR4 and activate NF-kβ, subsequently triggering the release of pro-inflammatory cytokines, production of reactive oxygen species, and induction of oxidative stress. Chronic overconsumption of alcohol also promotes small intestinal malabsorption and disruption of the balance of colonic bacteria, thereby modifying gut microbiota metabolism (131,132).

Comparatively, patients with chronic alcohol overconsumption display more significant variability in the composition of intestinal bacteria when compared to those with no or minimal alcohol intake history. Individuals who engage in alcohol overconsumption exhibit a potentially more inflammatory active microbiota, characterised by an over-representation of Proteobacteria at the phylum level and a notable increase in the genera *Clostridium*, *Holdemania* (Firmicutes), and *Sutterella* (Proteobacteria). Conversely, they demonstrate a lower abundance of the genus *Faecalibacterium*, which has been shown to protect against gastrointestinal conditions (131).

It is important to note that moderate alcohol consumption, particularly red wine, has been associated with certain health benefits attributed to specific polyphenols (133).

3.2 Microbiota modulation strategies

Given the pivotal role of the intestinal microbiota in the onset and pathogenesis of numerous diseases, modulation of the microbiota is a logical practice to restore and maintain host health. The different strategies of gut microbiota modulation, including probiotics, prebiotics, synbiotics, postbiotics and faecal microbiota transplantation, and their putative mechanisms of action are summarised.

3.2.1 Probiotics: autochthonous and allochthonous

Probiotics are living microorganisms that confer health benefits on the host when administered adequately (134). They can be classified as autochthonous (naturally occurring in the host) or allochthonous (originating from external sources). Autochthonous probiotics, such as certain strains of *Lactobacillus* and *Bifidobacterium*, are indigenous to the human gut and are believed to have a stronger impact on the gut microbiota. On the other hand, allochthonous probiotics are derived from external sources and may require continuous administration to maintain their presence in the gut.

Probiotic administration is suggested to restore microbial dysbiosis and maintain intestinal microbial balance by occupying host tissue and preventing the colonisation of pathogenic bacteria. Various studies have reported that ingesting specific probiotic strains diminishes the colonisation of pathogens, including *Clostridioides difficile* and *Staphylococcus aureus*, thereby supporting the use of probiotics to prevent intestinal infection. Both autochthonous and allochthonous probiotics

confer colonisation resistance by competing for nutrients and adhering to the surface of epithelial cells or mucus or by antagonising pathogen colonisation through aggregation with pathogens. In addition to direct interactions, probiotics can produce metabolites such as lactic and acetic acid or bacteriocin, inhibiting pathogen growth by lowering luminal pH and exerting direct antimicrobial activity. In this way, by excluding pathogenic invasion, probiotic intake helps reduce the risks of intestinal infection and subsequent inflammation. The most used microbes as probiotics include species from the genera *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*, although other genera such as *Bacillus*, *Propionibacterium*, *Streptococcus*, and *Escherichia* are also widely used. Probiotics are commonly used to treat acute diarrhoea and *Clostridioides difficile* infection (CDI) traveller's diarrhoea, IBS, IBD, and genitourinary infections, among others (135).

Fermented foods and beverages, such as fermented milk (e.g., yoghurts, cheese, and kefir) and plant-based foods (e.g., kimchi, sauerkraut, miso, and others), contain live microbes, although they are not considered probiotics. Probiotics must comply with specific criteria. When administered, the microbes must be alive in adequate numbers, and strains must be genetically identified and classified using the latest terminology and designated by numbers, letters, or names. Moreover, appropriately sized, and designed studies must be performed to designate a strain as a probiotic and determine the specific host to which the probiotics are intended. Strains shown to confer a benefit for one condition may not be probiotic for another application (136). Since most fermented foods do not comply with these criteria, they should not be considered probiotics but food containing live and active cultures (Figure 5) (134,137).

Probiotics are generally considered safe and well-tolerated for healthy subjects; however, their safety profile has been challenged in patients with underlying medical conditions. Probiotic translocation, which refers to the entry of viable bacteria into extraintestinal sites and the subsequent systemic or localised infections, is one of the biggest concerns. Although bacterial translocation occurs in healthy subjects, bacteria are typically sequestered and removed in the mesenteric lymph nodes under an intact immune system, conferring no detrimental effects. However, this physiological protection may fail in patients with a damaged intestinal barrier or compromised immunity. Various case reports of probiotic-associated bacteraemia, fungemia, endocarditis, liver abscess, and pneumonia have been published, even though the ingested probiotics are known to possess low-virulent and non-pathogenic properties (138).

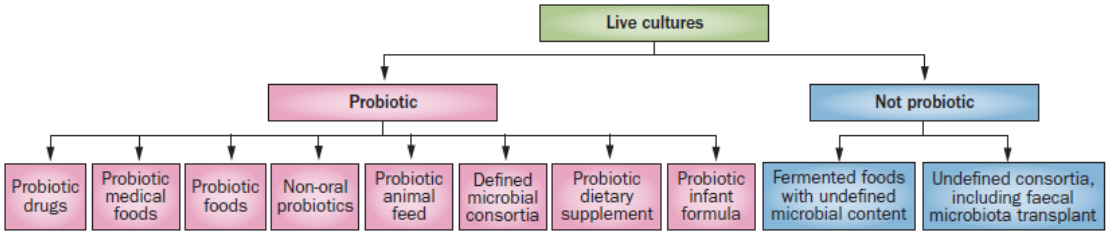

Figure 5. Overall framework for probiotics products.

3.2.2 Prebiotics

Prebiotics are substrates selectively utilised by host microorganisms, conferring health benefits (139). Various sources of carbohydrates, such as fruit, vegetables, cereals, and other plants like artichokes, bananas, asparagus, berries, garlic, onions, chicory, green vegetables, legumes, oats, barley, and wheat, among others, constitute potential prebiotics. Additionally, artificially produced prebiotics include lactulose, galactooligosaccharides (GOS), fructooligosaccharides (FOS), maltooligosaccharides (MOS), xylooligosaccharide (XOS) and fructans such as inulin and oligofructose (140). Furthermore, substances such as polyunsaturated fatty acids (PUFA), conjugated linoleic acid (CLA) and polyphenols also exhibit prebiotic activity (Figure 6) (138). To be classified as prebiotics, certain criteria must be met: (i) resistance to acidic pH in the stomach, resistance to hydrolysis by mammalian enzymes, and lack of absorption in the gastrointestinal tract; (ii) fermentability by intestinal microbiota; and (iii) selective stimulation of intestinal bacteria growth and/or activity to improve host health (139,141).

Prebiotics are selectively fermented by colonic probiotics, initially thought only to stimulate species of Lactobacillus and Bifidobacterium. However, research has revealed their impact on other autochthonous probiotics, such as species from the genera *Faecalibacterium*, *Akkermansia*, *Ruminococcus*, and *Roseburia*. Besides promoting probiotic growth and undergoing fermentation, most prebiotics possess antiadhesive properties against pathogens (138,139).

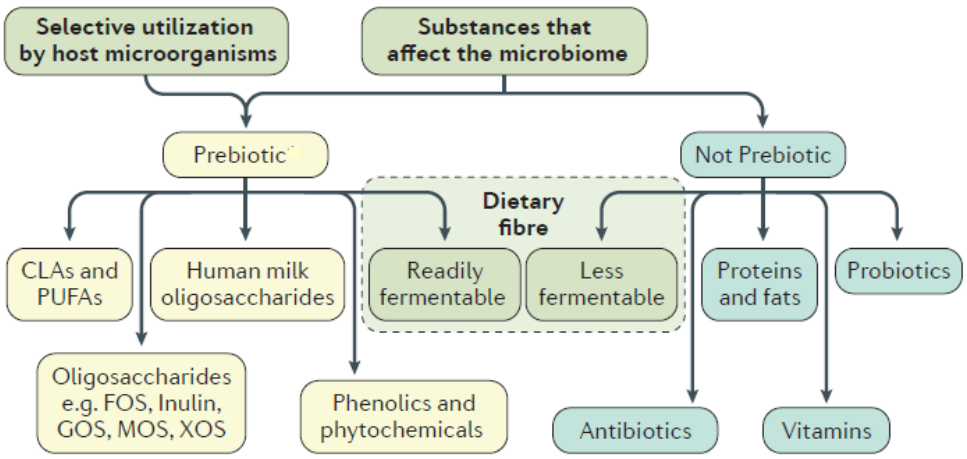

Figure 6. Distinguishing what is considered a prebiotic according to the official definition (139).

Common applications of prebiotics include the treatment of conditions such as constipation, travellers' diarrhoea, IBS, IBD, metabolic conditions like overweight and obesity, satiety, T2DM, metabolic syndrome, necrotising enterocolitis, and urogenital health, among others (139).

Prebiotics are generally considered to lack severe or life-threatening side effects. Due to their resistance to breakdown by intestinal enzymes, prebiotics are transported to the colon for fermentation by the gut microbiota. As a result, the side effects of prebiotics are primarily related to their osmotic functions, leading to symptoms such as osmotic diarrhoea, bloating, cramping, and flatulence (141).

3.2.3 Synbiotics

A symbiotic is a mixture of live microorganisms and substrate(s) selectively utilised by host microorganisms that confers a health benefit on the host. Therefore, a synbiotic is a product that contains both probiotics and prebiotics. There are two subsets of synbiotics. In "synergistic synbiotics", the substrate is designated to be selectively utilised by the co-administered microorganism(s). Conversely, a "complementary synbiotic" comprises a probiotic combined with a prebiotic designed to target autochthonous microorganisms (Figure 7) (142).

Synbiotics are often used to improve the survival rate of specific probiotics by providing them with a specific substrate, although the effects of synbiotics remain unclear. The health benefits of synbiotics likely depend on the specific combination of probiotics and prebiotics used (140).

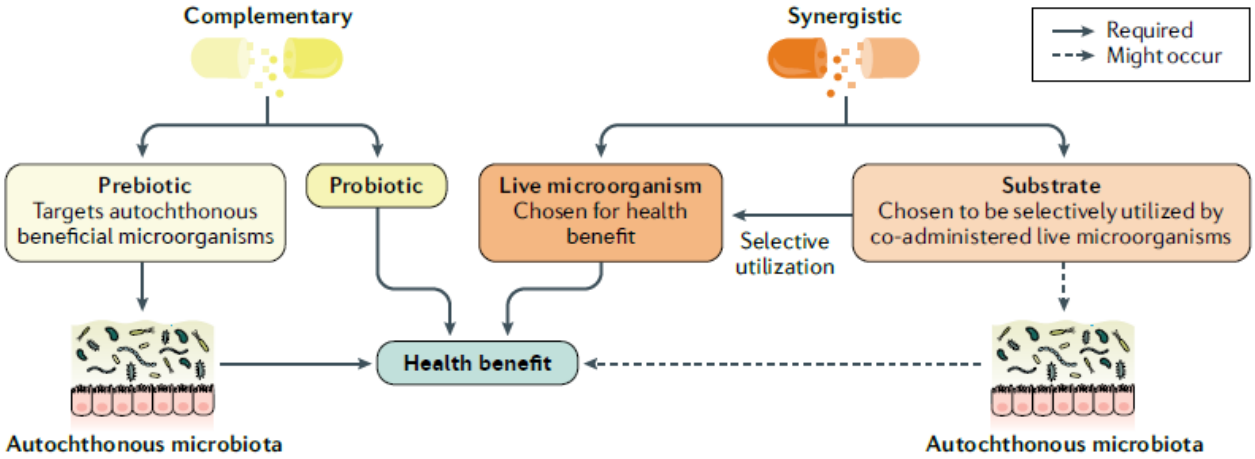

Figure 7. Design and mechanisms of action of complementary and synergistic synbiotics (142).

Prebiotics and probiotics tested to date have a strong safety record, and synbiotics formulated with them might also be presumed safe for the same intended uses.

3.2.4 Postbiotics

A postbiotic is a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host. Postbiotics are deliberately inactivated microbial cells with or without metabolites or cell components contributing to demonstrated health benefits (143). SCFA, produced from probiotic fermentation, is the most well-known postbiotic example. In addition to SCFAs, other examples of postbiotics include (Figure 8) (144):

- Heat-killed bacterial cells: *Lactobacillus* and *Bifidobacterium* species have been found to have beneficial functions in heat-killed formations.
- Cell-free supernatant (CFS): Biologically active metabolites secreted by microorganisms, mainly the supernatant bacteria, prepared after the bacterial cells were incubated, centrifuged, and removed. CFS has been reported to show antimicrobial, antioxidant and antitumour activity.
- Cell components: Two categories of cellular components have been investigated by far. Teichoic acids (TAs) are major constituents of bacterial cell walls, including both wall teichoic acids (WTAs) covalently linked to peptidoglycan and lipoteichoic acids (LTAs) anchored to the cytoplasmatic membrane. WTA was reported to be effective in modulating bacterial colonisation, while LTA was discovered to exert immune-modulatory activity by recognising TLRs and activating the host immune response.
- Bacteriocins: Antimicrobial peptides produced by certain bacteria, such as lactic acid bacteria, which can inhibit the growth of pathogens.

- Enzymes: Enzymes are recognised key regulators driving the metabolism of all organisms, playing the enzymes encoded by microbes' essential roles in host-microbe interactions.
- Exopolysaccharides (EPS): Complex carbohydrates produced by probiotic bacteria that have prebiotic-like effects, promoting the growth of beneficial bacteria in the gut.

Postbiotics are generally considered safe because they are derived from well-characterised probiotic microorganisms and their metabolic activities. Since postbiotics are typically isolated compounds, the risks associated with live microorganisms, such as probiotics, are minimised (143).

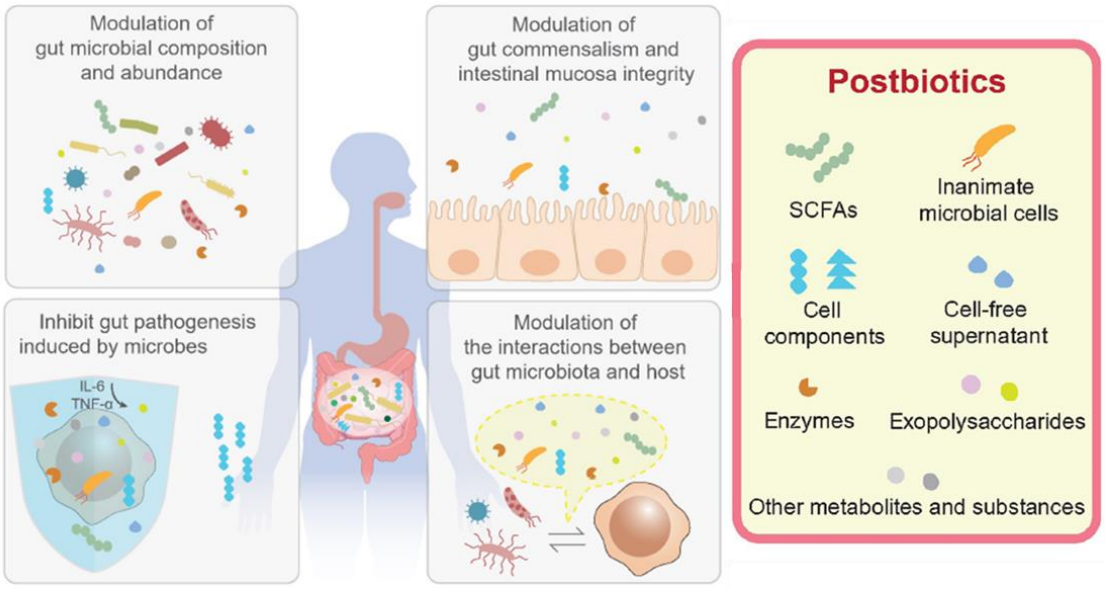

Figure 8. Classes of postbiotics and their biological activities and benefits (144).

3.2.5 Faecal microbiota transplantation

Faecal microbiota transplantation (FMT) aims to replace or reinforce a patient's gut microbiota by transferring faecal material from a healthy donor. FMT has demonstrated exceptional efficacy, particularly in treating recurrent CDI, a condition associated with dysbiosis. Efficacy rates of FMT in treating CDI range from 85% to 90%. CDI frequently gives rise to diseases such as IBS, nosocomial diarrhoea associated with antibiotic use, and colitis. In this way, the transferred microbiota can help restore a more balanced microbial community recipient's gut, reducing the overgrowth of harmful bacteria like CDI and promoting the growth of beneficial bacteria (Figure 9) (145,146).

The exact mechanisms by which FMT exerts its therapeutic effects are not yet fully understood, but there are several proposed mechanisms: (i) microbial restoration through the introduction of a diverse range of microorganisms from the donor, (ii) the introduced microbiota competes with and suppresses the growth of harmful bacteria such as CDI for resources and nutrients, (iii) the transplanted microbiota enhance metabolic functions and produce beneficial metabolites and (iv)

help modulate recipient's immune response by influencing the gut microbiota composition, promoting anti-inflammatory effects, and enhancing immune tolerance (147).

Several key aspects need to be considered in implementing FMT, including donor selection, preparation and preservation of the donor substance, receptor conditioning, and delivery techniques. The ideal stool donor should be a healthy volunteer without risk factors for infectious or chronic diseases. Donors should undergo periodic screenings to mitigate potential risks. Stool samples can be obtained in fresh, frozen, or lyophilised form and may come from either a single donor or multiple donors. Anaerobic stool processing is preferred as it enhances the growth of obligatory anaerobes while inhibiting the overgrowth of facultative anaerobes. Various administration routes can be employed for the FMT procedure, such as colonoscopy, nasoenteric tubes, or encapsulated formulations (148).

The most frequently reported adverse events associated with FMT are mild or moderate and include symptoms such as nausea and vomiting, diarrhoea, flatulence, abdominal distension, and pain. Instances of infectious agent transmission through FMT have also been documented (149).

While FMT has demonstrated remarkable success in specific conditions, further research is necessary to optimise donor selection, standardise protocols, and evaluate the long-term safety and efficacy of FMT in other diseases characterised by dysbiosis, such as IBD and metabolic disorders (148).

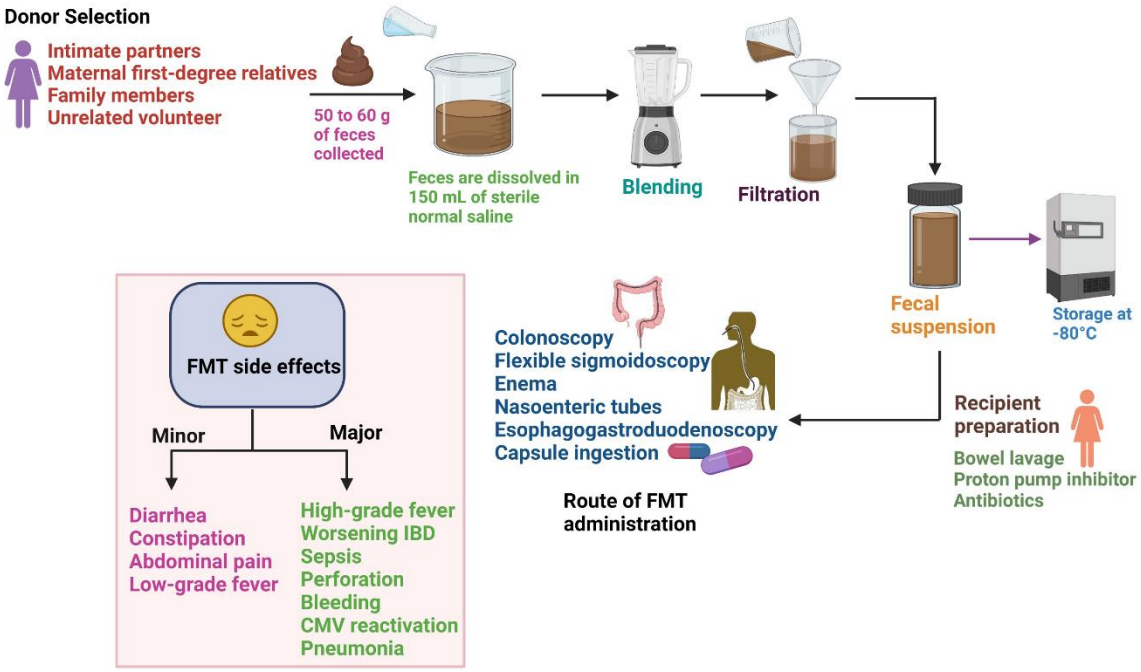

Figure 9. Diagram of the FMT procedure and the associated adverse events (150).

2

Objectives

The main objective of this thesis is to explore and investigate the role of intestinal microbiota, including dysbiosis in different symptoms and digestive disorders, as well as to assess the modulating capacity of a novel prebiotic.

To accomplish these challenges, faecal DNA extraction, quantitative polymerase chain reactions of specific microbial species and statistical analysis have been performed. The results are organised into three chapters, which present the following specific aims:

Chapter 1. To differentiate between pathological and temporary dysbiosis and to enhance our understanding of the underlying basis of symptomatology in various intestinal disorders.

Chapter 2. To analyse the *in vitro* effect of a novel prebiotic derived from grapes on the intestinal microbiota using samples from patients with inflammatory bowel disease, irritable bowel syndrome, and healthy controls.

Chapter 3. To describe intestinal microbiota alteration on chronic pancreatitis and determine whether this leads to a compensatory effect on the intestinal microbiota, increasing the abundance of those microorganisms with enzymatic activities.

3

Methodology

1 Patients and data sampling

The participants were recruited in two health centres: the Hospital Universitari Doctor Josep Trueta and the Bofill - Neupsi Clinic, both located in Girona, Spain.

Recruited patients were classified into five main groups:

1. Patients diagnosed with irritable bowel syndrome.
2. Patients diagnosed with inflammatory bowel disease, including those with Crohn's disease and ulcerative colitis.
3. Patients diagnosed with chronic pancreatitis who have developed exocrine pancreatic insufficiency.
4. Patients who presented various digestive symptoms but have not yet received a diagnosis.
5. Healthy subjects without digestive symptoms or previous diagnosis.

All patients with irritable bowel syndrome, inflammatory bowel disease, and chronic pancreatitis with exocrine pancreatic insufficiency were diagnosed using established clinical, pathological, and/or endoscopic criteria. All samples were included and processed following standard operating procedures with the corresponding approval of the Scientific and Ethics Committees (clinical research code of the studies performed: GG-TUG-1001, GG-PREVIPECT-1001 and GG-Creon).

Participants were asked to collect a stool sample from a bowel movement into a sterile container. The procedure followed later depended on the specific study:

- Chapter 1: The samples were kept at room temperature for a maximum of 48 hours. Once the samples were received at the GoodGut SLU facilities (Girona, Spain), they were homogenised, aliquoted, and frozen at -80°C until analysis.
- Chapter 2: The patients took the samples to the hospital, where they were kept at room temperature, and within a maximum of 4 hours, these samples arrived at GoodGut SLU for processing as part of the study.
- Chapter 3: The samples were kept at room temperature for a maximum of 12 hours, and then they were taken to the hospital and stored at -20°C. Subsequently, they arrived at the GoodGut SLU facilities, where they were preserved at -80°C until the analysis.

On the day of sample collection, subjects were also asked to complete a questionnaire about clinical data, such as age, gender, ethnicity, medical history, and medication usage, among other relevant information, depending on the study in which they participated.

2 Faecal DNA extraction

Genomic deoxyribonucleic acid (DNA) was extracted from all homogenised faecal samples using the NucleoSpin® Soil kit (Macherey-Nagel GMbH & Co., Duren, Germany), following the manufacturer's instructions. The sample was extracted with an approximate weight of 50 mg. In the case of liquid samples, such as those discussed in Chapter 2, an extraction volume of 250 µl was employed. The manufacturer provides the option of utilizing either SL1 or SL2 buffers for extraction. Following comprehensive analytical assessments, it was determined that the utilization of buffer SL1 yielded superior performance within the context of our methodology. Regarding DNA elution, the manufacturer specifies a range between 30 and 100 µl. Throughout this thesis, elutions were consistently executed with a volume of 100 µl and stored at -20°C until further use.

3 Microbial markers quantification through qPCR

Twenty microbial markers have been utilised in various studies encompassed within this thesis. Eubacteria (EUB) were quantified to assess the overall bacterial load. Each selected biomarker possesses a unique genetic sequence. The bacterial strains included in the analysis were *Faecalibacterium prausnitzii* (FAE), *F. prausnitzii* phylogroup I (PHG-I), *F. prausnitzii* phylogroup II (PHG-II), B46 (best BLAST match *Subdolinogranulum variabile*), *Roseburia* sp. (ROS), and *Ruminococcus* spp. (RUM), known for their butyrate-producing capabilities. Additionally, Gammaproteobacteria (GAM), the *Enterobacteriaceae* group (ENB), and *Escherichia coli* species (ECO) were included as representatives of proinflammatory members. *Akkermansia muciniphila* (AKK) was an indicator species for mucosal layer homeostasis and acetate production, while *Methanobrevibacter smithii* (MSM) represented methanogenic species. The proteolytic species comprised *Clostridium* cluster I (CLO), *Clostridium* cluster XIV, and *Enterococcus* sp. (ENT), whereas *Lactobacillus* sp. (LAC) and *Bifidobacterium* (BIF) were considered immunoprotective species. Additionally, opportunistic *Candida albicans* (CAN) and the phylogenetic groups Bacteroidetes (BAC) and Firmicutes (FIR) were included. Not all markers were employed in every chapter (Table 1).

Table 1. List of microbial markers used, code, functionality and chapters where they have been used.

Marker	Code	Functionality	Chapters
Akkermansia muciniphila	AKK	Mucosal layer homeostasis, acetate-producer	1, 2 and 3
B46 (*Subdolinogranulum variabile*)	B46	Mucosal layer homeostasis, butyrate-producer	2
Bacteroidetes	BAC	Phylum, balance, and diet indicator	1 and 3
Bifidobacterium	BIF	Immunoprotective	3
Candida albicans	CAN	Opportunistic yeast	1 and 3
Clostridium cluster I	CLO	Proteolytic	1 and 3
Clostridium cluster XIV	XIV	Proteolytic	1 and 3
Escherichia coli	ECO	Proinflammatory	1 and 3
Enterobacteriaceae	ENB	Proinflammatory	3
Enterococcus sp.	ENT	Proteolytic	1 and 3
Faecalibacterium prausnitzii	FAE	Mucosal layer homeostasis, butyrate-producer	1, 2 and 3
F. prausnitzii phylogroup I	PHGI	Mucosal layer homeostasis, butyrate-producer	2
F. prausnitzii phylogroup II	PHGII	Mucosal layer homeostasis, butyrate-producer	2
Firmicutes	FIR	Phylum, balance, and diet indicator	1 and 3
Gamma-proteobacteria	GAM	Proinflammatory	1 and 3
Lactobacillus sp.	LAC	Immunoprotective	1 and 3
Methanobrevibacter smithii	MSM	Methanogenic	1
Roseburia sp.	ROS	Mucosal layer homeostasis, butyrate-producer	1, 2 and 3
Ruminococcus spp.	RUM	Mucosal layer homeostasis, butyrate-producer	1, 2 and 3
Eubacteria	EUB	Overall bacterial load	1, 2 and 3

The quantification of AKK, B46, BAC, BIF, CAN, CLO, ENB, ENT, GAM, FIR, LAC, MSM, ROS, RUM, XIV, and EUB was conducted by preparing single reactions for each biomarker, utilising the GoTaq qPCR Bryt Master Mix (Promega, Madison, USA). FAE, PHGI, PHGII, and ECO were quantified on single reactions for each target using the GoTaq qPCR Probe Master Mix (Promega, Madison, USA). Each reaction consisted of a final volume of 10 µl containing the master mix and between 12 and 20 ng of genomic DNA template. The 16S and 18S rRNA gene-targeting primers and probes used in this study, along with their respective concentrations, are listed in Table 2. These primers and probes were procured from Macrogen (Seoul, South Korea). Accuracy was ensured by running samples in duplicate on the same plate alongside a non-template control reaction and a standard curve, which were included in each qPCR run. The mean of duplicate quantifications was

employed for data analysis. qPCRs were performed using the AriaDx thermocycler (Agilent Technologies, Santa Clara, CA, USA) under the quality standards of ISO13485.

Table 2. Forward (F) and reverse (R) primers and probes (PR) used in this work. All probes were 5'-labelled with FAM (6-carboxyfluorescein) as the reporter dye except PHGII, in which hexachlorofluorescein (HEX) was used. BHQ1 was used as quencher dye at the 3'-end for all probes. The base R can be A or G; W can be A or T, and Y can be C or T. Final qPCR master mix concentration in nmol/L.

Marker	Primers	Sequence 5'→3'	Concentration (nmol/L)	Reference
AKK	F	CAGCACGTGAAGGTGGGGAC	250	(151)
	R	CCTTGCGGTTGGCTTCAGAT		
B46	F	GTACGGGGAGCAGCAGTG	300	(70)
	R	GACACTCTAGA GCACAGTTTCC		
BAC	F	CCGGAWTYATTGGGTTTAAAGGG	100	(152)
	R	GGTAAGTTCCTGCGTA		
BIF	F	CTCCTGGAAACGGGTGG	250	(152)
	R	GGTGTTCTTCCCGATATCTACA		
CAN	F	CTGATTTATGGGTTCCTGAT	200	(153)
	R	GTTGATCAATTGAAGTAGAATC		
CLO	F	CTCAACTTGGGTGCTGCATTT	300	(154)
	R	ATTGTAGTACGTGTGTAGCCC		
ECO	F	CATGCCGCGTGTATGAAGAA	300	(50)
	R	CGGGTAACGTCAATGAGCAAA		
	PR	FAM-TATTAACTTTACTCCCTTCCTCCCCGCTGAA-BHQ1	100	
ENB	F	CAGGTCGTCACGGTAACAAG	150	(155)
	R	GTGGTTCAGTTTCAGCATGTAC		
ENT	F	TACTGACAAACCATTCATGATG	200	(156)
	R	AACTTCGTCACCAACGCGAAC		
FAE	F	TGTAAACTCCTGTTGTTGAGGAAGATAA	300	(50)
	R	GCGCTCCCTTTACACCCA		
	PR	FAM-CAAGGAAGTGACGGCTAACTACGTGCCAG-BHQ1	250	
FIR	F	GGCAGCAGTRGGGAATCTTC	100	(157)
	R	ACACYTAGYACTCATCGTTT		
GAM	F	TCGTCAGCTCGTGTYGTGA	100	(157)
	R	CGTAAGGGC CATGATG		
LAC	F	AGCAGTAGGGAATCTTCCA	200	(158)
	R	CGCCACTGGTGTTCYTCCATATA		
MSM	F	ACGCAGCTTAAACCACAGTC	150	(159)
	R	AAAGACATTGACCCRCGCAT		
PHGI and PHGII	F	CTCAAAGAGGGGGACAACAGTT	900	(160)
	R	GCCATCTCAAAGCGGATTG		
	PHGI_PR	FAM-TAAGCCCACGACCCGGCATCG-BHQ1	300	
	PHGII_PR	HEX-TAAGCCCACRGCTCGGCATC-BHQ1		

Marker	Primers	Sequence 5'→3'	Concentration (nmol/L)	Reference
ROS	F	TACTGCATTGGAAACTGTCG	125	(161)
	R	CGGCACCGAAGAGCAAT		
RUM	F	GGCGGCYTRCTGGGCTTT	250	(162)
	R	CCAGGTGGATWACTTATTGTGTTAA		
XIV	F	CGGTACCTGACTAAGAAGC	250	(162)
	R	AGTTTYATTCTTGCGAACG		
EUB	F	ACTCCTACGGGAGGCAGCAGT	200	(50)
	R	GTATTACCGCGGCTGCTGGCAC		

The thermal profiles varied based on the specific biomarker being analysed (Table 3). For probeless markers, a melting curve step was included at the end of each qPCR to verify the expected amplicon size and monitor dimer formation.

Table 3. qPCR conditions for each microbial marker. NA: not of application.

Microbial markers	Total cycles	Denaturing		Annealing and extension		Melting curve	
		Time (min)	Ta (°C)	Time (min)	Ta (°C)	Time (min)	Ta (°C)
B46	40	10:00	95	00:15 01:00	95 62	01:00 00:30 00:30	95 55 95
FAE and ECO	40	02:00 10:00	50 95	00:15 01:00	95 60	NA	NA
PHGI and PHGII	40	02:00 10:00	50 95	00:15 01:00	95 64	NA	NA
AKK, BAC, BIF, CAN, CLO, ENB, ENT, FIR, GAM, LAC, MSM, ROS, RUM, XIV and EUB	40	10:00	95	00:15 01:00	95 60	01:00 00:30 00:30	95 55 95

4 Abundance calculation

Once the qPCR results in Ct units for each marker were obtained, the data from chapters 1 and 3 were transformed into relative and total abundance values for statistical analysis.

The total abundance values (A, gene copies per gram of stool) for each marker were calculated using the following equation based on the standard curve included in each qPCR run:

$$A = \frac{\left(\frac{V_e}{V_c}\right) \cdot 10^{(Ct-b)/m}}{P \cdot C_g}$$

Where:

Ct is the threshold cycle, *b* is the y-axis intercept on the standard curve, *m* is the slope of the standard curve (Table 4), *Ve* is the volume of elution of the DNA extract (µl), *Vc* is the volume of

the DNA extract loaded in the PCR (µl), *P* is the weight of the stool analytical portion (g), and *Cg* is the number of copies of the 16S or 18S rRNA gene each indicator contains in its genome (Table 5).

Table 4. Main parameters of the standard curves used for calculating the abundance of microbial markers.

Marker	Efficiency (%)	Linearity (R2)	Slope	Intercept
Akkermansia muciniphila	93.500	0.998	-3.489	36.548
Bacteroidetes	103.006	1.000	-3.254	34.364
Bifidobacterium	109.732	0.995	-3.110	37.195
Candida albicans	90.298	0.995	-3.669	36.477
Clostridium cluster I	93.584	0.998	-3.490	36.900
Clostridium cluster XIV	72.710	0.999	-4.220	42.254
Escherichia coli	98.528	0.999	-3.359	36.992
Enterobacteriaceae	123.921	0.998	-2.857	35.950
Enterococcus sp.	102.658	0.999	-3.262	35.312
Faecalibacterium prausnitzii	98.700	0.999	-3.354	38.758
Firmicutes	83.030	0.979	-3.813	40.158
Gamma-proteobacteria	80.167	0.995	-3.295	39.207
Lactobacillus sp.	84.402	0.994	-3.764	41.616
Methanobrevibacter smithii	103.414	0.998	-3.243	34.974
Roseburia sp.	97.420	0.999	-3.386	35.164
Ruminococcus spp.	100.836	1.000	-3.303	35.384
Eubacteria	99.730	0.999	-3.329	35.734

The number of copies of the phyla Bacteroidetes and Firmicutes, as well as the total microbial load (Eubacteria), or markers that include different species, such as gamma-proteobacteria or *Clostridium*, was calculated using an average of the number of copies of the species that form these groups.

Table 5. Number of copies of 16S and 18S rRNA gene (Cg) for each marker.

Markers	Number of copies of 16S and 18S (Cg)	References
CAN	1	(163)
MSM	2	(164)
AKK	3	(165)
BIF	3.5	(166)
ENT	4	(164)
BAC, LAC, FIR, ROS, RUM, EUB	5	(164,166,167)
GAM	5.5	(166)
FAE, PHGI, PHGII	6	(164)
ECO, ENB	7	(166,168)
CLO, XIV	8	(166)

After obtaining the abundance of each marker, it undergoes logarithmic transformation to facilitate comprehension of the values. Following this transformation, relative abundance (RA) is computed. Relative abundance is determined by subtracting the logarithmic abundance value of each marker from that of Eubacteria (representing the total bacterial load).

5 Statistical analysis

The significance levels were established for p-values ≤ 0.05. Statistical analyses were conducted using the SPSS 23.0 statistical package (IBM, NYC, USA) for chapters 2 and 3, R Statistical software version 4.1.3 (R Core Team, Vienna, Austria) for chapters 1 and 3, and CoDaPack (version 2.02.21, Girona, Spain) for chapters 1 and 3. The CoDaPack program was employed for conducting centered logratio transformation (CLR) and generating the corresponding plots, as proposed by Aitchison (169).

Data normality was assessed through the Kolmogorov-Smirnov test. Alternatively, in cases where the sample size exceeded 30, normality was presumed based on the central limit theorem. For parametric data, ANOVA tests were employed for comparisons involving more than two categories, and the t-test was used for two-by-two comparisons. In the case of non-parametric data, the Kruskall-Wallis test was utilised for comparisons involving more than two categories, and the Mann-Whitney U test was applied for two-by-two comparisons. For multiparametric analyses, MANOVA and generalised regression models were employed. In Chapter 1, generalized regression models were utilized to quantify the impact of various factors on the abundance of microbial markers. When the predictor and outcome variables were categorical, the chi-square test was performed. Figure 10 shows the flowchart followed to choose a statistical test for each case.

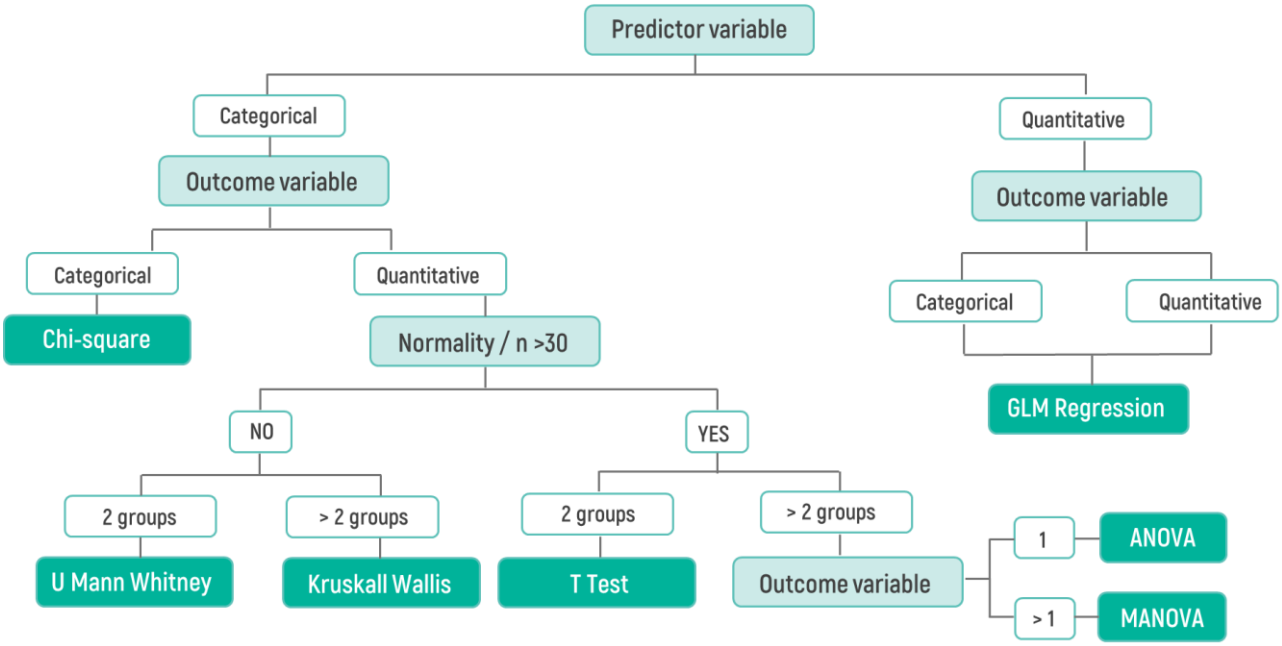

Figure 10. Statistical test flowchart followed in the analytical process of this study.

4

Results

CHAPTER 1

Categorizing and Characterizing Intestinal Dysbiosis: Evaluating Stool Microbial Test Clinical Utility

1 Background

The human gut microbiota, a complex ecosystem of trillions of microorganisms, plays a crucial role in maintaining human health. Emerging evidence has demonstrated the significance of the relationship between gut microbiota composition and various aspects of human health, including immune function, metabolism, mental health, and gastrointestinal disorders. Dysbiosis, characterized as an imbalance or perturbation in the gut microbial community, has been associated with various abnormal conditions, such as Inflammatory Bowel Disease (IBD), Irritable Bowel Syndrome (IBS), obesity, diabetes, and cardiovascular diseases, implicated in their development and progression (48,64).

Regulating the gut microbiome has emerged as a promising therapeutic approach for managing chronic diseases that burden healthcare systems significantly (60). Consequently, there has been a surge of interest in developing microbial stool tests that can accurately assess gut dysbiosis and provide insights into its implications for human health.

In recent years, extensive research has been dedicated to characterising the diversity and functional capacity of the gut microbiota, leading to the development of numerous microbial stool tests for dysbiosis assessment. However, the clinical applicability of these tests hinges on several factors, including the clinical and analytical validity of the assay, the interpretation of results by clinicians, and the successful translation of test outcomes into effective treatment strategies (60). Challenges associated with applying microbial stool tests in regular clinical practice have limited their widespread use. Factors such as the substantial volume of data generated from microbiome tests, the considerable interindividual variation in gut microbial composition, and the lack of disease condition-specific microbial profiles have hindered the seamless integration of these tests into routine clinical workflows (61,62).

Efforts are being made to overcome these challenges by improving the clinical validity of microbial stool tests, standardizing interpretation guidelines, and establishing disease-specific microbial signatures. These advancements aim to enhance the practical applicability of gut microbiome information, ultimately facilitating its effective utilization in clinical decision-making and personalised treatment approaches (63,64).

Nevertheless, the definition of a "healthy" gut microbiome remains a challenge, rendering the accurate determination of dysbiosis even more complex. The gut microbiota composition is highly individualised and influenced by various factors, including genetics, diet, lifestyle, and environmental exposures. Considerable interindividual variation in microbial diversity and abundance makes it difficult to establish a clear definition of a "normal" or "healthy" gut

microbiome (61,62,65). Consequently, identifying dysbiosis becomes subjective since it represents a deviation from an undefined healthy state (63).

A stool microbial test, TestUrGut®, has been developed to address this challenge. This test consists of a qPCR detection of a comprehensive set of 15 microbial markers that represent key functions of the gut microbiota, such as immune protection, mucosal homeostasis, proteolysis, and proinflammatory activity, among others. These markers were selected based on their association with dysbiosis-related disorders and their potential as diagnostic indicators. Additionally, from the analysis of these markers, 2 indices are derived. One is the Firmicutes/Bacteroidetes index, which has been related to the diet's characteristics (58) and body mass index (170). The other is an indicator index of dysbiosis, utilizing the relative abundance of two key microbial species, *Faecalibacterium prausnitzii* and *Escherichia coli*, known to be associated with dysbiosis (50).

This study aimed to evaluate the clinical utility and validate the representativeness of selected microbial markers and the dysbiosis index. Additionally, it aimed to distinguish between pathological and transient dysbiosis, contributing to our understanding of the underlying basis of symptomatology related to bowel patterns. Through the evaluation of clinical utility of the stool microbial test, we aim to assess the validity of the designed panel of markers, the robustness of the tolerance ranges and the concordance with the dysbiosis index. The results of this study provide valuable insights into the utility of the test in clinical practice and its potential contribution to the understanding and management of specific intestinal disorders.

2 Experimental design

2.1 Study population

The sample size of the study (N) was 154. The patients recruited previously underwent the faecal microbiota test after being visited by the gastroenterologist due to the presence of digestive discomfort at the NEUPSI-Clínica Bofill Centre in Girona, Spain. Of these 154 patients, 46 were men (29.87%) and 108 women (70.13%). Clinical data from the enrolled subjects at the time of the examination and their final diagnosis were recorded. The diagnosis was determined by the doctor following usual clinical guideline in practice.

The inclusion criteria were: i) being of legal age, ii) having conducted a TestUrGut® analysis, and iii) having duly signed informed consent. The exclusion criteria were: i) having received antibiotic treatment in the last month before collecting the faecal sample, ii) sampling the faeces more than 48 hours before arriving at laboratory facilities, and iii) being pregnant at the time of inclusion.

2.2 DNA extraction and qPCR analysis from stool samples

The DNA extraction performed is extensively detailed in Material and Methods section.

The abundance of 15 microbial markers representing the main phyla, groups, and genera present in gut microbiota was analysed by real-time quantitative Polymerase Chain Reactions (qPCR): *Akkermansia muciniphila*, Bacteroidetes, *Candida albicans*, *Clostridium* cluster I, *Escherichia coli*, *Enterococcus* sp., *Faecalibacterium prausnitzii*, Firmicutes, Gamma-proteobacteria, *Lactobacillus* sp., *Methanobrevibacter smithii*, *Roseburia* sp., *Ruminococcus* spp., *Clostridium* cluster XIV, and Eubacteria.

Quantification data of each microbial marker was collected and analysed with the Aria Software version 1.71 (Agilent Technologies, Santa Clara, USA).

After obtaining the results, the data were normalized for Eubacteria to facilitate comparison among samples with varying bacterial abundance.

2.3 Definition of tolerance values

To determine the tolerance range values indicative of a healthy population, as well as those at the borderline and beyond the accepted norms, we conducted an initial analysis on a cohort of healthy individuals. This analysis was later validated in a separate group comprising 24 healthy subjects and 6 patients with digestive diseases, including ulcerative colitis, Crohn's disease, and irritable bowel syndrome.

Within the healthy cohort, 14 samples belonged to female subjects, and the age range spanned from 21 to 69 years, with a mean age of 41.

The tolerance values were established by calculating the relative abundance of each microbial marker using logarithmic transformations (as defined in Methodology section 4 Abundance calculation). Specifically, the abundance of each marker was normalized with Eubacteria abundance. The mean and standard deviation (SD) of these ratios were then calculated, with SD either added or subtracted from the mean, depending on the nature of the microbial marker, as a borderline of the accepted norms.

SD was added to markers considered beneficial or protective — AKK, BAC, FAE, FIR, LAC, ROS, RUM, and EUB. Conversely, SD was subtracted from markers where abundances above established values could be harmful — CAN, ECO, ENT, GAM, and MSM. For the markers CLO and XIV, recognising that having too little or too much can be detrimental, SD was both added and subtracted from the mean, resulting in two tolerance limit values, above and below the average.

The resulting tolerance values demonstrating the limit beyond of the accept norms were obtained by adding or subtracting a unit, based on whether the microbial marker was beneficial or not to the borderline limit of the accepted norms.

Additionally, the relationship between the abundance of Firmicutes and Bacteroidetes was calculated by subtracting the logarithm of FIR from BAC. No tolerance values were assigned to this index, as its results are indicative of the type of diet rather than dysbiosis.

Lastly, the dysbiosis index involved the logarithmic subtraction of the abundance of the *F. prausnitzii* marker from the abundance of *E. coli* (72). The dysbiosis index, proposed by Lopez-Siles et al. in 2014, involves normalizing the number of human cells. In this thesis, akin to the doctoral thesis of Miquel-Cusachs in 2021 (171), which centred on faecal samples rather than biopsies, adjustments in the calculation of this index were made not to the count of human cells but rather to the DNA extraction weight (as specified in the abundance formula outlined in the Methodology section, specifically the Calculation of Abundance subsection). The average dysbiosis index value among all healthy controls defined the healthy range, with subsequent subtractions of SD establishing thresholds for tolerance levels to the borderline limit of accepted norms, indicating temporary dysbiosis, and beyond the limit of accepted norms signifying pathological dysbiosis.

2.4 Statistical analysis

2.4.1 Data preprocessing

The dataset included several variables, some of which had missing values. Missing values are a common issue in many clinical datasets that can introduce bias and reduce the statistical power of the analysis. The Multiple Imputation by Chained Equations (MICE) method was employed to estimate missing values based on the available data using the "mice" package in R (172). The imputation process occurs in multiple iterations, known as "cycles". In this study, five imputations were generated (m=5), the maximum number of iterations was set as 50 (maxit=50), and the imputation method was random forest (meth=rf). Each imputation represented a plausible completion of the missing data, capturing the uncertainty associated with the imputed values. The imputed values generated by the MICE algorithm were subsequently used for further analysis.

2.4.2 Statistical approach

For the analysis of the data, it was employed a combination of univariate analysis of variance (ANOVA), multivariate analysis of variance (MANOVA), and regression models. Regression models are mathematical tools to establish the relationship between a response variable (Y) and explanatory variables (X). Our study aimed to examine the relationship between the abundance of microbial markers and the occurrence of diseases and symptoms.

To accommodate the diverse nature of our response variables, which could be continuous or categorical, we utilized generalized linear models (glm) with a binomial link (equivalent to logistic regressions). These models are particularly advantageous as they can handle response variables with non-normally distributed errors, which aligns well with the characteristics of our dataset.

To quantify the impact of the independent variables on the dependent variable, we employed the Odds Ratio (OR). The OR provides a measure of effect and indicates the probability of an event occurring. Specifically, for each unit increase in the independent variable X, while holding other variables constant, the probability of the dependent variable Y occurring increases by Z%. A value of OR equal to 1 signifies no association between the variables.

All analyses were performed using version 4.1.3 of the R statistical software (173).

3 Results

3.1 Sex

The MANOVA analysis conducted to investigate the panel's microbial abundance revealed no statistically significant differences between sexes (Figure 11). However, upon conducting an ANOVA, a discrepancy between sexes emerged concerning the *A. muciniphila* marker (*p*-value 0.0481). Subsequently, this finding was further validated using a glm regression model (*p*-value 0.0104). The OR computed for the *A. muciniphila* marker was 2.54, indicating that women exhibit a notably greater abundance of this marker than men.

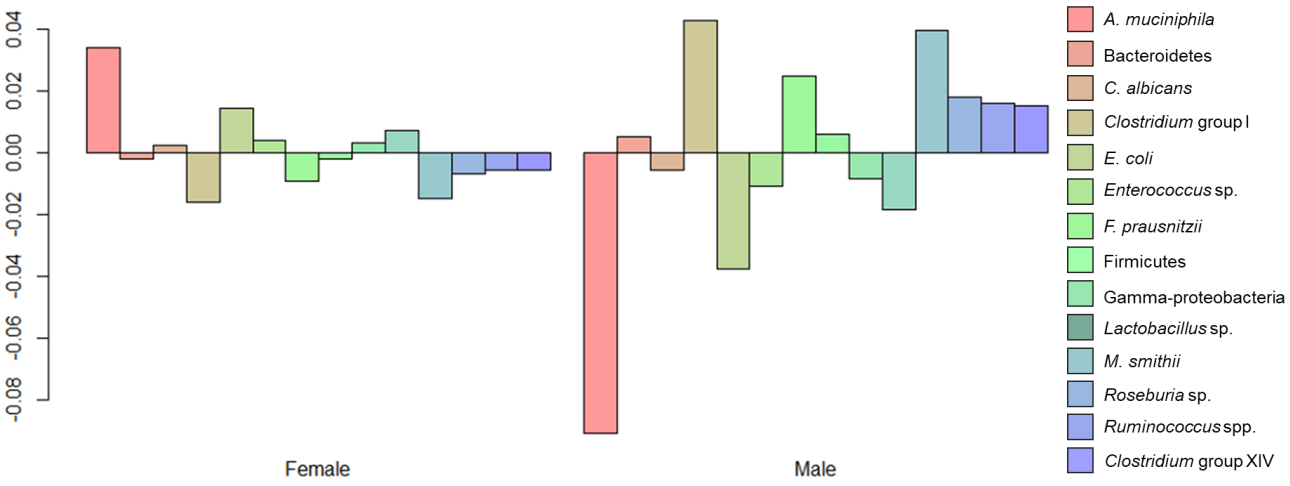

Figure 11. Geometric mean (differences between group mean and the average mean, Y axis) for the abundance of each microbial marker according to females and males.

3.2 Age

For analysis, patients were categorized into three age groups: young individuals (age 18 to 28, n=12), adults (age 29 to 59, n=89), and older individuals (age 60 and above, n=27).

In our investigation, neither the MANOVA, ANOVA, nor the glm model exhibited statistically significant differences across all microbial markers studied (Figure 12). These findings collectively indicate that age does not exert a conditioning effect on the abundance of any specific microbial marker analysed.

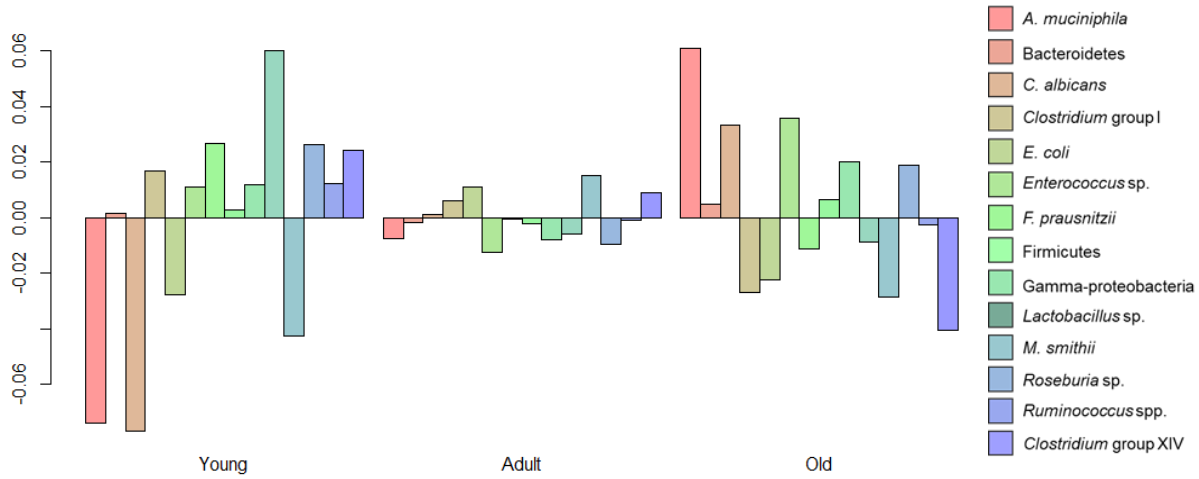

Figure 12. Geometric mean (differences between group mean and the average mean, Y axis) for the abundance of each microbial marker according to age classified into young, adult, and old.

3.3 Body Mass Index

Body mass index (BMI) data were available for 65 patients, and imputation was not conducted due to a considerable number of missing values. Based on their BMI values, patients were categorized into four groups: underweight (BMI < 18.5, n=4), normal weight (BMI 18.5 to 24.9, n=43), overweight (BMI 25 to 29.9, n=14), and obese (BMI ≥ 30, n=4).

The significant outcomes obtained from the MANOVA were further examined in conjunction with the ANOVA and glm analysis results. Our findings highlight *M. smithii* as the microbial marker most influenced by BMI, demonstrating significance in the glm model (p-value 0.0147), despite not achieving significance in the ANOVA (*p*-value 0.1643). *A. muciniphila*, a marker extensively linked to BMI in the literature, did not exhibit a significant p-value in the glm model; however, it yielded a noteworthy result in the ANOVA (*p*-value 0.0346).

Figure 13 illustrates a significant decrease in the abundance of *A. muciniphila* and *M. smithii* in low-weight patients, whereas *A. muciniphila* displayed increased abundance in those classified as obese.

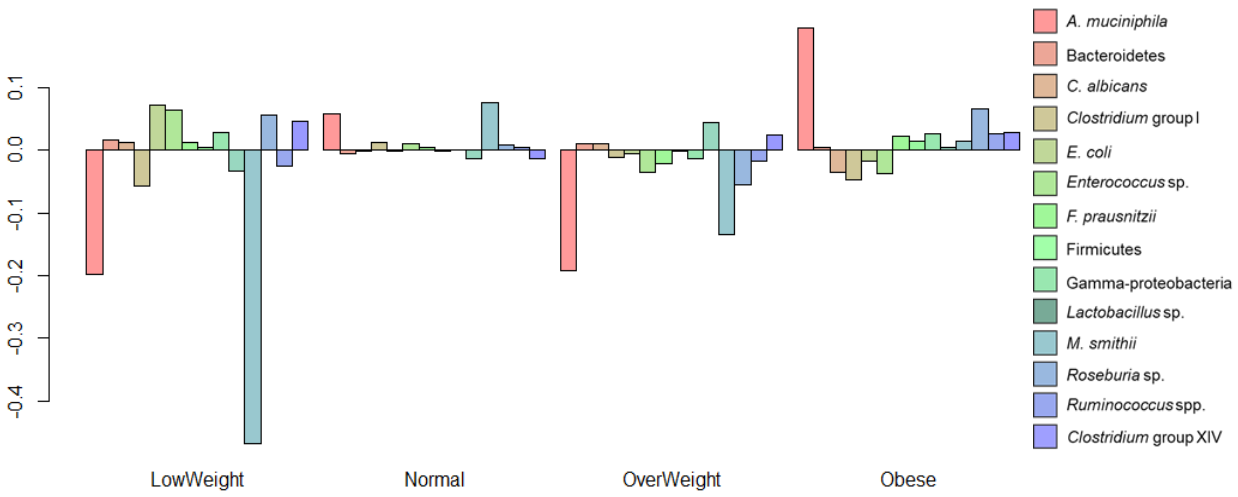

Figure 13. Geometric mean (differences between group mean and the average mean, Y axis) for the abundance of each microbial marker according to body mass index classified into low weight, normal, overweight, and obese.

3.4 Intestinal microbiome profile and digestive symptoms

Among the patients included in this study, 97% were diagnosed with IBS according to ROME IV Criteria, limiting the scope for analysing the relationship between marker abundance and other diseases such as mental, neurodegenerative, celiac disease, and intolerances, among others. As a result, our analysis focused solely on the behaviour of IBS.

3.4.1 IBS behaviour

Out of the 128 patients diagnosed with IBS, behaviour data were available for 62 individuals. Among them, 30 patients exhibited a diarrhoeal pattern (48.4%), 19 had constipation (30.6%), and 13 presented mixed behaviour (21.0%).

The MANOVA yielded significant results, further substantiated by ANOVA tests for the markers *A. muciniphila* and *M. smithii* (p-values of 0.009 and 0.004, respectively), as well as by the glm model.

Specifically, when examining the glm model for diarrheal behaviour, *A. muciniphila* displayed a significant association (p-value = 0.003) with an OR of 0.22, indicating that the abundance of *A. muciniphila* decreases by a factor of 4.54 (1/0.22=4.54) with the presence of diarrhoea. Regarding *M. smithii*, although not statistically significant, a trend was observed (p-value of 0.093) with an OR of 0.405, suggesting 2.5 times decrease in its abundance in the presence of diarrhoea.

Conversely, in the analysis of constipation, both *A. muciniphila* and *M. smithii* exhibited significant associations in the glm model (p-values of 0.011 and 0.001, respectively) with corresponding ORs of 3.932 and 5.659. These findings indicate that the abundance of these markers is increased by approximately 4 and 5.5 times, respectively, in the presence of constipation.

For patients displaying a mixed behaviour of diarrhoea and constipation, the glm model revealed a significant association only for *M. smithii* (*p*-value of 0.049) with an OR of 0.306, indicating a decrease in its abundance by a factor of 3.3.

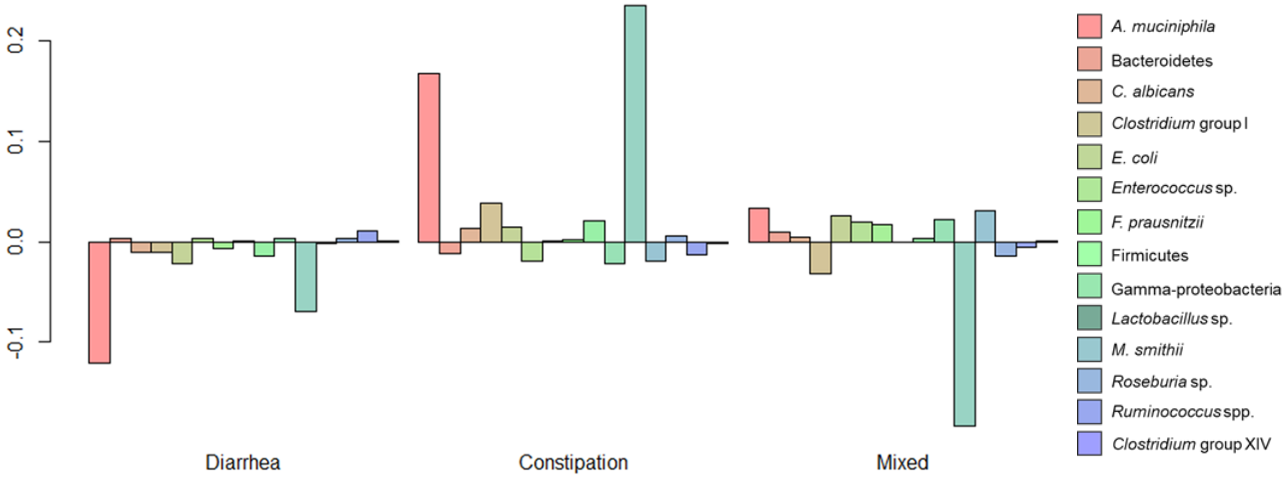

Figure 14. Geometric mean (differences between group mean and the average mean, Y axis) for the abundance of each microbial marker according to irritable bowel syndrome behaviour (diarrhoea, constipation and mixed).

3.5 Firmicutes/Bacteroidetes index

The FIR/BAC index is designated as predominantly Firmicutes when the index value is positive (greater than zero) and as predominantly Bacteroidetes when the value is negative (less than zero). Significant results were observed in the MANOVA analysis when comparing the abundance of microbial markers based on the predominance of Firmicutes or Bacteroidetes (p-value < 0.001, as depicted in Figure 15).

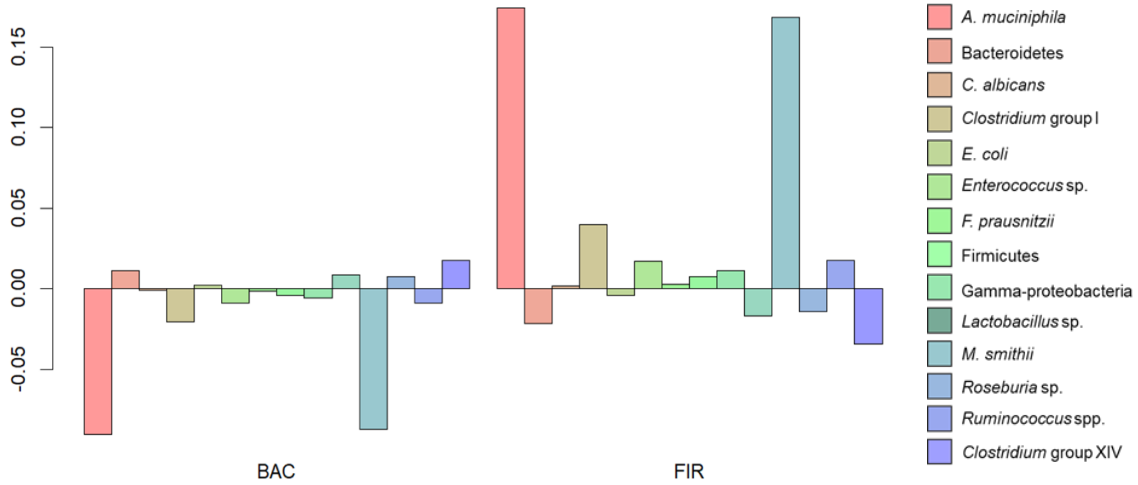

Figure 15. Geometric mean (differences between group mean and the average mean, Y axis) of the abundance of each microbial marker according to Firmicutes or Bacteroidetes predominancy.

In the MANOVA graph, notable differences in the abundance of *A. muciniphila* and *M. smithii* are evident, as confirmed by ANOVA analysis (p-values < 0.001 in both cases). A regression model was employed to assess and quantify this effect, yielding significant values for both markers. The OR indicates a substantial 2.72-fold increase in the abundance of *M. smithii* and a 5.08-fold increase in the abundance of *A. muciniphila* when there is a higher proportion of Firmicutes.

These findings closely parallel those observed in the context of IBS. Consequently, a potential correlation was investigated using a chi-square test, yielding a statistically significant result (p-value 0.008). Figure 16 illustrates a mosaic graph wherein a clear relationship is discerned: patients with diarrhoea exhibit a higher proportion of Bacteroidetes, patients with constipation show a higher prevalence of Firmicutes, and patients with mixed behaviour exhibit patterns akin to those with diarrhoea, coupled with a greater abundance of Bacteroidetes.

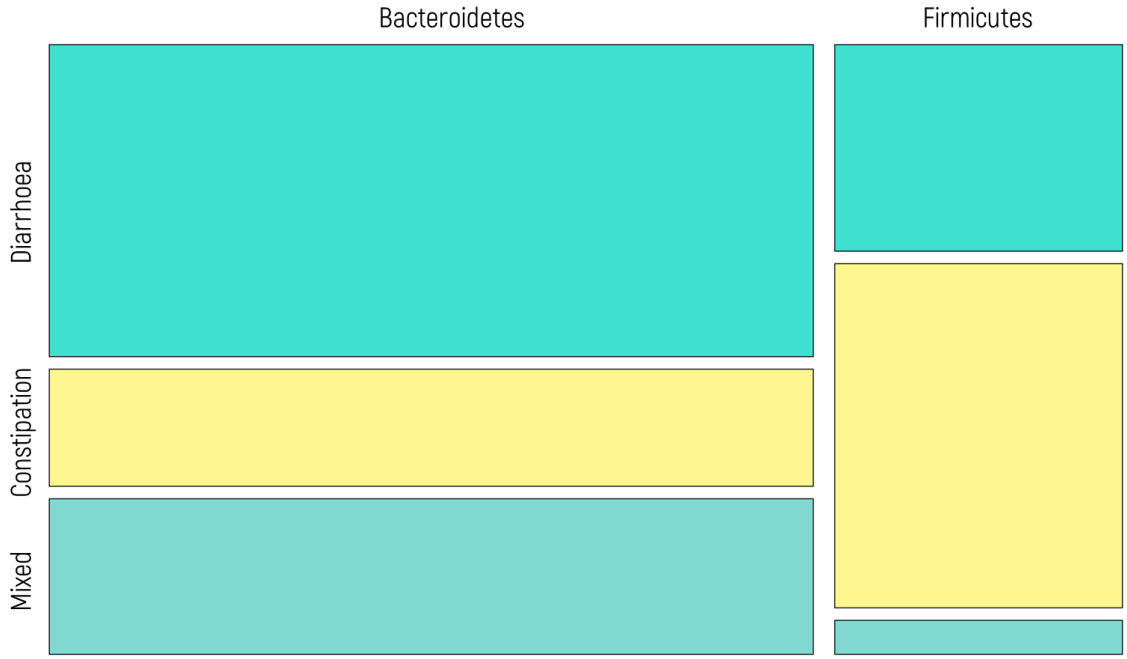

Figure 16. Mosaic graph depicting the dynamic shifts in Bacteroidetes and Firmicutes proportions among individuals with different manifestations of irritable bowel syndrome.

3.6 Dysbiosis index

The dysbiosis index was categorized into three groups: "healthy" when the value of the index is greater than 0.66 (n=109), representing values within the healthy range, "temporary" (n=17), indicating a slightly decreased index, values between 0.66 and -0.34; and "pathological" (n=5), denoting values well below the healthy range (< -0.34). Significant differences were observed in

the MANOVA analysis when comparing the abundance of microbial markers to the dysbiosis index (p-value <0.001).

Our findings demonstrate that a healthy dysbiosis index corresponds to minimal variation in the microbial markers panel (Figure 17). In cases of temporary dysbiosis, the variation increases, particularly with a notable rise in some microbial markers. Conversely, pathological dysbiosis is characterized by evident imbalances, displaying increased and decreased abundances of microbial markers, notably including decreases in beneficial microbial markers and increases in potential pathogenic species.

Further analysis using ANOVA revealed significant values for specific markers, namely *Clostridium* group I (p-value 0.007), gamma-proteobacteria (p-value <0.001), *E. coli* (p-value <0.001), and *F. prausnitzii* (p-value <0.001).

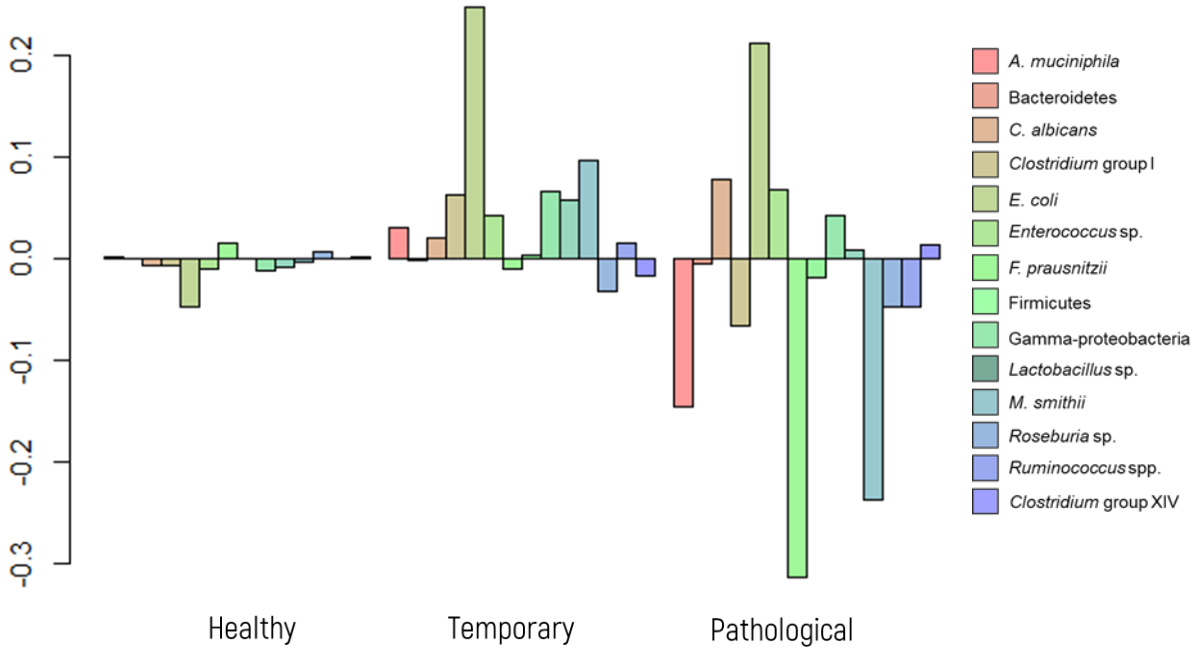

Figure 17. Geometric mean (differences between group mean and the average mean, Y axis) for the abundance of each microbial marker according to dysbiosis index classified into healthy, temporary, and pathological dysbiosis.

4 Discussion

In this study, we aimed to evaluate the clinical utility of the novel stool microbial test, TestUrGut®, which incorporates a set of 15 microbial markers representing critical functions of the gut microbiota. Additionally, two indexes are derived: a dysbiosis index derived from the abundance of *Faecalibacterium prausnitzii* and *Escherichia coli* was introduced as a quantitative measure to assess microbial imbalances in the gut, and the Firmicutes/Bacteroidetes index, which has been related to the type of diet.

Our cohort comprised 154 patients who sought medical consultation for digestive discomfort and underwent the faecal microbiota test TestUrGut®. We categorized the participants based on sex, age, and BMI to explore potential associations with microbial marker abundance and gut dysbiosis. A summary of the most significant findings from this study is found in Table 6.

Table 6. Summary of the most important results of this study.

Condition		**Finding**
Sex		*A. muciniphila* 2.54 times more abundant in women compared to men.
Age		No significant differences.
Body mass index		*M. smithii* and *A. muciniphila* decrease in abundance in underweight individuals, whereas *A. muciniphila* increases in obese individuals.
IBS behaviour	Diarrhoea	The abundance of *A. muciniphila* decreases 4.54 times. Related to a greater proportion of Bacteroidetes.
	Constipation	The abundance of *A. muciniphila* increased by 4.54 times, while that of *M. smithii* increased by 5.659 times. Related to a greater proportion of Firmicutes.
	Mixed	The abundance of *M. smithii* decreases 3.30 times. Related to a greater proportion of Bacteroidetes.
Dysbiosis index	Temporary	Increased abundance of some microbial markers.
	Pathological	Increased abundance of potentially pathogenic microbial markers along with decreased beneficial markers.

Significant associations were observed between sex and the *A. muciniphila* marker, with women displaying notably higher abundance than men. *A. muciniphila* belongs to the gram-negative bacteria within the Verrucomicrobia phylum and is characterized as a strict anaerobe capable of producing mucin-degrading enzymes. This bacterium utilizes mucins as a nitrogen and carbon source within the mucus layer of the epithelium. During mucin fermentation, *A. muciniphila* decomposes these substances into acetic and propionic acid and releases sulphate (174).The enrichment degree of *A. muciniphila* has been considered an indicator to evaluate body metabolic status, encompassing parameters such as glucose homeostasis, serum lipids, and adipocyte distribution in humans (175). Given that human sexes exhibit differences in fat distribution, often

linked to variations in sex hormone levels (176,177), it is plausible that the sex-related discrepancies observed in this study concerning *A. muciniphila* abundance may be influenced by how men and women differentially store excess energy and variations in fat body percentages.

In contrast, age did not appear to influence the abundance of any specific microbial marker, suggesting that age-related factors may not play a significant role in gut microbial composition in our cohort, consistent with previous findings (19,20).

Regarding BMI, *M. smithii* showed the most significant association with weight status, contradicting previous studies that reported an increase in *M. smithii* in patients with anorexia nervosa (178,179). However, the dataset is limited in terms of BMI data which it may have affected the ability to draw definitive conclusions regarding these results.

Our analysis of patients diagnosed with IBS identified significant associations between microbial markers and specific bowel behaviours. Specifically, *A. muciniphila* displayed a decrease in abundance in individuals with diarrhoeal behaviour, whereas both *A. muciniphila* and *M. smithii* exhibited increased abundance in the presence of constipation. These findings align with prior research demonstrating that methane gas production by *M. smithii* is correlated with slowing down intestinal transit, consequently leading to constipation (180,181). Regarding *A. muciniphila*, Gobert *et al.* were the first to observe an increased abundance of this marker in patients with IBS and constipation (182). However, the precise relationship between *A. muciniphila* and chronic constipation remains uncertain (183). Some studies have proposed that *A. muciniphila* induces a depletion of faecal water content through the degradation of intestinal mucin, resulting in impaired intestinal mucosal barrier function (184). Conversely, others have reported that the increase in *A. muciniphila* may be associated with stool firmness, making it more prevalent in individuals with slow transit (185).

The analysis of the FIR/BAC index, distinguishing patients with a higher proportion of Firmicutes or Bacteroidetes, revealed associations with *M. smithii* and *A. muciniphila* that closely mirrored the behavioural patterns observed in the analysis of IBS. A higher proportion of Bacteroidetes significantly correlated with a diarrhoeal or mixed pattern, while a higher proportion of Firmicutes was associated with patients experiencing constipation. These findings align with previous research, such as Zhuang's study in 2018 (186), which reported increased abundance of Bacteroidetes in patients with diarrhoea compared to controls, and other studies linked a greater abundance of Firmicutes to patients with constipation (187,188).

The dysbiosis index demonstrated its efficacy as a reliable representative of the general dysbiotic state. Furthermore, this study successfully distinguished between temporary and pathological

dysbiosis for the first time. Our findings reveal that pathological dysbiosis exhibits more pronounced variations in abundance than temporary dysbiosis within the specified tolerance values. The congruence between these variations allows for meaningful clinical inferences to be drawn. Specifically, temporary dysbiosis is characterized by an increased abundance of potentially pathogenic markers, while pathological dysbiosis is associated with decreases in the abundance of beneficial markers together with increased abundance of potentially pathogenic markers.

This study has several limitations that should be considered. First and foremost, further exploration and validation of these results are imperative to enhance the robustness and generalizability of the findings, primarily by including a larger sample size. Additionally, it is crucial to consider various factors that have been shown to influence the composition of the intestinal microbiota, such as tobacco use, exercise habits, and medication intake. Moreover, it is essential to recognize that digestive symptoms are often interconnected with mental health conditions, such as stress, anxiety, or depression, through the intricate gut-brain axis. Therefore, it would be advantageous to investigate and analyse the potential effects of these psychological factors on gut dysbiosis and symptomatology. By addressing these limitations and incorporating comprehensive analyses of relevant factors, future studies can provide a more thorough understanding of the complex relationships between gut microbiota, external influences, and human health. This understanding, in turn, may contribute to developing more effective and tailored therapeutic approaches for individuals affected by dysbiosis-related disorders.

In conclusion, our study yields valuable insights into the interplay between gut microbial markers, bowel behaviours, and the dysbiosis and FIR/BAC indexes. In this study, our analyses elucidate the utility and representativeness of the selected microbial markers within the TestUrGut® stool microbial test. These markers emerge as robust indicators of the overall state of the microbiota, demonstrating varying abundance intricately linked to the clinical symptomatology observed in patients.

Furthermore, our validation process extends beyond statistical analyses to encompass the translation of results into clinically relevant conclusions, particularly regarding symptomatology and patterns observed in IBS. The results of the dysbiosis index reinforce the robustness of our findings with the identification of alterations in the index, along with its correlation to the maximum deviations from the reference values in the panel. This indicates that both the tolerance levels and the index exhibit consistency and clinical utility, thereby providing precise and reliable support for the interpretation and decision-making process. The TestUrGut® stool microbial test is a promising diagnostic tool for discerning gut dysbiosis-related conditions, as the dysbiosis index has been shown to be a good indicator of the status of the overall microbiota. The potential of

TestUrGut® extends beyond digestive diseases, encompassing a broad range of conditions previously associated with dysbiosis. Implementing the TestUrGut® test offers a potential avenue for quantifying the intestinal microbiota, advancing towards more targeted and effective interventions. However, it is crucial to further validate and refine the test through additional research, encompassing diverse cohorts and larger sample sizes, to establish its clinical validity and broader applicability effectively.

CHAPTER 2

A novel grape-derived prebiotic selectively enhances the abundance and metabolic activity of butyrate-producing bacteria in faecal samples.

1 Background

In recent years, intestinal health has been increasingly linked to reducing the risk of several chronic diseases. Intestinal health increases interest in using prebiotics as functional food ingredients to improve health (189). Prebiotics aim to stimulate the selective growth of the potentially health-promoting indigenous microorganisms, hence, modulating the composition of the natural ecosystem (140). Besides, dietary prebiotics have the potential advantage of not being susceptible to antibiotics (190). Many food oligosaccharides and polysaccharides, such as dietary fibre, fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), and other related carbohydrates, have been reported to show prebiotic properties (191).

Prebiotics may promote a therapeutic effect in some intestinal diseases through different mechanisms. Numerous studies have revealed that faecal microbiota has a different composition in IBD and IBS patients compared to healthy controls (192–195), which reveals an overall reduction in biodiversity, especially in the Firmicutes phylum. This phylogenetic group includes several butyrate-producing bacteria, notably *F. prausnitzii,* a dominant species in the healthy human gut microbiota (53,196–199). In IBD patients, differences were observed between active and nonactive stages of the disease and between inflamed and non-inflamed regions of the intestine (200). Prebiotic intake influences intestinal microbiota composition and alters its metabolic properties by increasing the production of SCFA. This increase may lower the pH of the colonic environment and, thus, inhibit the growth of potentially pathogenic microorganisms (201). Among SCFA, butyrate stands out, playing a trophic role as a nutrient for colonocytes and enhancing the repair of the injured gut epithelium in IBD. Besides, evidence shows that butyrate acts directly as an anti-inflammatory agent by inactivating the intracellular transcriptional factor NFκB pathway, consequently attenuating the synthesis of inflammatory cytokines (202).

A novel prebiotic product denominated "Previpect" is composed of grape by-products from winemaking, specifically originating from the white grapes class of *Vitis vinifera* L. This novel prebiotic is obtained by drying the residues of the refuse from grapefruit pressing, removing seeds and other plant particles and further grinding. Previpect® has a high insoluble fibre content, making it an excellent prebiotic candidate.

This study aimed to evaluate the prebiotic properties of Previpect® by assessing fermentation profiles such as intestinal microbiota and bacterial SCFA production in an *in vitro* fermentation system. This experiment used fresh faecal samples from control subjects and patients suffering from IBD and IBS as inoculum. Besides, Previpect® fermentability was compared with a variant of our prebiotic, which is produced following the same procedure as Previpect® but with red grape

skins (Red Previpect), and three commercial prebiotics: inulin, grape pectin, and grape seed extract (GSPE).

2 Experimental design

2.1 Prebiotic treatments

The new prebiotic originated from the white class of *Vitis vinifera* L. Red Previpect was produced using the same methodology, but with the red type of *Vitis vinifera* L. its performance was compared with the following commercial preparations of prebiotic: Inulin and Grape Seed Extract (GSPE) (The Hut Group, Cheshire, UK), and Grapefruit Pectin (Source Naturals, Santa Cruz, CA, USA).

2.2 Compositional analysis of Previpect®

The chemical properties of Previpect® are listed in Table 7. Moisture, ash, and total fat contents were determined by gravimetry. Protein content was determined by the Kjeldahl method volumetric assay (203). The total carbohydrate content was calculated by subtracting from 100 the sum of moisture, ash, proteins, total fat, and fibre. Calories were calculated according to regulation 1169/2011 (204). Dietary and insoluble fibres were determined by gravimetry and enzymatic methods, from which soluble fibre was calculated. Calcium, phosphorus, magnesium, and potassium were measured by Mass Spectrometry with Inductively Coupled Plasma (ICP-MS). The analysis was performed at Laboratorio LINAS (Maçanet de la Selva, Spain).

Table 7. Chemical properties of Previpect®.

Main components	Average ± Standard deviation (n = 2)
Moisture (%)	9.80 ± 0.57
Ash (%)	4.54 ± 0.42
Protein (%)	6.29 ± 0.70
Total carbohydrate (%)	46.25 ± 6.01
Total fat (%)	3.70 ± 0.49
Dietary fibre (%)	30.30 ± 3.68
Soluble fibre (%)	2.90 ± 0.28
Insoluble fibre (%)	27.40 ± 3.39
Calories (Kcal/100g)	304 ± 9.90
Calcium (mg/Kg)	1913.5 ± 354.26
Phosphorus (mg/Kg)	1745 ± 247.49
Magnesium (mg/Kg)	623 ± 31.11
Potassium (mg/Kg)	15270 ± 2390

2.3 *In vitro* human digestion

Previpect® is intended to be administered using gastro-resistant capsules. In previous studies it was observed that i*n vitro* digested Previpect® lost much of its ability to stimulate the growth of butyrate-producing bacteria when compared with the values obtained with the undigested Previpect®, obtaining, in most cases a result similar to the negative control (Table 8). For this reason, all substrates, except Previpect®, were digested *in vitro* under appropriate conditions before being added into the faecal slurry and following the procedures described by Maccaferri et al. (205). In this experiment, digestion was not monitored by ion-exchange chromatography.

Table 8. Mean Ct abundances (n = 2) of bacterial markers (*S. variabile* (B46), *F. prausnitzii* (FAE), and its phylogroup I (PHGI) and phylogroup II (PHGII), *Roseburia* spp. (ROS) and *A. muciniphila* (AKK)).

Condition	B46	FAE	PHGI	PHGII	ROS	AKK
Negative control	20.26	16.04	15.94	20.06	17.17	17.01
Undigested Previpect®	18.37	13.72	12.96	18.91	15.09	17.36
Digested Previpect®	20.15	16.23	15.60	20.03	17.79	16.44

2.4 Study design

In this proof of concept, fifteen fresh faeces were collected at Hospital Doctor Josep Trueta (Girona, Spain), 9 of which were from patients with intestinal disorders (6 IBD and 3 IBS) and six from control subjects (CS) (Table 9). Control subjects were individuals without food intolerances, inflammatory bowel diseases, intestinal syndromes, neoplasms, and clinical symptomatology. IBD patients presented clinical activity according to the Partial Mayo score (206) for Ulcerative Colitis (UC, N=3) and according to the Harvey-Bradshaw index (207) for Crohn's Disease (CD, N=3), respectively. IBS patients were diagnosed according to Rome IV criteria. Participants followed regular diets and had not been treated with antibiotics, prebiotics, and/or probiotics for at least one month. Volunteers with severe comorbidities, pregnancy, previous surgeries that compromised the digestive system, or those who had received chemotherapy or radiation therapy in the past six months were excluded.

Table 9. Population characteristics.

	n	Age (Mean, range)	Gender, Female (%)	Clinical index
UC	3	59 (41-71)	100%	≥ 5†
CD	3	50 (37-61)	100%	≥ 5‡
IBS	3	45 (33-57)	33%	-
CS	6	34 (29-39)	33%	-

† Partial Mayo Score
‡ Harvey-Bradshaw index

2.5 Faecal slurry preparation and incubation

The samples were collected in sterile containers and kept at room temperature for less than 4 hours. Faecal samples were diluted 1:5 (w:v) with fermentation buffer (0.1 M KH_2PO_4, 0.05 mM NaOH; pH 7.0) (208) in a sterile plastic bag. The bags were carefully manually squeezed to mix the content.

All prebiotics (200 mg) were weighed in triplicates inside 20 ml screw-cap tubes with 10 ml of fermentation buffer previously degassed by increasing the temperature to 100 °C for 10 minutes. Faecal slurry (10 ml) was added to each tube up to a final concentration of 10% of the received stool sample. Tubes were tightly sealed and incubated at 37 °C under gentle agitation (120 rpm) for 72 hours. A blank without any fibre was used to control the *in vitro* fermentation process.

2.6 DNA extraction and bacteria quantification by real-time PCR

Once the incubation had finished, one aliquot from each fermentation triplicate was separated for DNA extraction. The DNA extraction performed is extensively detailed in the Material and Methods section.

The abundance of 6 microbial markers was analysed by qPCR: *Faecalibacterium prausnitzii* (FAE) and their two phylogroups (PHGI, PHGII), *Akkermansia muciniphila* (AKK), Roseburia (ROS), and B46 (best BLAST match *Subdolinogranulum variabile*).

All samples were amplified in duplicate and considered valid when the difference between threshold cycles (Ct) was less than 0.6. A non-template control reaction was included in each qPCR run.

2.7 Short-chain fatty acids

After separating the DNA extraction aliquots, the tubes were centrifuged for 30 minutes at 4500 x g at 4°C. The supernatants were transferred into new tubes and centrifuged at 4500 x g and 4°C for 15 minutes.

Supernatants from faecal incubations were sterilised by filtration using a pore size of 0.22 µm Ø. Acetate, propionate, and butyrate were analysed using a gas chromatograph (Agilent 7890A GC system, Agilent Technologies, Santa Clara, California, USA) equipped with a fuse-silica capillary column (DB-FFAP, 30 m x 0.32 mm x 0.5 µm) and a flame ionisation detector. Volatile fatty acids determination analyses were performed on an Agilent 7890A gas chromatograph equipped with a DB-FFAP capillary column 30mx0.32mmx0.50µm and a flame ionization detector, conducted at LEQUIA (Girona, Spain).

2.8 Statistical analysis

As stated before, all the experiments were conducted in triplicate. Goodness-of-fit of the bacterial population and SCFA data to normal distribution was tested using the Shapiro-Wilk W test. Due to the non-normal distribution, the Mann-Whitney U test was performed to compare the measurements between two treatments using SPSS (version 16.0, Chicago, United States). MANOVA (Wilks' Lambda test) was performed with RStudio (version 3.5.0, Boston, United States) after data transformation using CoDaPack (version 2.02.21, Girona, Spain). A p-value < 0.050 was considered statistically significant.

3 Results

3.1 Bacterial biomarkers

The effect on bacterial populations after the *in vitro* fermentation process in faecal samples supplemented with Previpect® and the other prebiotics was determined using qPCR (Table 10).

Several significant differences were found when the abundance of the different analysed bacterial markers was compared in all the tested fibres; the Wilks test was significant (p-value < 0.001) for all treatments (Figure 18). These results show that Previpect® enabled the growth of all the analysed bacteria except AKK, whose abundance was decreased in all inoculums. ROS was the bacterial marker with the most prominent increment in its abundance because of Previpect® incubation, followed by both *F. prausnitzii* phylogroups and B46. Previpect® did not show any effect in samples from CD patients compared to the negative control.

Table 10. Mean Ct values and standard deviation of bacterial markers *F. prausnitzii* (FPRA), *A. muciniphila* (AKK), *Roseburia* spp. (ROS), and *S. variabile* (B46), and *F. prausnitzii* phylogroup I (PHGI) and phylogroup II (PHGII), for control subjects (CS, N=6), irritable bowel syndrome (IBS, N=3), and Crohn's disease (CD, N=3) samples. Statistical comparisons between groups are indicated by superscript letters: different letters indicate statistically significant differences ($p < 0.05$) between groups.

Inoculum	Substrate	Fpra	AKK	ROS	B46	PHG I	PHG II
CS	Negative control	14.65 ± 1.21^{a}	21.73 ± 7.09^{a}	18.59 ± 1.94^{a}	19.01 ± 1.31^{a}	16.78 ± 1.50^{a}	17.33 ± 1.56^{a}
	Previpect	13.42 ± 0.59^{b}	21.25 ± 7.54^{a}	15.21 ± 2.44^{b}	18.31 ± 0.43^{a}	15.67 ± 1.46^{b}	16.42 ± 1.05^{a}
	Red Previpect	14.55 ± 1.36^{a}	21.16 ± 6.97^{a}	18.61 ± 2.09^{a}	18.93 ± 1.43^{a}	16.54 ± 1.48^{a}	17.33 ± 1.71^{a}
	Inulin	14.02 ± 1.24^{a}	19.70 ± 7.21^{a}	18.59 ± 3.21^{a}	18.63 ± 1.40^{a}	15.96 ± 1.51^{a}	16.87 ± 1.51^{a}
	Grape pectin	14.18 ± 1.36^{a}	20.27 ± 7.73^{a}	18.82 ± 3.16^{a}	18.86 ± 1.64^{a}	16.89 ± 2.36^{a}	17.25 ± 2.17^{a}
	GSPE	14.17 ± 1.21^{a}	21.34 ± 6.90^{a}	16.32 ± 1.01^{b}	18.61 ± 0.98^{a}	16.99 ± 2.33^{a}	16.66 ± 1.14^{a}
IBS	Negative control	16.44 ± 1.28^{abc}	14.21 ± 0.49^{abc}	18.93 ± 1.33^{a}	20.42 ± 0.76^{abc}	18.19 ± 0.87^{ab}	19.75 ± 1.58^{ab}
	Previpect	15.61 ± 1.19^{b}	14.44 ± 0.39^{b}	17.73 ± 1.49^{b}	20.06 ± 0.48^{b}	17.72 ± 0.82^{a}	18.97 ± 1.64^{a}
	Red Previpect	16.63 ± 0.83^{c}	14.09 ± 0.39^{a}	19.46 ± 0.99^{a}	20.68 ± 0.68^{c}	18.48 ± 0.53^{a}	19.87 ± 1.39^{a}
	Inulin	15.93 ± 1.27^{a}	14.24 ± 0.50^{a}	19.53 ± 0.45^{a}	20.28 ± 0.57^{a}	18.02 ± 1.02^{bc}	19.30 ± 1.76^{bc}
	Grape pectin	15.36 ± 1.23^{a}	14.26 ± 0.67^{a}	19.49 ± 1.09^{a}	19.92 ± 0.66^{a}	17.31 ± 0.92^{c}	18.64 ± 1.63^{c}
	GSPE	15.62 ± 1.42^{a}	13.80 ± 0.40^{c}	17.54 ± 1.72^{b}	19.72 ± 1.10^{a}	17.51 ± 1.16^{a}	18.98 ± 1.92^{a}
UC	Negative control	17.16 ± 0.90^{a}	21.83 ± 0.82^{a}	20.28 ± 10.03^{a}	21.42 ± 1.86^{a}	20.01 ± 1.07^{a}	20.40 ± 0.58^{a}
	Previpect	15.40 ± 2.11^{a}	24.87 ± 9.53^{a}	17.39 ± 1.76^{b}	21.81 ± 4.56^{a}	18.08 ± 1.88^{a}	21.01 ± 6.57^{a}
	Red Previpect	16.81 ± 0.97^{a}	23.75 ± 10.43^{a}	19.33 ± 1.70^{ab}	21.15 ± 1.30^{a}	19.59 ± 0.82^{a}	20.19 ± 1.86^{a}
	Inulin	16.40 ± 1.54^{a}	23.86 ± 10.40^{a}	20.32 ± 1.96^{a}	20.57 ± 1.89^{a}	18.61 ± 1.66^{ac}	19.63 ± 2.31^{a}
	Grape pectin	16.04 ± 1.48^{a}	24.92 ± 9.84^{a}	19.76 ± 1.94^{a}	20.44 ± 1.85^{a}	17.89 ± 1.58^{bc}	18.94 ± 2.29^{a}
	GSPE	16.47 ± 1.32^{a}	23.71 ± 10.54^{a}	19.68 ± 3.07^{ab}	20.78 ± 1.21^{a}	19.10 ± 0.96^{ac}	19.63 ± 1.89^{a}
CD	Negative control	15.70 ± 3.21^{a}	14.44 ± 2.35^{ab}	17.25 ± 1.94^{ab}	20.22 ± 3.08^{abc}	19.38 ± 3.13^{a}	20.31 ± 7.36^{a}
	Previpect	14.34 ± 2.69^{a}	16.41 ± 3.34^{a}	16.05 ± 1.43^{a}	18.86 ± 2.17^{a}	18.14 ± 3.58^{a}	19.07 ± 6.59^{a}
	Red Previpect	14.61 ± 1.45^{a}	14.10 ± 2.12^{b}	18.07 ± 1.30^{b}	19.01 ± 1.12^{b}	17.65 ± 2.44^{a}	19.14 ± 5.21^{a}
	Inulin	14.81 ± 1.06^{a}	14.32 ± 2.62^{ab}	19.21 ± 1.81^{bc}	19.21 ± 0.88^{b}	18.74 ± 3.92^{a}	19.26 ± 4.14^{a}
	Grape pectin	15.90 ± 1.27^{a}	14.68 ± 2.77^{ab}	20.13 ± 1.22^{c}	20.45 ± 1.04^{c}	19.73 ± 2.56^{a}	20.70 ± 4.83^{a}
	GSPE	16.08 ± 3.83^{a}	15.30 ± 4.27^{ab}	17.45 ± 2.23^{b}	20.37 ± 3.21^{a}	19.41 ± 3.29^{a}	20.92 ± 8.37^{a}

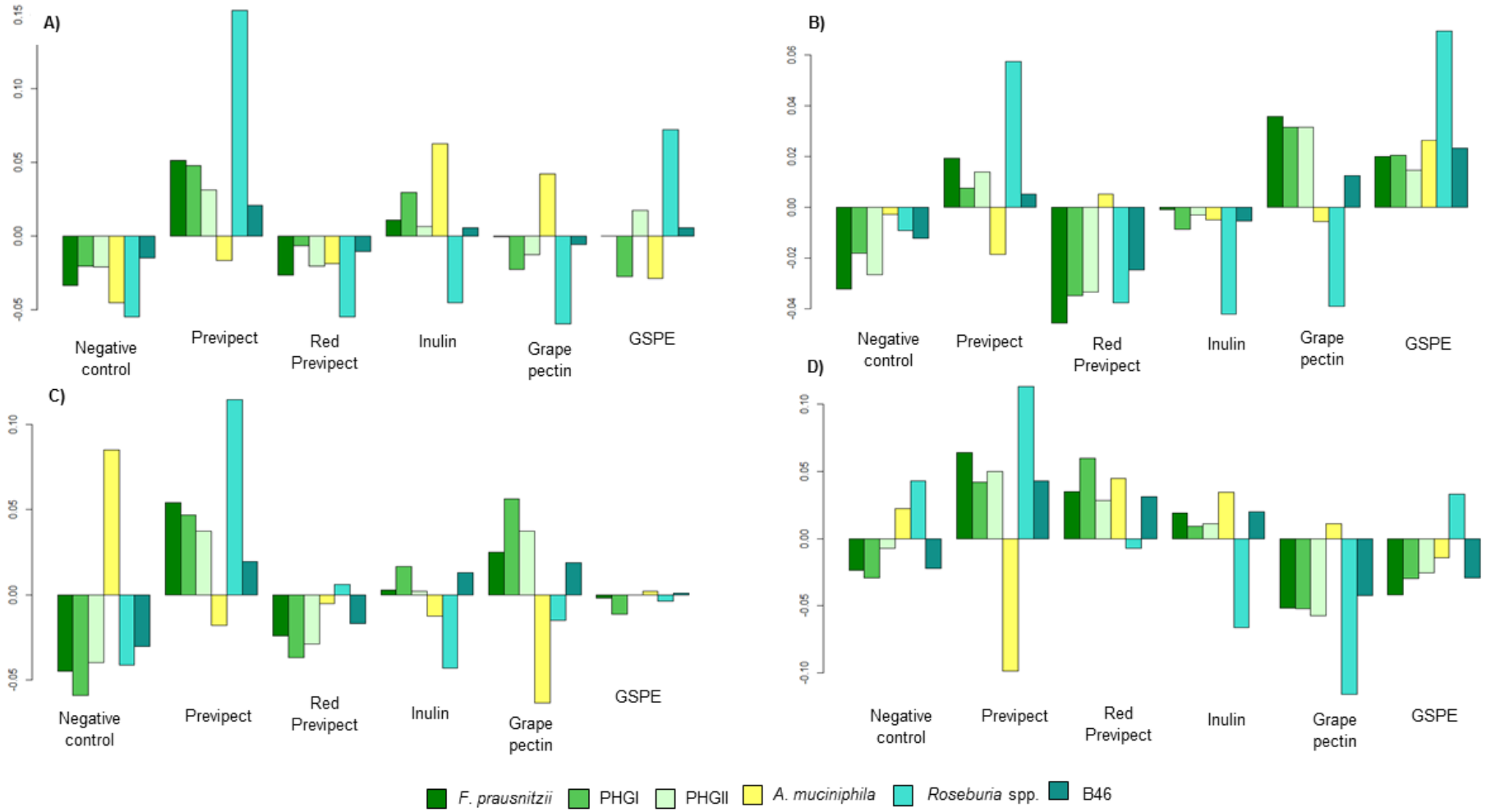

Figure 18. Geometric mean (differences between group mean and the average mean, Y axis) for each of the four population types: (A) control subjects, (B) irritable bowel syndrome, (C) ulcerative colitis, and (D) Crohn's disease; compared with the overall mean of each substrate and bacterial marker.

Furthermore, in samples from CS, Previpect® had a significant impact by boosting the abundance of FAE and its phylogroup I (p-value=0.001 and p-value=0.019, respectively). Although the repercussion caused by fibres in the analysed bacteria changed according to the condition of the sample donor, Previpect® demonstrated stability in all of them.

Previpect® induced a higher increment in the abundance of the studied bacterial markers than red Previpect® regarding ROS in CS (p-value=0.003), CD (p-value=0.019), and IBS samples (p-value=0.015), as with FPRA in CS and B46 in IBS patients (p-value=0.007 and p-value=0.031, respectively). Previpect® also showed significantly higher efficiency than inulin in stimulating the growth of ROS in all samples. Previpect® showed similar results as inulin on the ability to increase the abundance of ROS. Grape pectin did not show significant differences from Previpect® in any inoculum except for UC samples. Concerning GSPE, no differences were found in CD samples compared to Previpect®, although it was significantly better in increasing AKK abundance in IBS samples (p-value=0.012). However, Previpect® significantly augmented FAE abundance in CS (p-value=0.032).

3.2 Short-chain fatty acids

The fermentation of faecal slurry was carried out under standard conditions and led to the formation of the SCFAs acetate, propionate, and butyrate. Total SCFA, the sum of acetate, propionate, and butyrate, indicates fibre fermentability (209). Concentrations of SCFA after the *in vitro* fermentation experiment are presented in Table 11. The prebiotic fermentation cultures contained significantly higher concentrations of SCFA than the blank for all substrate conditions and inocula ($p < 0.001$). Previpect® produced the highest concentration of total SCFA in all inoculums from its fermentation, becoming the most suitable substrate for SCFA production. The lowest total SCFA yield was obtained from GSPE fibre, except for UC inoculum, where the lowest total SCFA was produced from Red Previpect®.

Acetate was the most abundant SCFA derived from Previpect® fermentation (Figure 19), which increased between 205.85-277.38% the amount produced by the blank, followed by butyrate (136.26-238.94% increase), and finally, propionate, whose yielding was comprised between 109.69-230.00% among inoculums. Total SCFA concentrations were increased 1.8-fold with respect to those of negative control in both UC and CD samples mean (p-value=0.001 and p-value<0.001 respectively), reaching a 2.3-fold increase in IBS and the highest increase in CS, being 2.5-fold (p-value=0.001).

Table 11. Butyrate, acetate, propionate, and total short chain fatty acids (SCFA) concentration (mg/L) after *in vitro* fermentation of Previpect®, red Previpect, inulin, grape pectin, and grape seed extract (GSPE) using faeces from control subject (n = 6), irritable bowel syndrome (n = 3), and Crohn's disease patients (n = 3) as inocula.

	Control subjects inoculum				Irritable bowel syndrome inoculum			
Condition	**Butyrate (mg/L)**	**Acetate (mg/L)**	**Propionate (mg/L)**	**Total SCFA (mg/L)**	**Butyrate (mg/L)**	**Acetate (mg/L)**	**Propionate (mg/L)**	**Total SCFA (mg/L)**
Negative control	308.84 ± 113.32***	573.13 ± 131.54***	291.56 ± 135.91***	424.41 ± 333.62***	293.33 ± 193.07***	648.05 ± 353.88***	260.05 ± 241.08***	1201.43 ± 699.48***
Previpect	1046.76 ± 405.03	2162.87 ± 648.14	852.65 ± 294.74	4062.28 ± 1147.52	767.75 ± 301.21	2376.85 ± 198.45	858.16 ± 50.08	4002.76 ± 497.45
Red Previpect	363.51 ± 165.90***	771.98 ± 159.42***	372.74 ± 167.04***	1508.23 ± 400.89***	385.26 ± 172.43*	1058.91 ± 568.44***	407.45 ± 158.61***	1851.61 ± 814.63***
Inulin	758.78 ± 329.93*	1399.43 ± 500.93***	609.49 ± 275.89**	2767.70 ± 1020.29***	611.29 ± 261.72	1585.68 ± 528.91***	637.40 ± 264.57	2834.37 ± 981.67**
Grape pectin	823.34 ± 426.60	1840.93 ± 663.95	736.47 ± 322.46	3400.74 ± 1222.30*	686.42 ± 255.15	2230.85 ± 305.34	818.42 ± 149.81	3735.69 ± 426.41
GSPE	358.20 ± 236.40***	696.37 ± 311.22***	337.19 ± 255.69***	1391.75 ± 787.56***	328.10 ± 159.95*	1098.00 ± 517.74***	339.85 ± 174.56***	1765.94 ± 785.88***
	Ulcerative colitis inoculum				**Crohn's disease inoculum**			
Condition	**Butyrate (mg/L)**	**Acetate (mg/L)**	**Propionate (mg/L)**	**Total SCFA (mg/L)**	**Butyrate (mg/L)**	**Acetate (mg/L)**	**Propionate (mg/L)**	**Total SCFA (mg/L)**
Negative control	321.30 ± 72.58***	581.18 ± 109.64***	359.57 ± 44.54***	1262.05 ± 196.05***	409.56 ± 161.82***	672.40 ± 384.30***	410.17 ± 126.48***	1492.13 ± 663.08***
Previpect	759.11 ± 130.34	1777.52 ± 322.23	1072.37 ± 371.19	3609.00 ± 724.51	1277.54 ± 442.09	2119.07 ± 451.17	860.10 ± 209.95	4256.71 ± 995.58
Red Previpect	354.44 ± 135.35***	725.15 ± 194.70***	401.67 ± 88.63***	1481.26 ± 398.82***	564.43 ± 189.43**	1000.37 ± 326.71***	550.92 ± 128.02**	2115.72 ± 622.36***
Inulin	479.00 ± 124.67**	1233.69 ± 448.17*	773.09 ± 370.06	2485.78 ± 924.24*	769.58 ± 334.83*	1168.70 ± 540.70**	630.37 ± 208.31*	2568.65 ± 1069.66**
Grape pectin	556.51 ± 151.98*	1465.65 ± 286.63*	709.42 ± 122.62*	2731.58 ± 495.27*	803.03 ± 389.87	1644.72 ± 566.68	752.09 ± 169.01	3199.83 ± 1078.88
GSPE	392.75 ± 75.89***	809.92 ± 221.67***	426.75 ± 153.07***	1629.42 ± 426.89***	404.82 ± 168.37***	742.60 ± 300.13***	365.31 ± 132.14***	1512.73 ± ***

Significant differences between fibres and Previpect are shown as *p-value < 0.05, **p-value ≤ 0.01, ***p-value ≤ 0.001.

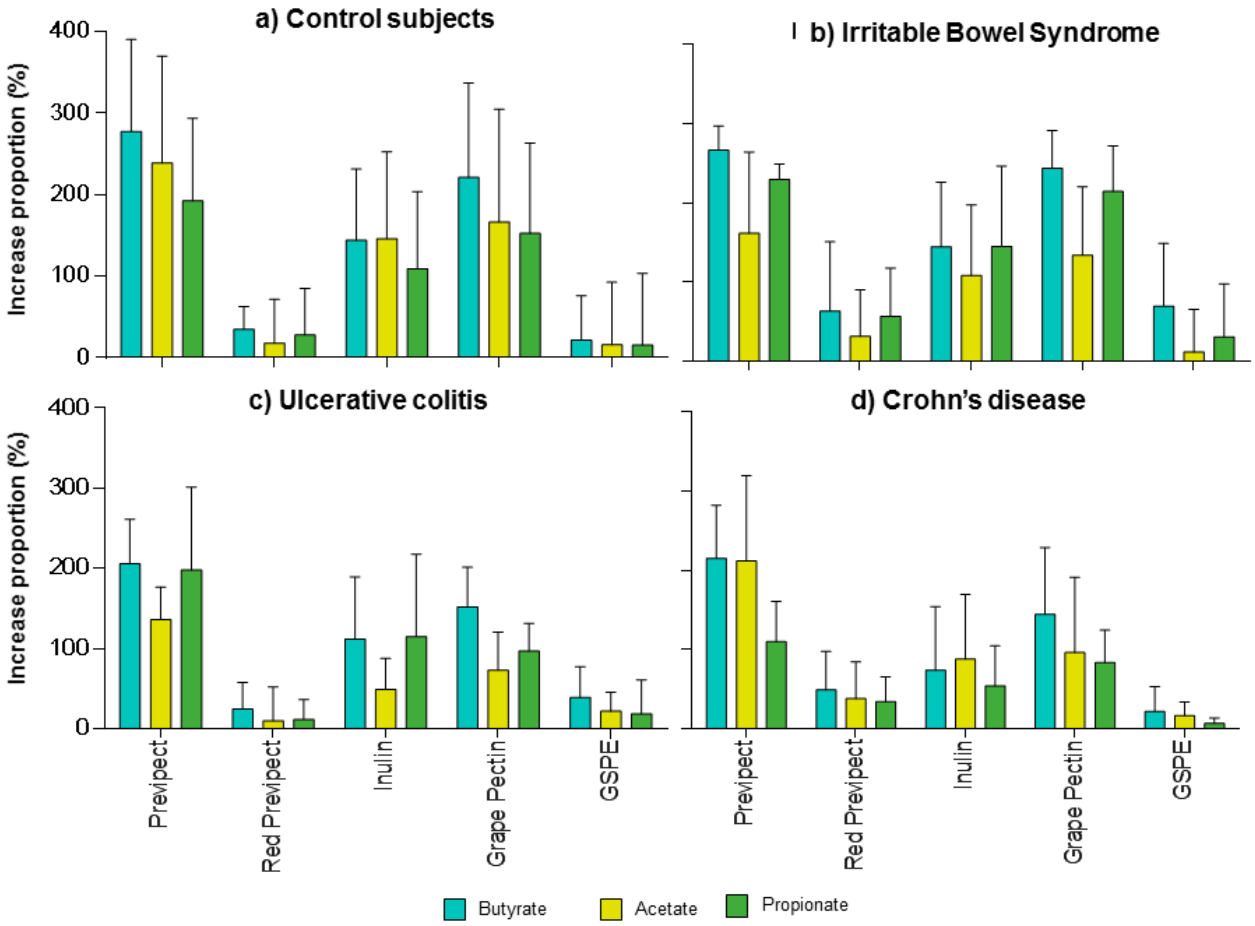

Figure 19. Mean and standard errors of the increase proportions (%) of the short-chain fatty acids: acetate (blue), butyrate (yellow), and propionate (green) regarding negative control of the process produced during the in vitro fermentation for Previpect®, red Previpect, inulin, grape pectin, and GSPE fibres using faecal inocula from (A) control subjects, (B) irritable bowel syndrome, (C) ulcerative colitis, and (D) Crohn's disease patients

No significant differences were observed in the production of SCFA when Previpect® was compared to grape pectin in CS, IBS, and CD samples. Nevertheless, Previpect® showed higher SCFA production than grape pectin in UC samples. Besides, Previpect® performed significantly better than inulin in CS and CD samples but showed no significant differences in propionate concentration in UC samples. Previpect® was also superior, enhancing acetate and butyrate production. In IBS inoculum, inulin showed no significant differences concerning butyrate and propionate. However, Previpect® was better at inducing acetate production. Previpect® fermentability resulted in significantly higher levels of SCFA than red Previpect® and GSPE substrates.

4 Discussion

Annually the processing of grapes (*Vitis vinifera* L.) for wine production leaves behind 14.5 million tons of grape by-products from wineries or "grape pomace" result in Europe. This pomace mainly consists of fruit skins and seeds, resulting from pressing the fruit (210). The skins of grapes are known to be rich sources of phenolic compounds (211). Grape skins represent about 5-10% of the total dry weight of the grape and are generally treated as a waste product, despite containing an array of flavonoids, polyphenols, and anthocyanins. These molecules have been shown to produce health benefits associated with antioxidant, cardioprotective, hepatoprotective, anticarcinogenic, and antidiabetic effects, among others (212). Grape skins also contain considerable amounts of potential prebiotic indigestible carbohydrates made up of 30% neutral polysaccharides (cellulose, xyloglucan, arabinan, galactan, xylan, and mannan), 20% of acidic pectin substances (62% of which are methyl esterified), ~15% insoluble proanthocyanidins, and <5% structural proteins (211). In addition to all the features mentioned above, this study has shown that Previpect® can also enhance the growth or metabolic activity of some beneficial bacterial species of the gut microbiota.

A summary of the most significant findings from this study is found in Table 12.

Table 12. Summary of the most important results of this study.

Condition		Previpect effect
Control subjects	Bacterial markers	Significant increase of *F. prausnitzii*, *F. prausnitzii* phylogroup I and *Roseburia* sp.
	Short-chain fatty acids	2.5-fold increase
Irritable bowel syndrome	Bacterial markers	Significant increase of *Roseburia* sp.
	Short-chain fatty acids	2.3-fold increase
Ulcerative colitis	Bacterial markers	Significant increase of *Roseburia* sp.
	Short-chain fatty acids	1.8-fold increase
Crohn's disease	Bacterial markers	No significant change of any bacterial marker.
	Short-chain fatty acids	1.8-fold increase

The analysed species were chosen because of the importance of their function in large bowel health. *F. prausnitzii* (together with its two phylogroups) is one of the leading components of the microbiota and the most recognisable butyrate-producing bacteria in the human colon (213); *S. variable* is a butyrate producer closely related to *F. prausnitzii* (214); *Roseburia* spp. comprises different butyrate-producing bacteria, which produce propionate, such as *R. inulinovorans*; and *A. muciniphila* is an acetate-producing bacteria (215). The results of this study demonstrate the great fermentative ability of Previpect®, enabling the growth of specially *Roseburia* spp., but also that of *F. prausnitzii*, its two phylogroups and *S. variabile* in all inocula. This fermentative potential has been reflected in a considerable increase in the three SCFA analysed (acetic, propionic, and butyric acid), produced not only by the quantified markers but also by all the SCFA-producing bacteria found in the intestinal microbiota of the analysed samples, increasing their concentration up to 2.5-fold. Acetic, propionic, and butyric acids are essential microbial fermentation products that benefit human health. Acetate is the most prominent SCFA and substrate for butyrate production reported to affect lipid metabolism, such as lipogenesis and cholesterogenesis (216). Propionic acid regulates glucose homeostasis in the liver (217). Lower faecal acetate and propionate concentrations have been observed in UC samples (218). Butyric acid plays a crucial role in maintaining human gut health, as it is the primary energy source for colonocytes (219), a regulator of gene expression, immune cell growth, and apoptosis in host cells (220), and protects against colitis and colonic cancer (221). Therefore, butyrate prevents mucosal atrophy by improving the mucosal barrier function and exhibits immunomodulatory effects and anti-inflammatory properties. Finally, butyrate strongly impacts IBD by improving the mucosal layer and inhibiting inflammation (222). Observations described in all these studies suggest that Previpect® may enhance the eubiotic state of the human gut microbiome.

As for IBS patients, since pathogenesis still is a matter of scientific debate, treatment focuses on relieving symptoms such as bloating, abdominal pain, diarrhoea, and constipation. The fact that the passive absorption of water in the colon depends on the presence of SCFAs may explain the potential role of butyrate in clinical conditions involving diarrhoea due to propionate decreasing colon motility (223). The relief of abdominal pain is an essential aspect of IBS treatment. Butyrate has a probable beneficial influence on the hypersensitivity of intestinal receptors, which decreases intraintestinal pressure by improving bowel peristalsis and retractability of the circular muscle layer (215,224,225).

Several investigations have shown that certain butyrate-producing firmicutes bacteria are reduced in IBD. In particular, the numbers of *F. prausnitzii* in faecal and gut mucosa samples are reduced in CD and UC (50,160,192,226). *Roseburia* spp. and *A. muciniphila* are also depleted in IBD mucosa and faecal samples from UC patients (227).

Inulin is a natural component in several foods, such as leek, asparagus, chicory, Jerusalem artichoke, garlic, artichoke, onion, wheat, banana, and oats (228). The prebiotic activity of inulin-type fructans has been extensively confirmed. These prebiotics target microorganisms like Bifidobacteria, which significantly increase in number after ingestion (229,230), and are the current market leaders. Concerning *F. prausnitzii* strains, they have not demonstrated the ability to ferment inulin (52) whereas Previpect® has proved it.

Pectin is a soluble dietary fibre and exerts physiological effects on the gastrointestinal tract, such as reducing glucose absorption, enhancing hypocholesterolaemia, and delaying gastric emptying. Pectin is found in sugar beet pulp, peach peels, pulps of grapes and pumpkin or apples, which has also demonstrated the ability to stimulate the growth of Bifidobacterium (231,232). Interestingly, most *F. prausnitzii* strains grow on apple pectin, although not on citrus pectin (233), revealing that not all pectins serve to stimulate their growth. This study shows that *F. prausnitzii* grows with grape pectin and that similar prebiotic effects are obtained compared to Previpect® since no significant differences were observed.

Previous studies have revealed several health-beneficial effects of wine grape seed flour or extract (GSF or GSPE), a by-product of winemaking, such as hypolipidemic and anti-obesity properties attributed to high contents of flavonoids (234). Health beneficial effects of GSPE are closely associated with modulation of the intestinal microbiota, mainly producing a prebiotic effect on *Akkermansia* sp. (235), confirmed by our results and unlike Previpect®, in which *A. muciniphila* was the only microorganism analysed presenting a significant decrease in the inoculum.

Since flavonoids are more abundant in red than white grapes, the effect of the Previpect® was compared with a Previpect® made from the red variety of grapes to see if its effect was distinct

and dependent on flavonoids (235). Thus, the ability exhibited by Previpect® goes beyond the flavonoids since it presented a higher capacity to increase the abundance of beneficial anaerobic bacteria species and to enhance the production of SCFA than Red Previpect®.

Despite these promising results, we acknowledge that a complete examination in larger cohorts is essential before commercial application and clinical studies *in vivo*.

In conclusion, Previpect® supplementation seems to be a promising tool for IBD and IBS treatment strategies, but also for CS to sustain good gut health and as a preventive measure for temporary dysbiosis.

CHAPTER 3

Increased abundance of gut microbiota with enzymatic activity in patients with pancreatic exocrine insufficiency.

1 Background

Chronic pancreatitis (CP) is a persistent inflammatory process of the pancreas that leads to fibrosis and loss of exocrine and endocrine parenchyma, causing atrophy (236). This syndrome is commonly characterised by clinical features such as abdominal pain, exocrine and endocrine insufficiency, and secondary pancreatic cancer, among other complications (237). Pancreatic exocrine insufficiency (PEI) is a frequent cause of maldigestion and a significant complication in chronic pancreatitis (238). In PEI, the exocrine pancreas secretes lesser pancreatic enzymes (such as lipases, amylases, or proteases) than the average levels (239). Deficiencies in amylase and proteases are not of significant clinical concern because other nonpancreatic sources of enzymes (such as salivary, gastric, and small intestinal enzymes) can generally compensate for these deficiencies (239). However, pancreatic lipase insufficiency is worrisome, as its synthesis and secretion are impaired earlier than other pancreatic enzymes (239). It is easily destroyed by gastric acid and luminal proteases, leaving gastric lipase as the only source of compensation (240,241). Thus, lipase insufficiency is the most concerning aspect of PEI.

Pancreatic enzyme replacement therapy (PERT) is the standard treatment for malabsorption resulting from PEI (242). This therapy aims to restore luminal enzymatic activity's quantity, composition, and availability in cases where pancreatic exocrine function is diminished. By correcting malabsorption, PERT facilitates nutritional improvement and body weight gain (243).

The gut microbiota plays a significant role in human physiology, affecting metabolism, modulating the immune system of the intestinal mucosa, producing vitamins, facilitating digestion, and influencing intestinal architecture (92). Dysbiosis, an imbalance in the gut microbiota, is associated with various digestive diseases, particularly inflammatory bowel diseases (244). Recent studies have shown that the exocrine pancreas is relevant in regulating gut microbiota composition in healthy individuals without pancreatic disease (93). Altered microbiomes have been found to have specific causal roles, and normalisation of the microbiome could have therapeutic applications (245). Certain groups of microorganisms possess enzymatic activities (246–248), so it is hypothesised that nutrient maldigestion in PEI, resulting from pancreatic enzymatic insufficiency, could lead to specific dysbiosis of the gut microbiota. This dysbiosis might eventually contribute to the metabolic abnormalities associated with PEI.

Based on these premises, the current study aimed to evaluate whether changes in the intestinal microbiota occur and to explore their associations with metabolic abnormalities in patients with PEI resulting from CP.

2 Experimental design

2.1 Study population

A series comprising 17 patients was included in this study and classified into two groups: (1) healthy subjects without risk factors for chronic pancreatitis, such as smoking and alcohol consumption, and (2) patients with CP who developed PEI without receiving any treatment (Table 13). All subjects were recruited at the Hospital Dr. Josep Trueta in Girona, Spain, following the clinical flow outlined in Figure 20. For patients with CP to be included, they had to have faecal elastase values <15 µg/g or between 15-200 µg/g and a breath test with C_{13}-triglycerides <29%.

Table 13. Patients' characteristics included in the study.

Characteristics	CP with PEI	Healthy subjects
n (%)	11 (65%)	6 (35%)
Age (mean, range)	53 (34-71)	50 (34-67)
Gender, male (%)	8 (73%)	5 (83%)

The inclusion criteria for participation in the study were as follows: (1) subjects aged over 18 years, (2) diagnosed with CP, except for the healthy group (certified by a gastroenterologist), and (3) provided signed informed consent. The exclusion criteria were: (1) receiving antibiotic treatment in the month preceding the study inclusion, (2) having severe comorbidity according to clinical criteria, (3) having undergone previous surgeries that affect the transit of the digestive system, such as colon resection, and (4) being pregnant at the time of inclusion.

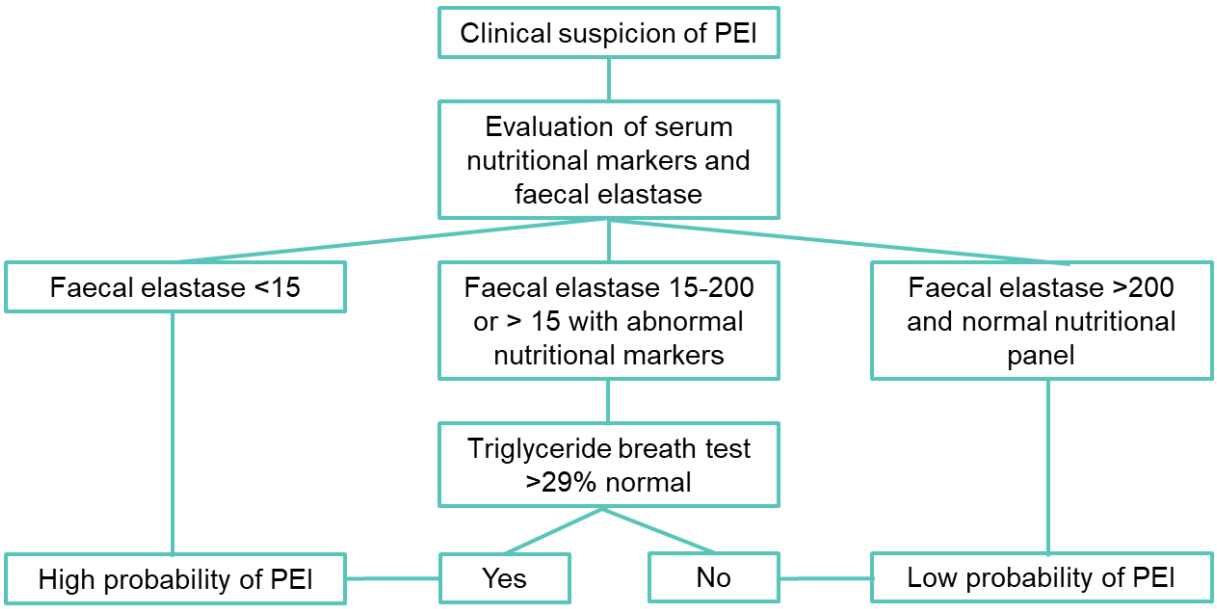

Figure 20. Algorithm followed to diagnose pancreatic exocrine insufficiency.

2.2 Faecal sample collection and DNA extraction

The sampling and DNA extraction performed is extensively detailed in the Material and Methods section.

2.3 qPCR analysis from stool samples

The abundance of 16 microbial markers representing the main phyla, groups, and genera present in gut microbiota was analysed by real-time quantitative Polymerase Chain Reaction (qPCR): Eubacteria (EUB), *Faecalibacterium prausnitzii* (FAE), *Akkermansia muciniphila* (AKK), *Escherichia coli* (ECO), Bacteroidetes (BAC), *Bifidobacterium* (BIF), *Clostridium* cluster I (CLO), Enterobacteriaceae (ENB), *Enterococcus* sp. (ENT), Gamma-proteobacteria (GAM), Firmicutes (FIR), *Lactobacillus* sp. (LAC), *Roseburia* sp. (ROS), *Ruminococcus* spp. (RUM), *Clostridium* cluster XIV (XIV), and *Candida albicans* (CAN).

2.4 Statistical analysis

Once the qPCR result in Ct units for each marker was obtained, they were transformed into relative and total abundance values to make the statistical analysis (as defined in Methodology section 4 Abundance calculation).

CoDaPack (version 2.02.21, Girona, Spain) was used for compositional data analysis and transformation. The statistical program RStudio v.4.0.4 (Boston, USA) was used to compare microbial abundances and clinical features between groups of patients, including the non-parametric tests Kruskal-Wallis and Mann-Whitney for groups comparisons and multivariate analysis (MANOVA). Significance levels were established for p-values ≤ 0.05.

2.5 Enzymatic indices definition

The microbial indices in this study were defined by grouping microbial taxa based on their putative enzymatic activities. As presented in Table 14, the markers were categorised into three distinct groups: (1) markers exhibiting all three pancreatic enzymatic activities (lipase, amylase, and protease), (2) markers with only amylase activity, and (3) markers lacking any enzymatic activity.

Table 14. The fifteen microbial markers classified according to their enzymatic activity.

With enzymatic activity		Without enzymatic activity
Amylase, protease, lipase	Amylase	
Lactobacillus sp. (LAC)(249–251)	*Roseburia* sp. (ROS)(246)	*F. prausnitzii* (FAE)
Gamma-proteobacteria (GAM)(248)	*Ruminococcus* spp. (RUM)(246)	*A. muciniphila* (AKK)
Enterobacteriaceae (ENB)(252)		*Clostridium* cluster I (CLO)
E. coli (ECO)(253)		*Clostridium* cluster XIV (XIV)
Bifidobacterium (BIF)(254)		
C. albicans (CAN)(247,255)		
Enterococcus sp. (ENT)(256)		

The markers EUB, FIR, BAC, CLO, and XIV were not specifically classified in the table because they included large bacterial groups with diverse enzymatic activities, making their classification challenging. However, the EUB marker was utilised to determine normalisation indexes for marker abundances. In cases where a marker encompassed another marker, such as *E. coli*, *Enterobacteriaceae*, and Gamma-proteobacteria, the broader group was selected as the representative marker. Consequently, Gamma-proteobacteria was chosen, while the other two were not included in the indices.

Based on this microbial classification, four indices were derived. Index 1 consisted of markers displaying all three enzymatic activities and formed by markers considered beneficial. Index 2 was similar to Index 1 but included potentially pathogenic markers, such as *C. albicans* and *Gamma-proteobacteria*. Index 3 comprised markers with only amylase enzymatic activity, specifically *Ruminococcus* spp. and *Roseburia* sp. Finally, index four comprised markers lacking enzymatic activity, such as F. *prausnitzii* and *A. muciniphila*.

$$\boldsymbol{Index\ 1}: \ [(RA\ LAC) + (AR\ ENT) + (RA\ BIF)]$$

$$\boldsymbol{Index\ 2}: \ [(RA\ CAN) + (RA\ LAC) + (RA\ ENT) + (RA\ BIF) + (RA\ GAM)]$$

$$\boldsymbol{Index\ 3}: \ [(RA\ RUM) + (RA\ ROS)]$$

$$\boldsymbol{Index\ 4}: \ [(RA\ FAE) + (RA\ AKK)]$$

The relative abundance values (RA) of each marker used to calculate the indices refers to the total abundance for each marker calculated as previously described.

3 Results

3.1 Microbial markers

Data normality was assessed using the Kolmogorov-Smirnov test, and since some significant values were observed, non-parametric tests were employed for data analysis. The Mann-Whitney test was conducted to examine differences between groups.

The analysis of microbial markers revealed significant differences in five markers: BAC (*p*-value 0.044), ENT (*p*-value 0.049), FIR (*p*-value 0.019), GAM (*p*-value 0.039), and LAC (*p*-value 0.005). All these markers were more abundant in patients with PEI than in control subjects (Table 15).

Table 15. Absolute abundance (gene copies per gram of stool, GC/g) means ± standard deviation for each microbial marker of each group of subjects (Control: control subjects, PEI: patients with chronic pancreatitis with pancreatic exocrine insufficiency) and *p*-value. Statistically significant differences between conditions are highlighted in colour.

Marker	PEI (GC/g)	Control (GC/g)	*p*-value
A. muciniphila	4.95 ± 2.09	6.63 ± 0.97	0.111
Bacteroidetes	10.37 ± 0.33	9.92 ± 0.41	0.044
Bifidobacterium	10.43 ± 1.07	9.81 ± 0.89	0.281
C. albicans	1.97 ± 1.17	1.40 ± 0.16	0.111
Clostridium cluster I	12.05 ± 0.67	12.55 ± 0.36	0.303
E. coli	5.45 ± 1.48	4.89 ± 1.13	0.454
Enterobacteriaceae	10.77 ± 1.13	9.93 ± 0.83	0.134
Enterococcus sp.	5.96 ± 0.63	5.33 ± 0.62	0.049
F. prausnitzii	8.33 ± 10.47	8.92 ± 0.67	0.708
Firmicutes	13.94 ± 0.44	13.39 ± 0.28	0.019
Gamma-proteobacteria	8.46 ± 0.59	7.84 ± 0.42	0.039
Lactobacillus sp.	7.36 ± 0.69	6.48 ± 0.31	0.005
Roseburia sp.	8.07 ± 1.12	8.12 ± 0.30	0.349
Ruminococcus spp.	7.80 ± 0.80	7.98 ± 0.24	0.779
Clostridium cluster XIV	9.64 ± 0.42	9.61 ± 0.42	0.925

In addition to observing significant differences in some markers when they were analysed individually during the compositional data analysis, some microbial markers were more associated with control subjects and others with those suffering from CP with developed PEI (Figure 21).

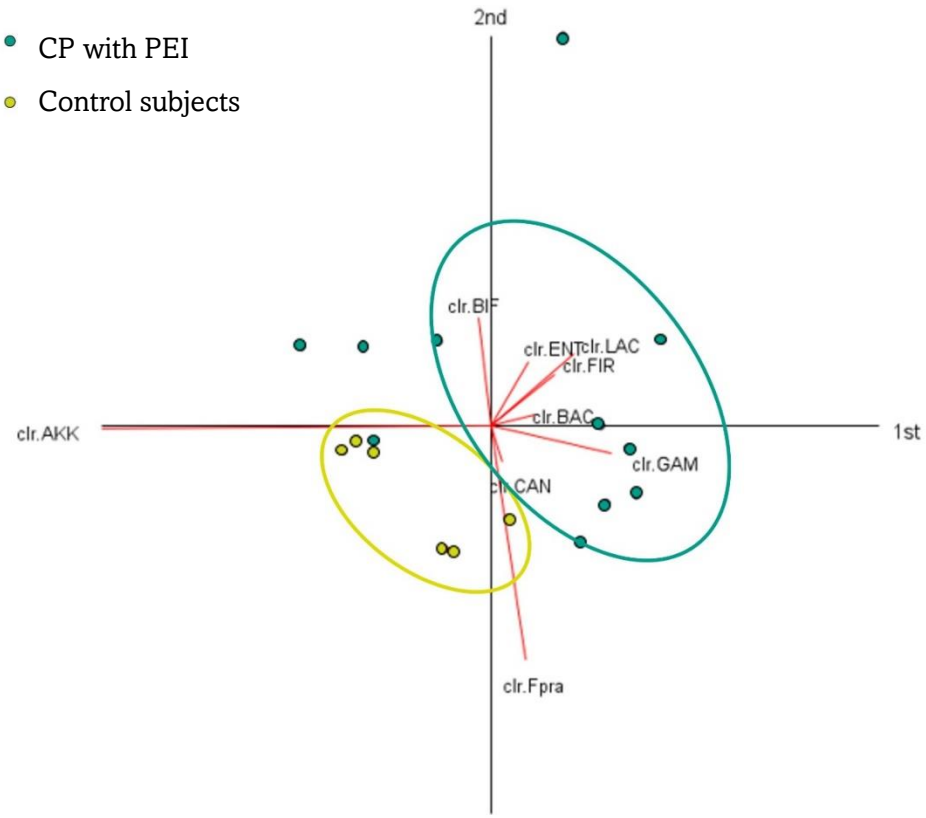

Figure 21. Centred log ratio transformation (CLR)-biplot (1st and 2nd axis) of dataset. Red lines are the vectors of the CLR variables showing the relationship between sample condition and the total abundance of analysed microbial markers.

Interestingly, the markers associated with control subjects lacked enzymatic amylase, protease, and lipase activity. In contrast, patients with CP and PEI exhibited a stronger association with markers possessing enzymatic amylase, protease, and lipase activity.

3.2 Enzymatic indices

To compare the values of the indices between patients with PEI and healthy subjects, the Mann-Whitney test was employed. Indices 1 and 2, calculated based on the abundance of microbial markers with putative enzymatic activities of amylase, protease, and lipase, exhibited significant differences (p-values of 0.044 and 0.032, respectively). Conversely, index 4, comprised of bacterial markers without any of the mentioned enzymatic activities but known to play a role in maintaining mucosal homeostasis (an indicator of eubiosis), was found to be significantly lower in control subjects compared to patients with PEI (p-value 0.039). Index 3, consisting of microbial markers

with only amylase as their enzymatic activity, did not show significant differences (*p*-value 0.4973) when comparing values between control subjects and those with PEI (Table 16, Figure 22).

Table 16. Mean index value + standard deviation for each index described in section 1.5 of each group of subjects (Control: control subjects, PEI: patients with chronic pancreatitis with pancreatic exocrine insufficiency). Different letters show statistically significant differences between Control and CP with PEI in each index.

Group	Index 1	Index 2	Index 3	Index 4
Control	11.24 ± 1.55[a]	13.82 ± 1.64[a]	3.90 ± 0.71[a]	4.31 ± 1.28[a]
PEI	9.47 ± 1.88[b]	12.46 ± 3.61[b]	4.07 ± 1.20[a]	6.70 ± 2.42[b]

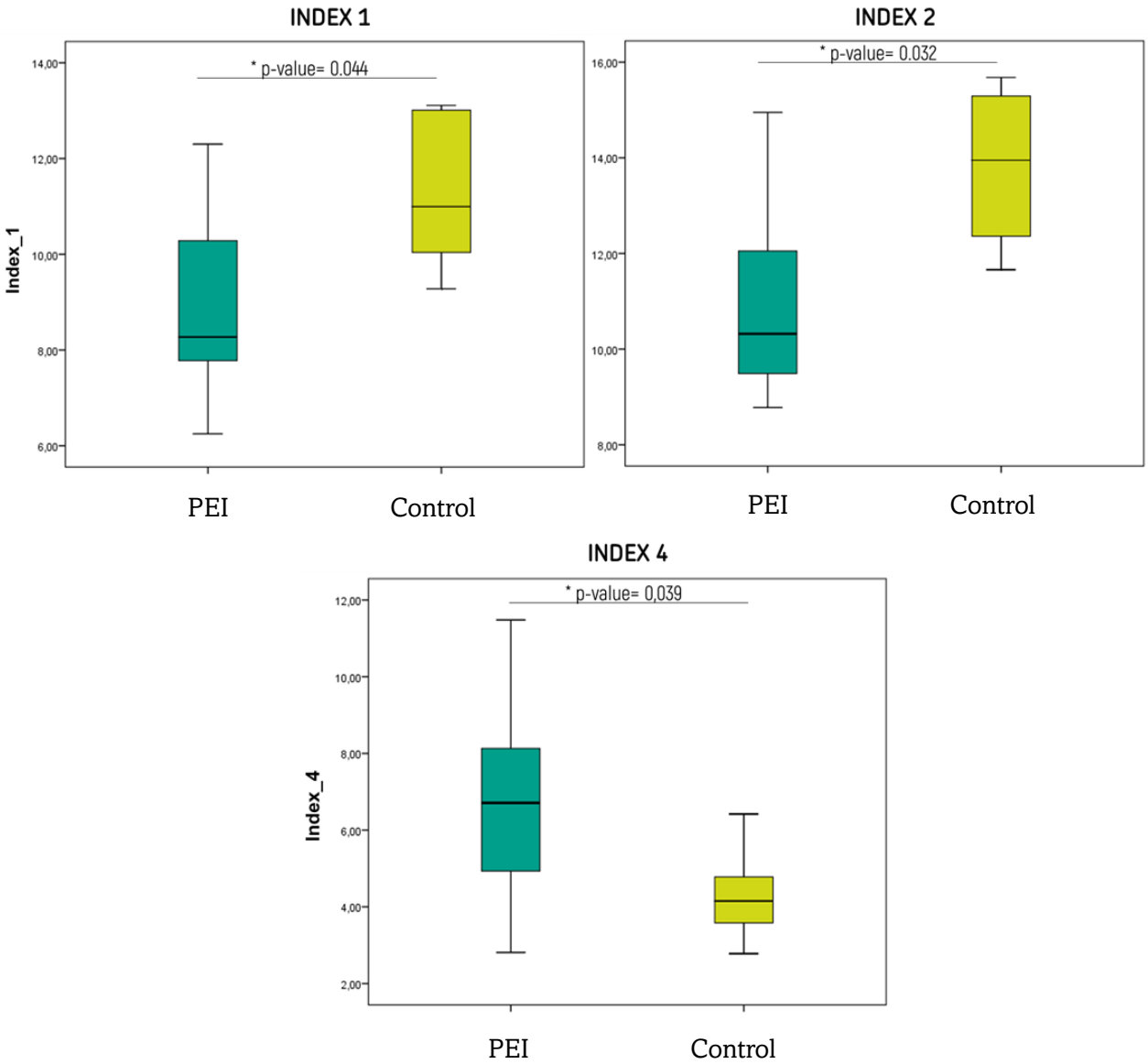

Figure 22. Values of indices 1, 2 and 4 with the *p*-value between the control group (yellow) and patients with chronic pancreatitis and developed exocrine pancreatic insufficiency (PEI, blue-green).

Furthermore, a MANOVA test was performed for the indices. Figure 23 shows how the three indices with significant differences (index 1, index 2, and index 4) display contrasting behaviour between patients with PEI and control subjects. The lower the value, the higher the abundance of the microbial markers.

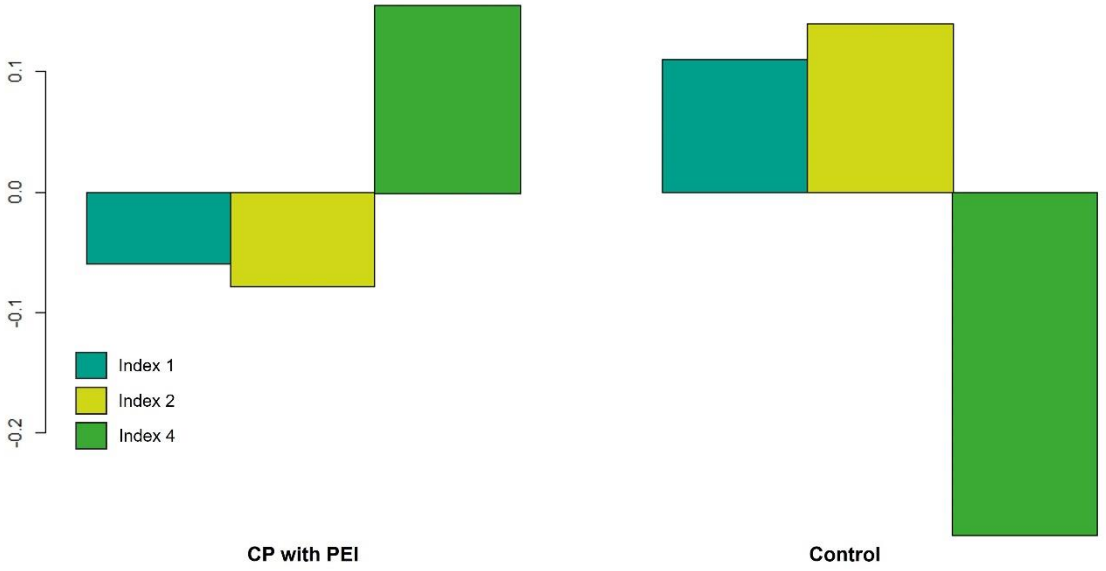

Figure 23. MANOVA test chart showing the behaviour of index 1, 2 and 4 with significant results between patients with PEI and controls.

4 Discussion

The pancreas plays a crucial role in regulating the gut microbiome by producing anti-microbial peptides, bicarbonate, and digestive enzymes (92). In cases of PEI, where pancreatic juice secretion is impaired, small intestinal bacterial overgrowth (SIBO) and gut dysbiosis can occur (257). Among the various pancreatic factors associated with alterations in the intestinal microbiome, reduced elastase levels have been identified as the most significant (93).

This study investigated alterations in the abundance of some selected microbial groups within the intestinal microbial community structure in individuals with CP and developed PEI. This was achieved by characterising 16 microbial markers representing the human gut microbiota's major phyla, groups, and genera. The findings suggest a significant impact of exocrine pancreatic function on the composition of the intestinal microbiome.

A summary of the most significant findings from this study is found in Table 17.

Table 17. Summary of the most important results of this study.

Condition		Finding
Bacterial markers individually		Significant increase of Bacteroidetes, *Enterococcus sp.,* Firmicutes, Gamma-proteobacteria *and Lactobacillus sp*. in patients with chronic pancreatitis and developed pancreatic exocrine insufficiency.
Enzymatic indices	With enzymatic activity	Significantly higher in patients with chronic pancreatitis and exocrine pancreatic insufficiency compared to controls.
	Without enzymatic activity	Significantly lower in patients with chronic pancreatitis and exocrine pancreatic insufficiency compared to controls.

When analysing the markers individually, the results align with those obtained in similar studies (92,245,258). Firmicutes, Bacteroidetes, *Lactobacillus* sp., and Gamma-proteobacteria were more abundant in patients with CP and PEI than in control subjects. Firmicutes and Bacteroidetes are two prominent phyla in the intestine, with Firmicutes being characterised by butyrate as the primary metabolic end product. Bacteria from Bacteroidetes play a critical role in maintaining gut homeostasis and are associated with specific metabolic functions related to nutrient digestion and calorie absorption (217). Increased abundance of Firmicutes and Bacteroidetes has also been observed in patients with other pancreatic diseases such as type 1 diabetes (259), wherein CP patients with PEI have a prevalence of impaired or diabetic glucose tolerance of 40-70%, with approximately half of these patients developing insulin-dependent diabetes mellitus (260). In cases of intestinal inflammation and subsequent dysbiosis, an overgrowth of pathobionts, particularly members of the Gamma-proteobacteria, has been observed in minipigs with PEI (261).

In contrast, beneficial bacteria such as *Lactobacillus* sp. exhibited a significant increase, as observed by Han et al. in mice with chronic pancreatitis (262). This increase could be attributed to the lipase activity of this bacterial group, although they have also been found to have a higher prevalence in certain gastrointestinal disorders, exerting anti-inflammatory effects by selectively degrading proinflammatory chemokines (263). While no significant differences in their abundance were observed, markers *F. prausnitzii* and *A. muciniphila* were correlated with control subjects compared to patients with CP and PEI. This observation is consistent with numerous studies that have reported a decrease in these microbial markers, indicative of intestinal homeostasis and beneficial for the host, in patients with CP and PEI compared to control subjects (261,264–267).

Four indices were derived to classify microbes based on their enzymatic activities rather than solely as beneficial or potentially pathogenic. Indices comprising microbial markers with amylase activity did not differ significantly between patients with CP and PEI and control subjects. This finding suggests that the amylase activity may be compensated by salivary, gastric, and/or small

intestinal enzymes (239), preventing dysbiosis resulting from the deficiency of this pancreatic enzyme.

Significant differences were observed between the two populations studied in indices composed of markers with all three pancreatic enzymatic activities (indices 1 and 2). Microbial markers included in these indices were more abundant in patients with PEI, indicating a compensatory response by the gut microbiota to offset the missing enzymatic activity, particularly lipase activity, which the human body cannot compensate for.

Although the underlying causality between the observed dysbiosis and metabolic or inflammatory conditions remains unclear, Frost *et al.* (93) hypothesised that the changes seen in the gut microbiota of patients with CP are a result rather than the cause of the disease. Given this perspective, we postulate that these disease-induced changes are nevertheless oriented to achieve functional compensations to impaired secretion of digestive enzymes due to PEI, hypothesizing that the changes seen in the gut microbiota of CP patients are a result rather than the cause of the disease.

Research on the gut microbiota and its correlation with pancreatic diseases is still in its infancy. However, our findings are consistent with those of Jandhyala *et al.* (268), who reported decreased abundances of *F. prausnitzii* and *R. bromii* in CP patients compared to the control group. Additionally, Kurdi *et al.* previously hypothesized that the dysbiosis observed in CP patients might stem from malabsorption and reduced pancreatic enzyme levels (269). These findings support our own results.

Despite these results, it is acknowledged that increasing the cohort size is advisable to investigate further whether the differences observed in this pilot study are maintained. Future studies should also consider additional factors influencing the microbiota, such as smoking, alcohol consumption, genetic factors (270), body mass index, or diabetes commonly associated with CP and PEI.

In conclusion, patients with PEI exhibit higher abundances of microbial markers with enzymatic activity, particularly lipase, than healthy subjects. Although no clinical or physiological implications were derived from these enzymatic activity differences, the results suggest the involvement of gut microbiota in the digestive tract to compensate for the lack of enzymatic activity characteristic of PEI. Establishing a clear association between alterations in the gut microbiota and pancreatic disease would be of significant clinical importance, as it could provide valuable options for clinical intervention. Although data is limited, if future studies continue to replicate these findings and provide explicit evidence of dysbiosis in pancreatic disease, it would support microbiota

manipulation as a new therapeutic frontier in CP with PEI, given its low cost and high manageability.

5

Discussion

The exploration of the human microbiome, now recognized as a pivotal area of study, traces its origins back to the observations of Antonie van Leeuwenhoek (1632–1723). Notably, it took centuries to examine into the complexities of these microbial communities beyond their role as mere pathogens. While the advancements in sequencing and technological tools certainly contributed to this expanding field, it is imperative to acknowledge the profound impact of diverse scientific disciplines. Noteworthy among these are environmental microbiology and microbial ecology and evolution, which have laid the groundwork for understanding that the majority of microorganisms residing within and on us are integral components, aiding in the maintenance of our health and well-being (271).

The concept of "Dysbiosis" has an extensive historical backdrop, tracing its roots to the analysis of the human gut "microflora" during the late 19th and early 20th centuries. Élie Metchnikoff, a Nobel Prize-winning zoologist-immunologist and longevity researcher, was the first to emphasize the significance of resident microorganisms and their varying effects on the human body, categorizing them as either "normal" or "pathological". The term "dysbiosis" initially surfaced in Carl Arthur Scheunert's 1920 paper on the link between intestinal "flora" and bone inflammation in horses. Scheunert asserted that gut dysbiosis played a role in equine disease and could be mitigated through improved hygiene practices in stables and water sources, potentially exerting a decisive influence (272).

Interestingly, despite early efforts to enhance human health through manipulation of the microbiota, this field of research remained relatively dormant for several decades, primarily due to technological limitations. Specifically, these constraints pertained particularly to the study of non-cultivable microorganisms of interest, compounded by the absence of comprehensive population-scale data that could capture the compositions and functions of the microbiota. However, the emergence of sequencing technologies and subsequent large-scale sequence-based microbiome initiatives, exemplified by projects such as the Human Microbiome Project and the Metagenomics of the Human Intestinal Tract consortium, has prompt the advancement of human microbiome research (273). Consequently, our understanding of human and environmental microbiomes has undergone an exponential expansion, yielding a continuous stream of discoveries that elucidate the composition and functions of microbiomes across diverse bodily regions, thereby unveiling potential associations with diseases, risks, and clinical symptomatology (274,275). This progression is evidenced by the

substantial body of scientific literature in this realm. In the year 2022 alone, an estimated 60,000 publications centred on the gut microbiota were disseminated. Over the preceding decade (2012-2022), the exploration of the gut microbiota has accumulated more than 554,000 dedicated publications. Remarkably, this encompasses roughly 75% of all publications concerning this subject within the past fifty years (since 1972). This observation not only underscores the flourishing state of this research domain but also underscores the urgency for further advancements.

The knowledge of the microbiome introduces avenues for interventional strategies, including personalised medicine and targeted ecological engineering controls within the environment. For instance, by discerning the "healthy" state of an individual's gut microbiome and understanding how it responds to external factors like diet, it becomes plausible to prescribe modulation strategies tailored to the individual, fostering the restoration of the gut microbiome from an "unhealthy" state to a "healthy" state (275).

In this context, this study has engaged in a comprehensive exploration of the intestinal microbiota in digestive diseases, encompassing three distinct dimensions.

Firstly, it was categorised and characterised the intestinal dysbiosis by evaluating a stool microbial test clinical utility. Alterations in the abundance of different microbial markers were correlated with symptoms such as diarrhoea or constipation, and conditions including gender, age, and body mass index. A noteworthy advancement is the successful differentiation between temporary and pathological dysbiosis, facilitated by a previously described index, thus contributing novel insights to the field.

Lately, people have been realizing of the importance that gut microbiota plays in overall health. This growing understanding has made people curious, leading to commercial availability of stool sample analysis kits worldwide. However, a challenge emerges from this proliferation. Patients often struggle to interpret test results, sometimes making decisions without specialist consultation. Even when consultations occur, specialists may lack training to comprehend the outcomes. In addition, many diagnostic tests lack rigorous validation and verification, with techniques sometimes falling short of robust scientific standards. Notably, numerous laboratories operate without adhering to ISO quality systems.

The merits of our test lie in adhering rigorously to ISO13485 standards, our approach embodies meticulous quality control. QPCR analysis lends credibility to our findings. In this case, we benefit from the use of the qPCR method as the number of species to be determined is below 30. We aim to quantify their abundance, and the known nature of the species makes qPCR an ideal choice. Sequencing would be more suitable if we were exploring new species or sequences, or if we needed to analyse more than 30 species. The qPCR method was chosen for its cost-effectiveness, simplicity, and applicability. It boasts the widest dynamic range, the lowest quantification limits, and the least biased results when compared to microarrays or RNA-seq (276).

A vital facet is the support framework established, aiding specialists in decoding outcomes, recognizing the necessity to contextualize results. Moreover, our validation process reinforces diagnostic test reliability, enhancing clinical utility and becoming a powerful tool to support personalised medicine and effective decision making.

Secondly, a prebiotic derived from grapes has been formulated, demonstrating the capacity to enhance the proliferation and metabolic activity of SCFA producing species. This effect is observed across both control groups and individuals afflicted with IBD and IBS. Notably, this prebiotic surpasses its competitors, including inulin - the current prebiotic market leader - as well as grape seed extract (GSPE), and equalling its performance with, and occasionally exceeding, pectin (277,278).

Prebiotics offer several advantages over their rivals, the probiotics. The specificity inherent to probiotics, predominantly comprising singular microorganism strains, contrasts with the non-specificity of prebiotics, which create a healthy environment for proliferation of beneficial bacteria. A pivotal challenge with probiotics pertains to their preservation; the vulnerability of these microorganisms to environmental stress during various phases - encompassing manufacturing, storage, and consumption - often jeopardizes their viability. Conversely, prebiotics are comparatively resilient, rendering their preservation and production markedly less intricate. Consequently, this aspect contributes to diminished manufacturing expenses, thereby enhancing accessibility and affordability within broader world regions (279).

Lastly, we have observed gut dysbiosis tailored to an abnormal condition. This marks the first instance where it is suggested that the gut's microbial inhabitants might be compensating for the lack of certain enzymes in patients with CP and resultant PEI. Particularly, we have found an increase

in bacteria with lipase activity among those patients facing pancreatic malfunction compared with control subjects. In contrast, the abundance of bacteria lacking enzymatic activity is notably diminished in comparison to the control group.

The study of the relationship between microbiota and pancreatic diseases remains a relatively new field of investigation. Nevertheless, alterations in the gut microbiota have been linked to several pancreatic diseases including type 1 diabetes, acute pancreatitis, pancreatic cancer, or CP. However, a definitive cause-and-effect connection between gut dysbiosis and pancreatic diseases has not been established yet (280,281).

Frost *et al.* suggested that exocrine pancreatic function is the most important host factor influencing the human gut microbiota. Their research identified significant correlations between pancreatic elastase levels and alterations in microbial diversity (93). In a separate study, Kurdi *et al.* postulated that the gut dysbiosis observed in CP patients with PEI could potentially indicate both malabsorption and decreased pancreatic enzyme levels (269). Jandhyala *et al.* found diminished abundances of *F. prausnitzii* and *Ruminococcus bromii* in CP patients relative to the control group. These alterations were linked to the metabolic changes characteristic of CP (258).

Collectively, these findings highlight the association between specific changes in the gut microbiota and CP. However, our study stands out as the first to infer positive effects of the observed dysbiosis. These insights should be further investigated in larger patient cohorts. Future approaches involving targeted strategies for gut microbiota modulation hold promise towards the precision medicine.

The findings presented in this thesis, while significant and pioneering in certain domains, require further validation across a broader population. Additionally, the factors outlined as influential on the microbiota – encompassing lifestyle, diet, and medication – merit consideration in future research studies.

Enhancing our comprehension of the human microbiome and its intricate interaction with the host holds undeniable value in interpreting the origins and underlying mechanisms of various human diseases. Furthermore, this understanding offers potential for the development of more effective therapeutic alternatives, addressing the limitations inherent in current treatment modalities.

The application of advances derived from gut microbiome studies, including FMT, probiotic and prebiotics, for the purpose of rectifying or restoring the perturbed gut microbiota inherent in

dysbiosis-associated disease states, holds great promise for alternative therapeutic options in managing symptomatic disorders. However, the beneficial therapeutic effect of microbiome-based therapy is largely dependent on the role of dysbiosis in contributing to the nature of the disease. Thus, accurately identifying crucial microbiota components, comprehending the underlying causes of dysbiosis, carefully selecting appropriate prebiotics to selectively enhance desirable commensals, and having reliable tools for monitoring the effect, are essential for successful integration of microbiome-based therapies into future clinical practices (273).

Finally, the findings detailed in this doctoral thesis can potentially serve as a guide to advance our understanding of the role played by the intestinal microbiota in a range of conditions, extending beyond digestive diseases (263,282,283) to encompass mental health, allergies (54), and metabolic disorders (284). Such advancements will surely lead to new understandings within this complex field, and it will enable continued growth in microbiome research for decades to come.

6

Conclusions

1. TestUrGut® demonstrates clinical utility for the management of digestive diseases or conditions.
2. Women exhibit a higher abundance of *A. muciniphila* compared to men in individuals with digestive symptoms.
3. Body mass index primarily influences the abundance of *M. smithii*, followed by *A. muciniphila*, with lower loads in cases of low weight and higher in instances of obesity in individuals with digestive symptoms.
4. Faecal consistency in irritable bowel syndrome patients strongly influences *M. smithii* and *A. muciniphila* levels, with higher abundance during constipation and lower during diarrhoea. This also pairs with the prevalence of Bacteroidetes in diarrhoea and Firmicutes in constipation.
5. The dysbiosis index that combines *F. prausnitzii* and *E. coli* allows us to differentiate between temporary and pathological dysbiosis.
6. Previpect®, a new prebiotic derived from grapes, stimulates the growth and metabolic activity of short-chain fatty acid-producing bacteria in patients with irritable bowel syndrome, inflammatory bowel disease, and healthy controls.
7. Patients with chronic pancreatitis and concomitant exocrine pancreatic insufficiency manifest an adapted dysbiosis wherein microorganisms exhibiting lipase enzyme activity may compensate for the characteristic deficiency in this condition.

7

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