

THE ROLE OF GUT MICROBIOTA-HOST INTERACTION IN OBESITY AND METABOLIC DISTURBANCES

EVA LAU GOUVEIA

TESE DE DOUTORAMENTO EM
METABOLISMO – CLÍNICA E EXPERIMENTAÇÃO, APRESENTADA À
FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

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No âmbito deste projeto de doutoramento:

- participei na definição de objetivos dos estudos, no seu desenho, definição de metodologia e na sua planificação;
- participei na sua concretização e recolha de dados;
- fui responsável pela redação da versão inicial e submissão final de todos os artigos incluídos nesta dissertação.

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Abreviaturas

AUC – Area under the curve

BMI – body mass index

BP – Blood pressure

GLP-2 – Glucagon-like peptide-2

HbA1c – hemoglobin A1c

HFD – High-fat diet

hsCRP – high-sensitivity c-reactive protein

I-FABP – Intestinal fatty-acid binding protein

LPS – Lipopolysaccharides

M – Months

NAFLD – Non-alcoholic fatty liver disease

NHANES – National Health and Nutrition Examination Survey

RCT – Randomized controlled clinical trial

RYGB – Roux-en-Y gastric bypass

T2DM – Type 2 diabetes

TLR4 – Toll-like receptor 4

SFCA – Short-chain fatty acids

U.S. – United States of America

WHO – World Health Organisation

95%CI – 95% confidence interval

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ABSTRACT

Obesity and associated metabolic diseases had spread worldwide in epidemic proportions. Obesity is closely associated with increased risk of insulin resistance, type 2 diabetes (T2DM), dyslipidemia, hypertension and non-alcoholic fatty liver disease. Recent data emphasize the role of gut microbiota in obesity, metabolic impairment, energetic storage dysfunction and increased low-grade systemic inflammation. This project arises from the need to consolidate and expand the clinical knowledge of gut microbiota-host interaction in obesity and associated metabolic disturbances. This understanding can potentially lead to the development of innovative therapy targets with the capacity to reduce these metabolic diseases.

For the abovementioned purpose, this PhD work plan involved: 1) Thematic review – consisting on a broad evidence review and synthesis (including a systematic review) of gut microbiota role and possible interventions to modulate it on obesity and metabolic impairments; 2) Original research – including an experimental study, a randomized controlled clinical trial (RCT), and a big data analysis. The experimental study was conducted in a high-fat diet (HFD) induced obesity rat model. The open-label RCT was performed in mild obese patients with T2DM. The big data, cross-sectional analysis was done with data from the National Health and Nutrition Examination Survey (NHANES).

In the original research, the first study (experimental study) showed lower Bacteroidetes levels ($\log_{10}$ rRNA gene copies/20ng of DNA: 5.45 in HFD vs. 6.43 in standard diet group, $p < 0.05$) and higher Firmicutes-to-Bacteroidetes ratio in the HFD induced obesity rat model (1.20 in HFD vs. 1.03 in standard diet group, $p < 0.05$). HFD fed rats had two-fold less insulin sensitivity and increased fecal levels of lipopolysaccharides (LPS), toll-like receptor 4 (TLR4) expression, and increased plasma proinflammatory cytokines, which reflected the proinflammatory status, typical of metabolic diseases. HFD may have also up-regulated the expression of intestinal fatty-acid binding protein (I-FABP) and may have increased glucagon-like peptide-2 (GLP-2) to improve gut barrier integrity.

To further achieve the purpose of this thesis, it was designed and conducted the first RCT that simultaneously studied clinical and microbiota changes in T2DM patients with class-1 obesity after Roux-en-Y gastric bypass (RYGB), compared to T2DM standardized medical

therapy (surgical arm: n=8 after 2 dropouts, vs. medical arm: n=10). Anthropometric and metabolic comparative analysis favored RYGB: at month 12 of follow-up, the percentage of weight loss was 25.5% vs. 4.9% ($p<0.001$), and hemoglobin A1c (HbA1c) was 6.2% vs. 7.7% ($p<0.001$), in surgical vs. medical arm, respectively. Also, only RYGB patients achieved diabetes improvement/remission (n=7, 87.5% of patients in surgical arm). It was observed a continuous increase of genus richness after RYGB during the 12 months of follow-up. In the medical arm, genus richness ended-up being significantly lower at month 12. Composition analysis indicated significant changes of the overall microbial ecosystem (permanova $p=0.004$, [$R^2=0.17$]) during the follow-up period after RYGB. There was also a strong association between improvement of anthropometric/metabolic/inflammatory biomarkers and increase in microbial richness and Proteobacterial lineages.

Lastly, it was performed a cross-sectional analysis on a large and representative U.S. population sample (38,802 adults), showing that 13.1% reported probiotic ingestion. Probiotic ingestion was associated with 17% lower prevalence of obesity and 21% lower prevalence of hypertension. Although there is increasing evidence of the potential benefits induced by probiotics in metabolic disturbances, there was lack of large cross-sectional studies to assess population-based prevalence of probiotic intake and metabolic diseases. The results of this study supported the possibility of gut microbiota modulation, by the use of probiotics, as an attractive therapeutic target to prevent and treat obesity and related metabolic disorders.

The aforementioned PhD data brought comprehensive knowledge on the clinical role of gut microbiota-host interactions in obesity and related metabolic diseases. The new insights in this metabolic crosstalk with gut microbiota may allow the development of integrated and effective strategies to prevent and treat obesity and its metabolic complications.

RESUMO

A obesidade, assim como as respectivas doenças metabólicas relacionadas, têm aumentado mundialmente em proporções epidêmicas. A obesidade está intimamente associada ao aumento do risco de insulinoresistência, diabetes tipo 2, dislipidemia, hipertensão arterial e esteatose hepática não alcoólica. Dados recentes enfatizam o papel da microbiota intestinal na obesidade, desregulação metabólica, disfunção energética e inflamação sistêmica de baixo grau. Este projeto surge da necessidade de consolidar e expandir a compreensão clínica da interação hospedeiro-microbiota intestinal na obesidade e distúrbios metabólicos associados. Este conhecimento poderá contribuir para o desenvolvimento de potenciais alvos terapêuticos inovadores, capazes de reduzir essas doenças metabólicas no futuro.

Para este propósito, o planejamento deste projeto de doutoramento envolveu: 1) Revisão temática – consistindo numa ampla revisão e síntese da evidência (incluindo uma revisão sistemática) sobre o papel da microbiota intestinal e possíveis intervenções para modular na obesidade e distúrbios metabólicos associados; 2) Investigação original – incluindo um estudo experimental, um ensaio clínico randomizado e controlado (RCT) e uma análise de *big data*. O estudo experimental foi realizado em ratos, num modelo de obesidade induzida por dieta rica em gordura (HFD). O RCT aberto foi desenhado para doentes com obesidade classe 1 e com diabetes tipo 2. A análise transversal de *big data* foi realizada com dados do *National Health and Nutrition Examination Survey* (NHANES).

Na investigação original, o primeiro estudo (estudo experimental) revelou níveis mais baixos de Bacteroidetes ($\log_{10}$ cópias de gene rRNA/20ng de DNA: 5,45 no grupo HFD vs. 6,43 no grupo de dieta normalizada, $p<0.05$) e maior razão Firmicutes para Bacteroidetes, nos ratos HFD (1,20 no grupo HFD vs. 1,03 no grupo de dieta normalizada, $p<0.05$). Os ratos alimentados com dieta hiperlipídica apresentaram duas vezes menos sensibilidade à insulina, níveis fecais aumentados de lipopolissacáridos (LPS), aumento da expressão de receptor do tipo *toll* 4 (TLR4) e de citocinas plasmáticas pró-inflamatórias, o que reflete o estado pró-inflamatório característico das doenças metabólicas. A dieta rica em gordura pode ainda ter regulado positivamente a expressão da proteína intestinal de ligação a

ácidos gordos (I-FABP) e ter aumentado o peptídeo 2 semelhante ao glucagon (GLP-2) para melhorar a integridade da barreira intestinal.

Para alcançar o objetivo desta tese, foi ainda desenhado e conduzido o primeiro RCT que estudou simultaneamente alterações clínicas e do microbioma intestinal em doentes diabéticos tipo 2 com obesidade de classe 1 após bypass gástrico em Y-de-Roux, em comparação com a terapia médica padronizada (grupo cirúrgico: n=8 após 2 abandonos, vs. grupo médico: n=10). Os resultados antropométricos e metabólicos comparativos favoreceram o bypass gástrico: aos 12 meses de seguimento, a percentagem de perda de peso foi 25,5% vs. 4,9% ($p<0,001$), e hemoglobina A1c (HbA1c) foi 6,2% vs. 7,7% ($p<0,001$), no grupo cirúrgico vs. médico, respetivamente. Adicionalmente, apenas doentes do grupo cirúrgico atingiram melhoria/remissão da diabetes (n=7, 87.5% dos doentes do grupo cirúrgico). Observámos um aumento contínuo da diversidade bacteriana após o bypass gástrico durante os 12 meses de seguimento. No grupo apenas sob terapêutica médica, a diversidade bacteriana foi significativamente menor após um ano de seguimento. A análise da composição do microbioma intestinal indicou alterações significativas no ecossistema microbiano global (permanova $p=0,004$, $[R^2 = 0,17]$) durante o período de acompanhamento após a cirurgia. Houve também uma forte associação entre melhoria dos biomarcadores antropométricos/metabólicos/inflamatórios e aumento da diversidade microbiana e das linhagens de Proteobacteria.

Por fim, foi realizada uma análise transversal usando *big data* de uma extensa amostra representativa da população dos Estados Unidos da América (38.802 adultos), tendo-se verificado que 13,1% relataram a ingestão de probióticos. A ingestão de probióticos foi associada a uma prevalência 17% inferior de obesidade e a uma prevalência de hipertensão 21% inferior, comparativamente à população não exposta a probióticos. Embora haja evidência crescente dos potenciais benefícios induzidos pelos probióticos nos distúrbios metabólicos, não existiam estudos transversais de larga escala para avaliar a prevalência da ingestão de probióticos e doenças metabólicas a nível populacional. Os nossos resultados indicam a possibilidade de modulação da microbiota intestinal, através do uso de probióticos, como um alvo terapêutico potencial para prevenir e tratar a obesidade e distúrbios metabólicos relacionados.

Os dados supracitados desta tese de doutoramento ampliaram o conhecimento sobre o papel clínico das interações microbiota-hospedeiro intestinal na obesidade e nas doenças

metabólicas relacionadas. Os novos conhecimentos desta interação metabólica com a microbiota intestinal podem permitir o desenvolvimento de estratégias integradas e eficazes para prevenir e tratar a obesidade e suas complicações metabólicas.

1. GENERAL PhD INTRODUCTION

1.1 Obesity: from definition to targeting new regulators

Obesity is a complex multifactorial metabolic disease that had spread worldwide in epidemic proportions. The World Health Organization (WHO) defines obesity as an excessive fat accumulation, objectively classified by a body mass index (BMI) measure $\geq 30 \text{ kg/m}^2$, that might impair health ¹. Obesity is a state of chronic, low-grade inflammation, which is now recognized to play an underlying pathogenic role in negative health outcomes of obesity ². The hallmark of this health impairment is the dramatic increase of the risk of metabolic diseases, such as type 2 diabetes (T2DM), non-alcoholic fatty liver disease (NAFLD), hypertension or dyslipidemia. In fact, 44% of T2DM and 23% of the ischemic heart disease are attributable to overweight and obesity, further associated with an increase in cardiovascular risk and a decrease of life expectancy ³. In this field, it was estimated a loss of 5- to 20-years in life expectancy, depending on the severity of the comorbid conditions ⁴⁻⁶. Importantly, the World Obesity Federation declared obesity a chronic progressive disease, clearly distinct from being just a risk factor for noncommunicable diseases ⁷.

The worldwide prevalence of obesity nearly tripled between 1975 and 2016 ¹. In 2016, it was estimated that 52% of the worldwide population were overweight or obese, which means over 650 million obese adults ¹. In Portugal, data from the First Portuguese Health Examination Survey 2015 showed that 38.9% of adult population was overweight and 28.7% was obese ⁸. Comparing to data from a Portuguese survey between 2003-2005, the prevalence of obesity had doubled in ten years ⁹. This global rise in obesity prevalence, accompanied by a growing burden of metabolic disturbances, turned it into a major health hazard ¹⁰. Having all this into account, more than just reduce obesity prevalence, it is mandatory to reverse the escalation of this disease, as well as the related metabolic disturbances.

Current health recommendations for obesity treatment mainly rely in three modalities: lifestyle intervention, pharmacotherapy and weight-loss procedures, including bariatric surgery ¹¹. Unfortunately, despite the advances in the knowledge and treatment of obesity, weight loss induces hormonal changes that can lead to weight regains and counteract the desirable long-term weight loss maintenance ¹². Ng *et al* conducted a systematic review of worldwide prevalence and trends of obesity in adults and children, from 1980 to 2013, and

found that no single country was successful in reducing obesity during those 33 years ¹³. The majority of real-life weight loss interventions are centered on caloric intake reduction and energy expenditure intensification. This ingestion-expenditure target is frequently not totally successful, suggesting that the countless etiological factors of obesity are not completely understood ¹⁴⁻¹⁶. Even after bariatric surgery, currently recognized as the best treatment strategy for weight loss, significant weight regain can occur ^{17,18}. Therefore, further understanding on the key regulators of body weight and metabolic status should be explored, since they may provide new insights into different targets for future interventions. The excessive fat accumulation that occurs in obesity results from a complex interaction between genetic, environmental and behavioral factors ¹⁹. Genetics has been associated to different aspects of energy homeostasis, such as body fat distribution, physical activity level, food intake, thermic effect of food, basal metabolic rate and environmental changes ^{20,21}. Besides these heritable factors, obesity arises as the result of an energy imbalance between calories consumed and the calories expended, creating an energy surplus and a state of positive energy balance resulting in excess body weight ²². New interactions, particularly related to food supply, eating behaviors, sedentary lifestyle, family-work culture/practices, built environments and public policies are also recognized as important obesogenic determinants ²³.

The complexity origin of this disease relies in the above-mentioned multifaceted etiologies. However, the obesity burden largely overcomes the one-disease hazard; instead, it is linked to a multi-organ disease with several complications. There is a need to expand the knowledge about the modifiable determinants of obesity. Better understanding of interactions between body weight and new regulators, could possibly attain a more robust decrease in obesity prevalence and in the related burden of metabolic diseases.

1.2 The Metagenome Hypothesis

Among different new molecular targets, gut microbiome has been pointed out as a potential crucial mediator of obesity and related metabolic disturbances ²⁴.

The term "microbiota" is defined as the collective microbial community inhabiting a specific environment, including bacteria, archaea, viruses and some unicellular eukaryotes.

The term “microbiome” is defined as the collective genome of the microorganisms inhabiting our body ²⁵. In humans, microorganisms reside in various parts of the body, such as the surface of skin or the gastrointestinal, genitourinary and respiratory tract ²⁶. The gastrointestinal tract harbors the largest collection of microorganisms, and, along the way, cellular density increases through the length of the gut, making colonic microbiota the densest and most diverse community in the gut ²⁶.

The humans host 10^{14} bacteria in the gut, a 150-fold of the human eukaryotic nuclear genome²⁷. The human gut microbiota is mainly composed by five bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia) and one Archaea (Euryarchaeota) ²⁶. Firmicutes and Bacteroidetes are the most prevalent phyla, representing more than 90% of the human intestinal bacteria ²⁸. Despite some gut bacterial species are potential pathogenic, the constant interaction between the host and its microbes usually remains beneficial to the health of the host ²⁶. The gut microbiota performs a variety of physiological functions and, for that reason, is considered a multifunctional organ. In fact, it has the ability to break down indigestible dietary polysaccharides, plays an important role in the biotransformation of conjugated bile acids, immune system maturation and intestinal response to epithelial intestinal injury ^{29–32}. It also has an active role in metabolic regulation, including glucose and lipid homeostasis ³³.

This crosstalk between host-gut microbiota has led to the emergence of ‘the metagenome hypothesis’. This theory postulate that gut microbiota is an important regulator of obesity and related metabolic disturbances ²⁴. The metagenome hypothesis is supported by the findings that composition of gut microbiota differs between healthy lean and obese subjects ²⁵. This deregulation on gut microbiota, characterized by modifications in bacterial metabolic activity, and/or a shift in local distribution of communities, is called ‘intestinal dysbiosis’.

Several elegant reports have suggested that the gut microbiota has a crucial role in the development of fat mass and altered energy homeostasis. The first study supporting this hypothesis was conducted by Backed *et al* and showed that germ-free mice (mice raised in the absence of any microorganisms) were leaner compared with mice that harboured microbiota since birth ³⁴. Furthermore, germ-free mice colonized with gut microbiota from obese animals had 60% more body fat mass, when compared to mice colonized with gut microbiota from lean donors ³⁵. Moreover, the occurrence of insulin resistance and glucose

intolerance was seen within 14 days post-microbiota colonization, even with a reduction in food intake (standard chow food), providing novel evidence that the bacteria community are able to control energy metabolism, in some way. Intestinal dysbiosis seen on the obese mice recipients was characterized by a greater proportion of Firmicutes and reduced levels of Bacteroidetes, showing that obesity-associated gut microbiome had an increased capacity of energy harvest from diet. These differences at the phylum level were partially confirmed in human studies ^{36,37}. Ley *et al* demonstrated that obese patients losing weight through low-calorie diets also had a shift of gut microbiota composition toward higher relative abundance of Bacteroidetes and decreased number of Firmicutes ³⁸. However, other studies have found diverging results ^{39,40}. The discrepancies observed between studies across the literature encouraged further analysis of the microbiota in human pathological conditions.

The aforesaid studies brought to discussion the cross-interaction between gut microbiota and host in the obesity genesis and maintenance. Several mechanisms have been proposed to further understand the relationship between microbiota regulation of fat storage and development of obesity-related diseases, including:

- a) The capacity of the “obese microbiome” to harvest energy from food – the storage hypothesis;
- b) The contribution of lipopolysaccharides (LPS), a component of the cell wall of gram-negative bacteria, to trigger a low-grade inflammatory state, described as metabolic endotoxemia, a common feature characterizing metabolic disorders – the metainflammatory hypothesis.

In spite of this growing evidence on the role of small bowel / gut microbiota on obesity and related metabolic diseases, there is still a gap of knowledge regarding the mechanisms responsible for these multifactorial and complex conditions. Identifying different gut microbiota-to-host interactions that might trigger the onset of metabolic diseases may offer new templates for discussion and innovative therapeutic targets.

2. PhD OVERVIEW

As previously stated, the gut microbiota is a novel player in obesity and in the obesity-related metabolic diseases. My interest in intestinal microbiota as a modulator of fat mass began in the first year of the doctoral program in metabolism - clinic and experimentation, in the module of '*Neuroendocrinia digestiva, microbioma e metabolismo*'. I felt that besides the advent of lots of novel data on the role of gut microbiota as an environmental factor of obesity pathophysiology, there was still need to acknowledge the complexity of those interactions, in clinical grounds, between gut microbiota and host, specifically in obesity and associated metabolic disturbances.

The studies from the present PhD thesis were designed to perform a comprehensive evaluation of the association between obesity, related metabolic disturbances and gut microbiota. The Figure 1 shows the work planning and connection of the PhD studies.

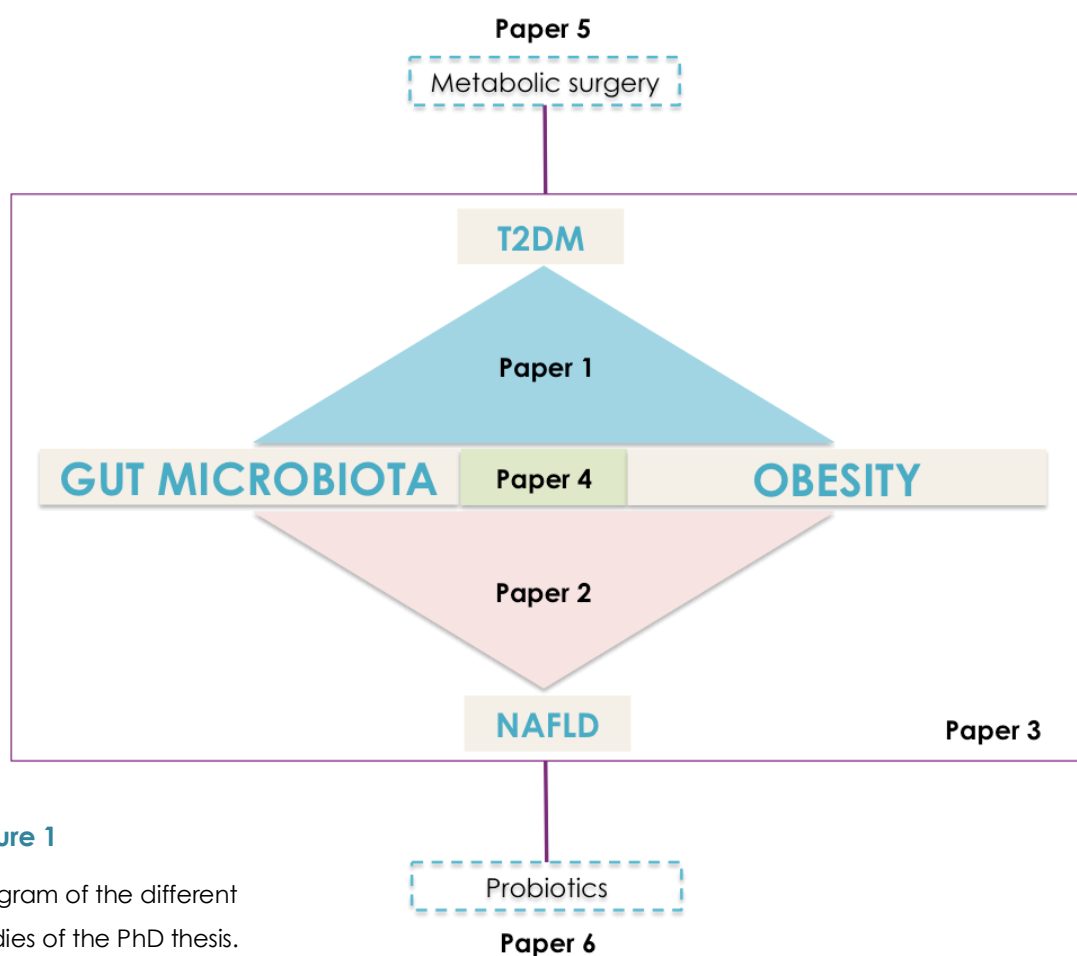


Figure 1

Diagram of the different studies of the PhD thesis.

The PhD work was divided in 4 phases:

- a) **First phase:** it was performed a detailed review on gut microbiota composition in obesity, T2DM and NAFLD and possible interventions targeting the gut microbiota modulation in metabolic diseases – **Paper 1, 2 and 3**. A comprehensive approach of this interaction was crucial to further understand obesity pathogenesis and related metabolic disturbances;
- b) **Second phase:** it was performed an experimental study in a high-fat diet (HFD) induced obesity rat model to characterize gut dysbiosis and to investigate the role of intestinal fatty-acid binding protein (I-FABP), a possible non-invasive marker of gut barrier dysfunction – **Paper 4**. Further acknowledgment of the crosstalk between gut microbiota-host and defining new and early non-invasive markers of gut barrier dysfunction might be of great interest in order to manage a safe modulation of the intestinal microbiota, before emergence of obesity and associated metabolic diseases;
- c) **Third phase:** it was performed an open-label randomized controlled clinical trial (RCT: ISRCTN53984585) to evaluate the association between gut microbiota changes and anthropometric, metabolic and inflammatory markers after metabolic surgery compared with medical therapy, in T2DM adults with mild obesity - BMI 30-35 Kg/m² – **Paper 5**. Metabolic surgery served as a biological model of gut microbiota modulation, to better understand the intricate role of gut microbiota in metabolic regulation. This study was carried out in partnership with the *Institut de Cardiométabolisme et Nutrition* (ICAN) in Paris, France. The project was directly discussed and developed with Professor Joel Doré and with Edi Prifti attending personal meetings at ICAN and periodically teleconferences;
- d) **Fourth phase:** it was designed a cross-sectional study using data from the National Health and Nutrition Examination Survey (NHANES), from 1999 to 2014, to evaluate the association of probiotic ingestion with obesity and metabolic-related disorders – **Paper 6**. This large population survey created powerful information about metabolic differences in subjects exposed to probiotics and the way as microbiota modulation, by probiotics, can regulate health status.

For PhD thesis systematization and presentation, the included studies were organized according to the following chapters:

1. Thematic Introduction: Bibliographic and Systematic reviews (First phase);
2. Original Research: Experimental study, Randomized Clinical Trial and Big Data analysis (Second, Third and Fourth phase).

2.1 Thematic Introduction: Bibliographic and Systematic Reviews

To understand the crosstalk between obesity and metabolic health the present thesis reviewed:

- a) The link between gut microbiota, obesity and T2DM, as well as innovative therapeutic approaches for obesity, by gut microbiota modulation - **Beyond gut microbiota: understanding obesity and type 2 diabetes - Paper 1;**
- b) The relationship between gut microbiota, NAFLD and metabolic disturbances - **Gut microbiota: association with NAFLD and metabolic disturbances - Paper 2;**
- c) The association between gut microbiota, T2DM and NAFLD, and possible interventions to modulate gut microbiota - **Disbiose e microbioma na obesidade, diabetes tipo 2 e esteatose hepática não alcoólica - Paper 3.**

2.2 Original Research: Experimental study, Randomized Clinical Trial and Big Data analysis

To further acknowledge the role of gut microbiota–host interaction in obesity and related metabolic disturbances the present thesis is composed of another 3 original studies:

- a) Experimental study – **The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity** – Paper 4;
- b) Randomized clinical trial – **Gut microbiota changes after metabolic surgery in adult diabetic patients with mild obesity: a randomized controlled trial** – Paper 5;
- c) Big Data Analysis - **Probiotic ingestion, obesity, and metabolic-related disorders: results from NHANES, 1999-2014** – Paper 6.

2.3 Support and Funding

The study "**The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity**", was supported by Fundação para a Ciência e Tecnologia (FCT) - *Fundo Social Europeu, Programa Operacional Potencial Humano (POPH)* from European Union, PTDC/AGR-TEC/2227/2012 and SFRH/BD/93073/2013.

DM² study - Gut microbiota changes after metabolic surgery in adult diabetic patients with mild obesity: a randomized controlled trial, was supported by a competitive grant from Sociedade Portuguesa de Endocrinologia, Diabetes e Metabolismo, and from Liga dos Amigos do Serviço de Endocrinologia do Centro Hospitalar Universitário de São João.

3.AIMS

The main objective of this research project was to further understand the role of gut microbiota-host clinical interactions in obesity and related metabolic disturbances.

As specific aims, we intended to assess:

1. The link between gut microbiota composition and obesity or related metabolic disturbances, as well as possible interventions to modulate gut microbiota (**Paper 1, 2 and 3**);
2. Gut microbiota characterization and the role of I-FABP as a possible plasma marker of intestinal injury and inflammation in a HFD-induced obesity rat model (**Paper 4**);
3. The association between gut microbiota changes and anthropometric, metabolic and inflammatory profiles after metabolic surgery compared with medical therapy, in T2DM adults with mild obesity - BMI 30-35 Kg/m² (**Paper 5**);
4. The association of probiotic ingestion with the prevalence of obesity and associated metabolic disturbances, namely T2DM, hypertension, and dyslipidemia (**Paper 6**).

Further specifications about the aims of the different studies are described next in each paper.

4. THEMATIC INTRODUCTION:

Bibliographic and Systematic Reviews

4.1 Paper 1

Eva Lau, Davide Carvalho, Cidália Pina-Vaz C, Adelino Barbosa, Paula Freitas

Beyond gut microbiota: understanding obesity and T2DM and innovative therapeutic targets

Hormones 2015;14(3):358-69

Review

Beyond gut microbiota: understanding obesity and type 2 diabetes

Eva Lau,¹ Davide Carvalho,¹ Cidália Pina-Vaz,² José-Adelino Barbosa,³ Paula Freitas¹

¹Department of Endocrinology, Diabetes and Metabolism, Centro Hospitalar São João; Faculty of Medicine University of Porto; Instituto de Investigação e Inovação em Saúde, Universidade do Porto; ²Department of Microbiology, Centro Hospitalar São João; Faculty of Medicine University of Porto; CINTESIS, Center for Health Technology and Services Research Porto; ³Department of Surgery, Centro Hospitalar São João; Faculty of Medicine University of Porto; Porto, Portugal

ABSTRACT

Obesity and type 2 diabetes are metabolic diseases that have reached epidemic proportions worldwide. Although their etiology is complex, both result from interplay between behaviour, environment and genetic factors. Within ambient determinants, human overall gut bacteria have been identified as a crucial mediator of obesity and its consequences. Gut microbiota plays a crucial role in gastro-intestinal mucosa permeability and regulates the fermentation and absorption of dietary polysaccharides, which may explain its importance in the regulation of fat accumulation and the resultant development of obesity-related diseases. The main objective of this review is to address the pathogenic association between gut microbiota and obesity and to explore related innovative therapeutic targets. New insights into the role of the small bowel and gut microbiota in diabetes and obesity may make possible the development of integrated strategies to prevent and treat these metabolic disorders.

Key words: Diabetes, Gut microbiota, Obesity

INTRODUCTION

Overweight and obesity are metabolic diseases that have spread worldwide, today reaching epidemic proportions and thus representing the fifth leading risk factors for global mortality. At least 2.8 million adults die each year as a result of being overweight

or obese. In addition, 44% of diabetes cases, 23% of ischemic heart disease cases and between 7% and 41% of certain cancer burdens are attributable to overweight and obesity.¹ Type 2 diabetes (T2D) is today following the trend of obesity and increasing globally. It is estimated that 347 million people worldwide have diabetes.² Although the etiology is complex, both obesity and diabetes result from interplay between behaviour, environment and genetic factors, while mutations seem to be responsible for less than 10% of phenotype variability. Thus, environmental factors stand out as the principal contributors to the obesity and diabetes epidemic. Within ambient determinants,

Address for correspondence:

Eva Lau, Alameda Professor Hernâni Monteiro,
4200-319 Porto, Portugal;
Tel.: +35122 551 2100, Fax: +351224220019,
E-mail: evalau.med@gmail.com

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human overall gut bacteria have been is thought to be a crucial mediator of obesity and diabetes pathogenesis.³ This new proposed model, the ‘metagenome hypothesis’, is based on the fact that humans host 10^{14} bacteria in the gut, representing an 150-fold of our eukaryotic nuclear genome.⁴ This “microbial organ” performs a variety of physiological functions, from protective to metabolic regulation, including an active part in glucose and lipid metabolism.⁵ New insights into the role of the small intestine and gut microbiota in diabetes and obesity are essential for the development of innovative therapeutic targets for the prevention, treatment and delay of obesity, T2D and metabolic associated disorders.

GUT MICROBIOTA

Gut microbiota is mainly composed of seven bacterial divisions, namely, Firmicutes, Bacteroides, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria and Actinobacteria. Firmicutes and Bacteroides are the most abundant species.⁶ Gut microbiota performs essential functions, critical for maintenance of health, namely metabolic roles, including vitamin production, amino acid synthesis and bile acid biotransformation, protective actions preventing pathogenic colonization and structural and histological functions regulating intestinal structure and function.⁷ Dysbiosis is a state characterized by alteration in microbiota composition, a change in bacterial metabolic activity and/or a shift in local distribution of communities. At present, intestinal dysbiosis is crucial in understanding the pathophysiology of several metabolic diseases including that of obesity and T2D (Figure 1).

1) Obesity and gut microbiota

Ley et al were the first to analyze bacterial 16S rRNA gene sequences from the distal intestinal (cecal) microbiota of genetically obese ob/ob mice and lean ob/+, all fed the same polysaccharide-rich diet. They found that ob/ob animals have a 50% reduction in the quantity of Bacteroidetes and a proportional increase in Firmicutes.⁸ Furthermore, this trait is transmissible: colonization of germ-free mice with an ‘obese microbiota’ results in a significantly greater increase in total body fat and insulin resistance than colonization with a ‘lean microbiota’.⁹ These results suggest that obesity is linked to a different gut micro-

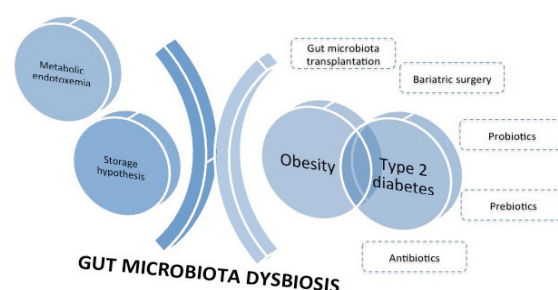


Figure 1. The pathogenic association between gut microbiota and obesity and type 2 diabetes and related innovative therapeutic targets that may treat these metabolic disturbances.

biota profile, characterized as intestinal dysbiosis. In addition, they have enabled identification of the gut microbiota as an additional contributing factor to the pathophysiology of obesity and insulin resistance. Subsequently, several studies have characterized gut microbiota in obese subjects. In obese humans, the relative proportion of Bacteroidetes was also found to be decreased by comparison with lean people.¹⁰ The same authors demonstrated a shift toward higher relative abundance of Bacteroidetes and decreased number of Firmicutes in obese patients losing weight through low-calorie diets.

One type of bariatric surgery, namely, Roux-en-Y gastric bypass (RYGB), has become a promising treatment for obesity, with double benefits: weight loss and metabolic improvement. Furet et al have profiled gut microbiota from fecal samples in 13 lean control subjects and in 30 obese individuals before and after RYGB. The Bacteroides/Prevotella group was lower in obese subjects before and increased 3 months (M3) after RYGB; *Escherichia coli* species also increased at M3 and were inversely correlated with fat mass and leptin levels, independently of food intake changes. Also, lactic acid bacteria including the Lactobacillus/Leuconostoc/Pediococcus group and Bifidobacterium genus decreased at M3.¹¹

In a group of obese or overweight subjects who underwent a 6-week program of energy restriction, followed by another 6-week weight stabilization period, subjects who lost less weight and thus more rapidly regained it had higher Lactobacillus/Leuconostoc/Pediococcus numbers in fecal samples at baseline.¹² The gut microbiota profile may, in the near future,

enable the prediction of which obese subjects will lose weight in response to an energy restriction diet, which is likely to be a helpful strategy in obesity treatment.

Furthermore, it was shown that low bacterial richness was linked to more marked overall adiposity and that obese adults who had lower bacterial richness had gained more weight over a 9-year follow-up period.¹³

2) Type 2 diabetes and gut microbiota

T2D is a metabolic disease characterized by a state of insulin resistance and low-grade inflammation. Microbial ecology can be an important regulator of energy homeostasis and glucose metabolism. Although animal experiments show clear differences between diabetic and non-diabetic gut microbiota, the huge variability in humans most likely masks these large-scale differences.

Diabetic leptin-resistant mice (db/db) presented a higher abundance of Firmicutes, Proteobacteria and Fibrobacteres phyla compared to lean mice.¹⁴ In humans, there is lack of uniformity in the gut microbiota profile in type 2 diabetic patients. Larsen et al have studied the fecal bacterial composition of 36 adult males, among whom 18 had T2D. The proportions of Firmicutes phylum and of the Clostridia class were significantly reduced in the diabetic group compared to the control group. Furthermore, they have found that the ratios of Bacteroidetes to Firmicutes and the ratios of the Bacteroides-Prevotella group to the *C. coccoides-E. rectale* group correlated positively with glucose plasma levels. Similarly, Betaproteobacteria was highly enriched in diabetic compared to non-diabetic persons and also positively correlated with glucose plasma.¹⁵

A protocol for a metagenome-wide study in 345 type 2 diabetic Chinese individuals was recently developed.¹⁶ Type 2 diabetic patients had increased opportunistic pathogens, such as *Bacteroides caccae*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium symbiosum*, *Eggerthella lenta* and *Escherichia coli*, and a decreased number of some types of butyrate-producers. An interesting finding was the moderate degree of this T2D-related dysbiosis: only 3.8±0.2% (mean 6 s.e.m.; n=5 344) of the gut microbial genes (at the relative abundance level) was associated with T2D in an individual.¹⁶ This observa-

tion has raised the concept of a 'functional dysbiosis' rather than a specific microbial species association with T2D pathophysiology.

Karrisson et al have analyzed the gut metagenome from normal women with impaired glucose tolerance and diabetic women.¹⁷ In the total cohort, *Lactobacillus* species correlated positively with fasting glucose and glycated hemoglobin (HbA1c) and *Clostridium* species correlated negatively with fasting glucose, HbA1c, insulin, C-peptide and plasma triglycerides, and positively with adiponectin and HDL. Based on sets of metagenomic clusters (MGCs), they found 26 differentially abundant clusters when comparing diabetics to subjects with normal glucose tolerance. They have also created a random forest model to examine whether microbiota composition can identify diabetes status. MGCs more accurately identified T2D (highest AUC 0.83) than microbial species (highest AUC 0.71).¹⁷

One type of bariatric surgery, namely RYGB, has also made possible a better understanding of the impact of gut microbiota modification and adaptation after the procedure.¹¹ *Fecalibacterium prausnitzii* species were lower in subjects with diabetes and associated negatively with inflammatory markers before and after the surgery and during the follow-up period, independently of changes in food intake.¹¹ In humans, blood 16S rDNA, a broadly specific bacterial marker, was found to be an important independent marker of the risk of diabetes and also predicted abdominal adiposity in a large sample of non-obese participants from a general population.¹⁸ This study reaffirms the concept that tissue bacteria are involved in the onset of diabetes in humans.

3) Mechanisms linking gut microbiota dysbiosis to obesity and type 2 diabetes

3.1) Metabolic endotoxemia

One of the mechanisms proposed to explain the crosstalk between gut microbiota, regulation of fat storage and development of obesity-related diseases is metabolic endotoxemia. The concept described as "metabolic endotoxemia" was first described in mice.^{19,20}

Bacterial lipopolysaccharides (LPS) are a component of the cell wall of gram-negative bacteria capable

of triggering an inflammatory state, which is present in metabolic disorders – the metabolic inflammation hypothesis. It was shown that high-fat feeding augments plasma LPS-containing microbiota at a concentration sufficient to increase body weight, fasting glycemia and inflammation.¹⁹ Moreover, oligofructose supplementation, which increases *Bifidobacteria* content, reduced the inflammatory tone, namely endotoxemia, plasma and adipose tissue proinflammatory cytokines, this strongly pointing to LPS as an important mediator of inflammatory response.²⁰

3.1.1) The LPS/CD14/TLR4 system

LPS in combination with CD14 serves as a ligand for toll-like receptor (TLR4). It was observed that CD14 knock-out mice, lacking functional LPS receptors, were hypersensitive to insulin. Obesity and diabetes were also delayed in response to high-fat feeding.¹⁹ Furthermore, increased endotoxemia was associated with increased CD14 expression and increased IL-6 levels after a mixed meal containing lipids in healthy humans.²¹ Accordingly, TLR4 inactivation reduced food intake and inflammatory response, despite no significant modification of body weight.²² It also blunted insulin resistance induced by LPS in differentiated adipocytes. Thus, the LPS/CD14/TLR4 system seems to set the threshold at which a high-fat diet induced insulin resistance and the onset of diabetes and obesity.

3.1.2) Mucosa permeability

Intestinal mucosa has an important role in the absorption of vital nutrients and in the regulation of barrier functions, thus preventing bacterial translocation. Intestinal mucosa integrity is ensured through intercellular tight junctions, mucus secretion, release of antimicrobial peptides from Paneth cells and immunoglobulin secretions from resident immune cells. Zonulin—a protein of the haptoglobin family released from liver and intestinal epithelial cells—is the main physiological regulator of intercellular tight junctions. Increased zonulin concentrations are related to changes in tight junction competency and increased GI permeability.²³ This “leak” in the paracellular absorption route enables invasion of antigens from the intestinal milieu, triggering an immune response and subsequent inflammation and oxidative stress.^{24,25}

A randomized, 14-week, double-blind, placebo-controlled trial with 23 endurance-trained men who took a supplement including six probiotic strains showed a significant reduction in fecal excretion of epithelial tight junction protein zonulin in response to treatment, suggesting increased integrity of the intestinal mucosa.²⁶ Similarly, a 9-week intervention trial in 93 obese volunteers supplemented with a prebiotic showed concomitant changes in the intestinal microbiota and a reduction in intestinal permeability, assessed with a dual-sugar-absorption test.²⁷ Increased plasma concentrations of zonulin have also been reported in a cohort of 25 patients with sepsis compared with a healthy control group, stressing the potential role of tight junction proteins in sepsis, resulting in disruption of the structural integrity of the intestinal mucosa and increased intestinal permeability.²⁸

A high-fat diet dramatically increased intestinal permeability via a mechanism associated with reduced expression of epithelial tight junction proteins, including zonulin and occludin.²⁹ Cani et al demonstrated that prebiotic-treated mice exhibited lower plasma LPS and cytokines and decreased hepatic expression of inflammatory and oxidative stress markers.³⁰ This decreased inflammatory tone was associated with lower intestinal permeability and improved tight junction integrity compared to controls. In these experiments, prebiotic supplementation increased endogenous intestinotrophic proglucagon-derived peptide (GLP-2) production, whereas the GLP-2 antagonist abolished most of the prebiotic effects.

GLP-2 plays a significant role in the adaptive regulation of bowel mass, mucosal integrity, stimulation of enterocyte proliferation and prevention of apoptosis.³¹ GLP-2's growth-promoting and cytoprotective properties in the gastrointestinal tract have aroused interest in its use as a therapeutic agent for the treatment of GI diseases involving malabsorption, inflammation and/or mucosal damage. It promotes expansion of the GI mucosal surface area, stimulates the uptake of luminal nutrients including carbohydrates and amino acids, enhances mucosal hexose transport, enhances the expression of genes encoding nutrient transporters and increases multiple enzymes involved in digestion along the GI tract. Interestingly, pharmacological GLP-2 treatment decreased gut permeability, systemic and hepatic inflammatory phenotype

associated with obesity to a similar extent as that observed following prebiotic-induced changes in gut microbiota.³⁰ In summary, a selective gut microbiota change can increase endogenous GLP-2 production and consequently improves gut barrier functions via a GLP-2-dependent mechanism, contributing to the improvement of gut barrier functions during obesity and diabetes. These data suggest that gut bacteria are involved in intestinal permeability control and in the occurrence of metabolic endotoxemia.

3.1.3) The endocannabinoid system

Several studies have suggested a close relationship between LPS, metabolic endotoxemia and the endocannabinoid (eCB) system.

Obesity and its metabolic complications are associated with macrophage infiltration, which is responsible for almost all adipose tissue TNF- α and IL-6 expression involved in inflammatory pathways.³² The activation of macrophages is dependent on LPS/CD14.¹⁹ On the other hand, LPS regulates the synthesis of eCB in macrophages and obesity is associated with increased eCB plasma levels.^{33,34} The endocannabinoid system is composed of endogenous bioactive lipids that act through cannabinoid receptor 1 (CB1) and 2.³⁵ Cannabinoid receptor 1 (CB1) blockage in obese mice improved gut barrier function by lowering alterations of tight junctions proteins (zonulin and occludin) and decreased plasma LPS levels.³⁶ Cannabinoid agonist administration significantly increased LPS and endotoxemia through changes in permeability.^{36,37} Consequently, gut microbiota may have a critical function in the regulation of gut permeability, contributing to endotoxemia, through the endocannabinoid system and LPS regulatory loop.

The ECB/LPS system also appears to have an important role in adipose tissue plasticity. Adipocyte differentiation and lipogenesis is reduced in the presence of physiological levels of LPS, whereas eCB activation increased adipogenesis in lean mice under physiological conditions.³⁶ Surprisingly, specific modulation of gut microbiota and CB1 blockage increased adipocyte differentiation and lipogenesis.³⁶ One possible hypothesis involves the fact that CB1 blockage reduces gut permeability, thus reducing LPS levels under pathophysiological conditions such as obesity, which counteracts their inhibitory effects on adipocyte

differentiation and lipogenesis that might paradoxically increase. These data show that the LPS/eCB system regulatory loops contribute to deregulation of adipogenesis, thereby perpetuating the disequilibrium and leading to a vicious cycle in obesity.

3.1.4) Intestinal alkaline phosphatase

Intestinal alkaline phosphatase (IAP) has a pivotal role in intestinal homeostasis. IAP is known as a regulator of lipid absorption across the apical membrane of enterocytes.³⁸ It also controls bacterial endotoxin-induced inflammation by dephosphorylation, detoxifying intestinal LPS, thus acting as a host defense factor against LPS.³⁹ IAP expression is not only modulated by dietary components, including fat, but also controlled by gut microbiota.^{40,41}

Consumption of a high-fat diet in conjunction with an obese phenotype was associated with changes in the gut microbiota, a decrease in IFA, an increase in LPS and ileal inflammation.⁴⁰ Ghoshal et al demonstrated that enterocytes internalize LPS from the apical surface and transport LPS to the Golgi complex. This complex also contains chylomicrons, the lipoproteins responsible for transport of dietary long-chain fat through blood and mesenteric lymph. It was observed that chylomicrons promote intestinal LPS absorption.⁴² Thus, excess chylomicron formation during high-fat feeding facilitates endotoxin translocation via a reduction in IAP activity, inducing intestinal inflammation that is present in obesity and insulin resistant states.⁴³

3.2) The 'storage' hypothesis

Energy metabolism can be profoundly regulated by host gut microbiota, that is, microbiota modulates energy balance. Adult germ-free conventionalization with a normal microbiota collected from the cecum of conventionally raised mice produced a 60% increase in body fat and insulin resistance, despite reduced chow consumption.⁴⁴ In this line, microbiota transplantation from the cecum of an obese donor (ob/ob) to adult germ-free mice resulted in a greater relative abundance of Firmicutes and a significant increase in body fat over two weeks, compared with mice colonized with a lean microbiota, despite no differences in food intake.⁹ As is well known, energy balance results from an equilibrium between energy

intake and energy expenditure. The above experiments gave rise to the hypothesis that obesity-associated gut microbiome has an increased capacity for energy harvest from the diet, the so called ‘storage effect’ hypothesis. This hypothesis is based on the following probabilities: microbial fermentation of dietary polysaccharides that cannot be digested by the host, intestinal absorption of monosaccharides and lipid metabolism regulation by microbiota.

3.2.1) Fermentation of dietary polysaccharides

Gut microbiota fermentation degrades non-digestible polysaccharides into short-chain fatty acids (SCFA), including acetate, propionate and butyrate, and other subproducts in the cecum and colon.⁴⁵ Gut microbiota is enriched by many enzymes involved in processing these otherwise indigestible dietary polysaccharides for the metabolism of, among others, starch/sucrose, galactose and butanoate.⁹ As predicted from these metagenomic analyses, the ob/ob cecum had a higher concentration of the major fermentation end-products – butyrate and acetate. These findings are consistent with the fact that Firmicutes are butyrate producers.^{46,47} SCFAs are ligands for the G-protein coupled receptors 41 and 43 (GPR41 and GPR43).⁴⁸ In concordance with previously published results, Samuel et al have shown that GPR41 knockout mice, colonized with a specific fermentative microbial community, were resistant to fat mass gain.⁴⁹ In addition, GPR43 knockout mice fed a high-fat diet presented significantly lower body fat mass and higher body lean mass compared to their littermate controls. These phenotype changes were accompanied by improved glucose control and lower HOMA index.⁵⁰ These data emphasize the potential role of GPRs and SCFA in fat mass development and regulation of glucose metabolism.

3.2.2) Intestinal absorption of monosaccharides

Conventionalization of germ-free mice results in a two-fold increase of the capillary density in the villus epithelium of the small intestine.⁵¹ Thus, gut microbiota shape the intestinal villus microvasculature, which increases the intestine’s absorptive capacity.⁵¹ Experimental data corroborate these observations, demonstrating that gut microbiota stimulates monosaccharides absorption from the gut lumen.⁴⁴

Furthermore, ob/ob mice also have less energy in their feces relative to their lean littermates.⁹

3.2.3) Lipid metabolism regulation

There is strong evidence that gut microbiota is a regulator of fat storage. A 14-d conventionalization of GF mice resulted in a 2.3-fold increase in liver triglyceride content and was accompanied by statistically significant increase in liver acetyl-CoA carboxylase and fatty acid synthase, two key enzymes in the de novo fatty acid biosynthetic pathway, known targets of sterol response element binding protein 1 (SREBP-1) and carbohydrate response element binding protein (ChREBP).⁴⁴ Moreover, it was concluded that increased capacity of storage triglycerides in adipocytes was mediated through the suppression of intestinal expression of a circulating lipoprotein lipase inhibitor. Lipoprotein lipase is a key regulator of fatty acid release from triglyceride-rich lipoproteins; in turn, adipocyte lipoprotein lipase activity is associated with an increase in fatty acids uptake and adipocyte triglyceride accumulation. FIAF (fasting-induced adipose factor), also named angiopoietin-like protein 4, is an inhibitor of lipoprotein lipase activity, suppressed in conventionalized germ-free mice, which explains the increased triglycerides storage in adipocytes in these mice. It therefore seems that microbiota coordinates increased hepatic lipogenesis, promoting storage of calories from the diet as fat, through suppression of FIAF, and resulting in increased lipoprotein lipase activity in adipocytes.

Furthermore, gut microbiota seems to be an active player in fatty acid oxidation. Besides the observation that germ-free knockout mice lacking FIAF were not protected against obesity, Backed et al demonstrated that germ-free mice were protected against obesity, despite consuming a western, high-fat, sugar-rich diet.⁵² This lean phenotype was related to increase skeletal muscle and liver levels of phosphorylated AMP-activated protein kinase and its downstream targets—acetylCoA carboxylase, carnitine-palmitoyl-transferase—that are involved in fatty acid oxidation.

4) Gut microbiota modulation

Understanding the metabolic impact of the complex interaction between gut microbiota and the host has driven interest in manipulating microbiota in order

to develop new therapeutic targets for the metabolic diseases, obesity and T2D

4.1) Gut microbiota transplantation

As has been described, gut microbiota profoundly affects metabolic homeostasis. Microbiota transplantation from mice fed a diet that induced obesity to lean germ-free mice promoted greater fat increase than transplants from lean donors.⁵³ The main principle of fecal transplant is therefore the possibility of this procedure replacing pathogenic microbes by beneficial communities, thus restoring the gut microbiota balance and enabling the cure of the disease. Although gut microbiota transplantation is a controversial issue, the metabolic effects of transplanting intestinal microbiota from lean donors to male recipients with metabolic syndrome were recently assessed.⁵⁴ Individuals were randomly assigned to receive intestinal infusions of allogenic or autologous microbiota. Six weeks after, subjects that received infusions of microbiota from lean donors had an insulin sensitivity increase, along with increase of butyrate-producing intestinal microbiota.

4.2) Bariatric surgery

Bariatric surgery, and specifically Roux-en-Y gastric bypass (RYGB), has become a promising treatment which, besides promoting weight loss, can also improve or resolve T2D in severely obese patients.^{55,56} Bariatric surgery seems, furthermore, an effective model to study the gut microbiota profile and its association with beneficial metabolic/inflammatory effects underlying the procedure, including loss of weight and resolution of T2D.

Zhang et al analyzed gut microbiota of 9 individuals, 3 in each of the categories of normal weight, obese and after gastric bypass surgery.⁵⁷ It was found that Firmicutes were dominant in normal-weight and obese individuals, but significantly decreased in those post bypass. After 3 months (M3) of RYGB, the Bacteroides/Prevotella group had increased in previously obese individuals. *Escherichia coli* species had also increased at M3 and were inversely correlated with fat mass and leptin levels independently of changes in food intake.¹¹ Interestingly, gut microbiota richness was also improved after RYGB and 202 white adipose tissue (WAT) genes changed following RYGB.⁵⁸ Furthermore, variations of gut

microbiota were associated with changes in WAT gene expression. To test the hypothesis that some of the effects of RYGB were caused by changes of the host-microbiota relationship, Liou et al used a mouse model of RYGB. Transferal of the gut microbiota from RYGB mice to non-operated ones resulted in weight loss and decreased fat mass, this corroborating the present hypotheses concerning the impact of gut microbiota modifications after RYGB in weight loss.⁵⁹

Although the available studies seem to bear out the relationship between gut microbiota modulation and underlying metabolic improvement after bariatric surgery, it is still necessary to confirm these observations in larger cohorts. It is also of great importance to understand whether microbiota changes are the cause or the consequence of weight loss and glycemic control after bariatric surgery.

4.3) Prebiotics

The concept of prebiotics was first introduced in 1995 by Gibson and Roberfroid.⁶⁰ A prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health, this especially applying to Bifidobacterium and Lactobacillus.⁶¹ Gut microbiota enables the fermentation of these non-digestible compounds so as to produce short-chain fatty acids.⁶²

High-fat feeding changes gut microbiota towards a reduced level of Bifidobacterium and increases metabolic endotoxemia, thus resulting in body weight gain and diabetes.¹⁹ Accordingly, feeding mice with a prebiotic, oligofructose, restored the levels of Bifidobacteria and normalized endotoxemia. Importantly, *Bifidobacterium* species were negatively correlated with endotoxemia.²⁰ In humans, oligofructose supplementation also promoted weight loss and improved glucose regulation in overweight adults.⁶³ Furthermore, it increased satiety and reduced hunger.⁶⁴ Thus, the mechanisms linking prebiotic-induced changes in gut microbiota and their beneficial metabolic effects seem to go beyond the reduction of metabolic endotoxemia.

Oligofructose supplementation decreased food intake, fat mass development and hepatic steatosis in normal and in obese rats; furthermore, it exerts / exerted an antidiabetic effect in streptozotocin-treated rats and high-fat-fed mice. The beneficial effects of

this prebiotic were associated with an increase in the number of L cells and of glucagon-like peptide-1 (GLP-1). Moreover, it was also found that oligofructose was able to modulate PYY and ghrelin, which could be involved in the control of food intake.⁶⁵ The relationship between the beneficial metabolic effects of prebiotics and GLP-1 were also supported by other studies.^{66,67} Endogenous intestinotrophic proglucagon-derived peptide (GLP-2) has also demonstrated its clinical relevance in association with the use of prebiotics. Cani et al have shown that prebiotic-fed mice had lower levels of several plasma cytokines, such as TNF α , IL1b, IL1 α , IL6, INF γ , known to promote tight junction disruption and a significant decrease of the chemokines MCP-1 and MIP-1^a.^{68,69} The decrease in these inflammatory markers was associated with improved tight junction integrity and lower intestinal permeability, which in turn resulted in lower LPS levels. In addition, GLP-2 production was increased, while the GLP-2 antagonist abolished the beneficial effects of prebiotics.⁶⁸ These data raised the hypothesis that a selective gut microbiota increases endogenous GLP-2 and consequently improves gut barrier integrity by a GLP-2-dependent mechanism, contributing to the improvement of the gut barrier in obesity and diabetes.

In humans, oligofructose supplementation also promoted beneficial metabolic effects, including weight loss and improved glucose regulation in overweight adults, these being associated with suppressed ghrelin and enhanced PYY.⁷⁰ Recently, T2D patients were supplemented with transglucosidase, resulting in a decrease in plasma glucose levels and in an increase in Bacteroidetes-to-Firmicutes ratio.⁷¹ However, there are conflicting data regarding prebiotic effects in humans.^{72,73}

It now appears that other constituents, besides the non-digestible carbohydrates of prebiotics, influence gut microbiota modulation, as shown by Isken et al. Indeed, the solubility of dietary fibres (ratio of soluble vs insoluble), when added to a high-fat western-style diet, differently affected body weight and insulin sensitivity in obesity-prone mice.⁷⁴

Regarding the presented data, nutritional approaches with prebiotics thus appear to be an important target to consider in the management of obesity and T2D.

However, it is essential to investigate which components may affect gut microbiota modulation and its impact on metabolic modulation.

4.4) Probiotics

Probiotics are food supplements that contain living bacteria, in particular those of the genera *Lactobacillus* and *Bifidobacterium* which act through diverse mechanisms to confer a health benefit to the host.⁷⁵ Probiotics appear to benefit T2D and obesity. However, a better insight into the bacterial strains and host metabolism is necessary for their efficient use. Rodent models have provided robust evidence for identifying human gut commensals that could be developed as probiotic therapies. Several studies have addressed the metabolic effects of *Bifidobacterium* supplementation. For instance, it was demonstrated that feeding *Bifidobacterium* improved several aspects of the metabolic syndrome reducing endotoxin concentration and intestinal inflammation.^{76,77} However, these effects seem to be strain-dependent, which requires a detailed understanding of each strain and the way it affects fat and glucose metabolism.⁷⁸ As concerns *Lactobacillus* supplementation, although there is experimental data showing its effects in reduction of body weight,^{79,80} some studies do not report these differences. Recently, *Akkermansia muciniphila* was shown to be decreased in obese and T2D mice.⁸¹ This study found that *A. muciniphila* treatment reversed high-fat diet-induced metabolic disorders, including fat-mass gain, metabolic endotoxemia, adipose tissue inflammation and insulin resistance. Moreover, *A. muciniphila* administration increased the intestinal levels of endocannabinoids, which, as has been previously described, control inflammation and the gut barrier.⁸¹ In humans there are increasingly well-controlled studies that seek to demonstrate the suggested potential benefits of probiotics in weight and diabetes management.⁸²⁻⁸⁵

4.5) Antibiotics

Antibiotics are agents that inhibit the growth of microorganisms. Administration of antimicrobial agents is another viable option for modulation of the gut microbiota profile. Regarding the fact that gut microbiota has been linked to obesity and T2D pathogenesis, Membrez et al studied the effects of norfloxacin and ampicillin in ob/ob mice.⁸⁶ After

a 2-week intervention, a significant improvement in fasting glycemia and oral glucose tolerance was observed. Concomitant reduction of plasma LPS and increase of adiponectin further supported the antidiabetic effects of the antibiotic treatment.⁸⁶ In high-fat fed mice, the modulation of gut microbiota by antibiotics administration resulted in improved glucose tolerance by reducing LPS levels and inflammatory status. In addition, it also increased the circulating levels of acetate, which activates AMPK and reduces macrophage infiltration.⁸⁷ Moreover, vancomycin-treated high-fat fed mice gained less weight after the treatment and had lower fasting blood glucose and plasma TNF α compared to the obese controls.⁸⁸ Importantly, in a NOD mouse model for diabetes treated with vancomycin, although the cumulative incidence of diabetes was lower, one single species, *Akkermansia muciniphila*, became dominant.⁸⁹

In humans, however, clinical studies are mainly limited to the characterization of the composition of gut microbiota after antibiotics administration.⁹⁰⁻⁹² However, there are some data that link antibiotic exposure to obesity risk.⁹³⁻⁹⁵ Thus, while in mice models the available data suggest that gut microbiota is a realistic target, in humans it remains uncertain whether antibiotics use might be associated with metabolic improvement.

CONCLUSION

Gut microbiota provides a broad field of increasing importance for the comprehension of obesity and T2D. As has been shown, this comprehension may make possible the development of new specific targets for the improvement, or even cure, of these prevalent metabolic diseases.

DISCLOSURE

There is no conflict of interest that would undermine the impartiality of this scientific work. There is no potential conflict of interest by any author of manuscript.

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4.2 Paper 2

Eva Lau, Davide Carvalho, Paula Freitas

Gut microbiota: association with NAFLD and metabolic disturbances

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Review Article

Gut Microbiota: Association with NAFLD and Metabolic Disturbances

E. Lau, D. Carvalho, and P. Freitas

Department of Endocrinology, Diabetes and Metabolism, Centro Hospitalar São João, Institute for Research and Innovation in Health Sciences, Faculty of Medicine, University of Porto, Alameda Professor Hernâni Monteiro, 4200-319 Porto, Portugal

Correspondence should be addressed to E. Lau; evalau.med@gmail.com

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Nonalcoholic fatty liver disease is the hepatic expression of metabolic syndrome, being frequently associated with obesity, insulin resistance, and dyslipidemia. Recent lines of evidence have demonstrated a role of gut microbiota in insulin resistance, obesity, and associated metabolic disturbances, raising the interest in its relationship with NAFLD pathogenesis. Therefore, intestinal microbiota has emerged as a potential factor involved in NAFLD, through different pathways, including its influence in energy storage, lipid and choline metabolism, ethanol production, immune balance, and inflammation. The main objective of this review is to address the pathogenic association of gut microbiota to NAFLD. This comprehension may allow the development of integrated strategies to modulate intestinal microbiota in order to treat NAFLD.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a very common disease, ranging from simple hepatic steatosis, characterized by excessive fat deposition in hepatocytes without any inflammation or necrosis to nonalcoholic steatohepatitis (NASH), characterized by steatosis and hepatic inflammation [1]. NAFLD is the hepatic expression of metabolic syndrome, being frequently associated with obesity, insulin resistance, and dyslipidemia [2]. Thus, its prevalence rises in parallel with the worldwide metabolic diseases epidemic, frequently developing on the background of obesity [3].

Although the pathogenesis of NAFLD is not completely understood, considerable progress has been made in recent years in elucidating the mechanisms responsible for liver injury. Initial theories were based on a “2-hit hypothesis” [4]. The “first hit” was characterized by hepatic triglyceride accumulation, which increases susceptibility of the liver to injury mediated by “second hits,” such as inflammatory cytokines/adipokines, mitochondrial dysfunction, and oxidative stress, which in turn lead to steatohepatitis and/or fibrosis [5].

The gastrointestinal tract harbors the largest number of bacteria, representing more than 150-fold their eukaryotic

nuclear genome [6]. This “microbial organ” is recognized to perform a variety of physiological functions, from protective functions to metabolic regulation, including an active part on glucose and lipid metabolism [7]. Recent lines of evidence suggest a role of gut microbiota in insulin resistance and obesity [8–11], raising the interest of gut microbiota as an active intervenient on NAFLD (Figure 1). Microbiota seems to induce obesity through several mechanisms: ability of microbial products such as acetate and propionate to signal via intestinal epithelial receptors; increased intestinal permeability with translocation of bacterial products resulting in high level of metabolic inflammation; and caloric salvage by some microbes being able to extract calories from food [12]. There is a close anatomical and functional relationship between gut and liver, through portal circulation, favoring bidirectional influences [13]. Liver receives approximately 70% of its blood supply from the intestine, representing the first line of defense against gut-derived antigens [13]. Thus, gut microbiome may play an important role in the maintenance of gut-liver axis health and in NAFLD pathogenesis.

In the background of obesity and insulin resistance, this systematic review aims to explore the relationship which links microbiota to NAFLD.

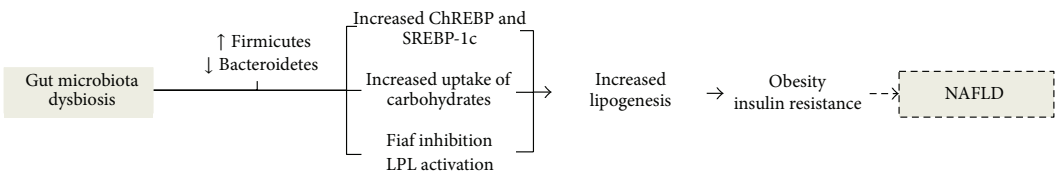


FIGURE 1: Schematic view of how the gut microbiota affects host fat storage and insulin resistance, which may result in NAFLD. The microbiota acts through an increase in the transactivation of lipogenic enzymes by liver carbohydrate response element binding protein (ChREBP) and sterol regulatory element binding protein 1 (SREBP-1), an increase in the uptake of dietary polysaccharides and through Fiaf inhibition with increased LPL activity in adipocytes, thereby promoting increase of hepatic lipogenesis and storage of calories harvested from the diet into fat.

NAFLD	Fatty liver [MeSH terms] or NASH or nonalcoholic steatohepatitis or nonalcoholic fatty liver or NAFLD or nonalcoholic fatty liver disease
Gut microbiota	Microbiota [MeSH terms] or gut microbiota
Exclusion of reviews	Not review [publication type]

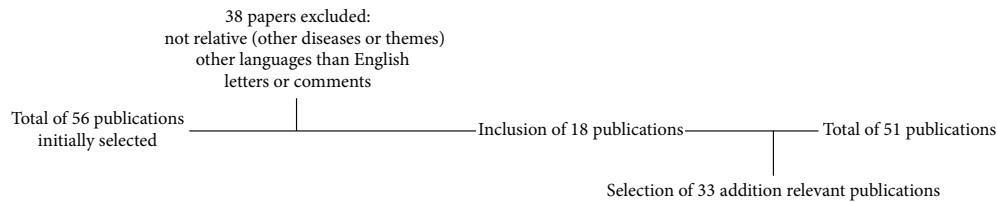


FIGURE 2: Search strategy in PubMed and studies selection.

2. Methods

A literature search was conducted with the aim of finding original experimental, epidemiological, and clinical studies on the association between gut microbiota and NAFLD. The search strategy used in PubMed, including the studies selection, is shown in Figure 2. Additional papers were identified in the reference lists of selected articles that met the inclusion criteria. Inclusion criteria were as follows: clinical studies with participants of any sex or ethnic origin with NAFLD/NASH diagnosed on the basis of radiological/histological evidence of fatty liver and epidemiological or experimental studies, regarding association between NAFLD/NASH and gut microbiota. Exclusion criteria were as follows: other causes of hepatic steatosis, such as alcoholic hepatic steatosis or viral hepatitis, and papers written in other languages than English.

All articles were read in full. Two independent investigators assessed papers for inclusion. Disagreement was resolved by discussion.

3. Results

Experimental and clinical studies have explored the pathogenic association between gut microbiota and NAFLD. A summary of studies, both in animal models and humans, are resumed in Tables 1 and 2, respectively.

3.1. Gut Microbiota Profile and Driven Mechanisms Associated with NAFLD

3.1.1. Experimental Data. Experimental data have addressed the role of gut microbiota in the regulation of immune balance, low-grade inflammation, gut permeability, and lipid metabolism on NAFLD.

Due to its anatomical links to the gut, the liver is constantly exposed to gut-derived bacterial products. Immune cells like Kupffer cells recognize molecular pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors, for example, toll-like receptors (TLR), thereby playing an important role in the protection against systemic bacterial [32].

Dietary fructose intake is associated with NAFLD development [33]. In a fructose-induced NAFLD mice model, hepatic steatosis was associated with a significant induction of TLR 1–4 and 6–8 [31]. Fructose-fed animals also had significantly higher number of F4/80 positive cells, a macrophages marker, and lower protein concentration of occludin, a tight junction protein [31]. Furthermore, the activation of TLRs is associated with an increase in levels of endotoxemia, produced by Gram-negative bacteria and lipopolysaccharides (LPS), emphasizing their role in intestinal permeability regulation and bacterial translocation [20]. Basically, LPS and other microbial components, in the intestine, bind to the specific receptor-activating TLRs signaling, triggering the

TABLE 1: Clinical studies on NAFLD and gut microbiota in humans.

Study patients and methodology	Outcomes	Reference number
Randomized controlled trial of 38 patients, 16 NASH patients (7 supplemented with probiotic versus 9 usual care group) versus 22 controls	NASH patients had lower fecal abundance of <i>Faecalibacterium</i> and <i>Anaerosporeobacter</i> but higher abundance of <i>Parabacteroides</i> and <i>Allisonella</i>	[14]
Cross-sectional study of 63 children, 16 controls versus 25 obese versus 22 NASH patients	Proteobacteria/Enterobacteriaceae/ <i>Escherichia</i> was similarly represented between healthy and obese microbiomes but was significantly elevated in NASH	[15]
Cross-sectional study of 60 patients, 30 NAFLD patients versus 30 controls	Lactobacillus and selected members of phylum Firmicutes (<i>Dorea</i> , <i>Robinsoniella</i> , and <i>Roseburia</i>) were higher in NAFLD patients; Oscillibacter was underrepresented	[16]
In-patient study of 15 female subjects placed on well-controlled diets in which choline levels were manipulated	Variations between levels of Gammaproteobacteria and Erysipelotrichi were directly associated with changes in liver fat in each subject during choline depletion	[17]
Randomized controlled trial of 48 children with NAFLD-22 supplemented with VSL#3 versus 22 placebos	A 4-month supplementation with VSL#3 improved NAFLD in children	[18]
Randomized controlled trial of 66 patients with NAFLD-34 supplemented with <i>Bifidobacterium longum</i> with fructooligosaccharides (FOS) and lifestyle modification versus 32 lifestyle modifications alone	<i>Bifidobacterium longum</i> with FOS and lifestyle modification significantly reduces endotoxin, hepatic steatosis, and NASH activity index	[19]

activation of pro-IL-1 β and pro-IL-18, which are processed into their active forms, and subsequently induce inflammation and fibrosis [20]. Taken together, these data support the notion that the onset of fructose-induced-NAFLD may be linked to an increase in intestinal translocation of microbial components, related to increased intestinal permeability and also dependent on an inflammatory response, through the increase of F4/80 positive cells and induction of several TLRs [20, 31, 33].

Inflammasomes are cytoplasmic multiprotein complexes consisting of caspase 1, PYCARD, NALP, and sometimes caspase 5, which act like sensors of endogenous or exogenous PAMPs or damage-associated molecular patterns (DAMPs). They regulate the activation of effector proinflammatory cytokines, such as pro-IL-1 β and pro-IL-18, and are expressed in myeloid cells and are a component of the innate immune system [21]. The exact composition of an inflammasome depends on the activator, which initiates inflammasome assembly; for example, dsRNA will trigger one inflammasome composition whereas asbestos will assemble a different variant. The inflammasome promotes the maturation of the inflammatory cytokines Interleukin 1 β (IL-1 β) and Interleukin 18 (IL-18). Thus, the inflammasome is responsible for activation of inflammatory processes and has been shown to induce cell pyroptosis, a process of programmed cell death distinct from the immunologically silent death mechanism that characterizes apoptosis. Pyroptosis is an intriguing inflammasome-mediated host defense mechanism, which prevents intracellular replication of pathogens, by releasing their intracellular content into circulation and therefore targeting the destruction of surviving bacteria by phagocytes and neutrophils. Different animal models reveal that inflammasome deficiency-associated changes in the gut microbiota composition were associated with exacerbated hepatic steatosis and inflammation through the influx of TLR4 and TLR9

agonists into the portal circulation. Subsequently, hepatic TNF- α expression was enhanced, inducing NASH progression [21]. Porphyromonadaceae was found to be increased in inflammasome-deficient mice and associated with exacerbated hepatic steatosis and inflammation [21]. le Roy et al. had also showed higher concentrations of Porphyromonadaceae in a mouse model of hepatic steatosis [22].

The microbiota also regulates energy and lipid metabolism, directing the host to a rapid increase in body fat content, despite reduced chow consumption, and to increase hepatic production of triglycerides [11]. Conventionalization of germ-free mice promoted absorption of monosaccharides and short chain fatty acids by fermentation and thus increased *de novo* hepatic lipogenesis and fat storage, by increasing liver carbohydrate response element binding protein (ChREBP) mRNA and regulating lipoprotein lipase activity [11]. Additionally, it seems that saturated fat stimulates hepatic steatosis and affects gut microbiota composition by an enhanced overflow of dietary fat to the distal intestine [23]. A saturated fat diet based on palm oil increased liver fat accumulation, reduced microbial diversity, and increased the Firmicutes-to-Bacteroidetes ratio [23]. A diet-induced elevation of lipid metabolism-related genes in the distal small intestine was also observed confirming the overflow of palm oil to the distal intestine [23]. Interestingly, a recent study has demonstrated that gut microbiota markedly impacts the lipid metabolism in the liver, independently of obesity [22].

The steatosis that first characterizes NAFLD may progress toward steatohepatitis, fibrosis, and cirrhosis [34]. To understand if gut microbiota is associated with liver fibrosis, a microbiota modification was induced by the creation of a bile duct ligation in high-fat mice models, taking into account that the bile acids have antimicrobial properties [20]. This process leads to decreased hepatic triglyceride (TG) content and increased fibrosis, thus allowing the correlation of microbiota

TABLE 2: Experimental studies on NAFLD and gut microbiota in mice.

Model	Outcome	Reference no.
High-fat diet- (HFD-) fed mice versus controls subjected to bile duct ligation (BDL) or hepatotoxin CCl ₄	HFD mice subjected to BDL had an increase of Bacteroidetes, Firmicutes, and Proteobacteria	[20]
Methionine-choline-deficient diet-fed mice versus HFD-fed mice	Inflammasome deficiency-associated changes in gut microbiota were associated with exacerbated hepatic steatosis and inflammation	[21]
HFD-fed germ-free mice colonized with intestinal microbiota from a responder donor (developed hyperglycaemia and higher proinflammatory cytokines) or a nonresponder	Responder-receiver developed hepatic macrovesicular steatosis and harbour distinct gut microbiota	[22]
Low-fat diet based on palm oil (LFD-PO) fed mice versus HFD based on palm oil (HFD-PO) versus olive oil (HFD-OO) versus safflower oil (HFD-SO)	The HFD-PO diet induced higher liver triglyceride content, reduced microbial diversity, and increased the Firmicutes-to-Bacteroidetes ratio	[23]
HFD-fed mice versus low-fat diet-fed mice	Quantitative variation in dietary choline induced an inverse quantitative variation in liver fat content; conversion of choline into methylamines by microbiota in mice on a HFD caused NAFLD	[24]
HFD-fed mice versus HFD supplemented with chitin-glucan (CG) versus controls	CG treatment significantly decreased hepatic triglyceride accumulation, which was negatively correlated with specific bacteria of clostridial cluster XIVa, that is, <i>Roseburia spp.</i>	[25]
High-fructose diet-fed mice supplemented with <i>Lactobacillus rhamnosus</i> GG (LGC) versus controls	Supplementation with LGC reduced liver fat accumulation and increased intestinal Firmicutes and Bacteroidetes	[26]
Methionine-choline-deficient-diet-fed mice (MCD) versus MCD-fed mice supplemented with <i>Lactobacillus casei</i> strain Shirota (LcS) versus controls	<i>Bifidobacterium</i> and <i>Lactobacillus</i> were markedly reduced by the MCD diet. Administration of LcS increased the <i>L. casei</i> subgroup and other lactic acid bacteria	[27]
HFD-fed rats supplementation with an herbal formula (HF) versus no supplementation versus controls	Supplementation of HF decreased hepatic steatosis; <i>Escherichia/Shigella</i> were enriched in HFD-fed rats but decreased to control levels after HF treatment	[28]
HFD-fed mice supplemented with <i>Bacteroides uniformis</i> CECT7771 versus controls	Supplementation with <i>Bacteroides uniformis</i> reduced NAFLD in HFD-mice; HFD resulted in marked changes in gut microbiota, partially restored by the intervention	[29]
N-3 PUFA-depleted diet-fed mice supplemented with fructooligosaccharides (FOS) versus controls	Supplementation with FOS reverses NAFLD induced by n-3 PUFA-depleted diet; FOS-treated mice exhibited higher caecal <i>Bifidobacterium spp.</i> and lower <i>Roseburia spp.</i> content and reduced hepatic triglyceride accumulation	[30]
Fructose-fed mice versus controls treated or not with antibiotics	Hepatic fat accumulation was associated with a significant induction of TLR 1–4 and 6–8. The effects of fructose were attenuated in antibiotic-treated mice. No systematic alterations of microbiota were found	[31]

to fibrosis and not to potential effects of fat liver content [20]. In this model, an increase in percentage of Gram-negative versus Gram-positive bacteria was also observed, a reduced ratio between Bacteroidetes and Firmicutes, as well as a dramatic increase of Gram-negative Proteobacteria. To further support the role of microbiota in liver fibrosis, high-fat-diet (HFD) microbiota was transplanted to control mice, resulting in an increase in liver injury [20].

3.1.2. Clinical Data. Some studies have documented small intestinal bacterial overgrowth (SIBO) in NAFLD and NASH patients, suggesting that increased exposure to intestinal bacterial products may contribute to their pathogenesis [35, 36]. Furthermore, hepatic steatosis has been associated with increased permeability caused by disruption of intercellular

tight junctions in the intestine, which was linked to SIBO [36]. Since then, growing lines of evidence have suggested that NAFLD patients are characterized by different gut microbiota composition, termed fecal dysbiosis.

To determinate the association between fecal microbiota and hepatic steatosis Wong et al. have analyzed fecal microbiota from 16 NASH patients and 22 controls [14]. NASH patients had lower fecal abundance of *Faecalibacterium* and *Anaerosporebacter*, but higher abundance of *Parabacteroides* and *Allisonella*. However, intrahepatic triglyceride content improvement was generally associated with a reduction in the abundance of Firmicutes and increase in Bacteroidetes, which reflects the contradictory data that still exists regarding the association between gut microbiota profile and hepatic steatosis.

In line, NASH children had unique characters in the composition, ecological diversity, and enterotyping patterns of gut microbiome [15]. However, when comparing NASH children, with healthy subjects and obese patients, fewer differences were observed between obese and NASH microbiomes. Among taxa with greater than 1% representation, Proteobacteria, Enterobacteriaceae, and *Escherichia* were the only phylum, family, and genus showing significant difference between obese and NASH microbiome [15]. *Escherichia*, under anaerobic conditions, is capable of converting sugars to a mixture of products by fermentation, including ethanol [37]. NASH patients had elevated blood alcohol, compared to healthy subjects and obese patients [15]. Taking into account that intestinal microflora is the major source of endogenous alcohol [38], these data have supported the hypothesis that elevated representation of alcohol-producing bacteria in NASH microbiome may cause liver inflammation in NASH by a constant supply of reactive oxygen species to the liver. However, Raman et al. failed to demonstrate an increase in *Escherichia* abundance in NAFLD patients and ethanol was identified as a ubiquitous fecal volatile organic component (VOC) in both obese NAFLD adults and healthy controls; besides ester VOC were more frequently present in fecal samples from obese NAFLD patients [16]. Nevertheless, neither blood nor breath alcohol concentrations were measured and population was limited to obese NAFLD and healthy subjects, turning difficult to conclude if the observed differences in ester VOC were a consequence of NAFLD rather than obesity [16].

Low-choline diets have been associated with NAFLD [24]. In this context, it was demonstrated that gut microbiota composition changes according to dietary choline levels. During choline depletion, the levels of Gammaproteobacteria and Erysipelotrichi were directly associated with liver fat content in each subject [17]. Thus, a model was created that accurately predicted the degree to which subjects developed fatty liver on a choline-deficient diet, taking into account these bacteria levels and changes in amount of liver fat and a single nucleotide polymorphism that affects choline. Taking into account that Gammaproteobacteria and Erysipelotrichi are Gram-negative bacteria, containing LPS, which was previously described as an inducer of chronic inflammation that characterize metabolic dysfunction, insulin resistance, and diabetes [39], it can therefore be hypothesized that LPS may contribute to NAFLD development in these patients.

3.2. Pre- and Probiotics Intervention and Their Mechanism of Action in NAFLD

3.2.1. Experimental Data. Prebiotics and probiotics are known modulators of gut microbiota. The role of intestinal microbiota in NAFLD has garnered significant attention, by demonstration of the beneficial effects of pre- or probiotics administration in NAFLD models. This finding is sustained at different levels, including gut microbiota profile, gut barrier function, LPS and low-grade inflammation, lipid metabolism, and energy balance.

Supplementation of a HFD with fungal chitin-glucan (CG) decreased hepatic triglyceride accumulation and

restored the number of bacteria from clostridial cluster XIVa including *Roseburia spp.*, which were decreased due to HFD [25]. CG treatment also significantly decreased HFD-induced body weight gain, fat mass development, fasting hyperglycemia, glucose intolerance, and hypercholesterolemia, independently of the caloric intake. These beneficial effects were correlated with specific bacteria of clostridial cluster XIVa, that is, *Roseburia spp.*, and did not appear to be mediated by incretin glucagon-like peptide 1 (GLP-1) [25].

Lactobacillus rhamnosus GG (LGC) supplementation in high-fructose-induced NAFLD mice had strongly reduced liver fat accumulation [26]. The fat liver content improvement by LGC was associated with manipulation of gut microbiota: LGC have increased the total numbers of Firmicutes and Bacteroidetes; LGC attenuated the expression of the proinflammatory cytokines TNF- α and IL-1 β and IL-8R in the liver. To determine whether changes in portal LPS levels and intestinal inflammation were associated with changes in intestinal barrier, the levels of occludin and claudin-1, tight junction proteins, were measured [26]. Occludin and claudin-1 expression was reduced in mice fed high-fructose diet compared to control diet and restored after LGC supplementation. These data support the hypothesis that the associated beneficial effects of increased members of the Firmicutes are due to the fact that they produce butyrate, which is known to regulate gut barrier function [40].

LPS role was further supported by other experimental studies. *Lactobacillus casei* strain (LcS) administration suppressed LPS elevation and protected against methionine-choline-diet-induced NASH development in a mice model [27]. Thus, gut modulation by LcS administration may contribute to the normalization of tight junction proteins, protects against impairment of gut permeability, and subsequently diminishes inflammation and reverse hepatic steatosis. Furthermore, treatment with a Chinese herbal formula (CHF) supplementation ameliorated NAFLD and resulted in a reduction in *Escherichia/Shigella* levels, Gram-negative bacteria containing LPS in their cell walls that may impair the gut barrier and trigger a low-grade chronic inflammation state [28]. CHF supplementation increased *Collinsella*, short chain fatty acid (SCFA) producers [28]. SCFA may also be responsible for the beneficial effects of CHF treatment, as SCFA can stimulate epithelial cell proliferation, which may improve gut barrier integrity [41].

Immune modulation was also observed after oral administration of *Bacteroides uniformis* CECT 7771, which reduced liver steatosis in HFD-fed mice, improved immune defense mechanisms on macrophages and dendritic cells, and reduced the gut inflammatory signals [29].

To support the effects of gut microbiota in modulation of fat storage and host metabolism, the expression of ChREBP, a transcription factor required for glucose-induced expression of the lipogenic genes acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS), as well as that of ACC2 and FAS was found to be significantly increased in mice fed with fructose-rich diet and significantly reduced after LGC [26]. Moreover, in another mice model of NAFLD, fructooligosaccharides (FOS) supplementation reduced hepatic triglyceride accumulation through changes in microbiota composition, thus

leading to an increase in GLP-1, which stimulates fatty acid oxidation by peroxisome proliferator-activated receptor- α and lessened cholesterol accumulation by inhibiting sterol regulatory element binding proteins (SREBPs) [30].

3.2.2. Clinical Data. In human, few prospective, randomized, and controlled clinical trials have yet been designed to address the potential role of intestinal microbiota in NAFLD and the potential beneficial effects from modulation of gut microbiota, by pre- or probiotics intervention.

VSL#3 is a mixture of eight probiotic strains (*Streptococcus thermophilus*, *bifidobacteria* [*B. breve*, *B. infantis*, *B. longum*], *Lactobacillus acidophilus*, *L. plantarum*, *L. paracasei*, and *L. delbrueckii subsp. bulgaricus*) [18]. In children, a 4-month supplementation with VSL#3 has improved NAFLD [18]. Therefore, it is conceivable that the effects of VSL#3 in these patients could be dependent on the restoration of normal gut microbiota. Also, circulating levels of GLP-1, both in total and in active form, have significantly increased after the 4-month supplementation, which may have improved fat metabolism. GLP-1 is an incretin secreted by L-cells in the small intestine in response to food intake, whose main roles are stimulation of glucose-dependent insulin secretion, inhibition of postprandial glucagon release, delay of gastric emptying, and induction of pancreatic β -cell proliferation [42]. Besides improving hepatic glucose metabolism, GLP-1 seems to be a novel target against NAFLD, by increasing fatty acid oxidation, decreasing lipogenesis, and improving hepatic glucose metabolism [42], and may also be an active intervenient, establishing the link between NAFLD and gut microbiota.

Treatment with *Bifidobacterium longum* plus fructooligosaccharides (Fos) reduced HOMA-IR and NASH activity in association with reduced endotoxin, C-reactive protein, and TNF- α levels [19]. These data further support the hypothesis that endotoxin-induced activation of macrophages plays a key role in the pathogenesis of liver injury in NAFLD patients.

4. Discussion

NAFLD is an emerging complex multifactorial disease resulting from the interaction of genetic, environmental, metabolic, and inflammatory factors. Both obesity and diabetes are major risk factors for NAFLD [43]. As it has been previously described, the gut microbiota exerts a profound influence on fat deposition, being a key regulator of energy storage [11, 44].

Germ-free mice colonized with gut microbiota from obese animals showed body fat mass and liver triglyceride content and an insulin resistance increase. Microbiota promoted absorption of monosaccharides from the gut lumen, with resulting induction of *de novo* hepatic lipogenesis, by increased activity of acetyl-CoA carboxylase and fatty acid synthase [11]. In humans, this relationship is further reinforced by the demonstration of the relative fewer proportion of Bacteroidetes in obese people by comparison with lean people and the shift toward higher relative abundance of Bacteroidetes and decreased number of Firmicutes in obese patients losing weight through low-calorie diets [9]. These named "obese microbiomes" have increased capacity of

harvesting energy from food, resulting in fat accumulation. However, the relationship between Bacteroidetes and Firmicutes levels and obesity and associated metabolic disturbances is still controversial.

According to what has been described in Section 3, differences in gut microbiota profile may also have impact on the liver, on the background of obesity and insulin resistance. Most of the available data demonstrating this association is based on association studies, lacking human intervention studies, which would further improve the knowledge of gut microbiota influence on NAFLD.

As it has been described, insulin resistance is a common feature of metabolic syndrome and NAFLD. Thus, the decrease of the inhibitory effects of insulin on peripheral lipolysis increases the availability of free fatty acids, playing a critical role in the development of fatty liver [45]. Metabolic endotoxemia triggers insulin resistance, obesity, and diabetes, through LPS, which in combination with CD14 serves as ligand for TLR [46]. LPS and other endotoxins also can activate TLRs, inducing an inflammatory response, linked to hepatic fat accumulation [20, 21, 31, 32]. An interesting finding was the observation that small intestinal bacterial overgrowth predicted severe hepatic steatosis [47]. In fact, bacterial overgrowth may increase intestinal permeability, by disruption of intercellular tight junctions, subsequently exposing liver surface to bacterial products, resulting in hepatic fat deposition [36].

An additional contributor is the modulation of choline metabolism by intestinal microbiota. Choline and methionine-deficient diets have been associated with hepatic steatosis [17, 48]. The gut microbiota catalyzes the conversion of choline to dimethylamine and trimethylamine [49]. A high-fat diet in a mice model susceptible to impaired glucose homeostasis and NAFLD reduces the bioavailability of choline, mimicking the effect of choline-deficient diets [24]. These results establish a possible association between choline bioavailability and hepatic steatosis, through metabolic activity of gut microbiota, which is affected by diet. In addition, Gammaproteobacteria and Erysipelotrichi levels were associated with hepatic steatosis, during choline depletion [17]. As these bacteria are Gram-negative, these data further support the role of LPS as an active player on NAFLD development.

Endogenous production of ethanol by bacteria also seems to mediate hepatic fat accumulation. In an obese mouse model, in the absence of ethanol ingestion, ethanol was detected in exhaled breath [50]. Hence, intestinal production of ethanol may contribute to the genesis of obesity-related fatty liver, triggering inflammatory signals [15].

Interventional studies with pre- and probiotics gave further support to the possible effects of intestinal microbiota modulation on NAFLD pathogenesis. Besides the impact on fat storage and host metabolism, GLP-1 may be an important contributor, linking NAFLD, insulin resistance, and gut microbiota. FOS supplementation in a mice model of hepatic steatosis reduced fatty liver accumulation, through changes in gut microbiota, responsible for GLP-1 increase [30]. GLP-1 stimulated fatty acid oxidation by peroxisome proliferator-activated receptor- α and inhibited SREBPs [30]. In children, VSL#3 supplementation improved NAFLD and had

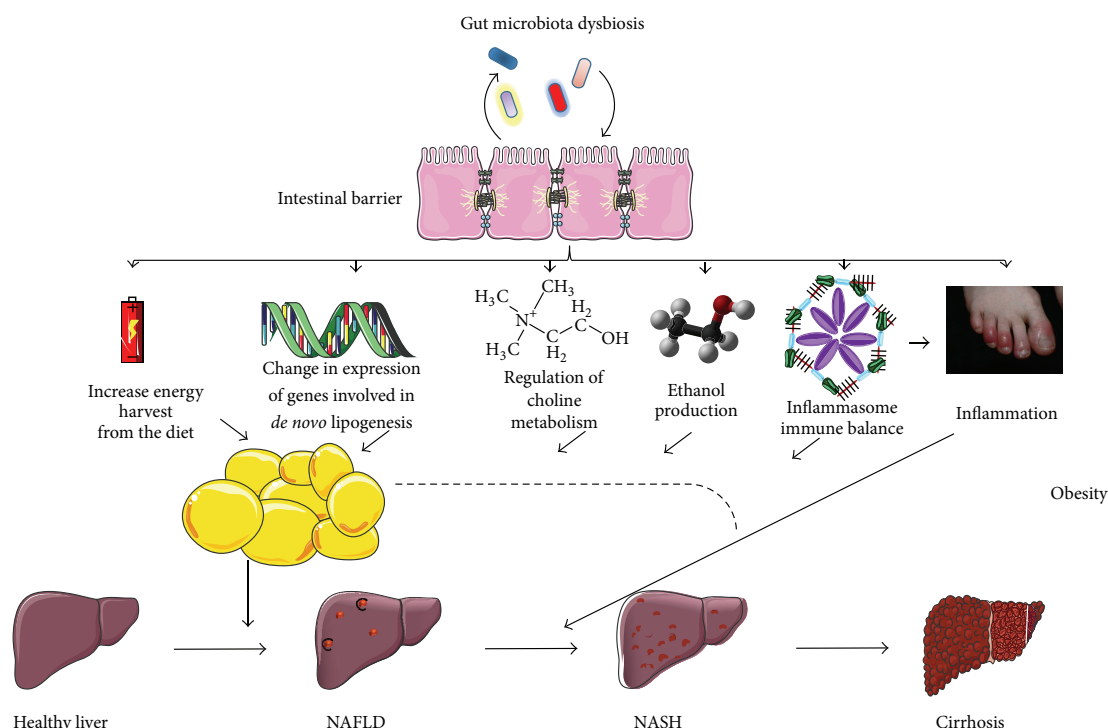


FIGURE 3: Possible pathways involved in NAFLD pathogenesis, by gut microbiota.

increased GLP-1, supporting the impact of gut microbiota modulation on fat metabolism [18].

Therefore, intestinal microbiota, beyond its capacity to regulate body fat gain and insulin resistance, seems to play a fundamental role on NAFLD, through different pathways (Figure 3), including

- (i) increasing energy harvest from diet,
- (ii) change in expression of genes involved in *de novo* lipogenesis,
- (iii) regulation of choline metabolism,
- (iv) ethanol production,
- (v) inflammasome and innate immunity,
- (vi) inflammation.

However, the majority of studies were conducted under experimental conditions, namely, under fat rich diets, which limits the demonstration of a definitive role of gut microbiota in hepatic steatosis, especially in NAFLD nonobese patients. Further comprehension of the relationship between gut microbiota and hepatic steatosis will allow the development of new specific targets and integrated strategies to modulate intestinal microbiota, including prebiotics and probiotics, in order to improve or even cure this prevalent metabolic disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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4.3 Paper 3

Eva Lau, Davide Carvalho, Paula Freitas

Dysbiosis and microbiota in obesity, T2DM and NAFLD

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Disbiose e microbioma na obesidade, diabetes tipo 2 e esteatose hepática não alcoólica

Eva Lau

Davide Carvalho

Paula Freitas

RESUMO

O microbioma intestinal tem sido proposto como regulador do metabolismo energético, assumindo-se como fator preponderante na fisiopatologia da obesidade, insulinoresistência e esteatose hepática. Desse modo, sua modulação poderá contribuir para a regulação da disbiose intestinal que caracteriza esses distúrbios metabólicos e, conseqüentemente, do metabolismo glicídico e lipídico e reversão do estado inflamatório sistêmico que caracteriza essas complicações.

INTRODUÇÃO

A obesidade é um problema de importância crescente em nossa sociedade, sendo considerada pela Organização Mundial da Saúde a epidemia global do século XXI. Em 2014, estimou-se que, globalmente, 39% dos adultos eram obesos ou apresentavam excesso ponderal, o que representa o dobro da população atingida em 1980.¹

A obesidade é um fator de risco preponderante para complicações metabólicas, nomeadamente diabetes melito tipo 2 e esteatose hepática não alcoólica. A par do aumento da prevalência da obesidade, a prevalência do diabetes quadruplicou para 422 milhões de adultos em 2014.² A esteatose hepática não alcoólica, por sua vez, representa a expressão hepática da síndrome metabólica, pelo que se compreende que sua prevalência aumento, paralelamente à tendência dos outros distúrbios metabólicos.³

O trato gastrointestinal contém uma biodiversidade de bactérias que codificam cerca de 150 vezes mais genes, comparativamente ao genoma humano eucariótico.⁴ O intestino destaca-se por concentrar um maior número de bactérias, isto é, até 100 trilhões de microrganismos, sendo composto majoritariamente por bactérias pertencentes aos filos Bacteroidetes e Firmicutes.⁵ Este designado “órgão microbiano” é reconhecido por desempenhar diversas funções, desde proteção até regulação metabólica, incluindo um papel crucial no metabolismo glicídico e lipídico, o que pode constituir um dos elos de ligação entre obesidade, diabetes e esteatose hepática não alcoólica.

MICROBIOMA NA OBESIDADE, DIABETE E ESTEATOSE HEPÁTICA NÃO ALCOÓLICA

Em condições fisiológicas, a composição do microbioma intestinal é estável. O termo disbiose designa a perturbação desse equilíbrio, o que se pode traduzir em alterações da regulação do metabolismo do hospedeiro. A obesidade se associa a uma redução da proporção de Bacteroidetes e aumento proporcional de Firmicutes e a perda de peso resulta na inversão dessa razão.^{4,6} Para além disso, menor biodiversidade bacteriana parece corresponder a maior ganho ponderal ao longo do tempo.⁷ Num programa de restrição energética aplicado a doentes obesos, verificou-se ainda que os que apresentavam maior número de *Lactobacillus/Leuconostoc/Pediococcus* ganhavam mais rapidamente o peso perdido, o que poderá abrir portas para a predição da evolução ponderal, com base no perfil do microbioma intestinal.⁸

O perfil em diabéticos ainda não reúne total consenso, apesar de alguns agentes oportunistas terem se associado, nomeadamente *Bacteroides caccae*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium symbiosum*, *Eggerthella lenta* e *Escherichia coli*.⁹ Nesse estudo, verificou-se, no entanto, que apenas $3,8 \pm 0,2\%$ dos genes codificados pelo microbioma se associavam ao diabetes, de onde emergiu o conceito de “disbiose funcional”, em vez de apenas se considerarem as alterações do conteúdo do microbioma como condicionantes do diabetes. Um modelo que inclui agrupamentos metagenômicos permitiu prever o estado glicêmico, o que, a confirmar-se em estudos de maior escala, poderá permitir a adoção de estratégias preventivas com base no risco individual de alterações metabólicas.¹⁰

O perfil da flora parece influenciar o desenvolvimento de esteatose hepática, tendo-se verificado que o transplante fecal de doadores animais submetidos a dieta rica em gordura, para um grupo controle, resultava em lesão hepática. Um dos mecanismos propostos é o aumento da translocação bacteriana, mediado por aumento da permeabilidade intestinal, com condizente resposta inflamatória.^{11,12} Além disso, a síndrome do supercrescimento bacteriano no intestino delgado foi estabelecida como fator preditor de esteatose hepática grave¹³ (Figura 1).

As alterações do microbioma podem conduzir a um aumento do lipopolissacarídeo (LPS), um componente da parede celular das bactérias Gram-negativas, cuja translocação para circulação sistêmica é facilitada por diminuição da permeabilidade intestinal, decorrente de rotura das proteínas de junção intercelular do epitélio – ocludina e zonulina-1. Os elevados níveis de LPS ativam os macrófagos por meio do sistema CD14 acoplado ao ligante *toll-like receptors 4* (TLR4), desencadeando uma resposta inflamatória designada por endotoxemia metabólica com consequente desenvolvimento de insulinoresistência e promoção de acúmulo de gordura.¹⁴

O sistema endocanabinoide também parece regular a permeabilidade intestinal. Se, por um lado, o LPS regula a síntese de endocanabinoides em macrófagos, por outro, foi demonstrado que o bloqueio dos receptores endocanabinoides tipo 1 melhorava a função barreira intestinal ao diminuir as alterações das proteínas de junção intercelular, com consequente redução dos níveis de LPS e redução do estado pró-inflamatório crônico.^{15,16}

Além da endotoxemia metabólica, o microbioma é reconhecido por sua capacidade de extração energética de componentes não digeríveis da dieta que são fermentados em ácidos graxos de cadeia curta/AGCC. Estes induzem a expressão de acetilcoenzima A carboxilase e a síntese de ácidos graxos, enzimas-chave na lipogênese hepática, e bloqueiam a ativação da AMP quinase, envolvida na oxidação lipídica, o que contribui para a deposição de gordu-

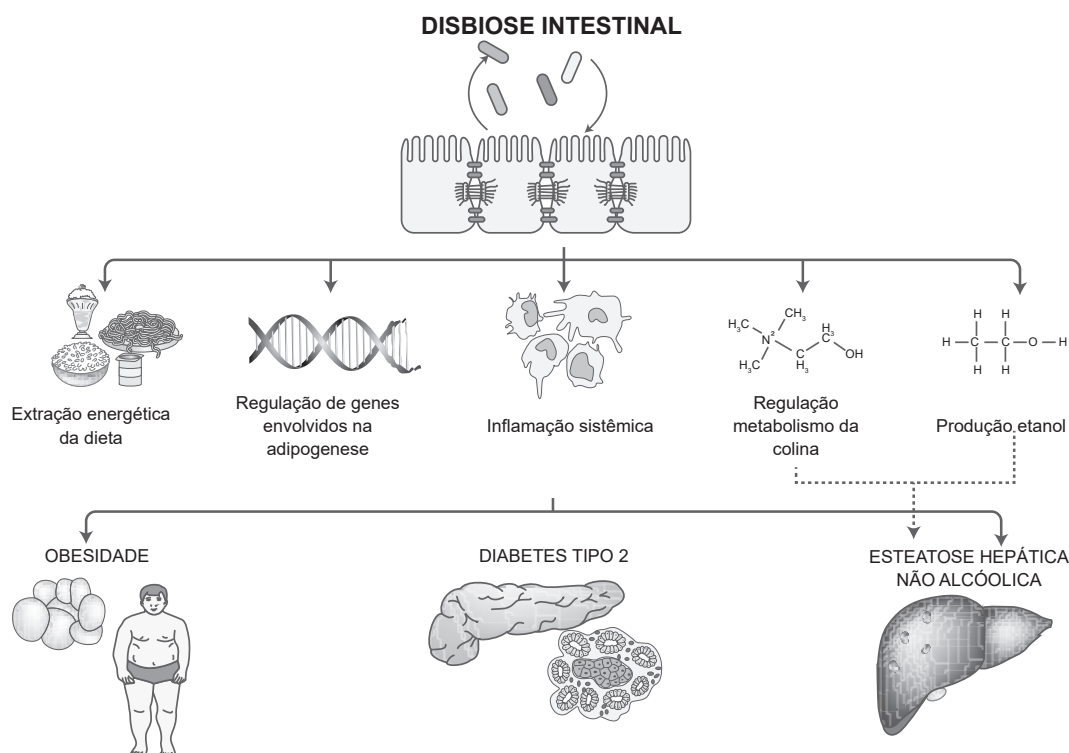


Figura 1 Mecanismos fisiopatológicos envolvidos na associação da disbiose intestinal com a obesidade, o diabetes e a esteatose hepática não alcoólica.

ra corporal. Simultaneamente, a disbiose intestinal suprime a expressão do fator adipocitário induzido pelo jejum, o que aumenta a atividade da lipoproteína lipase, permitindo a liberação de ácidos graxos dos triglicerídeos circulantes e favorecendo a adipogênese.¹⁷ A contribuir para a esteatose hepática, destaca-se ainda a depleção de colina e a produção de etanol bacteriano, que podem resultar das alterações do microbioma.^{18,19} Estratégias de intervenção terapêutica para estes distúrbios metabólicos são ilustradas na Figura 2.

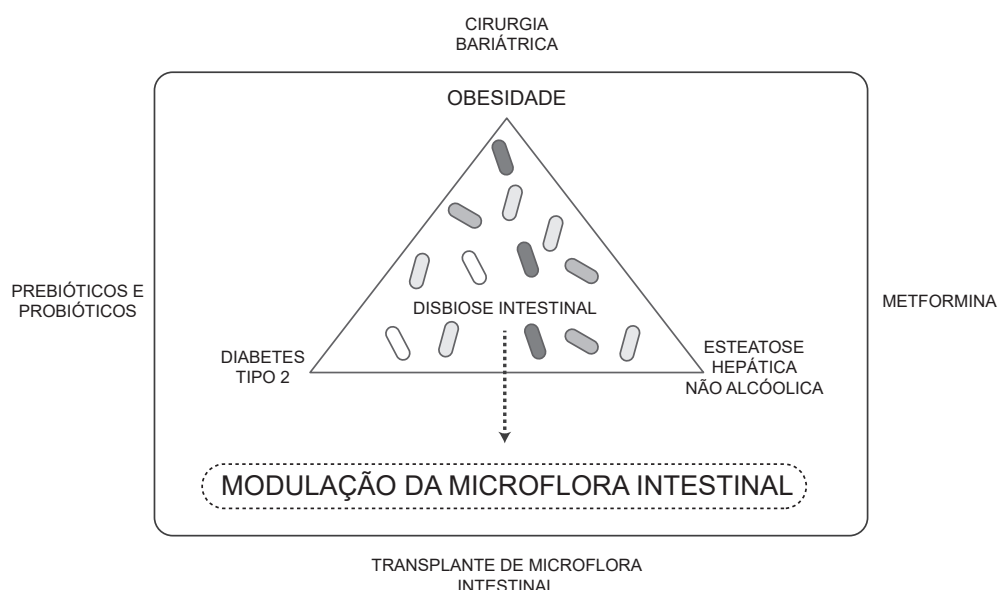


Figura 2 Estratégias de intervenção terapêutica na obesidade, diabetes e esteatose hepática não alcoólica com base na modulação da flora microbiana intestinal.

Intervenção terapêutica/prebióticos e probióticos

O termo prebiótico designa componentes não digeríveis da dieta, capazes de estimularem seletivamente a proliferação de populações bacterianas benéficas.²⁰ Os probióticos correspondem a microrganismos vivos não patogênicos, que também conferem vantagens ao hospedeiro.²¹ Podem afigurar-se parceiros importantes no tratamento do diabetes, obesidade e esteatose hepática não alcoólica.^{22,23}

Os efeitos metabólicos dos prebióticos derivam em parte da redução do estado inflamatório sistêmico e da regulação da adipogênese. A suplementação com oligofrutose demonstrou restauro dos níveis de *Bifidobacterium* num modelo de camundongos submetidos à dieta de cafeteria, com consequente redução da endotoxemia metabólica e da melhoria de parâmetros metabólicos, incluindo diminuição da massa gorda, da esteatose hepática e da melhoria da tolerância à glicose.^{24,25} O aumento dos níveis do peptídeo semelhante ao glucagon-2 (GLP-2), que desempenha um efeito trófico sob a parede intestinal, resulta na diminuição da permeabilidade intestinal e redução dos níveis de LPS, com menor resposta inflamatória e melhoria dos distúrbios metabólicos.²⁶

Além disso, o efeito benéfico dos prebióticos, nomeadamente diminuição do apetite, parece ser também mediado pela sua capacidade de regulação do peptídeo semelhante ao glucagon-1 (GLP-1), peptídeo YY e grelina. Destaca-se ainda sua capacidade de estimular a oxidação lipídica, o que se reflete em menor acúmulo de gordura, nomeadamente em nível hepático.

A administração de probióticos também se tem revelado eficaz no restauro do equilíbrio da microbiota intestinal. A suplementação com *Bifidobacterium* demonstrou melhoria de vários componentes da síndrome metabólica ao reduzir a concentração de LPS e a inflamação intestinal.²⁶ O aumento das proteínas de junção ocludina e claudina-1 poderá ser um dos mecanismos envolvidos, o que se verificou após a administração de *Lactobacillus rhamnosus*, com diminuição da expressão de citocinas proinflamatórias no fígado, como o TNF-alfa e a interleucina 8, e a redução do acúmulo de gordura hepática.²⁴ O *Lactobacillus rhamnosus* também comprovou o potencial dos probióticos de regular o armazenamento de gordura, tendo-se verificado que sua suplementação é capaz de diminuir a expressão do fator de transcrição ChREBP – proteína de ligação responsiva a carboidratos, necessária à expressão de enzimas-chave na adipogênese, isto é, acetil-coA-carboxilase 1 e sintase de ácidos graxos.²⁴

Recentemente verificou-se que a *Akkermansia muciniphila* se encontrava diminuída em obesos diabéticos e que sua administração diminuía a endotoxemia metabólica, além de reverter aumento da massa gorda e insulinoresistência num modelo de camundongos. O aumento dos endocanabinoides intestinais poderá ser um dos contribuintes para a melhoria da função barreira intestinal após a suplementação com esse probiótico.²⁷

Cirurgia bariátrica

Um dos mecanismos propostos para compreender a melhoria dos parâmetros metabólicos são as alterações do perfil microbiano intestinal após a cirurgia, não necessariamente vinculado à perda de peso. A proporção de Firmicutes está significativamente reduzida.²⁸ Verificou-se ainda que, após o procedimento cirúrgico, a redução de leptina se correlacionava inversamente com a concentração de *Escherichia coli* e *Bacteroides/Prevotella*, que aumentou, e, positivamente com *Bifidobacterium* e *Lactobacillus/Leuconostoc/Pediococcus*, que

experimentaram redução. A espécie *Faecalibacterium prausnitzii*, reduzida em obesos diabéticos, aumentou após a cirurgia e seus níveis se encontravam inversamente correlacionados com interleucina 6 e proteína C reativa de alta sensibilidade, conhecidos marcadores inflamatórios.²³ Desse modo, o aumento dos níveis dessa população bacteriana, que se revelou independente do aporte alimentar, poderá traduzir o potencial terapêutico da *Faecalibacterium prausnitzii* na redução do estado inflamatório crônico associado a obesidade e diabete.

A cirurgia bariátrica, a par das alterações do microbioma, aumenta os níveis de GLP-1, ao reduzir os níveis da enzima dipeptidase tipo 4.³⁰ Apesar de ainda não se ter comprovado que o perfil microbiano poderia ser responsabilizado, tal poderá se confirmar. O aumento da biodiversidade microbiana, após a cirurgia, também poderá estar na base da perda sustentada de peso, e melhoria metabólica associada.³¹ O transplante da microflora intestinal, proveniente de humanos submetidos a esse tipo de cirurgia, para camundongos, conduziu a redução da deposição de gordura e quociente respiratório, o que indica menor utilização de glicídios como fonte energética.³²

Transplante de microflora intestinal

O transplante de microbioma para roedores desprovidos de flora (*germ-free*) promove aumento da gordura corporal e da insulinoresistência.¹⁷ (17)absorption, or storage are crucial given the worldwide epidemic of obesity. The gut microbial community (microbiota. Pacientes com síndrome metabólica receberam, de forma randomizada, transplante das próprias fezes (controles) ou de humanos magros saudáveis. Após 6 semanas, verificou-se melhoria da insulinoresistência, com aumento da biodiversidade, e de bactérias produtoras de butirato, que poderiam prevenir a translocação bacteriana e impedir a endotoxemia metabólica.³³

Metformina

A metformina é um antidiabético oral insulinoresistente que suprime a neoglicogênese hepática e melhora a captação periférica de glicose em nível hepático e no músculo esquelético. Recentemente, a modulação do microbioma intestinal foi apontada como fator interveniente.³⁵ Em camundongos sob dieta rica em gordura tratados com metformina durante 6 semanas, verificou-se aumento da população de *Akkermansia muciniphila* e, simultaneamente, melhoria do perfil glicêmico. Ao mesmo tempo, verificou-se aumento dos níveis de mucina produzida pelas células caliciformes, o que poderá traduzir uma melhoria da função de barreira intestinal, justificando, conjuntamente com os mecanismos já conhecidos desse antidiabético, a melhoria da sensibilidade à insulina. A administração oral de *Akkermansia muciniphila* a esse modelo melhorou, também, a tolerância à glicose, e atenuou a inflamação no tecido adiposo. Esses dados abrem portas à possibilidade de manipulação farmacológica, com o sentido de aumentar a população dessas bactérias para o tratamento do diabetes tipo 2 e distúrbios relacionados.

CONSIDERAÇÕES FINAIS

O conhecimento cada vez mais aprofundado e a identificação de genes regulados pelo microbioma intestinal permitirá o desenvolvimento de novas estratégias, com base na mo-

dulação e restauro do seu equilíbrio. Poderá ainda permitir a previsão do risco de desenvolvimento dessas complicações metabólicas e a adoção de estratégias individuais de acompanhamento e prevenção desses distúrbios.

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5. ORIGINAL RESEARCH:

**Experimental study, Randomized
Clinical Trial and Big Data analysis**

5.1 Paper 4

Eva Lau, Cláudia Marques, Diogo Pestana, Mariana Santoalha, Davide Carvalho, Paula Freitas, Conceição Calhau

The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity

Nutr Metab 2016;13(31):1-7

RESEARCH

Open Access



The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity

Eva Lau^{1,2,3†}, Cláudia Marques^{3,4†}, Diogo Pestana^{3,4}, Mariana Santoalha³, Davide Carvalho^{1,2,5}, Paula Freitas^{1,2,5} and Conceição Calhau^{3,4,6*}

Abstract

Background: Intestinal fatty-acid binding protein (I-FABP) is expressed in epithelial cells of the mucosal layer of the small intestine tissue. When intestinal mucosal damage occurs, I-FABP is released into the circulation and its plasma concentration increases. In the context of obesity, the gut barrier integrity can be disrupted by dietary fat while intestinal permeability increases.

Objective: To investigate whether intestinal fatty acid binding protein (I-FABP) is a suitable plasma marker of intestinal injury and inflammation in obesity.

Methods: Twelve male Wistar rats were randomly divided into two groups of six animals each: standard (St) and high-fat (HF) diet fed groups for 12 weeks.

Results: HF fed animals developed obesity, insulin resistance and seemed to present increased plasma levels of proinflammatory cytokines (MCP-1 and IL1 β). The gut microbiota composition of these animals was also altered, with lower number of copies of Bacteroidetes, Prevotella spp. and Lactobacillus spp., in comparison with those from St diet group. Fecal lipopolysaccharide (LPS) concentrations tended to be increased in HF fed animals. Intestinal expression of TLR4 seemed to be also increased in HF fed animals suggesting that HF diet-induced dysbiosis may be behind the systemic inflammation observed. However, in contrast to other intestinal inflammatory diseases, plasma I-FABP levels were decreased in HF fed rats whereas I-FABP expression in jejunum tended to be increased.

Conclusions: HF diet-induced obesity is characterized by dysbiosis, insulin resistance and systemic inflammation. In this context, plasmatic I-FABP should not be used as a marker of the intestinal barrier dysfunction and the low-grade chronic inflammatory status.

Keywords: Inflammation, Intestinal fatty acid binding protein, Intestinal permeability, Obesity, Gut microbiota

Background

The new concepts on the pathophysiology of obesity and insulin resistance highlight the role of intestinal microbiota and intestinal barrier in the development of these disorders [1, 2]. Microbiota seems to mediate obesity and associated metabolic disturbances through several

mechanisms including energy storage and metabolic inflammation [1, 3]. On the other hand, intestinal mucosa plays not only an important part in the absorption of vital nutrients, but also in anatomical/barrier and immune functions, preventing bacterial translocation. High-fat diet changes gut microbiota composition and increases intestinal permeability, by a mechanism associated with a reduced expression of epithelial tight junction proteins [4]. The altered intestinal barrier and the subsequent translocation of bacteria or bacterial products, namely lipopolysaccharide (LPS) are now recognized as key

* Correspondence: ccalhau@med.up.pt

[†]Equal contributors

³Departamento de Bioquímica, Faculdade de Medicina, Universidade do Porto, Porto, Portugal

⁴CINTESIS, Centro de Investigação em Tecnologias e Serviços de Saúde, Porto, Portugal

Full list of author information is available at the end of the article



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mediators of the low-grade inflammation state, which characterize metabolic disorders.

Intestinal fatty-acid binding protein (I-FABP) is an intracellular protein specifically and abundantly expressed in the epithelial cells of the mucosal layer of the small and large intestine tissue [5]. The location of I-FABP in the mature epithelium of villi facilitates its leakage into the circulation from enterocytes when intestinal mucosal damage occurs [5]. It has been shown that I-FABP is released into the circulation following small intestinal mucosal injury and its plasma concentration has been associated with small intestinal diseases - necrotizing enterocolitis and celiac disease [6–8]. Therefore, I-FABP has emerged as a possible non-invasive marker for evaluating gut wall integrity loss and inflammation. Defining new and early non-invasive markers of gut barrier dysfunction might be of great interest in order to manage a safe modulation of the intestinal microbiota before emergence of obesity and associated metabolic diseases.

The aim of this study was to investigate the role of I-FABP as a possible plasma marker of intestinal injury and inflammation and its relationship with microbiota dysbiosis in a high-fat diet-induced obesity Rat model.

Methods

Animals and housing

Twelve male Wistar rats were purchased from Charles River (Barcelona, Spain) and kept under controlled environmental conditions (22–24 °C and 12 h light/dark cycles), for at least 1 week before starting the experiments. Animals, 8 weeks of age, were randomly divided into two groups of six animals each: standard (St) and high-fat (HF) diet group. The diets were respectively “St” (Teklad 2014, Harlan Laboratories, Santiga, Spain) and “HF” with 45 % of energy from lipids and 17 % of energy from sucrose (D12451 Research Diets, New Brunswick, NJ, USA). Animals were subjected to different experimental conditions for a total of 12 weeks. The water and chow were supplied *ad libitum*. Food and beverage consumption and body weight were monitored weekly, to carefully characterize energy ingestion and weight gain.

At the end of the 12 weeks, food was removed 4–6 h before sacrifice and the animals were anesthetized with a mixture of ketamine (50 mg/kg) and medetomidine (1 mg/kg) and maintained with isoflurane. Meanwhile, using a Quantum/S bioelectrical impedance analyzer (RJL Systems, Akern SRL, Florence, Italy), the body composition of each rat was determined by bioelectrical impedance, according to the procedure already described in the literature [9]. Before perfusion of the vascular compartment with a saline solution (NaCl 0.9 %, w/v), blood was drawn from the left ventricle into tubes with or without heparin to obtain plasma and serum, respectively. Aliquots were frozen at –80 °C until further

analysis. Colon and jejunum were dissected, pat dried and snap-frozen in liquid nitrogen. Fresh fecal samples were collected directly from the colon of all animals and snap-frozen in liquid nitrogen. Both tissues and fecal samples were stored at –80 °C until further analysis.

Animal handling and housing protocols followed European Union guidelines (Directive 2010/63/EU) for the use of experimental animals in scientific research. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine of University of Porto.

Oral glucose tolerance test (OGTT)

After 7 weeks of treatment, rats were fasted over 5 h and a baseline blood draw from the saphenous vein was collected for plasma fasting glucose and insulin measurements. Animals were gavaged with a glucose solution of 2 g/kg body weight and blood droplets from the saphenous vein were collected to measure glycaemia thereafter at 30, 60, 90 and 120 min. Glucose levels were measured with Precision Xtra Plus test strips and an Optium Xceed device (Abbott Diabetes Care, Ltd., Maidenhead, UK). Plasma insulin levels were measured using a Rat/Mouse Insulin ELISA kit (Merck Milipore, Madrid, Spain). The homeostasis model assessment (HOMA) was used to calculate approximate insulin resistance using the formula: glucose (mg/dL) × insulin (ng/mL)/405.

Blood and biochemical analysis

Biochemical evaluation of plasma was performed at the end of the study at São João Hospital Center Clinical Pathology Department. Routine biochemical parameters were measured using conventional methods with an Olympus AU5400* automated clinical chemistry analyzer (Beckman-Coulter, Izasa, Porto, Portugal).

Plasma content in leptin, adiponectin, monocyte chemo-attractant protein-1 (MCP-1), I-FABP and glucagon-like peptide-2 (GLP-2) were determined using, respectively, Rat Leptin ELISA Kit (Merck, Milipore, Madrid, Spain), Human/Mouse/Rat Adiponectin Enzyme Immunoassay Kit (RayBiotech, Norcross, GA, USA), Rat MCP-1 ELISA Kit (RayBiotech, Norcross, GA, USA), Rat (FABP2) ELISA Kit (Shanghai Sunred Biological Technology Co., Ltd, Shanghai) and GLP-2 ELISA Kit (Merck, Milipore, Madrid, Spain). Serum interleukin-1 *beta* (IL-1 β) was determined by Luminex assay using custom Miliplex Rat Kits (Merck Milipore, Madrid, Spain), according to the manufacturer's protocols using the Luminex Xmap Multiplexing Technology platform.

Tissue RNA isolation and qRT-PCR

Total RNA from jejunum and colon was isolated using NZYol reagent (NZYTech, Portugal) according to

manufacturer's instructions. RNA samples were treated with DNase I (RQ1 RNase-free DNase; Promega, Portugal). cDNA was synthesized from 1 µg of treated mRNA with NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal). Quantitative real-time polymerase chain reaction (qRT-PCR) was run on Lightcycler96 (Roche Applied Science, Indianapolis, ID, USA). Cycling conditions were as follows: denaturation (95 °C for 10 min), amplification and quantification (95 °C for 10 s, annealing temperature for 10 s and 72 °C for 10 s, with a single fluorescence measurement at the end of the 72 °C for a 10-s segment) repeated for 45 cycles and a final melting step with a temperature ramp from 60 to 97 °C. Rat-specific primer sequences (Sigma-Aldrich, St. Louis, MO, USA) used are described in Table 1. The Cq values obtained were transformed into relative quantification data using the formula $2^{-(\Delta Cq)}$.

DNA extraction from stool

Genomic DNA was extracted and purified from stool samples using NZY Tissue gDNA Isolation Kit (NZYtech, Lisbon, Portugal) with some modifications. Briefly, feces (170–200 mg) were homogenized in TE buffer (10 mM Tris/HCl; 1 mM EDTA, pH 8.0) and centrifuged at 4000 x g for 15 min. The supernatant was discarded and the pellet was resuspended in 350 µL of buffer NT1. After an incubation step at 95 °C for 10 min, samples were centrifuged at 11000 x g for 1 min. Then, 25 µL of proteinase K were added to 200 µL of the supernatant for incubation at 70 °C for 10 min. The remaining steps followed manufacturer's instructions. DNA purity and quantification were assessed with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Microbial analysis of Rat stool by qRT-PCR

qRT-PCR was performed in sealed 96-well microplates using a LightCycler FastStart DNA Master SYBR Green kit and a LightCycler96 instrument (Roche Applied Science, Indianapolis, ID, USA). Primer sequences (Sigma-Aldrich, St. Louis, MO, USA) used to target the 16S

rRNA gene of the bacteria and the conditions for PCR amplification reactions were previously described in Marques C et al. [10]. To verify the specificity of the amplicon, a melting curve analysis was performed via monitoring SYBR Green fluorescence in the temperature ramp from 60 to 97 °C. Data were processed and analyzed using the LightCycler software (Roche Applied Science, Indianapolis, ID, USA). Standard curves were constructed using serial tenfold dilutions of bacterial genomic DNA, according to the following webpage <http://cels.uri.edu/gsc/cndna.html>. Bacterial genomic DNA used as a standard was obtained from DSMZ (Braunschweig, Germany). Genome size and the copy number of the 16S rRNA gene for each bacterial strain used as a standard was obtained from NCBI Genome database (www.ncbi.nlm.nih.gov). Data are presented as the mean values of duplicate PCR analysis.

Fecal LPS quantification

Quantification of LPS was performed using the Chromo-Limulus Amebocyte Lysate (Chromo-LAL) reagent (Associates of Cape Cod, Inc., Falmouth, MA, USA). Briefly, 1 mL of sterile saline solution (NaCl 0.9 %) was added to 100 mg feces, vortexed and centrifuged (10 min, 10000 g, 4 °C) twice. Total supernatant (fecal water) was filtered with 0.45 µm filter and then with 0.22 µm filter. Fecal water and Chromo-LAL (1:1) were incubated at 37 °C for 20 min and absorbance was read every 10 s at 405 nm.

Statistical analysis

Values are expressed as the arithmetic mean ± standard error of the mean (SEM). Given the small sample size, a non-parametric test (Mann-Whitney test) was used to analyze the differences between St and HF groups. Correlation between variables was established using two-tailed Pearson's correlation test. The differences were considered statistically significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism 6 statistical software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Energy and metabolic parameters

Energy intake was increased in the HF diet fed group when compared with St diet fed group (72.60 ± 0.15 vs. 57.9 ± 0.47 kcal/day, $P < 0.05$). Consequently, HF diet fed animals gained more weight during the 12 weeks of the study (205.5 ± 37.8 vs. 134.7 ± 22.5 g, $P < 0.05$) (Table 2). To assess whether these differences in weight gain were related to alterations in adiposity, we decided to evaluate the body composition of the animals of both groups. Our results showed that rats under HF diet had more body fat mass comparing to rats under St diet (206.3 ± 21.03 vs. 166.8 ± 21.26 , $P < 0.05$).

Table 1 Primer sequences and real-time PCR conditions used for gene expression analysis by qRT-PCR

Gene name	Primer Sequence (5'-3')	AT
I-FABP	ATGGAAAGGAGCTGATTGCT	59 °C
	TTGGCTCTCCACTCTTCATA	
TLR4	GATGCCTCTCTGCATCTGG	60 °C
	TCATGAGGGATTCTGCTGAGA	
GAPDH	GGCATCGTGAAGGGCTCATGAC	62 °C
	ATGCCAGTGAGCTTCCCGTTACG	

AT annealing temperature, GAPDH glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene), I-FABP intestinal fatty acid binding protein, TLR4 Toll-like receptor 4

Table 2 Energy ingestion, body composition and metabolic parameters of Wistar rats fed either with standard (St) or high-fat (HF) diet during 12 weeks

	St	HF	P value
Energy ingested (Kcal/day)	57.9 ± 0.5	72.60 ± 0.1	<0.05
Weight gain (g)	134.7 ± 9.2	205.5 ± 15.4	<0.05
Fat mass (g)	166.8 ± 8.7	206.3 ± 8.6	<0.05
Total cholesterol (mg/dL)	64.3 ± 3.1	69.8 ± 6.6	0.78
Triglycerides (mg/dL)	79.3 ± 11.5	79.2 ± 8.3	0.83
Leptin (ng/mL)	6.2 ± 1.2	19.1 ± 2.6	<0.05
GLP-2	7.0 ± 0.4	9.8 ± 0.8	<0.05

Values are presented as mean ± SEM ($n = 6$ rats per group). GLP-2 glucagon-like peptide-2

To assess glycemic response, we performed an oral glucose tolerance test (OGTT). The total area under the curve (AUC) of the glycemic response was increased by HF diet feeding (Fig. 1a, b). In addition, HF diet fed rats had almost two fold less insulin sensitivity, as determined by homeostasis model assessment (HOMA) of insulin resistance (Fig. 1c).

We also examined plasma concentrations of leptin, which has postulated roles in obesity and insulin action (Table 2). HF diet fed group presented significantly higher leptin levels (19.1 ± 2.6 vs. 6.2 ± 1.2 , $P < 0.05$) than St diet fed group.

Total cholesterol and triglycerides did not differ between groups (Table 2).

Gut microbiota, LPS and inflammatory status

To study whether HF diet could induce modifications within the intestinal microbiota, we quantified some of the main bacterial groups presented in fecal samples. Analysis of the bacterial 16S rDNA revealed that, at the phylum level, animals from the HF diet group were characterized for having lower Bacteroidetes and higher Firmicutes to Bacteroidetes ratio (Table 3). Firmicutes/Bacteroidetes ratio was positively correlated with weight gain ($r = 0.829$, $P < 0.05$) and AUC ($r = -0.723$, $P < 0.05$) while Bacteroidetes were negatively correlated with weight gain ($r = -0.800$, $P < 0.05$) and AUC ($r = -0.716$, $P < 0.05$).

HF diet feeding also resulted in a decrease in the number of copies of *Prevotella* spp. and *Lactobacillus* spp. (Table 3). We further determined whether fecal LPS levels could be altered as a result of the changes in the gut microbiota. Our data showed that fecal LPS levels seemed to be more elevated in HF diet fed animals (Fig. 2a). In addition, LPS levels were positively correlated with Firmicutes/Bacteroidetes ratio ($r = 0.787$, $P < 0.05$) and negatively correlated with Bacteroidetes ($r = -0.670$, $P < 0.05$).

Afterwards, we evaluated the colonic expression of toll-like receptor 4 (TLR4) which is capable to recognize LPS. In agreement with our LPS findings, TLR4 expression

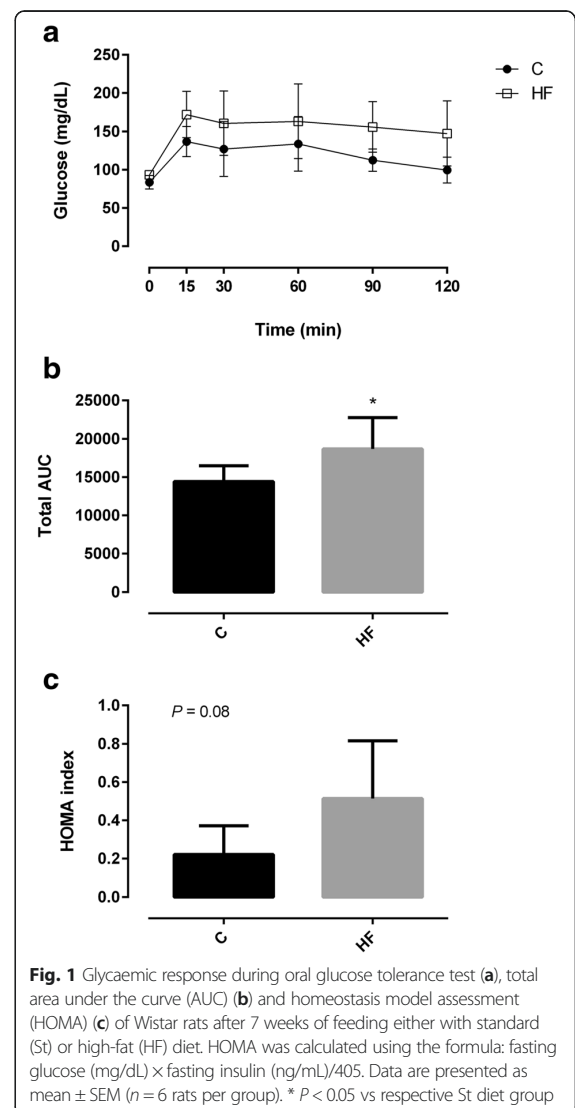


Fig. 1 Glycaemic response during oral glucose tolerance test (a), total area under the curve (AUC) (b) and homeostasis model assessment (HOMA) (c) of Wistar rats after 7 weeks of feeding either with standard (St) or high-fat (HF) diet. HOMA was calculated using the formula: fasting glucose (mg/dL) × fasting insulin (ng/mL)/405. Data are presented as mean ± SEM ($n = 6$ rats per group). * $P < 0.05$ vs respective St diet group

tended to be increased in the colon of animals fed with HF diet (Fig. 2b). Next, we investigated whether HF diet and microbiota changes were associated with systemic inflammation. In accordance, the chemokines MCP-1 and IL-1 β appeared to be more elevated in the plasma of HF diet fed rats (Fig. 2c, d). LPS was positively correlated with MCP-1 ($r = 0.726$, $P < 0.05$).

I-FABP and GLP-2 in high fat-diet induced obesity

To understand the relationship between I-FABP, HF diet feeding and systemic inflammation we quantified plasma I-FABP levels and its intestinal expression in both groups of rats. Surprisingly, plasma I-FABP levels were decreased after HF diet feeding (Fig. 3a). On the other

Table 3 Quantification of gut microbiota phyla, genera and species in different experimental groups

	St	HF	P value
Firmicutes/Bacteroidetes	1.03 ± 0.01	1.20 ± 0.03	<0.05
Firmicutes	6.61 ± 0.08	6.50 ± 0.09	0.35
Bacteroidetes	6.43 ± 0.10	5.45 ± 0.15	<0.05
Bacteroides spp.	4.36 ± 0.23	4.15 ± 0.50	0.18
Prevotella spp.	3.59 ± 0.29	2.21 ± 0.13	<0.05
Lactobacillus spp.	4.86 ± 0.27	3.77 ± 0.17	<0.05
<i>Clostridium leptum</i>	5.53 ± 0.04	5.43 ± 0.12	0.65
Bifidobacterium spp.	2.00 ± 0.24	2.12 ± 0.22	0.75

Values are presented as mean ± SEM and expressed as log₁₀ 16S rRNA gene copies/20 ng of DNA (n = 6 rats per group). HF high-fat diet group, St standard diet group

hand, I-FABP relative expression in jejunum tended to be higher in HF diet fed rats (Fig. 3b) which could be considered an adaptive response to the increased dietary fat content of the diet. To determine intestinotrophic status of the animals we quantified GLP-2 plasma levels. GLP-2 plasma levels were significantly increased after HF diet feeding (Table 2). GLP-2 and intestinal expression of I-FABP seemed positively correlated with energy ingested ($r = 0.719$, $P < 0.05$ and $r = 0.770$, $P = 0.07$, respectively).

To determine whether I-FABP plasma levels could be used as a marker of the metabolic alterations and inflammatory status associated with obesity, we evaluated the correlation between plasma I-FABP and host metabolic and inflammatory parameters (Fig. 4). Plasma I-

FABP levels were negatively correlated with fecal LPS ($r = -0.806$, $P < 0.05$) and IL-1B ($r = -0.623$, $P < 0.05$).

Discussion

Several studies have provided compelling evidence suggesting an association between gut microbiome dysbiosis, obesity and low-grade inflammatory state [11].

In consonance with previous reports, we found that the animals fed with HF diet had lower Bacteroidetes and higher Firmicutes to Bacteroidetes ratio [10]. This dysbiosis pattern might lead to an increased capacity of harvesting energy from food [12, 13]. HF diet also induced obesity and may have triggered intestinal inflammation since fecal levels of LPS and TLR4 expression tended to be increased in the animals fed with this diet. TLR4 is the LPS primary receptor that mediates its proinflammatory effects [14]. As a result, HF fed animals seemed to present higher plasma levels of proinflammatory cytokines and developed insulin resistance. As supported by other authors, it appears to be a causative role for the gut bacteria-induced proinflammatory state to the development of weight gain and insulin resistance in rats under HF diet [15, 16]. In this context, the gut barrier has an important role in the prevention of LPS leakage from the intestinal lumen to the portal blood. However, in animal models of diet-induced obesity, intestinal barrier function seems to be compromised [17].

In different intestinal diseases, I-FABP has emerged as a potential biomarker of intestinal barrier dysfunction

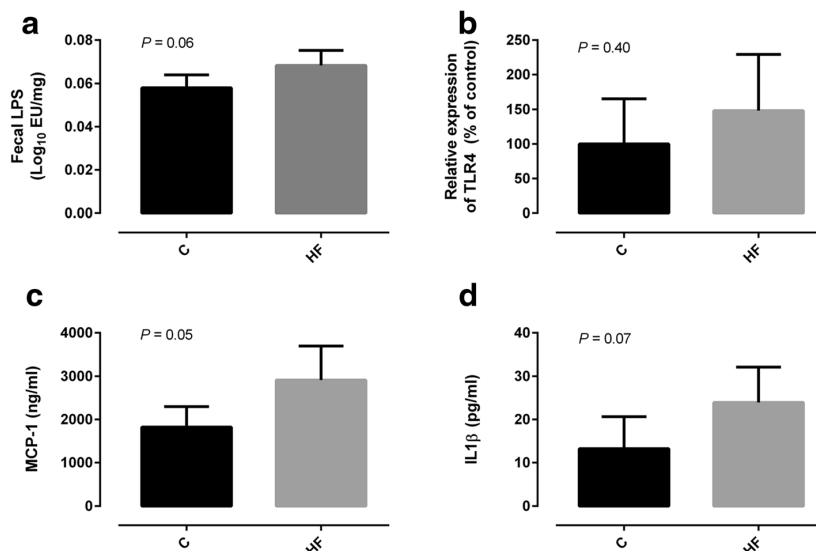


Fig. 2 Fecal lipopolysaccharide (LPS) (a), intestinal expression of toll-like receptor 4 (TLR4) (b), plasma monocyte chemoattractant protein-1 (MCP-1) (c) and serum interleukin-1 beta (IL-1β) (d) of Wistar rats fed either with standard (St) or high-fat (HF) diet during 12 weeks. Data are presented as mean ± SEM (n = 3–6 rats per group). * $P < 0.05$ vs respective St diet group

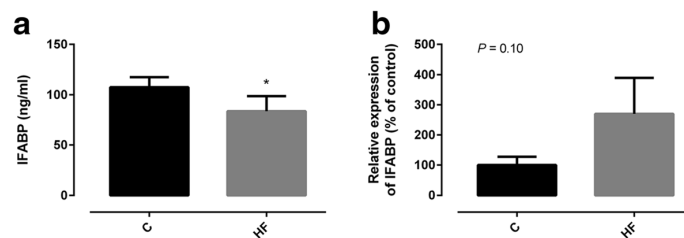


Fig. 3 Levels of plasma (a) and intestinal expression of intestinal fatty-acid binding protein (I-FABP) (b) of Wistar rats fed either with standard (St) or high-fat (HF) diet during 12 weeks. Data are presented as mean $\pm$ SEM ($n = 3-6$ rats per group). * $P < 0.05$ vs respective St diet group

[18, 19]. Basal I-FABP plasma levels may reflect the physiological turnover rate of enterocytes, whereas elevated levels might indicate intestinal epithelial cell damage [20]. Nevertheless, in the case of obesity-associated metabolic diseases, the existent data about I-FABP and intestinal barrier dysfunction is limited.

Verdam et al. have reported that chronically elevated glucose levels in obese individuals were associated with increased enterocyte loss, assumed by the increase on I-FABP levels [21]. It was therefore speculated that the increased enterocyte loss observed in subjects with chronic hyperglycemia might have contributed to the impaired intestinal barrier function, thereby promoting endotoxin-induced low-grade inflammation. However, increased I-FABP levels could also be a result of an increased production of I-FABP by enterocytes rather than enterocyte loss.

Interestingly, in our study we found that the relative expression of I-FABP tended to be increased in HF diet

fed rats. As intestinal absorption capacity can be adapted to the dietary fat content, we hypothesize that HF diet may have up-regulated several genes known to play an important role in long-chain fatty acids uptake such as I-FABP [22]. The animals fed with HF diet also showed increased GLP-2 levels. GLP-2 is a 33 amino acid peptide associated with intestinal growth and adaptation in a variety of pathological conditions [23]. As suggested by other authors, GLP2/GLP2R system may be increased after HF diet to further promote fat absorption in the intestine [23]. On the other hand, the inflammatory state induced by microbiota changes after HF diet feeding might have increased GLP-2 production in order to improve the mucosal barrier integrity and, therefore, blunt the inflammatory stress [2].

Conclusions

To our best knowledge, this is the first study demonstrating that, inversely to what happens in other

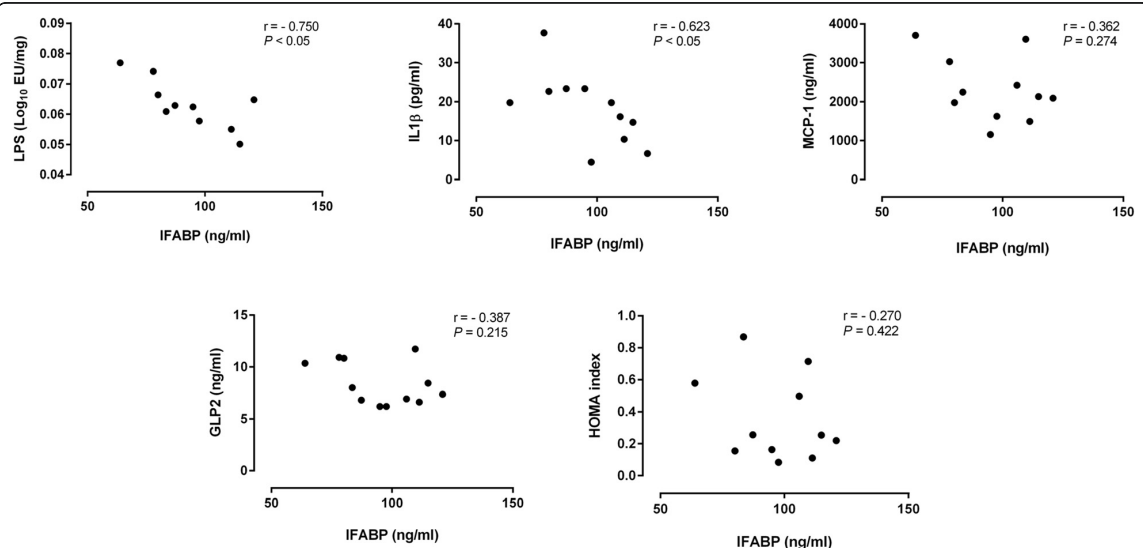


Fig. 4 Correlations between plasma levels of intestinal fatty-acid binding protein (I-FABP) and host metabolic and inflammatory parameters. Data of all experimental groups were gathered and analyzed using two-tailed Pearson's correlation test

intestinal inflammatory diseases, plasma I-FABP does not positively correlates with the inflammatory status presented in obesity. Instead, I-FABP is decreased in plasma but probably increased in jejunum in order to face dietary fat content. The search for noble biomarkers has to continue since it is extremely important to anticipate the progression of obesity-associated metabolic diseases and, thus, allowing the prevention or monitoring of the therapeutic strategies use.

Abbreviations

AUC: area under the curve; GLP-2: glucagon-like peptide-2; HF: high-fat; HOMA: homeostasis model assessment; I-FABP: intestinal fatty-acid binding protein; IL-1 β : interleukin-1 beta; LPS: lipopolysaccharide; MCP-1: monocyte chemoattractant protein-1; OGTT: oral glucose tolerance test; qRT-PCR: quantitative real-time polymerase chain reaction; St: standard; TLR4: toll-like receptor 4.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EL, CM, DC, PF and CC designed the study. EL, CM, DP and MS performed laboratory work. EL and CM analyzed and interpreted data and wrote the manuscript; DC, PF, CC critically revised the manuscript. All authors have read and approved the final manuscript.

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Author details

¹Serviço de Endocrinologia, Diabetes e Metabolismo, Centro Hospitalar São João, Porto, Portugal. ²Instituto de Investigação e Inovação em Saúde, Porto, Portugal. ³Departamento de Bioquímica, Faculdade de Medicina, Universidade do Porto, Porto, Portugal. ⁴CINTESIS, Centro de Investigação em Tecnologias e Serviços de Saúde, Porto, Portugal. ⁵Departamento de Medicina, Faculdade de Medicina, Universidade do Porto, Porto, Portugal. ⁶Nutrição e Metabolismo, NOVA Medical School/Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisboa, Portugal.

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5.2 Paper 5

Eva Lau, Eugeni Belda, Paul Picq, Davide Carvalho, Manuel Ferreira-Magalhães, Isaac Barroso, Flora Correia, Isabel Miranda, Cidália Pina Vaz, Eduardo Lima Costa, Adelino Barbosa, Karine Clément, Joel Doré, Paula Freitas, Edi Prifti.

Gut microbiota changes after metabolic surgery in adult diabetic patients with mild obesity: a randomised controlled trial.

[Full-paper; Submitted]

Gut microbiota changes after metabolic surgery in adult diabetic patients with mild obesity: a randomised controlled trial

Eva Lau^{1,2}, Eugeni Belda³, Paul Picq³, Davide Carvalho^{1,4}, Manuel Ferreira-Magalhães^{2,5}, Isaac Barroso^{6,7}, Flora Correia^{8,9}, Cidália Pina Vaz^{2,10}, Isabel Miranda¹¹, Eduardo Lima Costa¹², Adelino Barbosa¹², Karine Clément¹³, Joel Doré¹⁴, Paula Freitas^{1,4}, Edi Prifti^{3,15*}

¹ Department of Endocrinology and Nutrition, Centro Hospitalar de S. João, Porto, Portugal;

² CINTESIS - Center for Health Technologies and Information Systems Research - Faculty of Medicine, University of Porto, Porto, Portugal;

³ Integromics, Institute of Cardiometabolism and Nutrition, ICAN, Paris, France

⁴ I3S – Instituto de Investigação e Inovação em Saúde, Faculty of Medicine, University of Porto, Porto, Portugal;

⁵ Health Information and Decision Sciences Department - Faculty of Medicine, Porto University, Porto, Portugal;

⁶ Department of Biochemistry, Centro Hospitalar de S. João, Porto, Portugal;

⁷ EpiUnit – Instituto de Saúde Pública, University of Porto, Porto, Portugal;

⁸ Department of Nutrition, Centro Hospitalar de S. João, Porto, Portugal;

⁹ Faculty of Nutrition and Food Science, Porto, Portugal;

¹⁰ Department of Pathology, Division of Microbiology, Faculty of Medicine, University of Porto, Porto, Portugal;

¹¹ Surgery and Physiology, Cardiovascular Research Center, Faculty of Medicine, University of Porto, Porto, Portugal;

¹² Department of Surgery, Centro Hospitalar de S. João, Porto, Portugal;

¹³ Sorbonne Université, INSERM, NutriOmics research unit, Pitié-Salpêtrière hospital, Paris France;

¹⁴ Université Paris-Saclay, INRA, MetaGenoPolis, AgroParisTech, MICALIS, 78350, Jouy-en-Josas, France;

¹⁵ Unité de Modélisation Mathématique et informatique des Systèmes complexes, IRD, Sorbonne Université, UMMISCO.

* Correspondence:

Dr. Edi Pifti

edi.pifti@gmail.com

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Abstract

Background/Aims: Roux-en-Y gastric bypass (RYGB) surgery is one of the most efficient procedures for the treatment of obesity, also improving metabolic and inflammatory status, in patients with mild obesity. The underlying mechanisms have not been fully understood, but gut microbiota is hypothesized to play a key role. Our aim was to evaluate the association between gut microbiota changes and anthropometric, metabolic and inflammatory profiles after metabolic surgery compared with medical therapy, in type 2 diabetic (T2DM) adults with mild obesity (BMI 30-35 Kg/m²).

Subjects and Methods: DM² was an open-label, randomised controlled clinical trial (RCT: ISRCTN53984585) with 2 arms: (i) surgical, and (ii) medical. The main outcome was gut microbiota changes after: metabolic surgery (Roux-en-Y gastric bypass - RYGB) *versus* standard medical therapy. Secondary outcomes included anthropometric, metabolic and inflammatory profiles. Clinical visits, blood workup, and stool samples were collected at baseline and months (M)1, 3, 6, 12. Gut microbiota was profiled using 16S rRNA targeted sequencing.

Results: Twenty patients were included: 10 in surgical and 10 in medical arm. Anthropometric and metabolic comparative analysis favoured RYGB over medical arm. At M12, the percentage of weight loss was 25.5% *vs.* 4.9% ($p<0.001$) and HbA1c was 6.2% *vs.* 7.7% ($p<0.001$) respectively.

We observed a continuous increase of genus richness after RYGB up until M12. In the medical arm, genus richness ended-up being significantly lower at M12. Composition analysis indicated significant changes of the overall microbial ecosystem (permanova $p=0.004$, [$R^2=0.17$]) during the follow-up period after RYGB. There was a strong association between improvement of anthropometric/metabolic/inflammatory biomarkers and increase in microbial richness and Proteobacterial lineages.

Conclusions: This was the first RCT studying composite clinical, analytic, and microbiome changes in T2DM patients with class 1 obesity after RYGB *versus* standard medical therapy. The remarkable phenotypic improvement after surgery occurred concomitantly with changes in the gut microbiome, but at a lower level.

Introduction

Obesity and type 2 diabetes mellitus (T2DM) are both metabolic diseases that have expanded worldwide, reaching epidemic proportions and increasing patients' morbidity and mortality (1). Close interaction and imbalance between epigenetic and environmental factors are the primers for the complex etiological pathways of both diseases. Recent studies have emphasised the role of gut microbiota in metabolic regulation - specifically in energetic storage dysfunction and systemic inflammation (2,3).

The "Microbiome Hypothesis" is based on the fact that humans host 10^{14} bacteria in the gut, which perform a variety of physiological functions, ranging from protective to metabolic regulation, including carrying out an active part in glucose and lipid metabolism (4,5). Intestinal dysbiosis, characterised by changes in gut microbiota composition, has been shown to play an essential role in obesity and T2DM (6,7). Ley *et al.* showed a shift towards a higher relative abundance of *Bacteroidetes* and a decreased quantity of *Firmicutes* in patients with obesity who lost weight through low-calorie diets (8). Furthermore, experimental data has highlighted the role of gut microbiota in nutrient absorption, as well as in the maintenance of gut barrier integrity and lipogenesis and hormonal status, leading to an increasing interest in shaping human gut microbiota composition in order to prevent and treat obesity and restore glucose homeostasis (9,10).

Recent clinical trials reported that bariatric surgery, namely Roux-en-Y gastric bypass (RYGB) or bilio-pancreatic diversion, was the most effective treatment for combined weight loss and improvement/remission of T2DM in patients with severe obesity [body mass index (BMI) $\geq 35 \text{ kg/m}^2$] (11,12). Such encouraging results led to further research adopting the same approach in patients with mild obesity (BMI $30\text{--}35 \text{ kg/m}^2$) and the outcomes also proved the efficacy of this surgery in this subgroup of patients with obesity (13). The mechanism by which T2DM improves rapidly after RYGB, before significant weight loss, has not yet been completely understood, but recent studies have shown that gut microbiota modulation contributes to beneficial metabolic effects (14,15). Furet *et al.* demonstrated a shift toward an increase of *Faecalibacterium prausnitzii* in diabetic patients which was associated with a reduction in low-grade inflammatory state in obesity and diabetes (14).

To our knowledge, no randomised controlled clinical studies have been carried out assessing the association between gut microbiota changes and metabolic outcomes after RYGB in diabetic patients with mild obesity, compared to standard medical therapy. The main aim of our study was to evaluate gut microbiota changes after metabolic surgery *versus* standard medical therapy in diabetic adult patients with class 1 obesity. Secondary aims included: 1) the assessment of anthropometric, metabolic and inflammatory changes after interventions; and 2) the study of associations between gut microbiota alterations with anthropometric, metabolic, and inflammatory changes. This research contributes to improve our understanding of the intricate role of the gut microbiota in metabolic regulation.

Methods

Study design and participants

The Diabetes, Microbiota and Metabolic surgery (DM²) study was an open-label, randomised controlled clinical trial (RCT) carried out at the Centro Hospitalar São João (CHSJ) in Porto,

Portugal, which was designed to assess gut microbiota changes and T2DM resolution in patients with mild obesity after metabolic surgery *versus* standard medical therapy. The Ethics Committee from CHSJ approved the clinical protocol and all patients provided written informed consent. This RCT was registered in International Standard Randomised Controlled Trial Number Registry (ISRCTN), with the number ISRCTN53984585.

We used electronic medical records to identify candidates to participate in the trial, and between May 2014 and August 2014 we screened 42 patients at the CHSJ Endocrinology outpatient centre. Assuming rates of diabetes resolution to be 83%, with a 20% dropout rate in each study arm, required an enrolment of 10 patients in each category. This provided a power of >90% to detect differences between the 2 groups, using a 2-sided alpha of 0.05. Consequently, we included 20 participants who were randomly assigned to one of the two study arms, with a 1:1 ratio, using a computer-generated randomisation procedure, to receive either RYGB or standard medical therapy. Blinding was unsuitable for use, due to the major differences between the two treatment therapies; however, participants and researchers were only aware of the study arm of each participant after patients' informed consent and the random allocation concealment.

Inclusion criteria were as follows: age ≥ 20 and ≤ 65 years old; BMI ≥ 30 and $< 35\text{Kg/m}^2$; previous diagnosis of T2DM, according to the American Diabetes Association (ADA) definition, and under antidiabetic medical therapy (16); duration of diabetes > 3 months; overnight-fasting C-peptide > 0.7 ng/ml; negative anti-GAD autoantibody; possible eligibility for general anaesthesia; ability and willingness to participate in the study, with an understanding of the requirements of each arm of the study (written informed consent). The exclusion criteria were: specific contraindication to obesity surgery; diabetes secondary to a specific disease (maturity-onset diabetes of the young, latent autoimmune diabetes in adult or pancreatitis); having taken any antibiotic, probiotic, or prebiotic agents during the month before randomisation; pregnancy; debilitating disease; any psychological condition which could hamper a patient's cooperation; any condition which, in the opinion of the researcher, could have meant that participation in the study was risky or could have biased the results.

Surgical arm: Roux-en-Y gastric bypass surgery

Participants in the surgical arm underwent RYGB – whereby a 30 ± 10 mL capacity subcardial gastric pouch was created on a nasogastric 36F calibrating tube by sectioning the stomach with a linear stapler 3–4 cm horizontally on the lesser curve, 4 cm distant from the e-g junction, and then vertically until attainment of the angle of Hiss. After identification of the Treitz ligament, the jejunum was transected at 100 cm from the ligament of Treitz and the two stumps were closed. The distal stump was anastomosed to the distal end of the gastric pouch. Finally, the proximal stump of the transected bowel was joined end-to-side to the jejunum 150 cm distant from the gastroenterostomy.

Medical arm: Standard medical therapy

Participants in the medical arm were managed with antidiabetic standard medical therapy. This was defined as the use of lifestyle (nutrition and exercise counselling) guidelines by ADA to optimise weight loss and glycaemic control, along with frequent glucose self-monitoring and titration strategies, and also drug therapy for hyperglycaemia and restoration of pancreatic cells function (16). In addition, all subjects were treated according to ADA guidelines for lipid and blood pressure targets.

Follow-up

The follow-up period ran from October 2014 to May 2016. Clinical visits and laboratory tests were carried out at six points in time: i) during the screening period; ii) at baseline, after randomisation (M0); iii) and at four moments of follow-up - (Months M1; M3; M6; M12 after the RYGB in surgical arm, or after the first appointment in medical arm).

Clinical visits were carried out at all six moments of assessment. Patients were evaluated by an endocrinologist who performed a complete medical history and physical examination, including anthropometric measurements (body weight, height, waist circumference, visceral fat area, body fat mass and fat-free mass) using bio-impedance analysis (Inbody[®], model 720). Dietary records were also evaluated by a nutritionist, who carried out a detailed dietary intake (quality and quantity) for the 24-hour period before the interview. Patients also maintained dietary records for the 72-hour period before the clinical visit. Laboratory tests included blood biochemical tests and gut microbiota analysis. Blood biochemical tests were conducted at all six moments of assessment and patients had at least eight hours of fasting, with trained nurses collecting blood samples at the beginning of the clinical visit. Gut microbiota analyses were conducted at M0, M1, M3, M6 and M12, and faecal samples were collected by participants at home, after appropriate exemplification and training of the collection procedure. Faecal samples were collected in the morning, before breakfast. Whole stools were self-collected in sterile boxes and stored at -20°C. Samples were treated in the laboratory and stored as 200 mg aliquots at -80°C until further analysis.

Biochemical tests

Routine chemical analyses were evaluated using an enzymatic colorimetric test using the Olympus AU 5400 clinical chemistry analyser (Beckman Coulter[®], USA), including glucose (fasting glucose, insulinemia and C-peptide) and lipid profile components of the serum [total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and Triglycerides (TG)]. Low-density lipoprotein cholesterol (LDL-C) was calculated according to the Friedewald equation (17). The measurement of hemoglobin A_{1C} (HbA_{1C}) was performed using ion-exchange high-performance liquid chromatography (HPLC) with a Variant[™] Turbo HbA_{1C} (Bio-Rad[®], USA). Serum high-sensitivity C-Reactive Protein (hsCRP) was assessed using particle-enhanced immunonephelometric tests with a BN[™] II laser nephelometer (Siemens[®], Erlangen, Germany). Insulin was assayed using an electrochemiluminescence immunoassay with a Cobas e411 automated analyser (Roche[®], Germany). C-Peptide was assessed using electrochemiluminescence immunoassay with a Cobas e411 automated analyser (Roche[®], Germany).

Gut microbiota data processing

Gut microbiota profiling was carried out using 16S rRNA targeted sequencing, using a methodological approach developed by GenoScreen[®] (France). This process consists of three steps: 1) the preparation of libraries of amplicons according to the Metabiote[®] tool, limiting the bias of amplification between samples and including a positive control (the artificial bacterial community "ABC control"), with a first negative control (background of the whole process of libraries construction) and a second negative control (background of the stool extraction step); 2) sequencing of the 16S amplicons libraries on an Illumina MiSeq "paired-end" 2x250bp run; and 3) sorting by sample indexes and reassembly of the two "paired-end" reads to obtain full length 16S rDNA sequences. The 16S library constructions were carried out using 5 ng of gDNA, extracted according to the Metabiote[®] protocol developed by GenoScreen[®].

The raw demultiplexed sequences were processed using *mothur*, with default parameters. Abundance tables at the genus level were rarefied at 2,059 reads and were normalised before statistical analyses, using the total count procedure. The Bray-Curtis beta-diversity distance matrix was computed using the *vegdist* function of a *vegan* R package from the rarefied genus abundance matrix (18). Principal Coordinate Analysis (PCoA) from Bray-Curtis beta-diversity matrix was carried out with the *cmdscale* function of a *vegan* R package.

Enterotype classification was carried out following the Dirichlet Multinomial Mixture (DMM) method of Holmes et al. (31), using the rarefied genus abundance matrix as the input. The DMM approach groups samples together if their taxon abundances can be modelled by the same Dirichlet-Multinomial (DM) distribution. Genus richness was estimated using different methods. Rarefaction to 6,000 reads per sample and upsizing procedure were used to estimate genus diversity. This consisted in a 10-times rarefaction procedure, with different alpha diversity metrics (observed genera, Chao1, ACE as richness estimators; Shannon, Simpson and InvSimpson as evenness estimators). We observed differences in sequencing depth between samples from each group, however this variability was omitted after the rarefaction procedure.

Outcomes

The main outcome of the DM² study was the quantification of gut microbiome diversity and composition after RYGB, compared with standard medical therapy. The secondary outcomes included the relation between T2DM remission/improvement (as measured by anthropometric, metabolic and inflammatory biomarkers) and gut microbiota composition and modification throughout the intervention. Both outcomes were assessed at M0, M1, M3, M6 and M12. Diabetes remission was defined as HbA_{1c} < 6.5%, without using any antidiabetic medication. Diabetes improvement was considered if patients still required non-insulin antidiabetic drugs, but at lower doses compared to the baseline (without insulin use), as well as HbA_{1c} levels ≤ 7.0% (13).

Statistical analysis

All values are described as mean and standard deviation (SD). Homeostasis model assessment of insulin resistance (HOMA-IR), percentage of total weight lost (%WL) and BMI were calculated, in accordance with the literature. The composition of microbiota was expressed with the mean of the normalised relative abundance values. PCoA transformation of the multidimensional data at the genus level was carried out, using the Bray-Curtis beta-diversity distance matrix. A PERMANOVA test on *vegan* R package with n=999 permutations was carried out to test for differences in microbiome composition after surgery and interventions for the medical arm. Statistical analyses of the microbiome were carried out using the *momr* and *relome* R packages developed at Institute of Cardiometabolism and Nutrition (ICAN), France. Non-parametric tests (Wilcoxon, Kruskal-Willis or Spearman correlations) were performed when analyzing microbiome data. Benjamini-Hochberg multiple testing adjustment was applied (FDR<0.1 indicates significance).

Paired Mann-Whitney tests were performed to analyse the changes in these parameters between baseline and the various points in time, for each study arm category. Linear regression was used to analyse associations between continuous variables in different timepoints, for each study arm, and R² effect size was also calculated. All inferential statistical computations were considered to be significant when p-values were <0.05. Statistical analysis was carried out using SPSS version 25 (SPSS IBM, New York, NY, USA) and R.

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Results

Clinical and biological baseline characteristics

We screened 42 patients, from which we randomised 20 participants in the DM² study (see **Figure 1**). There were two dropouts in the surgical arm: one patient quit the study after allocation and the other was excluded after surgery, due to newly-diagnosed tuberculosis.

Patient's characteristics are described in **Table 1**. The average age was 53 vs. 58 years old, BMI was 33.6 kg/m² vs. 32.0 kg/m², HbA_{1c} was 8.7% vs. 8.2%, and women were 50% vs. 30%, for the surgical arm vs. medical arm, respectively. No significant differences were observed in BMI, HbA_{1c}, fasting glucose, insulin, C-peptide levels, or HOMA-IR between the two arms. However, patients who underwent RYGB had significantly higher weight, visceral fat area, body fat mass, and serum hsCRP at baseline (**Table 1**).

Bariatric surgery outperforms medical therapy in improving patients' anthropometric, metabolic and inflammatory profiles

One month after the beginning of the study, there was no evidence of differences in BMI between arms, however, %WL was significantly higher in the surgical category (10.5% vs. 3.1%, $p<0.001$; **Table 2**). Neither were any significant differences observed at this time in fasting glucose, HbA_{1c}, or C-peptide between arms. Nonetheless, we observed lower insulin levels (6.6 mg/dL vs. 18.5 mg/dL, $p=0.026$) and HOMA-IR (2.5 vs. 6.5, $p=0.035$) in the surgical arm.

At three months of follow-up, differences in %WL were even greater (17.7% vs. 5.5%, $p<0.001$), and differences in BMI (27.7 kg/m² vs. 30.3 kg/m², $p=0.007$) and waist circumference (97.9 cm vs. 103.3 cm, $p=0.034$) were observed, whereas none were for fasting glucose, HbA_{1c}, or C-peptide between both arms. However, differences between arms increased as far as insulin levels (6.0 mg/dL vs. 17.2 mg/dL, $p=0.019$) and HOMA-IR (1.9 vs. 6.1, $p=0.013$) were concerned.

At six months, differences in BMI and %WL continued to be even more pronounced (BMI: 25.7 kg/m² vs. 30.4 kg/m², $p<0.001$; %WL: 23.4% vs. 5.2%, $p<0.001$) and body fat measures were significantly lower in the surgical arm (visceral fat area, $p=0.029$; and body fat mass, $p=0.003$). Measures of insulin resistance were also significantly reduced in the surgical arm (HbA_{1c}: 6.2% vs. 7.3%, $p=0.038$; HOMA-IR: 1.6 vs. 7.2, $p<0.001$), as were triglycerides (91.9 mg/dL vs. 143.6 mg/dL, $p=0.049$), while HDL-C was significantly increased (54.4 mg/dL vs. 40.7 mg/dL, $p=0.014$).

Finally, at 12 months of follow-up, the average BMI was 24.6 kg/m² vs. 30.5 kg/m² ($p<0.001$), and HbA_{1c} was 6.2% vs. 7.7% ($p<0.001$) in the surgical vs. medical arm, respectively (**Table 1**). The %WL was 25.5% in the surgical arm and 4.9% in the medical arm ($p<0.001$). Waist circumference, visceral fat area and body fat mass were statistically lower in the surgical arm ($p<0.001$, $p=0.007$ and $p=0.002$, respectively). In addition, fasting glucose, insulinemia, C-peptide, and HOMA-IR were significantly lower in the surgical arm ($p=0.007$, $p=0.020$, $p=0.020$ and $p=0.027$, respectively), and HDL-C was higher ($p=0.004$). See **Table 1** for absolute values for each parameter and **Figure 2** for summarised trajectories. At the final endpoint, all participants from the medical arm failed to achieve diabetes remission or improvement, while in the surgical arm, 5 participants (62.5%) experienced remission from their diabetes ($p=0.007$ for comparison with medical arm, Chi² test), 2 participants (25%) improved their phenotypes, and only 1 showed no improvement. Additional analyses were performed using the relative change between baseline and 12 months of follow-up. These results are

shown in the supplementary material (**Supplementary Table S1, Table S2 and Table S3, and Figure S1**).

Microbiota composition and clinical phenotypes before interventions

We hypothesised that the clinical changes that occurred in the study cohort may have been accompanied by changes in the gut microbiome. Consequently, we characterised the latter at different levels of specificity - such as richness, community type, and the genus taxonomic levels. In this study, we computed richness as being the number of present/observed genera. First, we observed no saturation at the genus level during the rarefaction analysis (**Supplementary Figure S2A**), which indicates that the sequencing depth may be insufficient to capture all of the complexity of the microbiome at the genus level. However, the limited sequencing depth would nevertheless allow for the capture of the signal for the most prevalent and abundant rates. We observed differences in sequencing depth between study arms (lower in surgical) (**Supplementary Figure S2B**). These differences were levelled out with a rarefaction procedure.

At baseline, we observed a lower genus richness (trend) in the surgical arm, compared with the medical arm (61.37 ± 9.16 vs 71.3 ± 11.19 respectively, $p=0.055$) (**Supplementary Figure S3A**). Moreover, when correlating genus richness with clinical variables (**Supplementary Table S4**), we observed that the waist circumference ($p=0.014$) and hsCRP inflammatory marker ($p=0.023$) were negatively associated, as previously observed (19,20), (**Supplementary Figure S3B-D**).

Next, we analysed the microbiome data using a community-based approach. Enterotypes were documented as interesting describers of microbiome abundance data (21). Indeed, recently an improved enterotyping approach, based on DMM has shown to be better for capturing a significant signal in the microbial communities (22). We applied the DMM method, despite difficulties in determining the same optimal group number in the Laplacian profile — we fixed it for $k=4$ for comparison of the results with the original study (**Supplementary Figure S2C**). The potential drivers of enterotype composition for different groups are: **K1**: Synergistes (2 genera), Desulfovibrio (delta-proteobacteria), Ruminococcaceae genera Clostridium IV, and Unclassified Ruminococcus; **K2**: Lachnospiraceae, Ruminococcus; **K3**: Actinobacteria (Bifidobacterium), Bacillales (even less abundant), Enterobacteriales (potentially pro-inflammatory). Additionally, decreased levels of the following were observed: *Faecalibacterium* (potentially anti-inflammatory) and Oscillibacteria (Ruminococcaceae); **K4**: Lachnospiraceae, *Roseburia*, and *Ruminococcus* (all Clostridiales) (data not shown).

These enterotype profiles are different from what is described in previous studies (23–26). Moreover, no significant differences are observed in genus richness across enterotypes when considering the entire study cohort (data not shown) (22). Contrary to what is expected, no significant associations are observed between enterotypes and clinical variables at baseline.

Next, we searched for compositional changes between study arms at baseline by applying a PERMANOVA test (non-parametric MANOVA from Bray-Curtis beta diversity distance matrix computed with genus abundance data). No significant difference was observed ($p=0.095$; **Supplementary Figure S2D**). When testing for differentially abundant features between surgical and medical arms at baseline, five genera were observed (not resisting multiple testing adjustment; **Supplementary Figure S4**).

Microbiota changes during the intervention

Despite lower baseline genus richness in the surgical arm (61 +/- 9 prokaryotic genera), we observed a significant continuous increase until attaining 76 +/- 9 prokaryotic genera at M12 ($p=0.03$ on paired Wilcoxon tests). On the contrary, genus richness did not change throughout the intervention in the medical arm (71 +/- 11 at baseline; 71 +/- 10 at M12; $p=0.92$ on paired Wilcoxon tests) (**Figure 3A**). These results confirm previous findings in patients with severe obesity where microbial gene richness increases after RYGB (27).

The enterotype analyses did not allow us to observe any significant change throughout the intervention, which is probably a consequence of the small number of patients and stratification in the statistical tests. In the surgery arm, the composition analysis indicated significant changes in the microbiome ($R^2=0.17$; permanova $p=0.004$) (**Figure 3C**). Changes in the medical arm were not significant (**Figure 3B**), which confirmed the observations at the richness level.

Furthermore, we searched for specific genera that changed in abundance at the study endpoint, compared to baseline. We observed that *Ruminococcus*, *unclassified_Lachnospiraceae* family and *Faecalibacterium* significantly decreased, while *Klebsiella*, *Gammaproteobacteria*, *Enterobacter*, *unclassified_Gammaproteobacteria*, *unclassified_Veillonellaceae* increased after 12 months of RYGB. In the medical arm, *unclassified_Lachnospiraceae* and *Sutterella* significantly decreased, while *unclassified_Clostridiales* and *unclassified_Bacteria* increased, when comparing baseline with M12 (**Supplementary Figure S5**). None of these changes did resist multiple testing adjustment though (**Supplementary Table S5**).

Finally, we explored the differences in the Firmicutes/Bacteroides ratio (rFB) during the interventions' follow-up. This score is largely used in microbiome studies as a marker of ecosystem health. No significant differences between the two categories or through time were observed in this case (**Supplementary Figure S6A**). However, in agreement with previous findings (28), the Proteobacteria/Firmicutes ratio (rPF) significantly increased after RYGB, but not in the medical arm (**Supplemental Figure S6B**).

How do microbiome changes relate to phenotypic evolution?

We analysed the associations between relative changes in metabolic parameters and microbial richness. There was a strong inverse relationship between changes in body composition and anthropometric/metabolic markers (waist circumference, diastolic blood pressure, HbA_{1c}), as well as inflammation (hsCRP) along with changes in microbial richness (Figure 4).

Similar analyses looking for associations of relative changes in clinical parameters with changes in target genera (those showing significant changes one year after the interventions from univariate tests) indicated significant associations ($FDR<0.05$) between changes in anthropometric variables, glucose sensitivity variables (glycated haemoglobin, insulin), and inflammatory variables (hsCRP), and also changes in Proteobacterial lineages, mostly of the gamma clade (**Figure 4 and supplementary Figure S7**). We observe that improvement in anthropometric, metabolic and inflammatory profiles represented by decrease in these variables after the interventions was associated with an increase in gamma-proteobacterial lineages, which were mostly driven by the RYGB surgery. Similarly, improvements in levels of HDL-C after intervention was positively associated with increases in these same gamma-proteobacterial clades, which was significant ($FDR<0.05$) only for the *Enterobacter* genus. On the other hand, we observed a positive association (although not significant) between changes in Firmicutes genera (*Ruminococcus*, *Faecalibacterium*)

and changes in these same clinical variables explained by the phenotypic improvement, along with a decrease in these Firmicutes genera after one year of follow-up (**Figure S5**).

Discussion

Bariatric surgery treatment has increased worldwide, being the most efficient procedure for the treatment of severe obesity. The underlying beneficial metabolic effects go beyond weight loss, which has led to the consideration of RYGB for diabetic patients with milder forms of obesity. This is the first randomized controlled clinical trial that simultaneously explored clinical and microbiota changes in diabetic patients with class-1 obesity after RYGB *versus* standard medical therapy. We found that both RYGB and standard medical therapy groups improved anthropometric outcomes at 1 year of follow-up, with RYGB patients having significantly higher improvements. However, only RYGB patients achieved improvement/remission in diabetes status ($n=7$, 87.5%) and, significantly improved anthropometric and glycaemic profiles, independently and progressively, during the first year of follow-up and simultaneously had gut microbiota changes.

Gut microbiota plays a relevant role in the complex causes of obesity and T2DM, and is hypothesised to be involved in the modulation of metabolic status after RYGB. Microbial richness is a simple descriptive parameter of the microbiome. A healthy microbial ecosystem is usually characterised by an elevated level of microbial richness (19,20). In addition, one of the key features of the microbiome that characterises obesity is a low level of microbial richness, which is correlated with metabolic disorders, such as low-grade inflammation, insulin resistance, and adipocyte size (19,29). Here, microbial richness was measured at the genus level. Despite a trend of lower baseline genus richness in patients in the surgical arm, we observed a continuous increase after RYGB during the 12 months of follow-up. Our results confirm previous observations that RYGB increases microbiome richness, not only in patients with morbid obesity, but also for a broader BMI range, including patients with a BMI 30-35 Kg/m² (27).

As expected, there was a strong inverse association between increase in microbial richness and improvement of clinical phenotype, including anthropometric, metabolic (waist circumference, diastolic blood pressure, HbA1c), and inflammatory (hsCRP) biomarkers. These results suggest that systemic and anatomical changes induced by RYGB can restore a putative loss of microbial richness with an improvement of metabolic profile. On the contrary, genus richness did not change in the standard medical therapy arm and ended-up being significantly lower at M12, with no differences in glycaemic profile, comparing to baseline. This suggests that standard medical therapy optimisation does not target the gut microbiota, which reinforces the hypothesis that modulation of gut microbiota by pre or probiotics could be a complementary strategy for improving glycaemic status in this context.

Across enterotypes, there were no significant differences in genus richness, nor in clinical variables at baseline. We hypothesise that as the sequencing depth was relatively low in this study, it provided a low number of observed genera, which significantly influenced the enterotyping outcome. Indeed, the evaluation of the Bray-Curtis distance is mostly driven by the most abundant genera, which affects the sensitivity of the analyses. Finally, the number of individuals in each group (four for the baseline K4 therapy, and five for the baseline K4 bypass) decreased the statistical power, making the interpretation difficult.

One year after surgery, we observed a significant decrease in three bacterial taxa belonging to the Firmicutes phylum (*Ruminococcus*, *Lachnospiraceae_unclassified* and *Faecalibacterium*), which are recognised as having anti-inflammatory properties with a beneficial impact on metabolic health (14,27). This leads us to hypothesise that, despite RYGB improving the metabolic status in T2DM patients with mild obesity, RYGB did not have the ability to restore a healthy microbiome composition, especially if it started with a highly dysbiotic microbiome state. We also observed an increase in abundance of bacteria of the phylum Proteobacteria (*Klebsiella*, *Gammaproteobacteria*, *Enterobacter*, *Gammaproteobacteria_unclassified*), one year after the surgical intervention, as well as of *Veillonellaceae_unclassified* (Firmicutes phyla). Indeed, an increase in gamma-proteobacteria after bariatric surgery is a common finding - both in humans and in mice (28,30,31). In our study, we also observed an increase of the Proteobacteria/Firmicutes ratio after RYGB. The increase of bacteria belonging to the phylum Proteobacteria was associated with the improvement of metabolic and inflammatory parameters after bariatric surgery (32). In an animal model, the increase of proteobacteria was also accompanied by a reduction in inflammatory response and glucose homeostasis improvement (33). The phylum Proteobacteria is composed of facultative anaerobes, and consequently oxygen increase (34) combined with higher pH after RYGB (in the gut) could contribute to an increase of these bacteria with an impact on metabolic health.

On the other hand, we did not observe any significant differences with regards to ratio Firmicutes/Bacteroidetes (rFB). The concept that obesity is associated with a lower percentage of Bacteroidetes and a higher percentage of Firmicutes in obese individuals is contradicted by several studies, which demonstrate that there is no difference in relative abundance of Firmicutes and/or Bacteroidetes and no association of weight loss with the rFB (35,36) in obese individuals. Furthermore, T2DM has been linked to a decreased abundance of Firmicutes and an increase in bacteria belonging to Bacteroidetes and Proteobacteria, when compared to obese patients (37). However, it is difficult to validate these links from the results of our study, which included T2DM patients with mild obesity, knowing the Firmicutes phylum and Bacteroidetes contain at least 250 and 20 genera, respectively. Higher taxonomic levels may not necessarily reflect specific bacteria changes, however, the results of our study are in line with those of Campisciano et al., as they corroborate that rFB is not a predictive biomarker of the outcome for metabolic surgery (38).

Strengths and Limitations

This study assessed patients' evolution at different points in time over the first year of follow-up, including the first and third months after interventions, contrary to the majority of published studies which lack comprehensive data regarding the first six months following baseline and through a 12-month period. These intermediate timepoint assessments allowed us to monitor a clear evolution of the clinical profile.

The sample size could have impaired to a degree the multivariate analysis - however, the results are consistent throughout the timepoints as well as with other studies. Of note is the fact that our observations can be limited by different technical aspects of microbiome analysis, including the collection, generation, and quantification of the abundance profiles. In addition, heterogeneity in dietary profiles and physical activity can also explain part of phenotypic outcomes during the treatment. Dietary data were mainly recorded for the medical arm, but not for the surgical arm throughout the different time-points, which limits our ability to control for food intake in the changes in microbiome profiles. Consequently, dietary analyses were not shown.

444 In conclusion, our research suggests that there is a remarkable phenotypic improvement after
 445 metabolic surgery which occurs simultaneously with gut microbiota changes. Nevertheless, gut
 446 microbiome changes alone cannot explain the beneficial metabolic health impact of RYGB. Other
 447 mechanisms such as diet, hormonal changes, bile acids metabolism, and physical activity need to be
 448 further explored in this equation in order to better explain the metabolic improvement of T2DM
 449 patients with mild obesity after RYGB.

450 **Conflict of Interest**

451 *The authors declare that the research was conducted in the absence of any commercial or financial*
 452 *relationships that could be construed as a potential conflict of interest.*

453 **Author Contributions**

454 EL, MFM, DV and PF were responsible for the conception and the design of the study; EL, IB, FC,
 455 IM, CPV, ELC, AB and PF conducted the study; EP, EB, PP and MFM designed the analytical
 456 approach, processed and analysed the data; EL, EP, EB, DC, MFM, KC, JD and PF reviewed data
 457 and wrote the paper; EL and EP had primary responsibility for final content. All authors read and
 458 approved the final manuscript.

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462 **Reference**

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Table 1. Clinical and biological characteristics at baseline and at final endpoint.

Figure 1. Study flowchart.

M, month

Figure 2. Clinical trajectories throughout the follow-up time points by study arms.

Average values +/- standard error values are depicted for each time point (baseline, 1, 3, 6 and 12 months). Blue and red colours indicate the medical and the surgical arm respectively. A-C: corpulence measurements; D-G: metabolic measurements.

Figure 3. Gut genus richness throughout the follow-up time points and arms.

(A): genus richness distribution across time in each study arm. (B): PCoA analysis of microbiome changes through time in the medical arm. (C): PCoA analysis of microbiome changes through time in the surgical arm. PERMANOVA tests were carried out over beta-diversity distance matrices computed from rarified genus abundance data testing for differences between time points in each study arm (R² and P values shown).

Figure 4. Heatmap of associations of changes between phenotypes and genera between baseline and 12 months of follow-up.

Heatmap of spearman correlation coefficients between relative change associations of genus richness and target genera with changes in clinical variables. */#: p/q values<0.05 in spearman correlation tests.

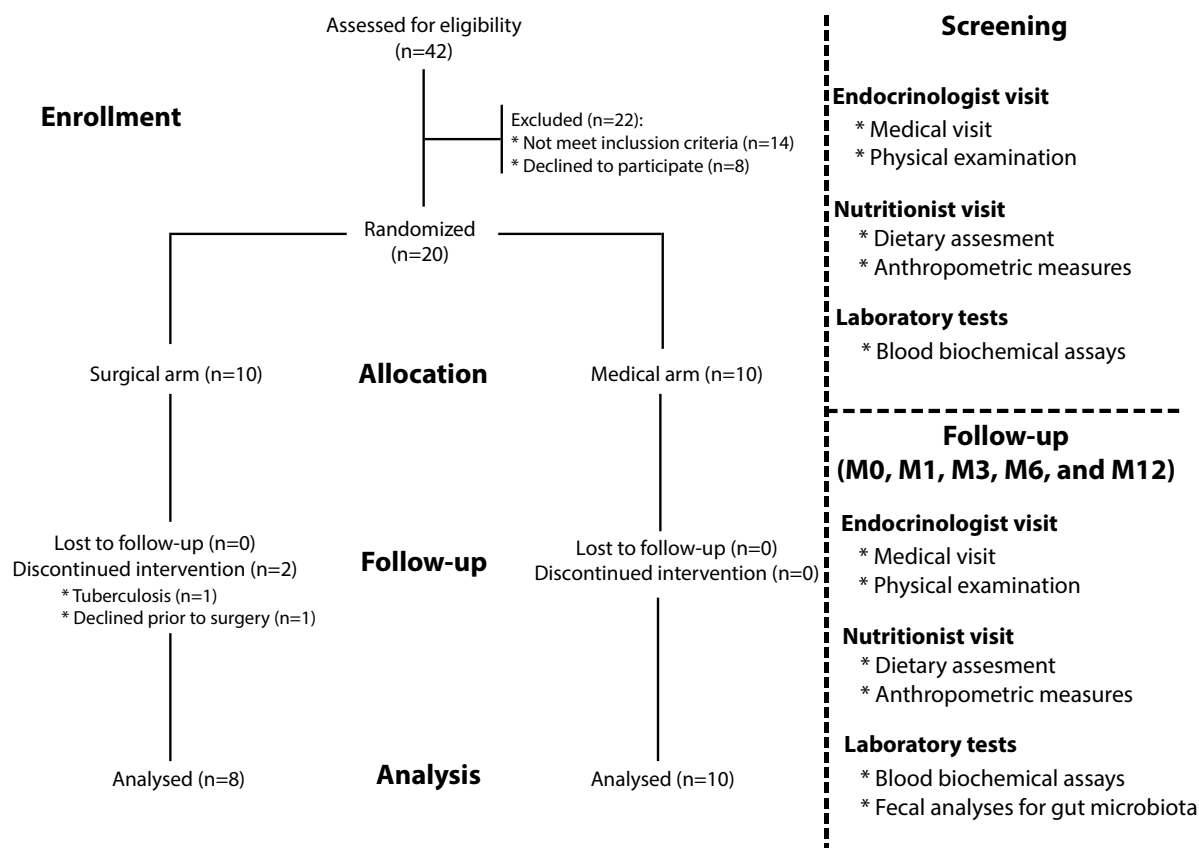


Figure 1. Study flowchart

M, month

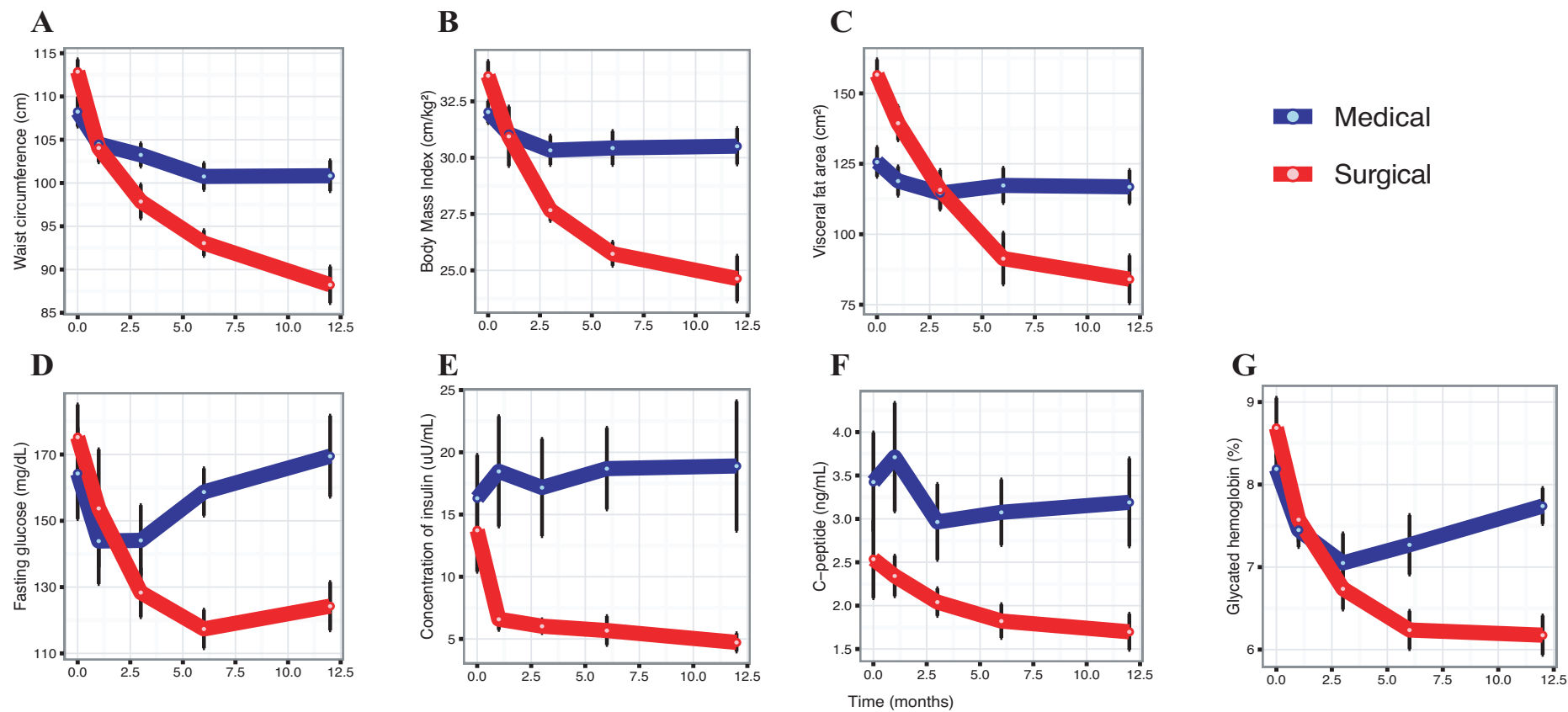


Figure 2. Clinical trajectories throughout the intervention by study group.

Average values $\pm$ standard error values are depicted for each time point (baseline, 1, 3, 6 and 12 months). Blue and red colors indicate respectively the medical and the surgical arm. A-C: corpulence measurements; D-G: metabolic measurements.

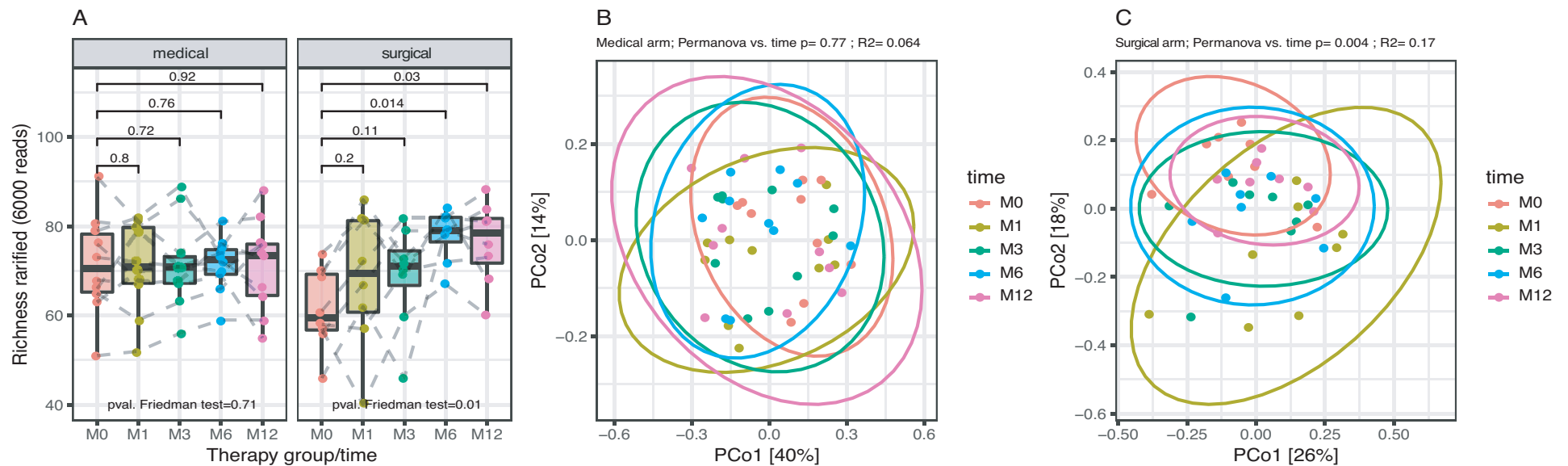


Figure 3. Gut genus richness throughout the follow-up time points and arms.

(A): genus richness distribution across time in each study group. (B): PCoA analysis of microbiome changes through time in the medical arm. (C): PCoA analysis of microbiome changes through time in Bypass group. PERMANOVA tests were carried out over beta-diversity distance matrices computed from rarefied genus abundance data testing for differences between time points in each study arm (R^2 and P values shown).

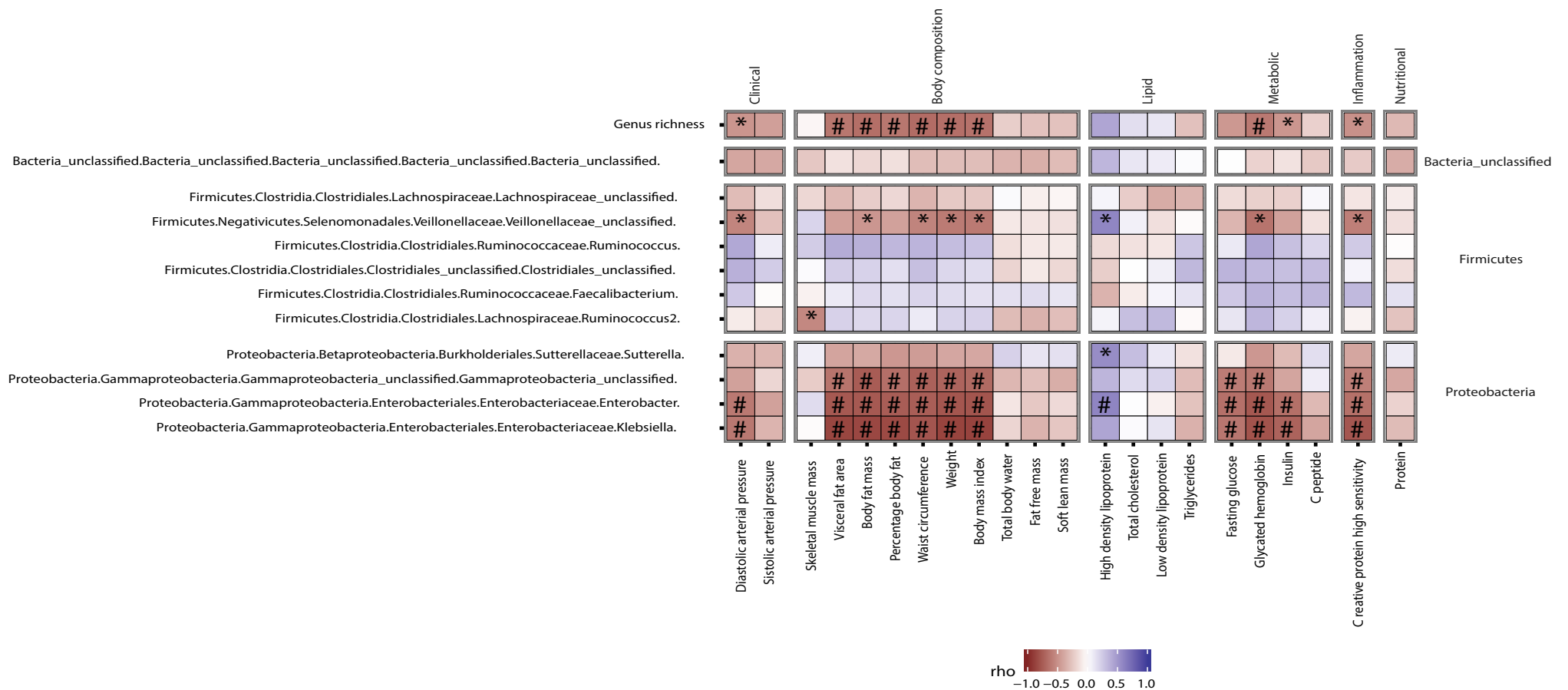


Figure 4. Heatmap of associations of changes between phenotypes and genera between baseline and 12 months of follow-up.

Heatmap of spearman correlation coefficients between relative change associations of genus richness and target genera with changes in clinical variables */#
p/q values<0.05 in spearman correlation tests.

Table 1. Clinical and biological characteristics at baseline and at final endpoint.

	Baseline				Month 12			
	Surgical arm N=8	Medical arm N=10	p	q	Surgical arm N=8	Medical arm N=10	p	q
Age, (years)	53.4 (8.78)	58.2 (4.73)	0.191	0.48	.	.	.	.
Diabetes duration (years)	8.12 (4.73)	10.0 (4.42)	0.404	0.62	8.12 (4.73)	10.0 (4.42)	0.404	0.43
Weight (Kg)	92.5 (7.65)	83.9 (9.10)	0.046	0.22	69.2 (11.8)	79.7 (9.07)	0.058	0.096
BMI (%)	33.6 (1.84)	32.0 (1.56)	0.069	0.23	24.6 (2.87)	30.5 (2.55)	<0.001	0.0023
%WL (%)	.	.	.	.	25.5 (8.56)	4.88 (5.53)	<0.001	0.0018
Waist circumference (cm)	113 (4.01)	108 (5.47)	0.055	0.22	88.2 (5.99)	101 (5.64)	<0.001	0.0023
Visceral fat area (cm ²)	157 (15.3)	126 (16.7)	0.001	0.0087	84.0 (24.0)	117 (18.8)	0.007	0.019
Body fat mass (Kg)	39.9 (4.60)	30.2 (3.89)	<0.001	0.0064	17.9 (5.71)	27.4 (4.71)	0.002	0.0086
Fat-free mass (Kg)	52.5 (9.69)	53.7 (9.48)	0.802	0.86	51.2 (9.60)	112 (193)	0.343	0.38
Fasting glucose (mg/dL)	175 (27.9)	164 (43.6)	0.528	0.75	124 (20.6)	170 (38.7)	0.007	0.019
HbA _{1c} (%)	8.69 (1.03)	8.19 (0.56)	0.246	0.49	6.17 (0.68)	7.74 (0.69)	<0.001	0.0021
Insulin (uU/mL)	13.7 (9.45)	16.3 (11.0)	0.602	0.8	4.72 (2.09)	18.9 (16.5)	0.024	0.048
C-peptide (ng/mL)	2.54 (1.27)	3.43 (1.80)	0.239	0.49	1.70 (0.59)	3.19 (1.60)	0.019	0.042
HOMA-IR	5.99 (4.38)	6.58 (4.27)	0.780	0.86	1.43 (0.66)	8.60 (8.60)	0.027	0.05
Total cholesterol (mg/dL)	170 (36.0)	165 (56.1)	0.821	0.86	173 (26.3)	155 (41.4)	0.286	0.37
Triglycerides (mg/dL)	124 (47.7)	186 (175)	0.308	0.56	100 (42.2)	169 (124)	0.131	0.19
HDL-c (mg/dL)	52.0 (15.9)	39.7 (11.8)	0.093	0.27	62.9 (9.64)	41.5 (15.5)	0.003	0.0088
LDL-c (mg/dL)	93.4 (23.1)	97.9 (48.7)	0.799	0.86	89.8 (19.8)	79.9 (32.6)	0.441	0.44
Systolic BP (mmHg)	132 (11.6)	132 (10.5)	1.000	1	120 (10.4)	124 (8.56)	0.311	0.37
Diastolic BP (mmHg)	70.6 (4.84)	66.8 (11.8)	0.370	0.62	63.2 (5.97)	67.0 (8.65)	0.294	0.37
hsCRP (mg/dL)	6.89 (2.95)	2.61 (1.50)	0.004	0.027	0.82 (0.61)	1.39 (0.84)	0.118	0.18

BMI: body mass index; %WL: percentage of weight lost; HbA_{1c}: Glycated hemoglobin; HOMA-IR: homeostatic model assessment for insulin resistance; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; BP: blood pressure; hsCRP: high sensitivity C-reactive protein; n.a.: not applicable.

Bold values are statistically significant. Values between parentheses are standard deviation of the respective mean value.

Supplementary material

Clinical changes during intervention

Anthropometric and inflammatory measures were significantly reduced at any given endpoint in both arms (Supplemental Table S1 and Table S2). However, in the medical arm, differences between baseline and endpoints remained stable (with a BMI mean change from M0 to M1 and M12 in the medical arm: -0.98 kg/m^2 and -1.52 kg/m^2 , respectively; a plasma hsCRP mean change from M0 to M1 and M12 in the medical arm: -1.25 mg/L and -1.22 mg/L , respectively), while in the surgical arm, the differences increased from one endpoint to another (BMI mean change from M0 to M1 and M12 in the surgical arm: -2.68 kg/m^2 and -9.00 kg/m^2 , respectively; plasma hsCRP has a mean change from M0 to M1 and M12 in the surgical arm: -0.64 mg/L and -6.06 mg/L , respectively).

Metabolic profile was globally unchanged through endpoints in the medical arm (**Supplementary Table S1 and Table S2**). In the surgical arm, metabolic profile significantly improved at each timepoint (HbA_{1c} mean change from M0 to M1 and M12 in the surgical arm: -1.11% and -2.51% , respectively; with a mean change for triglycerides from M0 to M1 and M12 in the surgical arm: -10.00 mg/dL and -23.88 mg/dL , respectively).

Systolic and diastolic blood pressure levels were similar between the medical arm and the surgical arm at any given timepoint. However, only participants in the surgical arm showed a significant reduction of systolic and diastolic blood pressure from M0 to M12 (systolic blood pressure mean change from M0 to M12 in the surgical arm: -12.50 mmHg ; diastolic blood pressure mean change from M0 to M12 in the surgical arm: -7.38 mmHg). The linear regression analysis focused on the surgical arm only, where we found no association between HbA_{1c} and BMI at any given time point (at M1: $r^2 0.019$, $p=0.745$; at M12: $r^2 0.013$, $p=0.786$).

Table S1. Mean changes between baseline and time points (1, 3, 6, and 12 months of follow-up) in the medical arm.

	Month 1 (mean change)	p	q	Month 3 (mean change)	P	q	Month 6 (mean change)	p	q	Month 12 (mean change)	p	q
Body weight (kg)	-2.67	0.005	1.18	-4.66	0.001	1.54	-4.40	0.005*	1.15	-4.19	0.022*	0.88
BMI (kg/m ²)	-0.98	0.004	1.22	-1.70	0.001	1.63	-1.60	0.005*	1.18	-1.52	0.022*	0.88
%WL (%)	-3.15	0.003	1.26	-5.49	<0.001	1.68	-5.18	0.004	1.21	-4.89	0.021	0.88
Waist circumference (cm)	-3.75	<0.001	1.91	-5.00	0.001	1.58	-7.49	0.002*	1.39	-7.41	0.001*	1.54
Visceral fat area (cm ²)	-6.77	0.003	1.31	-11.07	0.002	1.39	-8.34	0.047*	0.73	-8.83	0.042*	0.75
Body fat mass (kg)	-2.08	<0.001	2.06	-3.35	<0.001	1.81	-2.01	0.112	0.56	-2.80	0.018*	0.91
Fat-free mass (kg)	-0.60	0.221	0.42	-1.32	0.055	0.70	-1.50	0.073	0.64	-1.32	0.123	0.54
Fasting glucose (mg/L)	-20.40	0.128	0.53	-20.20	0.224	0.41	-5.60	0.661	0.14	-5.20	0.725	0.11
HbA _{1c} (%)	-0.74	0.001	1.46	-1.14	0.009	1.06	-0.92	0.028*	0.82	-0.45	0.114	0.55
Insulin (I U/mL)	2.16	0.181	0.46	0.86	0.756	0.10	2.38	0.196	0.43	2.59	0.458	0.25
C-peptide (ng/mL)	0.29	0.333	0.32	-0.46	0.187	0.45	-0.35	0.253	0.39	-0.24	0.594	0.17
HOMA-IR	-0.06	0.938	0.03	-0.46	0.616	0.16	0.63	0.386	0.29	2.02	0.373	0.30
Total cholesterol (mg/dL)	-11.80	0.162	0.48	-19.50	0.291	0.35	-5.50	0.642	0.15	-10.10	0.421	0.27
Triglycerides (mg/dl)	-4.00	0.754	0.10	-9.90	0.836	0.07	-42.50	0.401	0.28	-17.30	0.564	0.19
HDL-c (mg/dL)	0.80	0.665	0.14	0.30	0.855	0.06	1.00	0.409	0.27	1.80	0.347	0.31
LDL-c (mg/dL)	-10.80	0.145	0.50	-27.30	0.100	0.58	-7.50	0.551	0.20	-18.00	0.216	0.42
Systolic BP (mg/dL)	-10.70	0.039	0.76	-15.30	0.030	0.81	-8.00	0.088	0.61	-7.70	0.192	0.45
Diastolic BP (mg/dL)	-0.10	0.980	0.01	-0.90	0.804	0.08	1.40	0.665	0.14	0.20	0.968	0.01
hsCRP (mg/L)	-1.25	0.021	0.89	-1.18	0.010	1.02	-1.31	0.038*	0.77	-1.22	0.035*	0.78

BMI: body mass index; %WL: percentage of weight lost; HbA_{1c}: Glycated hemoglobin; HOMA-IR: homeostatic model assessment for insulin resistance; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; BP: blood pressure; hsCRP: high sensitivity C-reactive protein; n.a.: not applicable.
Bold values are statistically significant.

Table S2. Mean changes between baseline and time points (1, 3, 6, and 12 months of follow-up) in the surgical arm.

	Month 1 (mean change)	p	q	Month 3 (mean change)	p	q	Month 6 (mean change)	p	q	Month 12 (mean change)	p	q
Body weight (kg)	-9.85	<0.001	2.92	-16.41	<0.001	4.71	-21.48	<0.001*	5.55	-23.29	<0.001*	3.22
BMI (kg/m ²)	-2.69	0.040	0.89	-5.96	<0.001	5.08	-7.90	<0.001*	4.41	-9.00	<0.001*	2.50
%WL (%)	-10.46	<0.001	3.71	-17.67	<0.001	3.60	-23.37	<0.001*	5.06	-25.49	<0.001*	2.98
Waist circumference (cm)	-8.81	0.004	1.50	-15.00	<0.001	2.93	-19.81	<0.001*	5.43	-24.64	<0.001*	3.29
Visceral fat area (cm ²)	-17.23	0.001	2.11	-40.89	<0.001	3.27	-65.29	<0.001*	3.82	-72.61	<0.001*	3.86
Body fat mass (kg)	-6.33	<0.001	2.54	-13.55	<0.001	4.44	-20.06	<0.001*	5.89	-21.98	<0.001*	3.52
Fat-free mass (kg)	-3.51	<0.001	2.56	-2.86	0.009	1.27	-1.41	0.081	0.72	-1.31	0.171	0.54
Fasting glucose (mg/L)	-21.50	0.280	0.41	-46.88	0.004	1.49	-57.88	0.001*	2.12	-51.00	0.001*	1.96
HbA _{1c} (%)	-1.11	0.007	1.35	-1.95	0.002	1.71	-2.45	<0.001*	2.45	-2.51	<0.001*	2.22
Insulinemia (I U/mL)	-6.83	0.090	0.76	-7.71	0.043	0.87	-8.06	0.042*	0.88	-9.01	0.014*	1.15
C-peptide (ng/mL)	-0.19	0.553	0.22	-0.50	0.166	0.55	-0.71	0.043*	0.88	-0.84	0.019*	1.07
HOMA-IR	-3.53	0.100	0.73	-4.09	0.035	0.92	-4.40	0.025*	1.01	-4.56	0.013*	1.15
Total cholesterol (mg/dL)	-10.13	0.465	0.27	-3.13	0.784	0.10	-7.88	0.446	0.29	2.63	0.758	0.11
Triglycerides (mg/dl)	-10.00	0.392	0.32	-16.25	0.168	0.54	-32.50	0.011*	1.22	-23.88	0.004*	1.46
HDL-c (mg/dL)	-4.63	0.247	0.45	-1.00	0.692	0.15	2.38	0.514	0.24	10.88	0.010*	1.23
LDL-c (mg/dL)	-2.50	0.857	0.07	1.13	0.921	0.04	-3.75	0.694	0.15	-3.63	0.666	0.16
Systolic BP (mg/dL)	-15.25	0.022	1.03	-10.75	0.073	0.75	-13.25	0.002*	1.73	-12.50	<0.001*	2.30
Diastolic BP (mg/dL)	-7.88	0.008	1.31	-5.00	0.068	0.76	-5.00	0.010*	1.25	-7.38	0.005*	1.45
hsCRP (mg/L)	-0.64	0.848	0.07	-4.95	0.001	1.91	-5.76	<0.001*	2.41	-6.06	<0.001*	2.49

BMI: body mass index; %WL: percentage of weight lost; HbA_{1c}: Glycated hemoglobin; HOMA-IR: homeostatic model assessment for insulin resistance; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; BP: blood pressure; hsCRP: high sensitivity C-reactive protein; n.a.: not applicable.
Bold values are statistically significant.

Table S3. Clinical and biological characteristics at intermediate time points after surgical arm *versus* medical arm.

	Month 1				Month 3				Month 6			
	Surgical arm N=8	Medical arm N=10	p	q	Surgical arm N=8	Medical arm N=10	p	q	Surgical arm N=8	Medical arm N=10	p	q
Weight (Kg)	82.6 (4.44)	81.2 (8.82)	0.677	0.86	76.0 (5.77)	79.3 (8.56)	0.358	0.57	71.0 (8.64)	79.5 (8.93)	0.058	0.084
BMI (%)	30.9 (3.74)	31.1 (1.78)	0.946	0.95	27.7 (1.36)	30.3 (2.07)	0.005	0.048	25.7 (1.51)	30.4 (2.38)	<0.001	0.0012
%WL (%)	10.5 (2.82)	3.15 (2.51)	<0.001	<0.001	17.7 (2.96)	5.49 (3.27)	<0.001	<0.001	23.4 (4.62)	5.18 (4.28)	<0.001	<0.001
Waist circumference (cm)	104 (4.74)	104 (4.48)	0.845	0.89	97.9 (5.59)	103 (4.29)	0.043	0.16	93.1 (4.21)	101 (4.93)	0.003	0.0098
Visceral fat area (cm²)	139 (17.2)	119 (16.5)	0.022	0.16	116 (19.9)	115 (17.2)	0.897	0.93	91.4 (26.0)	117 (19.8)	0.037	0.061
Body fat mass (Kg)	33.6 (5.96)	28.1 (4.19)	0.049	0.19	26.4 (5.84)	26.9 (4.06)	0.838	0.93	19.9 (6.31)	28.2 (4.02)	0.007	0.02
Fat-free mass (Kg)	49.0 (8.80)	53.1 (9.32)	0.356	0.6	49.7 (9.10)	52.4 (8.47)	0.529	0.63	51.1 (10.5)	52.2 (8.80)	0.821	0.92
Fasting glucose (mg/dL)	154 (50.4)	144 (41.0)	0.662	0.86	128 (21.1)	144 (33.6)	0.244	0.46	117 (16.5)	159 (22.6)	<0.001	0.0024
HbA_{1c} (%)	7.57 (0.78)	7.45 (0.66)	0.722	0.86	6.74 (0.71)	7.05 (1.14)	0.487	0.62	6.24 (0.66)	7.27 (1.14)	0.030	0.056
Insulin (uU/mL)	6.59 (2.28)	18.5 (14.0)	0.026	0.16	6.03 (1.58)	17.2 (12.4)	0.019	0.091	5.67 (3.28)	18.7 (10.4)	0.003	0.01
C-peptide (ng/mL)	2.34 (0.66)	3.71 (1.98)	0.064	0.2	2.04 (0.45)	2.96 (1.38)	0.072	0.23	1.82 (0.55)	3.08 (1.20)	0.011	0.024
HOMA-IR	2.54 (1.12)	6.52 (5.01)	0.035	0.17	1.90 (0.53)	6.11 (4.31)	0.013	0.082	1.59 (0.81)	7.20 (3.66)	0.001	0.0039
Total cholesterol (mg/dL)	160 (33.5)	153 (42.1)	0.712	0.86	167 (31.1)	146 (53.9)	0.309	0.53	162 (28.2)	160 (41.5)	0.874	0.92
Triglycerides (mg/dL)	114 (49.0)	182 (151)	0.210	0.57	108 (32.4)	176 (116)	0.106	0.23	91.9 (30.6)	144 (62.6)	0.038	0.061
HDL-c (mg/dL)	47.4 (10.9)	40.5 (14.9)	0.275	0.57	51.0 (12.2)	40.0 (13.2)	0.086	0.23	54.4 (7.89)	40.7 (12.0)	0.011	0.024
LDL-c(mg/dL)	90.9 (27.1)	87.1 (33.6)	0.795	0.89	94.5 (25.0)	70.6 (34.3)	0.107	0.23	89.6 (20.8)	90.4 (32.7)	0.952	0.95
Systolic BP (mmHg)	117 (9.11)	121 (8.71)	0.300	0.57	121 (10.3)	117 (14.7)	0.453	0.61	119 (10.1)	124 (8.29)	0.256	0.35
Diastolic BP (mmHg)	62.8 (4.17)	66.7 (12.7)	0.376	0.6	65.6 (5.90)	65.9 (7.46)	0.932	0.93	65.6 (4.37)	68.2 (8.69)	0.428	0.54
hsCRP (mg/dL)	6.25 (10.8)	1.36 (0.89)	0.242	0.57	1.94 (1.35)	1.43 (1.21)	0.421	0.61	1.12 (0.82)	1.30 (0.93)	0.677	0.8

BMI: body mass index; %WL: percentage of weight lost; HbA_{1c}: Glycated hemoglobin; HOMA-IR: homeostatic model assessment for insulin resistance; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; BP: blood pressure; hsCRP: high sensitivity C-reactive protein; n.a.: not applicable.
 Bold values are statistically significant. Values between parentheses are standard deviation of the respective mean value.

Table S4. *Microbial richness relationship with clinical phenotypes*

Table S5. Differentially abundant genera in each group

(Table S4 and Table S5 are annexed to this PhD thesis in excel format)

Supplementary Figures

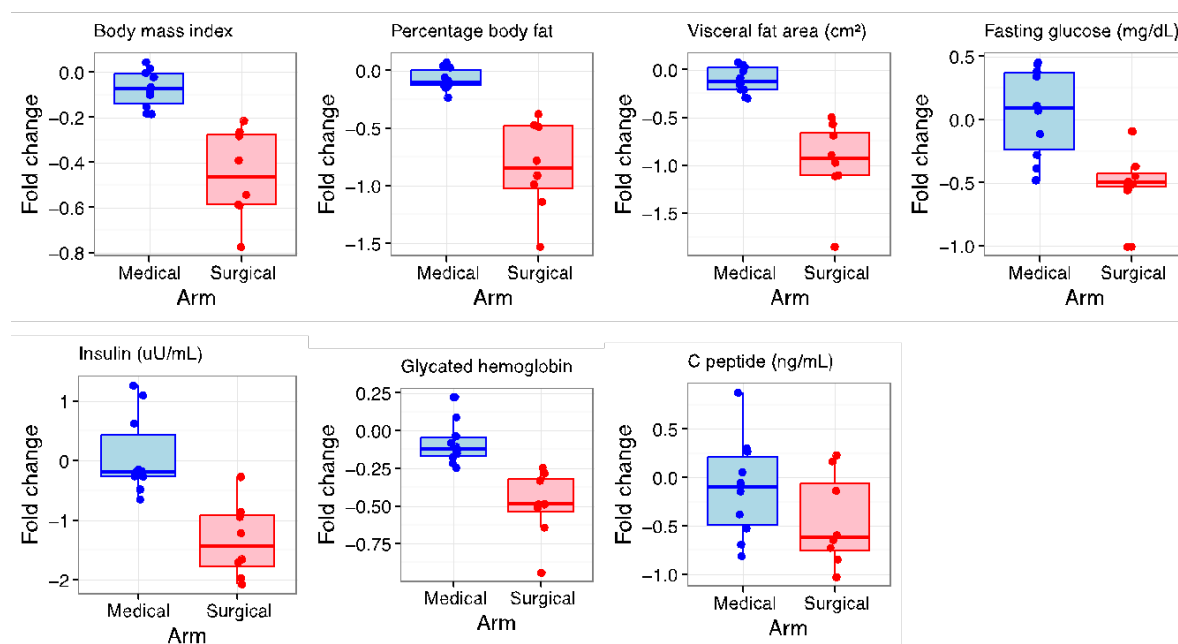


Figure S1. Fold change computed as value at (M12-M0)/M0 is presented for the clinical variables above.

We can easily notice a greater improvement in percentage in the RYGB arm.

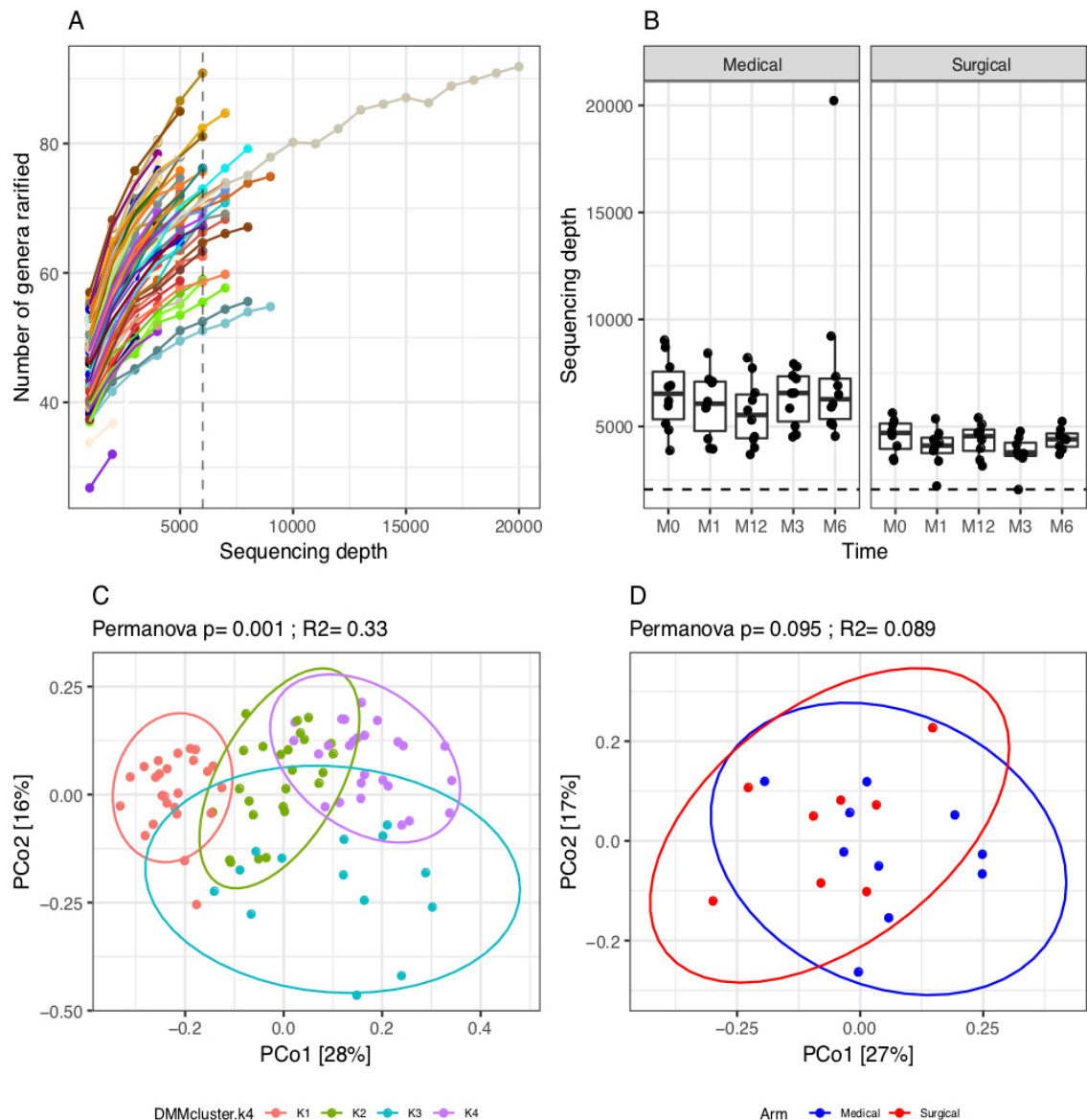


Figure S2. rarefaction analyses, enterotypes in DMM k-4

A: Genus richness as a function of rarefaction for each sample. The threshold of 6000 reads is depicted by the vertical dashed line. **B:** Boxplots of sequencing depth by time point and study group. We notice an overall lower depth in the surgical group. **C:** Principal Coordinates Analyses of all samples coloured by enterotype classification using DMM approach with $k=4$. Permanova test was used to evaluate the impact of enterotype classification in microbiome composition. **D:** Principal Coordinates Analyses of baseline samples coloured by clinical group. Non-significant differences were observed in microbiome composition between both clinical arms (medical, surgical) according with Permanova test.

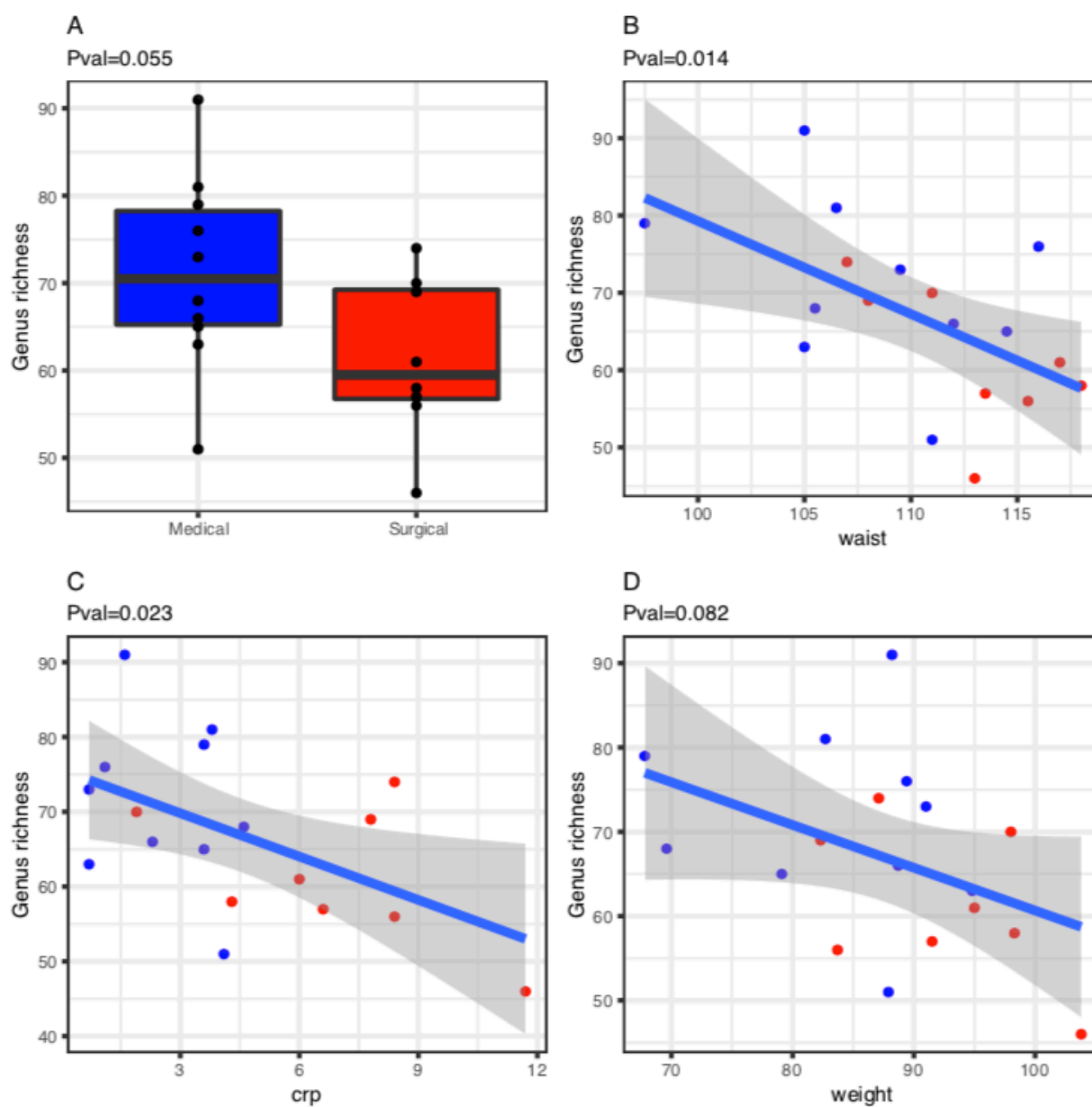


Figure S3. Microbial richness at baseline and relationship with clinical phenotypes.

(A): genus richness distribution at baseline by study group. (B-D): scatter-plots between significant clinical phenotypes and genus richness (p-value in Spearman correlations<0.1).

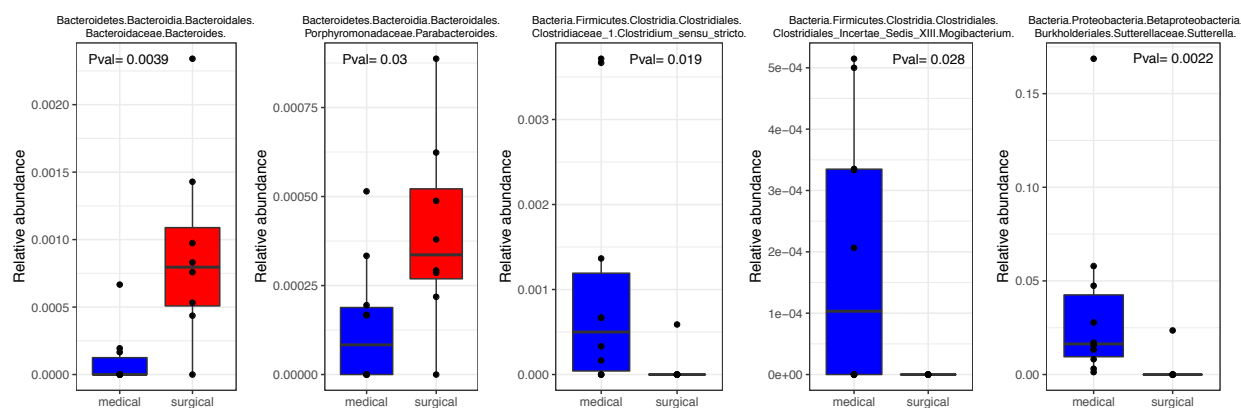


Figure S4. Microbial genus with significant differences between both study groups.

P-values comes from non-parametric Wilcoxon tests. None of them resist P-value adjustment for multiple comparisons (Benjamini-Hochberg method).

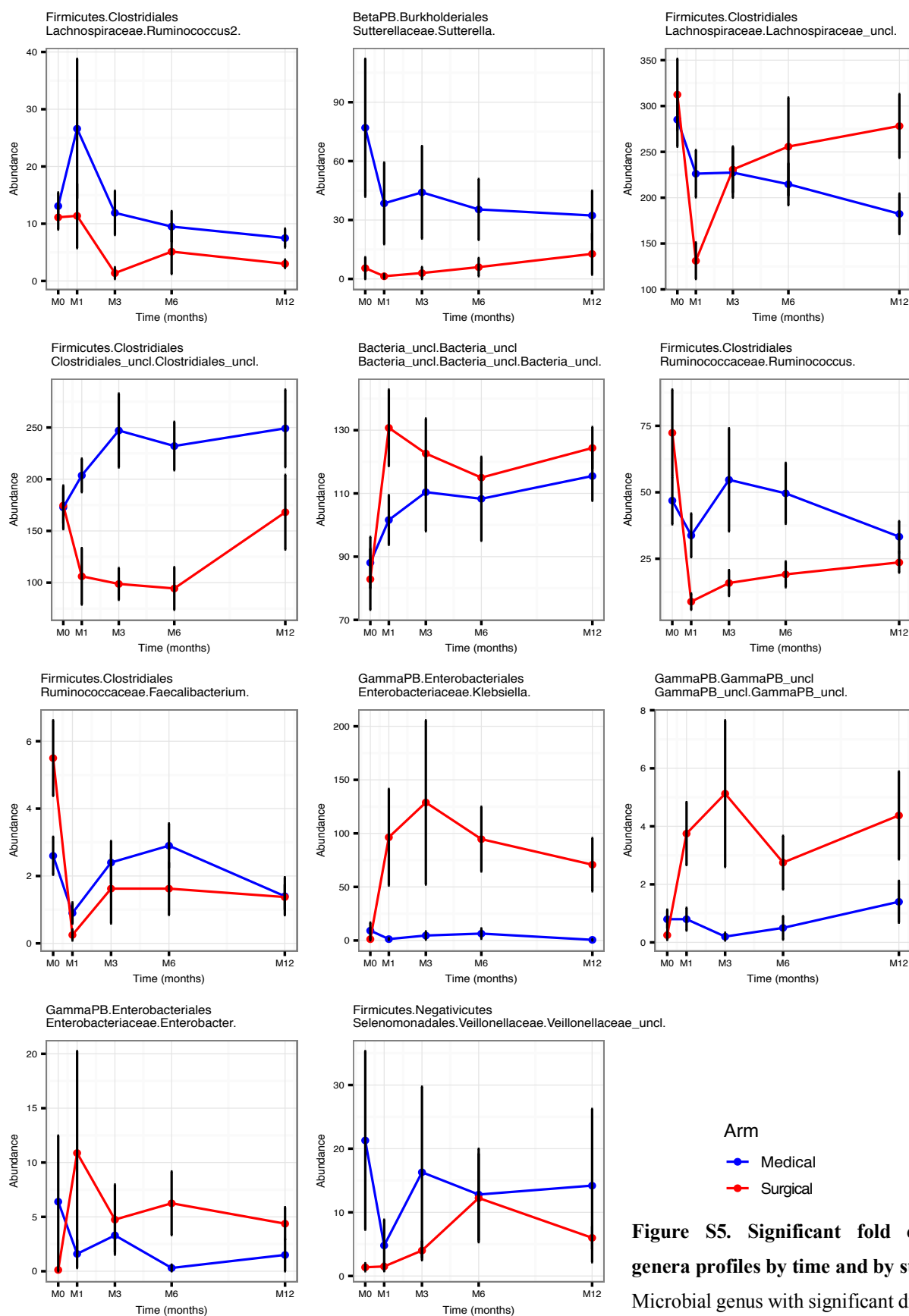


Figure S5. Significant fold changes in genera profiles by time and by study arms. Microbial genus with significant differences in abundance distributions between baseline and month 12 in medical or surgical arms (p-value Paired Wilcoxon tests <0.05; Table S5).

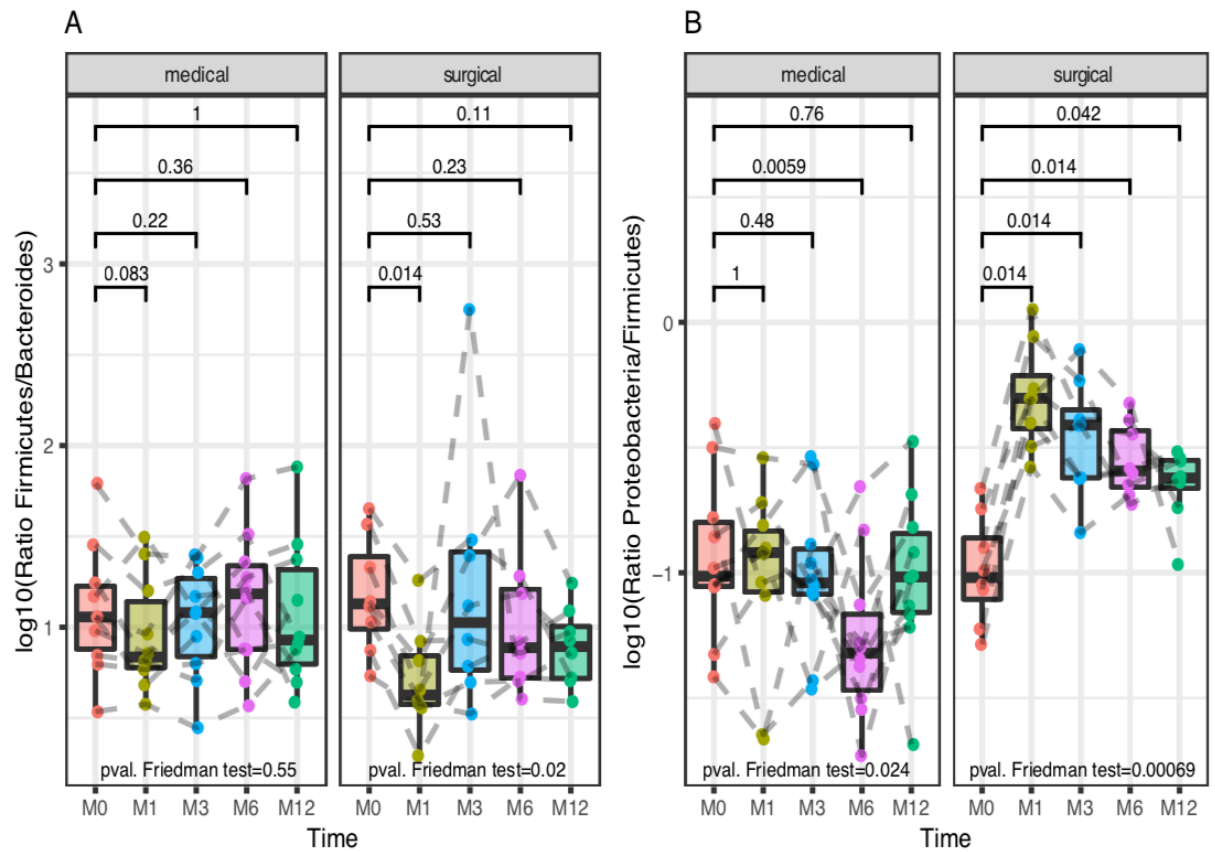


Figure S6. Ratio marker evolution through time and study arms.

A: Boxplots indicating the Firmicutes/Bacteroidetes ratio across time and study arm **B:** Boxplots indicating the Proteobacteria/Firmicutes ratio across time and study arms. P-values of non-parametric paired Wilcoxon tests between baseline and other time points are indicated..

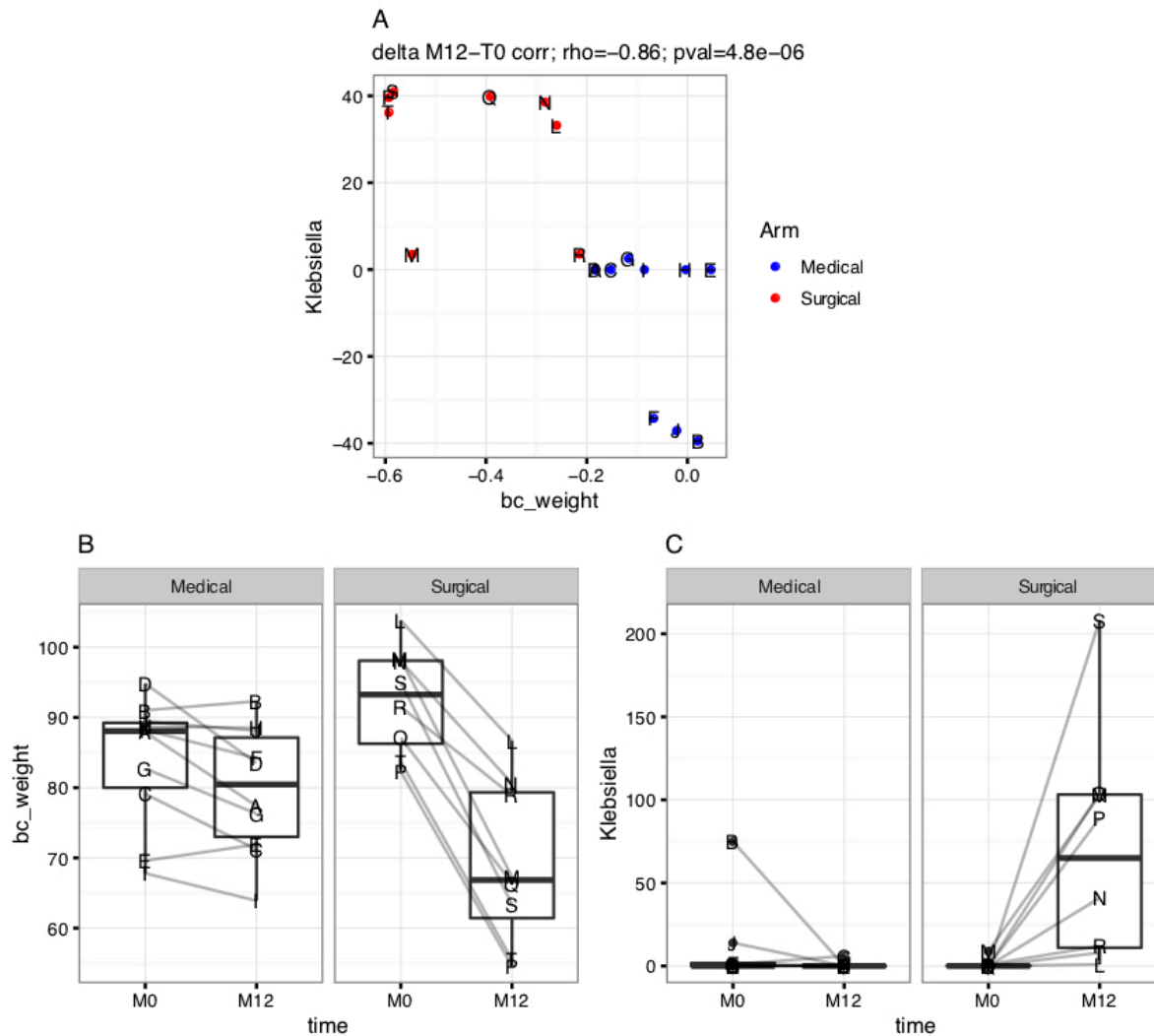


Figure S7: Association between changes in weight and changes in *Klebsiella* genus abundance after interventions

A: Correlation of relative changes in *Klebsiella* genus abundance and changes in Weight between baseline and month 12. **B:** Boxplots illustrating distribution of weight and **C:** boxplot indicating distribution of *Klebsiella* genus abundance between baseline (M0) and month 12 (M12) stratified by study arm.

Flyer with a short study information for patients, medical physicians and research collaborators

Porquê o estudo *PM²* ?

É um estudo que visa compreender as alterações da flora microbiana intestinal na diabetes tipo 2, assim como o seu papel na resolução da diabetes tipo 2 após cirurgia metabólica. Modelos recentes indicam que esta cirurgia resulta numa possível resolução da diabetes tipo 2, assim como numa redução significativa do peso corporal. Pensa-se que a microbiota intestinal tem um papel fulcral nesta modificação do curso da diabetes.

Em que consiste o estudo *PM²* ?

É um estudo em que participantes com diabetes tipo 2 e obesidade moderada serão divididos em 2 grupos (terapêutica médica vs cirurgia metabólica) e onde se avaliarão as alterações metabólicas, assim como a flora microbiana intestinal, em cada grupo e ao longo do tempo.

Onde decorre o estudo *PM²* ?

Faculdade de Medicina da Universidade do Porto
Centro Hospitalar S. João

O estudo *PM²* está aprovado por uma comissão de ética?

Este estudo está de acordo com a Declaração de Helsínquia e foi aprovado pela Comissão de Ética do Centro Hospitalar S. João.

Quem pode entrar no estudo *PM²* ?

Os participantes têm que cumprir os seguintes requisitos:

- Idade entre 20 e 65 anos
- Diagnóstico de diabetes mellitus tipo 2
- IMC entre 30 e 35 Kg/m²

Quem devo contactar ?

Investigadoras responsáveis:
Paula Freitas, MD, PhD | Eva Lau, MD
919 624 956 | evalau.med@gmail.com

Colabore!
Compreender e curar a diabetes
poderá ser uma realidade

DMicrobiota
Metabolic

5.3 Paper 6

Eva Lau, João Sérgio Neves, Manuel Ferreira-Magalhães, Davide Carvalho, Paula Freitas.

Probiotic ingestion, obesity, and metabolic-related disorders: results from NHANES, 1999-2014

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Article

Probiotic Ingestion, Obesity, and Metabolic-Related Disorders: Results from NHANES, 1999–2014

Eva Lau ^{1,2,*}, João Sérgio Neves ^{1,3}, Manuel Ferreira-Magalhães ^{2,4}, Davide Carvalho ^{1,5}
and Paula Freitas ^{1,5}

¹ Department of Endocrinology, Diabetes and Metabolism, Centro Hospitalar Univesrsitário São João, 4200 Porto, Portugal; joaosergioneves@gmail.com (J.S.N.); davidecarvalho@gmail.com (D.C.); paula_freitas@sapo.pt (P.F.)

² CINTESIS—Centre for Health Technologies and Information Systems Research, Faculty of Medicine, University of Porto, 4200 Porto, Portugal; ferreirademagalhaes@gmail.com

³ Department of Surgery and Physiology, Cardiovascular Research Centre, Faculty of Medicine, University of Porto, 4200 Porto, Portugal

⁴ Health Information and Decision Sciences Department, Faculty of Medicine, Porto University, 4200 Porto, Portugal

⁵ I3S—Instituto de Investigação e Inovação em Saúde, Faculty of Medicine, University of Porto, 4200 Porto, Portugal

* Correspondence: evalau.med@gmail.com; Tel.: +351-919-624-956

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Abstract: Gut microbiota dysbiosis has been recognized as having key importance in obesity- and metabolic-related diseases. Although there is increasing evidence of the potential benefits induced by probiotics in metabolic disturbances, there is a lack of large cross-sectional studies to assess population-based prevalence of probiotic intake and metabolic diseases. Our aim was to evaluate the association of probiotic ingestion with obesity, type 2 diabetes, hypertension, and dyslipidemia. A cross-sectional study was designed using data from the National Health and Nutrition Examination Survey (NHANES), 1999–2014. Probiotic ingestion was considered when a subject reported consumption of yogurt or a probiotic supplement during the 24-hour dietary recall or during the Dietary Supplement Use 30-Day questionnaire. We included 38,802 adults and 13.1% reported probiotic ingestion. The prevalence of obesity and hypertension was lower in the probiotic group (obesity-adjusted Odds Ratio (OR): 0.84, 95% CI 0.76–0.92, $p < 0.001$; hypertension-adjusted OR: 0.79, 95% CI 0.71–0.88, $p < 0.001$). Accordingly, even after analytic adjustments, body mass index (BMI) was significantly lower in the probiotic group, as were systolic and diastolic blood pressure and triglycerides; high-density lipoprotein (HDL) was significantly higher in the probiotic group for the adjusted model. In this large-scale study, ingestion of probiotic supplements or yogurt was associated with a lower prevalence of obesity and hypertension.

Keywords: intestinal microbiota; probiotics; nutrients

1. Introduction

Obesity is a pro-inflammatory state that plays a central role in the progression of several diseases, such as type 2 diabetes, hypertension, and dyslipidemia [1]. The pathophysiology of obesity and metabolic-related diseases is complex, resulting from the imbalance between environmental and genetic factors. The human gastrointestinal tract is populated by a complex ecosystem—the gut microbiota—which is responsible for the regulation of essential functions for the maintenance of health, including protective, structural, and histological functions [2]. New insights emphasize the role of gut microbiota in energy homeostasis, giving rise to the “The Metagenome Hypothesis” as a

key player in the comprehension of metabolic diseases [3–5]. Accordingly, recent studies have shown the relationship between intestinal dysbiosis, which is defined as a change in the composition of gut microbiota and glucose and lipid metabolism deregulation in obesity and type 2 diabetes [6,7]. Obesity has been linked to an increase in Firmicutes and a decrease in Bacteroidetes [5,8]. Likewise, two large metagenome-wide association studies reported that type 2 diabetes had a lower proportion of butyrate-producing *Clostridiales* (*Roseburia* and *Faecalibacterium prausnitzii*) and greater proportions of *Clostridiales* that do not produce butyrate [7,9]. On one hand, distinct differences in gut microbiota result in a greater increase in harvesting energy from the diet by fermentation and the absorption of dietary polysaccharides, promoting hepatic lipogenesis. On the other hand, gut microbiota regulate intestinal permeability and an increase in the translocation of lipopolysaccharide-containing gut microbiota increases the inflammatory state, which is named metabolic endotoxemia, accompanied by weight gain and insulin resistance [10]. Understanding this interplay between gut microbiota and the host has created interest in shaping microbiota to prevent, treat, or delay obesity, type 2 diabetes, and metabolic-associated complications.

Probiotics are food components or supplements with living microorganisms that confer health advantages to the host [11]; specific strains have been increasingly studied as a potential therapeutic approach to shape gut microbiota composition, with possible benefits for weight control and diabetes management [12–15]. The administration of probiotics may restore the crosstalk between human host and gut microbiota, controlling homeostatic functions during obesity and metabolic-related disorders. Although there is increasing evidence of the potential benefits of probiotics in metabolic diseases, there is a lack of large cross-sectional studies to evaluate the population-based prevalence of probiotic intake and metabolic differences in those exposed to probiotics compared to those who are not. Large population surveys can create powerful information about population health status and trends. To date, there are no published studies about the large-scale use of probiotic supplements and yogurts and possible associations with metabolic diseases. This type of analysis can produce high-quality data for the real-life use of these types of food and supplements. Our aim was to assess the association of probiotic ingestion, through yogurt or supplements, with the prevalence of obesity and associated metabolic disturbances, namely type 2 diabetes, hypertension, and dyslipidemia.

2. Materials and Methods

2.1. Study Design and Settings

We designed a cross-sectional analysis, using data from the National Health and Nutrition Examination Survey (NHANES). NHANES is a national research survey designed to collect demographic, socio-economic, health, and nutritional statuses from a representative sample of the non-institutionalized civilian resident population of the United States of America. NHANES is a major program of the National Centre for Health Statistics (NCHS), which is part of the Centers for Disease Control and Prevention (CDC), and the detailed methodology is described in the literature [16]. NHANES was approved by the NHANES Institutional Review Board (IRB) and the NCHS Research Ethics Review Board (ERB) (after 2003).

2.2. Participants and Data Collection

We included adults aged 18 years or older, who had been included in NHANES between 1999 and 2014. Pregnant women were excluded. NHANES participants without physical examination or laboratory data and with no dietary data or implausible dietary data (24-hour dietary recall) were also excluded. Figure S1 (supplementary material) shows the flowchart of the study population. NHANES data collection was performed through an in-home interview for demographic and basic health information data collection, together with a health examination in a Mobile Examination Centre (MEC), where participants were examined and surveyed. NHANES MEC examinations included anthropometric measurements, blood pressure assessment, and blood workup. Data were collected by a trained interviewer who had completed an intensive training course administered by the US Department of Agriculture and the US Department of Health and Human Services.

2.3. Assessment and Definition of Probiotic Exposure

In all NHANES cycles, from 1999 to 2014, a 24-hour dietary recall was collected. Using an automated multiple-pass method, a detailed dietary intake (quality and quantity) for the 24-hour period before the interview was recorded. For participants in the 1999–2002 NHANES, only one in-person 24-hour dietary recall was performed. From 2003 onward, an additional telephone dietary recall interview was also performed 3 to 10 days following the in-person dietary interview. For the participants of the 2003–2014 NHANES, we used the mean of the nutritional information from both recalls (in-person recall and telephone recall). To assess probiotic supplementation exposure, we also used the Dietary Supplement Use 30-Day (DSQ), which assesses food supplement use during the preceding 30 days. Table S1 (supplementary material) lists the probiotic supplements included.

Probiotic ingestion was considered when a subject reported consumption of a probiotic supplement or yogurt (as a dietary source of probiotics) during the 24-hour dietary recall or of a probiotic supplement during the DSQ. Non-yogurt foods containing probiotics were classified as probiotic supplements.

2.4. Definition of Metabolic Comorbidities, Smoking, and Physical Activity

Obesity was defined as a body mass index (BMI) ≥ 30 Kg/m². Type 2 diabetes was defined as glycated hemoglobin (HbA1c) $\geq 6.5\%$, fasting plasma glucose level ≥ 126 mg/dL, or current glucose-lowering drug use. Dyslipidemia was assumed if participants had low-density cholesterol (LDL) ≥ 160 mg/dL, high-density cholesterol (HDL) < 40 mg/dL, triglycerides ≥ 200 mg/dL, total cholesterol ≥ 240 mg/dL, or if they were being treated with lipid-lowering drugs. Systolic and diastolic blood pressures (BP) were determined by the mean of 3 or 4 consecutive blood pressure readings. Hypertension was defined as systolic blood pressure ≥ 140 mmHg, or diastolic blood pressure ≥ 90 mmHg, or current medication for hypertension.

Smoking status was classified as former and current smokers. Current smokers were those who reported smoking at least 100 cigarettes during their lifetime and were currently smoking every day, or some days. Former smokers were those who reported smoking at least 100 cigarettes during their lifetime, but do not currently smoke.

Physical activity was measured differently along NHANES cycles. We classified participants using variables that allowed categorization of physical activity level into three categories (low, intermediate, and high). From 1999 to 2006 the physical activity level was assessed with the question “compare activity with others of the same age” (participants answering “less active” were classified into category “low”, “about the same” into category “intermediate”, and “more active” into category “high”). From 2007 to 2014, the weekly metabolic equivalents (MET) minutes of physical activity (accounting for vigorous work-related activity, moderate work-related activity, walking or bicycling for transportation, vigorous leisure-time physical activity, and moderate leisure-time physical activity) was divided into tertiles (participants were classified as “low” if included in the lower MET-minute tertile, as “intermediate” if in the middle MET-minute tertile, and as “high” if in the higher MET-minute tertile).

2.5. Statistical Analysis

Statistical analysis took into account the complex survey design of the NHANES dataset and was performed according to the CDC analytic recommendations [17].

Continuous variables were described as mean $\pm$ standard deviation (SD) and categorical variables were described as absolute and relative frequencies. To assess the association between probiotic exposure and metabolic comorbidities (obesity, diabetes, hypertension, and dyslipidemia), we performed unadjusted and adjusted logistic regression models. To evaluate the association between probiotic exposure and cardiometabolic parameters (BMI, HbA1c, fasting plasma glucose, systolic BP, diastolic BP, LDL, HDL, and triglycerides), we performed unadjusted and adjusted multivariate linear regression models. We excluded those participants who were being treated with anti-hypertensive drugs from systolic and diastolic BP analysis, participants treated with anti-

dyslipidemic drugs from analysis concerning lipid profiles, and patients treated with antidiabetic drugs from HbA1c and fasting plasma glucose analysis.

In the adjusted analyses, we used the following models (Table S2): Model 1, including age, sex, ethnicity (Mexican American, other Hispanic, non-Hispanic white), annual family income (<\$25,000, \$25,000 to \$75,000, >\$75,000), and education (<9th grade, ≥9th grade); model 2, including all model 1 covariates plus alcohol intake, smoking status (never a smoker, current smoker, or former smoker), physical activity (low, intermediate, high), ingested kcal per day, ingested carbohydrates/kcal per day, ingested protein/kcal per day, ingested fiber/kcal per day, and ingested polyunsaturated/saturated fatty acids ratio. In model 2, we also included BMI in all analyses except in the obesity analysis and sodium intake per day only in the hypertension and blood pressure analyses. As a supplementary analysis, we performed an additional model (model 3) that classified the diet pattern using the Dietary Approaches to Stop Hypertension (DASH) score. In model 3 we included all model 1 covariates plus alcohol intake, smoking status, physical activity, and the DASH dietary pattern score. Model 3 also included BMI in all analyses except the obesity analysis and sodium intake per day only in the hypertension and blood pressure analyses. The DASH score is based on 9 target nutrients (sodium, total fat, saturated fat, protein, fiber, cholesterol, calcium, magnesium, and potassium), as previously described [18]. Individuals meeting the DASH goal were given a score of 1.0 for that nutrient; if they attained an intermediate goal, they were given a score of 0.5 for that nutrient. The DASH score is the sum of the score for each individual nutrient. Logistic regression results were expressed as an odds ratio (OR) and a 95% confidence interval (95% CI). A two-sided *p*-value of <0.05 was considered statistically significant. Analyses were performed with Stata (version 14.2).

3. Results

3.1. Baseline Characteristics According to Probiotic Consumption

We included 38,802 adults, of whom 13.1% had exposure to probiotic supplements or yogurt. Baseline population characteristics according to probiotic consumption are described in Table 1. Participants in the group exposed to probiotic supplements or yogurt were more likely to be female, older, non-Hispanic white and to have a higher income and education level. Ingestion of kcal/day was similar between groups.

Table 1. Baseline population characteristics according to probiotic consumption (*n* = 38,802).

	No Exposure to Probiotics	Exposure to Probiotics	<i>p</i> Value
Participants, <i>n</i> (%)	33,719 (86.9%)	5083 (13.1%)	n.a.
Socio-economic characteristics			
Male gender, %	50.2 %	35.0 %	<0.001 *
Age, years ± SD	46.0 ± 15.3	48.9 ± 13.2	<0.001 *
Annual family income <\$25000, %	30.7%	19.6%	<0.001 *
Education level less than 9 th grade, %	6.4%	2.7%	<0.001 *
Ethnicity			<0.001 *
Non-Hispanic White, %	68.4%	79.5%	
Non-Hispanic Black, %	11.9%	5.4%	
Mexican American, %	8.5%	5.0%	
Other Hispanic, %	5.2%	4.1%	
Other ethnicities, %	6.0%	5.9%	
Risk factors			
Current smokers, %	20.2%	7.6%	<0.001 *
Former smokers, %	28.3%	31.7%	0.002 *
Alcohol consumption >20 g/day, %	15.7%	13.9%	0.028 *
Physical activity level #			0.028 *

Low	28.8%	26.9%	
Intermediate	36.6%	39.3%	
High	34.6%	33.8%	
Nutritional characteristics			
Kcal/day, kcal $\pm$ SD	2060.7 $\pm$ 648.9	2042.4 $\pm$ 529.3	0.163
Carbohydrates/day, g/100 kcal $\pm$ SD	12.3 $\pm$ 2.3	12.6 $\pm$ 1.8	<0.001 *
Protein/day, g/100 kcal $\pm$ SD	3.9 $\pm$ 0.9	4.2 $\pm$ 0.8	<0.001 *
Fiber/day, g/100 kcal $\pm$ SD	0.8 $\pm$ 0.3	0.9 $\pm$ 0.3	<0.001 *
Polyunsaturated/saturated fatty acids ratio $\pm$ SD	0.7 $\pm$ 0.3	0.8 $\pm$ 0.3	0.001 *
Sodium per day, mg	3389.2 $\pm$ 1272.3	3282.3 $\pm$ 1024.4	<0.001 *
DASH score (0–9)	2.66 $\pm$ 1.15	3.33 $\pm$ 1.12	<0.001 *
Cardiometabolic parameters			
BMI, kg/m ²	28.5 $\pm$ 5.8	27.8 $\pm$ 5.1	<0.001 *
HbA1c, %	5.6 $\pm$ 0.8	5.5 $\pm$ 0.6	<0.001 *
Glucose, mg/dL	97.9 $\pm$ 28.4	96.0 $\pm$ 22.4	0.001 *
Systolic BP, mmHg	122.8 $\pm$ 15.7	120.5 $\pm$ 13.9	<0.001 *
Diastolic BP, mmHg	71.2 $\pm$ 10.1	70.2 $\pm$ 8.4	<0.001 *
HDL, mg/dL	51.9 $\pm$ 13.6	56.7 $\pm$ 12.6	<0.001 *
LDL, mg/dL	116.5 $\pm$ 29.5	115.5 $\pm$ 25.5	0.398
Triglycerides, mg/dL	139.1 $\pm$ 100.1	121.1 $\pm$ 61.5	<0.001 *

BMI: body mass index; BP: blood pressure; HDL: high-density lipoprotein; LDL: low-density lipoprotein; n.a.: Not applicable; * statistically significant. # See methods regarding physical activity level definition.

3.2. Prevalence of Metabolic Comorbidities According to Probiotic Consumption

The prevalence of metabolic comorbidities according to probiotic supplement or yogurt exposure is represented in Figure 1. All four studied comorbidities were lower in the exposed group. The comorbidities that showed higher differences in prevalence between groups were obesity and hypertension; 5.4% and 2.9% lower, respectively. Diabetes prevalence difference was only 1.6%, but this was still significant in our analysis.

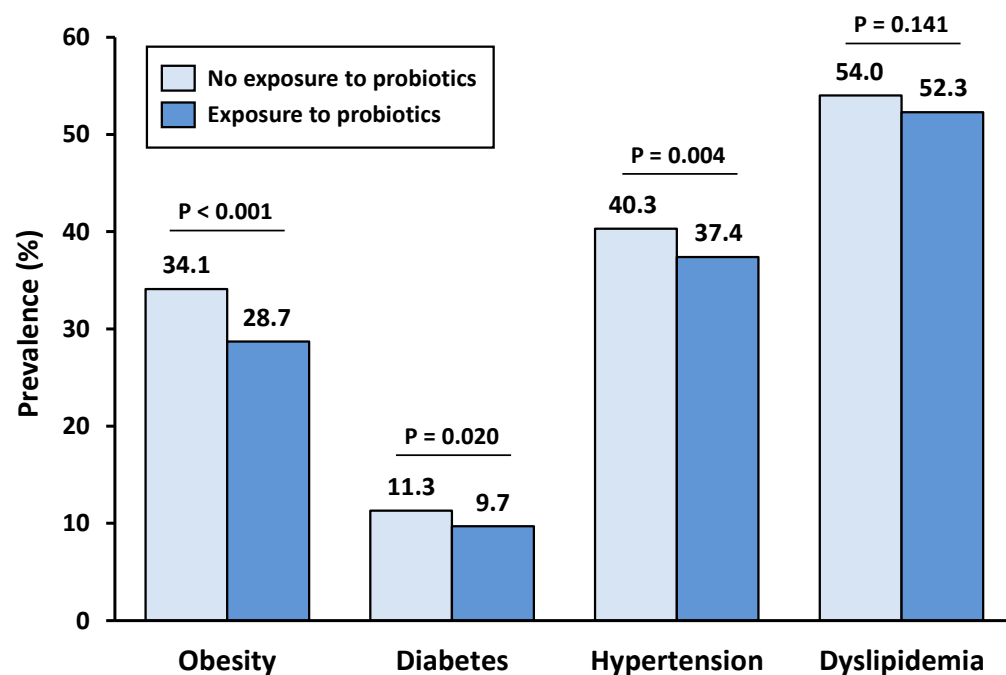


Figure 1. Prevalence of obesity, diabetes, hypertension, and dyslipidemia, according to probiotic exposure.

3.3. Modulation of Metabolic Comorbidities According to Probiotic Consumption

Table 2 summarizes the results of metabolic disturbances by using unadjusted and adjusted models of the prevalence of comorbidities according to probiotic supplement or yogurt exposure. For unadjusted analysis, participants exposed to probiotics manifested a 22% reduction in the odds of having obesity (OR: 0.78, 95% CI 0.71–0.86; $p < 0.001$), a 16% reduction in the odds of having diabetes (OR: 0.84, 95% CI 0.73–0.96; $p = 0.020$), and a 12% reduction in the odds of having hypertension (OR: 0.88, 95% CI 0.81–0.96; $p = 0.004$). No significant differences were found in dyslipidemia prevalence.

After adjusting for potential confounders, obesity and hypertension prevalence remained significantly lower in the probiotic exposed group (Table 2). In turn, diabetes prevalence became similar between groups.

Table 2. Odds ratios of disease in subjects exposed to probiotics compared to non-exposed subjects.

	Unadjusted, OR (95% CI)	<i>p</i> Value	Model 1, OR (95% CI)	<i>p</i> Value	Model 2, OR (95% CI)	<i>p</i> Value
Obesity	0.78 (0.71–0.86)	<0.001	0.82 (0.75–0.90)	<0.001	0.83 (0.76–0.92)	<0.001 *
Diabetes	0.84 (0.73–0.97)	0.020	0.96 (0.82–1.13)	0.650	0.97 (0.81–1.17)	0.783
Hypertension	0.88 (0.81–0.96)	0.004	0.76 (0.68–0.84)	<0.001	0.79 (0.71–0.89)	<0.001 *
Dyslipidemia	0.93 (0.85–1.02)	0.141	0.95 (0.86–1.05)	0.356	1.01 (0.90–1.13)	0.863

Model 1: Age, sex, race, income and education; model 2: Model 1 + alcohol intake, smoking status, physical activity, kcal per day, carbohydrates/kcal per day, protein/kcal per day, fiber/kcal per day, and polyunsaturated/saturated fatty acids ratio. Model 2 also includes BMI in all analyses except in the obesity analysis and includes sodium intake per day only in the hypertension analysis. * Statistically significant.

Table 3 summarizes the results of cardiometabolic parameters according to probiotic supplement or yogurt exposure. In the unadjusted analysis, all the studied markers were lower in the probiotic-exposed group, with the exception of HDL (which was higher in the exposed group) and LDL (no differences were seen). After adjusting for potential confounders, BMI, and systolic BP, diastolic BP and triglycerides remained significantly lower in the probiotic-exposed group and HDL remained significantly higher (Table 3). Table S3 and S4 summarize the odds ratios of disease and the variation of cardiometabolic parameters, respectively, according to probiotic supplement or yogurt exposure, after accounting for the DASH dietary pattern score. The associations of probiotic supplement or yogurt ingestion with cardiometabolic parameters were not different after adjusting for DASH diet adherence.

Table 3. Variation of cardiometabolic parameters in participants exposed to probiotics compared to non-exposed participants.

	Unadjusted	<i>p</i> Value	Model 1	<i>p</i> Value	Model 2	<i>p</i> Value
BMI, kg/m ²	−0.74 (−1.01 to −0.46)	<0.001 *	−0.47 (−0.75 to −0.20)	0.001 *	−0.41 (−0.67 to −0.15)	0.002 *
HbA1c ^a , %	−0.03 (−0.05 to −0.01)	0.003 *	−0.01 (−0.03 to 0.01)	0.382	0.01 (−0.01 to 0.03)	0.590
Glucose ^a , mg/dL	−0.94 (−1.71 to −0.18)	0.016 *	−0.55 (−1.29 to 0.19)	0.146	−0.17 (−0.90 to −0.55)	0.641
Systolic BP ^b , mmHg	−2.43 (−3.33 to −1.53)	<0.001 *	−1.99 (−2.83 to −1.16)	<0.001 *	−1.48 (−2.31 to −0.66)	<0.001 *
Diastolic BP ^b , mmHg	−0.92 (−1.46 to −0.38)	0.001 *	−1.13 (−1.71 to −0.55)	<0.001 *	−0.86 (−1.45 to −0.27)	0.005 *
LDL ^c , mg/dL	0.03 (−2.53 to 2.59)	0.980	−0.93 (−3.50 to 1.63)	0.472	−0.02 (−2.59 to 2.55)	0.988
HDL ^c , mg/dL	4.93 (4.10 to 5.75)	<0.001 *	1.89 (1.12 to 2.66)	<0.001 *	1.43 (0.69 to 2.17)	<0.001 *
Triglycerides ^c , mg/dL	−16.82 (−22.64 to −10.99)	<0.001 *	−11.74 (−18.14 to −5.33)	<0.001 *	−8.52 (−15.18 to −1.86)	0.013 *

Model 1: Age, sex, race, income and education; model 2: Model 1 + alcohol intake, smoking status, physical activity, kcal per day, carbohydrates/kcal per day, protein/kcal per day, fiber/kcal per day, and polyunsaturated/saturated fatty acids ratio. Model 2 also includes BMI in all analyses except in the BMI analysis and includes sodium intake per day only in the BP analyses. ^a Excluding participants treated with anti-hypertensive drugs. ^b Excluding participants treated with anti-dyslipidemic drugs. ^c Excluding participants treated with antidiabetic drugs. * Statistically significant.

The odds of metabolic comorbidities according to the origin of probiotics is presented in Figure 2. Among those participants exposed to probiotics, 95.7% were exposed to yogurt and 5.4% were exposed to probiotic supplements. The odds of obesity, diabetes, hypertension, and dyslipidemia for participants exposed to yogurt or to probiotic supplements alone were similar to the odds for participants exposed to any type of probiotic. Although the confidence intervals were wider in the analysis of probiotic supplements (due to the smaller number of exposed individuals), the point estimates for the association with comorbidities was similar to the association with exposure to any probiotic or exposure to yogurt, with the exception of diabetes. Although the association in both analyses was not significant, the odds ratio for diabetes was 0.92 (95% CI 0.77–1.10) among participants exposed to yogurt and 1.28 (95% CI 0.71–2.23) among those exposed to probiotic supplements.

Figure 2

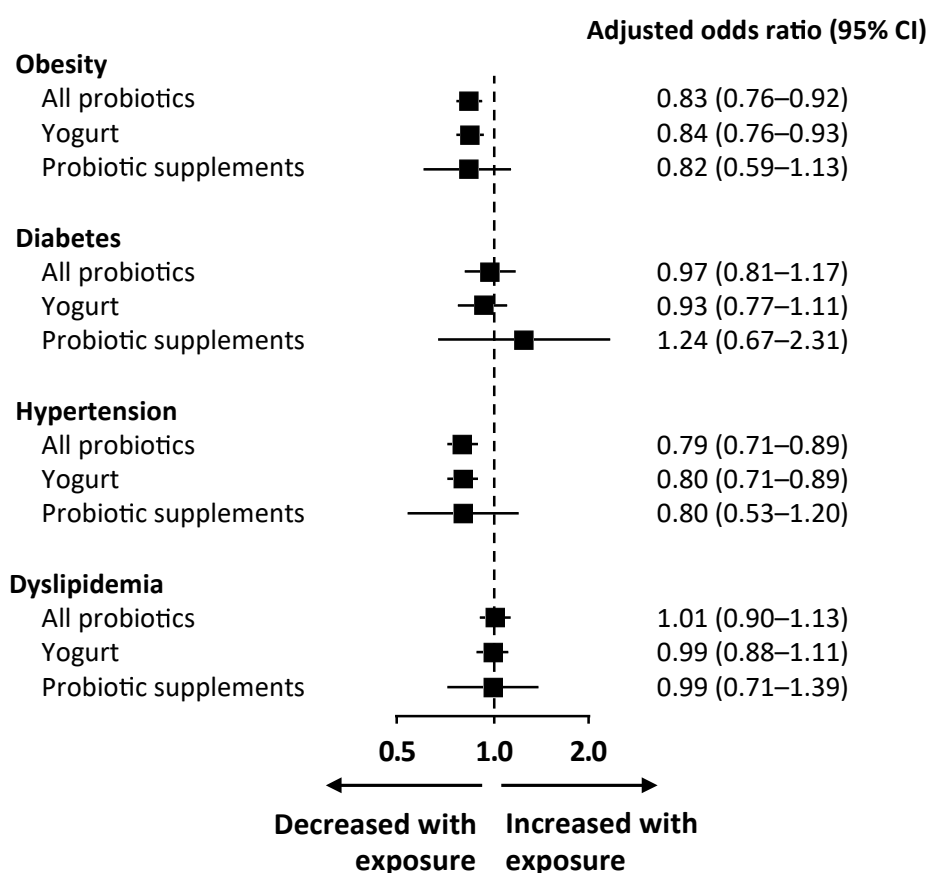


Figure 2. Odds ratio of disease in participants exposed to probiotics compared to those not exposed, according to the origin of probiotics (all probiotics, yogurt, or probiotic supplements). Logarithmic regression models adjusted for age, sex, race, income, education, alcohol intake, smoking status, physical activity, carbohydrates/kcal per day, protein/kcal per day, fiber/kcal per day, and polyunsaturated/saturated fatty acids ratio (model 2). Model 2 also includes BMI in all analyses except in the obesity analysis and includes sodium intake per day only in the hypertension analysis.

4. Discussion

We conducted a cross-sectional analysis on a large and representative US population, for a total of 38,802 adults, and found that 13.1% reported the use of probiotic supplements or yogurt ingestion. Although there are several studies addressing the possible beneficial associations of probiotic

ingestion and several metabolic outcomes, there is a lack of large cross-sectional studies to objectively assess population-based prevalence of probiotic intake and metabolic differences in those exposed and not exposed to probiotics. To our knowledge, this was the first large cross-sectional analysis aiming to assess the association of probiotic ingestion, either by probiotic supplements or yogurt, with metabolic disturbances. Probiotic ingestion was associated with a 17% lower prevalence of obesity and a 21% lower prevalence of hypertension. Furthermore, HDL cholesterol was significantly higher and triglyceride levels were significantly lower in the probiotic group.

Probiotics modulate gut microbial communities, exerting beneficial metabolic effects through the regulation of multitudinous physiological metabolic pathways. Among the molecular mechanisms, the regulation of adipogenesis, stimulation of insulin signaling, improvement of gut barrier function, reduction of metabolic endotoxemia, and down-regulation of cholesterol levels are some of the suggested key players in the crosstalk between probiotics and metabolic disorders [19].

4.1. Obesity

We found that probiotic ingestion, via supplements or yogurt, was associated with a lower prevalence of obesity (17% reduction), before and after adjusting for demographics and potential confounders. Although subjects that consumed probiotics had a higher consumption of carbohydrates, fiber, and protein, the effects of probiotics on BMI were significant, even after adjustment for confounders (-0.41 Kg/m^2 between groups in model 2). Furthermore, there were no differences in total energy intake per day between groups and, also, there was no linear association of physical activity level (low, intermediate, and high) and probiotic ingestion. The only thing observed was a higher proportion of intermediate physical activity level, but lower proportions of high and low physical activity levels in the probiotic-exposed group. Putting this all together, these results support our hypothesis of the beneficial impact of probiotic ingestion per se on metabolic health, namely its effects on the regulation of body weight. In agreement with our study, a recent meta-analysis of randomized clinical trials with 957 subjects, with a mean BMI of 27.6 kg/m^2 , showed that probiotic administration significantly reduced body weight by -0.60 kg and BMI by -0.27 kg/m^2 [20]. NHANES's data between 1999–2004 also showed that yogurt consumption was associated with a lower likelihood of having obesity (OR: 0.57, 95% CI 0.40–0.82; $p < 0.05$) [21]. A prospective study including 120,877 US individuals evaluated lifestyle factors and weight change at four-year intervals, with multivariable adjustments. The four-year weight change was negatively associated with yogurt ingestion [22], which further supports our results.

4.2. Diabetes

In the unadjusted analysis, probiotic supplement and yogurt consumers had lower odds of having diabetes and, accordingly, lower glycemia and HbA1c levels. However, when adjusted for individual characteristics and confounders, the difference was no longer significant. One possible explanation for this is that diabetes is largely determined by individual demographic characteristics, such as age, ethnicity and, mainly, BMI [23]. Therefore, there are no longer differences after adjusting for individual factors. Furthermore, in another meta-analysis of randomized controlled trials, Ruan I et al. [14], concluded that probiotics had a greater effect on fasting blood glucose for people with diabetes. On the contrary, those without diabetes only show a trend of a glucose-lowering effect, which shows that probiotic supplementation may have a greater benefit for individuals with higher fasting glucose levels [14]. In three prospective cohorts in the US, yogurt intake was consistently and inversely associated with type 2 diabetes risk [24]. Probiotic supplementation seems to be more effective in reducing HbA1c in diabetic patients with higher baseline BMIs and, furthermore, probiotic supplements with greater bacterial species may be more effective [25], which we could not evaluate in our study. Yao K et al. [26] conducted a meta-analysis in patients with type 2 diabetes to investigate the effects of probiotics on glucose metabolism, and, similar to our results, they did not find a significant effect on fasting blood glucose levels, although HbA1c was improved with probiotic supplementation. In the analysis according to the origin of probiotics, we found a non-significant trend to higher odds of diabetes in participants exposed to probiotic supplements, which was not

seen in participants exposed to yogurt. This may be explained by a reverse-causality relationship. Participants with diabetes may be more prone to consuming probiotic supplements due to their known potential to improve glucose control in diabetes.

4.3. Dyslipidemia

We did not find differences in the odds of dyslipidemia according to probiotic ingestion, however, we did observe some beneficial aspects in the lipid profile of the probiotic-exposed group. HDL was significantly increased and triglycerides were significantly decreased in the probiotic group, even after adjustment. Our results are in line with a study by Fu et al. [27], with 893 subjects, which showed that gut microbiota was associated with a 4.5% variance in BMI, a 6% variance in blood levels of triglycerides, and a 4% variance in HDL, but had little effect on LDL or total cholesterol. In contrast, a meta-analysis including 1624 participants (828 in the probiotic and 796 in the placebo group) demonstrated that probiotics reduced total cholesterol and LDL cholesterol by 7.8 mg/dL and 7.3 mg/dL, respectively but had no significant effects on HDL cholesterol or triglycerides [28]. Human clinical studies have yielded different results on the association of lipid profiles with probiotic supplementation. Differences in the type of probiotics and in the experimental designs, including the clinical heterogeneity of participants, namely their baseline levels of blood lipids, may affect the role of probiotics in lipid metabolism and explain the different results. Given the clinical correlation between obesity and related metabolic disorders, it is possible that the observed associations between gut bacterial composition and lipid levels can be mediated, in part, through the effects on BMI.

4.4. Hypertension

Ingestion of probiotic supplements or yogurt resulted in a 21% reduction in the odds of hypertension after adjusting for potential confounders. Both systolic BP and diastolic BP were significantly lower. A meta-analysis of randomized clinical trials supports our results, showing that probiotic consumption significantly reduced systolic BP by -3.56 mmHg and diastolic blood pressure by -2.38 mmHg, compared with control groups [29]. In our study, for the adjusted model, probiotic ingestion was associated with a lower systolic BP by 1.48 mmHg and a lower diastolic BP by 0.86 mmHg. The modulation of BP by probiotics may be linked to several mechanisms, including their capacity to improve lipid profiles, to reduce BMI, and to produce bioactive peptides with angiotensin-converting inhibitory activity [30–32].

4.5. Strengths and Limitations

Our study has a number of strengths and limitations, which need to be highlighted. We conducted an analysis of a large cross-sectional survey, which was representative of the US population. To the best of our knowledge, this is the first cross-sectional study aiming to assess the association of probiotic ingestion, through supplements or yogurt, and metabolic disturbances. A previous study on the NHANES population was carried out, aiming to evaluate the association between dairy products (which included yogurt ingestion) with obesity and other disturbances of metabolic syndrome; however it did not evaluate probiotic supplementation [21]. We also developed an analytic strategy that included adjusted logistic regression models to obviate confounders such as physical activity, alcohol consumption, and smoking status. One of the limitations of this type of analysis is that we cannot deduce causation; however, we were able to show strong associations between probiotic ingestion and the prevalence of some metabolic disturbances. The participants in the probiotic-exposed group were more likely to be non-Hispanic white. It has been previously stated that gut microbiome varies by geographic ancestry [33], which may limit the extrapolation of these results to other ethnic groups. Furthermore, inter-individual differences in the composition of gut microbiota were previously associated with different responses to probiotics [34], including non-responders to gut microbiota modulation. The absence of individual gut microbiota analyses in our study may have hampered the analysis of these type of responders. The assessment of probiotic exposure was based on self-reported information, however, NHANES only provides dietary

information that is considered to be reliable [35]. In our study, we defined probiotic ingestion as being either yogurt or probiotic supplement consumption. Assuming that there could be differences between the types of ingestion, we performed a sensitivity analysis based on the origin of probiotics. There were no significant differences in associations according to the source of exposure to probiotics. Probiotic supplements and yogurts vary in the amounts of bacteria per serving they are composed of. In our study, the population was classified according to whether or not they were exposed to probiotic supplements or yogurt. The duration and quantity of exposure were not taken into account, which may have diluted the magnitude of the association between probiotic consumption and metabolic disturbance.

In summary, our study supports the beneficial association of probiotic supplement or yogurt ingestion with metabolic health, specifically obesity and hypertension. Furthermore, probiotic ingestion was significantly associated with higher HDL cholesterol and lower triglyceride levels. Our study supports the possibility of gut microbiota modulation by the use of probiotics as an attractive therapeutic target to prevent and treat obesity and related cardiometabolic disorders. Future research should focus on understanding the gut microbiota ecosystem and on identifying individuals who benefit the most from selective modulation of microbiota.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Figure S1: Flowchart of the study population; Table S1: Probiotic supplements included; Table S2: Variables included in the adjusted models; Table S3: Odds ratio of disease in subjects exposed to probiotics compared to non-exposed (model 3); Table S4: Variation of cardiometabolic parameters in participants exposed to probiotics compared to non-exposed (model 3).

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Supplementary data

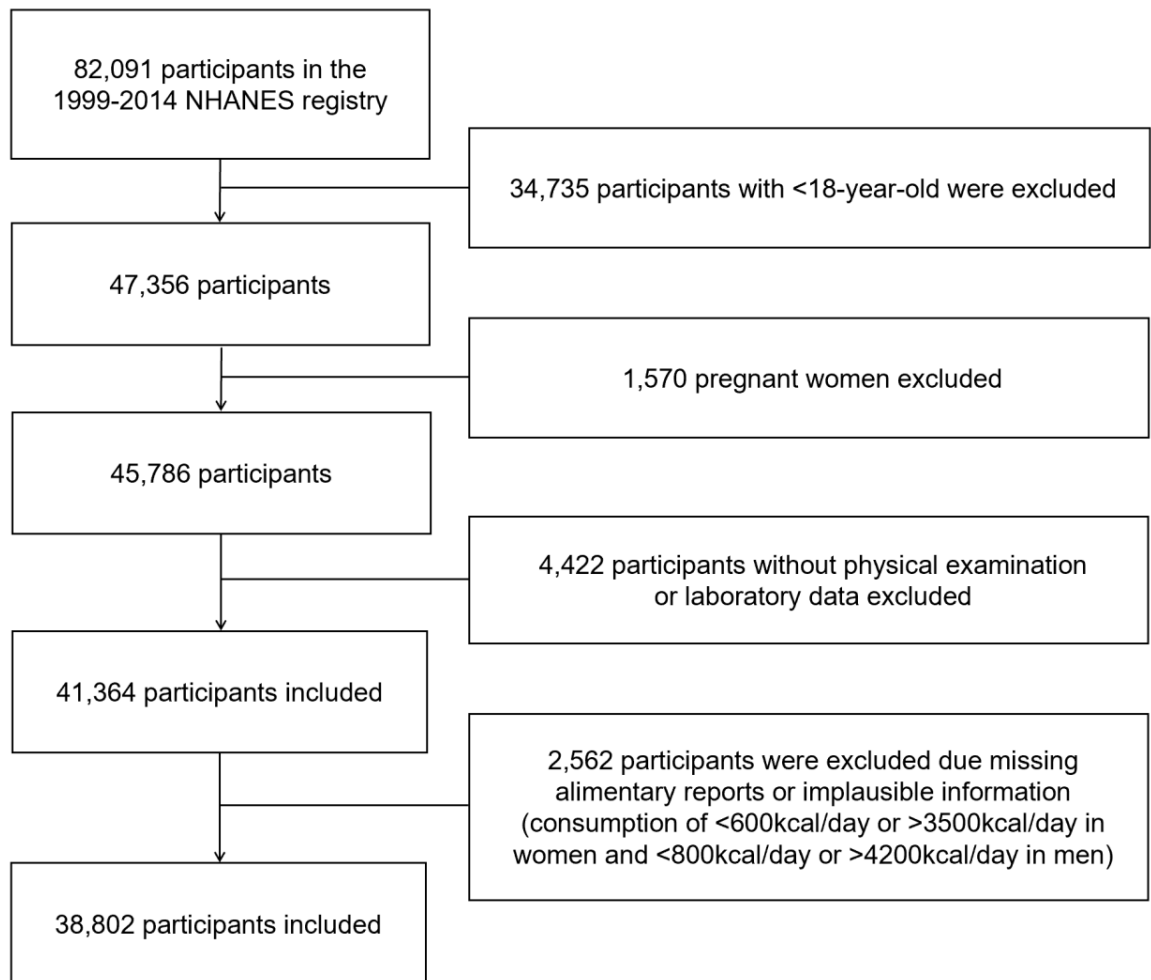


Figure S1. - Flowchart of the study population

Table S1. – Probiotic supplements included.

21ST CENTURY HIGH POTENCY ACIDOPHILUS
365 PROBIOTIC COMPLEX WITH ACIDOPHILUS DOUBLE STRENGTH
4X PROBIOTIC UP&UP CONTAINS 4 STRAINS OF NATURAL, BENEFICIAL BACTERIA WITH B. INFANTIS 10 MG
ACCUFLORA ADVANCED CD PROBIOTIC 5 PROBIOTIC STRAINS ADVANCED CD: CONTROLLED DELIVERY
ADVANCED NATURALS FLORAMAX ADVANCED PROBIOTIC FORMULA 12 BILLION CELLS OF 7 STRAINS OF MICROFLORA WITH ACIDOPHILUS, BIFI
ALIGN PROBIOTIC
ALIGN PROBIOTIC B. INFANTIS 35624 WITH: UNIQUE B. INFANTIS 35624
ALLERGY RESEARCH GROUP BIFIDOBOTICS WITH L. SPOROGENES
ALLERGY RESEARCH GROUP GI FLORA L.ACIDOPHILUS, L.CASEI, L. RHAMNOSUS, B. LONGUM HYPOALLERGENIC
AMERICAN HEALTH CHEWABLE ACIDOPHILUS WITH BIFIDUS MILK FREE
AMERICAN HEALTH CHEWABLE MILK FREE ACIDOPHILUS AND BIFIDUM ONE BILLION ORGANISMS VEGETARIAN FORMULA
BACID PROBIOTIC CONTAINS A BLEND OF BENEFICIAL BACTERIA INCLUDING LACTOBACILLUS ACIDOPHILUS
BASIC'S NATURAL ACIDOPHILUS BASIC VITAMINS
BIOGAIA PROBIOTICS GERBER SOOTHE COLIC DROPS PROBIOTIC
BIORAY CYTOFLORA PROBIOTIC IMMUNITY TONIC DAILY
BIOSPEC PROBIOTIC PLUS HIGH POTENCY, MULTI-STRAIN PROBIOTIC
BLUEBONNET MILK-FREE ACIDOPHILUS PLUS FOS L. ACIDOPHILUS, L. BULGARICUS, BIFIDUS
BREAKTHROUGH FORMULATIONS MEGA-FLORA DIGESTIVE TRACT FLORA
CELL NUTRITIONALS PROBIOTICS AR ACID RESISTENT 16 STRAIN FORMULA COMPREHENSIVE, DAIRY FREE FORMULA PROVIDING 12 BILLION VIABLE CELLS PER CAPSULE AT TIME OF MANUFACTURE
COUNTRY LIFE DAIRY-FREE ACIDOPHILUS WITH PECTIN
COUNTRY LIFE POWER-DOPHILUS WITH FOS HYPOALLERGENIC MILK FREE
CULTURELLE DIGESTIVE HEALTH PROBIOTIC PROBIOTIC WITH NATURALLY SOURCED DAIRY-FREE LACTOBACILLUS GG ONCE DAILY CAPSULES 10 BILLION ACTIVE CULTURES
CULTURELLE KIDS! PACKETS PROBIOTIC PROBIOTIC WITH NATURALLY SOURCED LACTOBACILLUS GG
CULTURELLE WITH LACTOBACILLUS GG
CVS PHARMACY EXTRA STRENGTH ACIDOPHILUS
DDS-100 ACIDOPHILUS WITH FOS CHEWABLE TABLETS (2 BILLION CFU/G) UAS LABORATORIES
DEFAULT LACTOBACILLUS ACIDOPHILUS
DEFAULT PROBIOTIC
DESIGNS FOR HEALTH PROBIOTIC SYNERGY POWDER 20 BILLION ORGANISMS PER SERVING

DIGESTIVE ADVANTAGE LACTOSE DEFENSE FORMULA DUAL-ACTION! ENZYME PLUS PROBIOTICS TAKE EVERY DAY
DIGESTIVE ENZYMES & PROBIOTICS WITH ENZYGUARD-D 4LIFE
DIGESTZEN PB ASSIST+ PROBIOTIC DEFENSE FORMULA DOTERRA
ECOQUEST / INFINITY ESSENTIALS FOR LIFE 2 ENZYME, FLORA AND CHROMIUM COMPLEX
ENERGETIX FLORA SYNERGY
ENZYMATIC THERAPY NATURAL MEDICINES ACIDOPHILUS PEARLS WITH L. ACIDOPHILUS AND B. LONGUM
ENZYMATIC THERAPY PROBIOTIC PEARLS HIGH POTENCY ADVANCED 3-LAYER SOFTGEL ONCE DAILY LACTOBACILLI & BIFIDOBACTERIA
FINEST NUTRITION ACIDOPHILUS L. ACIDOPHILUS 1 BILLION CFUS (LIVE ORGANISMS) PER TABLET ONE PER DAY
FLORA BILBERRY EXTRACT 25% STANDARDIZED ANTHOCYANIDINS WITH FREEZE-DRIED BLUEBERRY POWDER
FLORA CERTIFIED ORGANIC FLAX OIL COLD-PRESSED & UNREFINED
FLORA FLOR-ESSENCE
FLORA UDO'S CHOICE OIL BLEND CERTIFIED ORGANIC BLEND OF FLAX AND OTHER NUTRITIONALLY SUPERIOR OILS
FLORA UDO'S CHOICE UDO'S OIL DHA 3-6-9 BLEND BASED ON THE IDEAL 2:1:1 RATIO OF OMEGA FATTY ACIDS
FLORA WHITE WILLOW BARK 55 MG STANDARDIZED SALICIN
FLORABABY ADVANCED PROBIOTIC FORMULA FOR INFANTS & TODDLERS 5 STRAINS OF BENEFICIAL PROBIOTICS 4 BILLION LIVE CULTURES PER SERVING PROBIOTIC RENEW LIFE
FLORADIX CALCIUM-MAGNESIUM WITH ZINC, VITAMIN D AND HERBS
FLORADIX FLORAVITAL IRON + HERBS LIQUID EXTRACT FORMULA RICH IN IRON AND B-VITAMINS VEGETARIAN LIQUID FORMULA
FLORADIX IRON + HERB TABLETS HIGH POTENCY IRON WITH VITAMINS, YEAST AND HERB EXTRACTS
FLORADIX KINDER LOVE CHILDREN'S MULTIVITAMIN LIQUID EXTRACT FORMULA VITAMINS A, B, C, D AND E WITH CALCIUM, HERBAL EXTRA
FLORADIX TABLETS WITH IRON, VITAMINS, YEAST AND HERB EXTRACTS
FLORAJEN3 HIGH POTENCY PROBIOTIC ACIDOPHILUS BIFIDUM LONGUM 15 BILLION LIVE CULTURES PER CAPSULE
FRIENDLY FLORA PREBIOTIC AND PROBIOTIC COMPLEX FOS AND LACTOBACILLUS 5 BILLION CFU PROCAPS LABORATORIES
FUSION PLUS IRON / FOLIC ACID / VITAMIN / PROBIOTIC CAPSULES
FUTUREBIOTICS COLON GREEN GENTLE, ALL-NATURAL FIBER WITH PROBIOTICS AND ENZYMES NOW WITH SUPER-STABLE PROBIOTICS
GARDEN OF LIFE PRIMAL DEFENSE HSO PROBIOTIC FORMULA
GARDEN OF LIFE PRIMAL DEFENSE ULTRA ULTIMATE PROBIOTIC FORMULA 15 BILLION CELLS DAILY 13 BENEFICIAL CULTURES WITH HSOS W

GARDEN OF LIFE RAW BEYOND VITAMINS & MINERALS VITAMIN CODE 50 & WISER MEN RAW WHOLE FOOD MULTI LIVE PROBIOTICS & ENZYMES RAW FOOD-CREATED NUTRIENTS WITH CODE FACTORS RAW FOOD
GARDEN OF LIFE RAW PROBIOTICS WOMEN 50 & WISER 85 BILLION LIVE CULTURES 33 PROBIOTIC STRAINS PROBIOTIC-CREATED VITAMINS, MINERALS, ENZYMES & PREBIOTICS CONTAINS TARGETED PROBIOTICS RAW WHOLE FOOD
GARDEN OF LIFE RAW VITAMIN CODE 50 & WISER WOMEN RAW WHOLE FOOD MULTI LIVE PROBIOTICS & ENZYMES RAW FOOD-CREATED NUTRIEN
GENERIC20 GR8-DOPHILUS 8 STRAINS & 4 BILLION POTENCY WITH FOS
GENERIC36 ACIDOPHILUS PROBIOTIC BLEND
GENERIC7 PROBIOTIC ACIDOPHILUS
GENERIC8 ACIDOPHILUS & PROBIOTIC COMPLEX
GNC NATURAL BRAND ACIDOPHILUS 2 BILLION CFU
GNC NATURAL BRAND ACIDOPHILUS 4 BILLION CFU
GNC NATURAL BRAND MEGA ACIDOPHILUS
GNC NATURAL BRAND POTENT ACIDOPHILUS
GNC NATURAL BRAND ULTRA ACIDOPHILUS 350 MG
GNC PROBIOTIC COMPLEX CHEWABLE TABLET 1.5 BILLION CFUS
GNC PROBIOTICS PROBIOTIC COMPLEX 4 4 BILLION CFUS
GNC PROBIOTICS ULTRA 25 BILLION CFUS PROBIOTIC COMPLEX
GNC ULTRA 50 BILLION CFUS PROBIOTIC COMPLEX GUARANTEED POTENCY 50
GNC WOMEN'S ULTRA MEGA WITH PROBIOTICS MULTIVITAMIN DELIVERS 1 BILLION LIVE, ACTIVE CULTURES WITH 1,600 IU OF VITAMIN D-3
HEALTH PLUS INC. SUPER COLON CLEANSE WITH HERBS & ACIDOPHILUS
HEALTHWAY MEDICAL, P.C. PRO-C 750 MG VITAMIN C WITH PROBIOTICS VEGETARIAN
HEALTHY ORIGINS NATURAL PROBIOTIC 30 BILLION CFU'S 8 STRAINS AND 30 BILLION COLONY FORMING UNITS FLORAFIT
HERBALIFE FLORAFIBER LACTOBACILLUS ACIDOPHILUS AND FIBER
JARROW FORMULAS BABY JARRO-DOPHILUS +FOS WITH CLINICALLY DOCUMENTED STRAINS 3 BILLION PROBIOTIC
JARROW FORMULAS BABY'S JARRO-DOPHILUS WITH CLINICALLY DOCUMENTED STRAINS 3 BILLION PER GRAM HYPOALLERGENIC NON-DAIRY PRO
JARROW FORMULAS ENHANCED PROBIOTIC SYSTEM JARRO-DOPHILUS EPS ENTERIC COATED
JARROW FORMULAS JARRO-DOPHILUS + FOS 6 BENEFICIAL PROBIOTIC STRAINS WITH CLINICALLY DOCUMENTED STRAINS 3.4 BILLION PER CAPSULE PROBIOTIC
JARROW FORMULAS JARRO-DOPHILUS ORIGINAL WITH CLINICALLY DOCUMENTED STRAINS 6 BENEFICIAL PROBIOTIC STRAINS 3.4 BILLION PER CAPSULE PROBIOTIC
JUICE FOR LIFE POWER FRUIT FORMULA WHOLE FOOD CONCENTRATES WITH ENZYMES, PROBIOTICS & ANTIOXIDANTS
JUICEFESTIV FRUITFESTIV 23 FRUITS, ANTIOXIDANTS, PROBIOTICS & DIGESTIVE ENZYMES FEATURES: ACAI, POMEGRANATE, BLUEBERRY AND NONI NATROL

KID'S KYO-DOPHILUS PROBIOTIC ONE PER DAY
KLAIRE LABS THER-BIOTIC INFANT FORMULA 10+ BILLION CFUS MULTI-SPECIES PROBIOTIC
KYO-DOPHILUS
KYO-DOPHILUS PROBIOTICS PLUS CRANBERRY EXTRACT
L'IL CRITTERS PROBIOTIC ACIDOPHILUS DAILY
L.A. FARMACIA NATURAL MICROENCAPSULATED ACIDOPHILUS
LIFETIME LIQUID ACIDOPHILUS SOY BASE MILK FREE
MASON NATURAL CHEWABLE SOLUBLE FIBER WITH PROBIOTICS ADULTS & KIDS
MEGAFLORA OPTIMAL POTENCY PROBIOTIC FORMULA 100% WHOLE FOOD MEGAFOOD
METAGENICS LACTO VIDEN ID DAIRY FREE, STRAIN MODIFIED LACTOBACILLUS BLEND
METAGENICS ULTRA FLORA PLUS DF CAPSULES
METAGENICS ULTRAFLOA IMMUNE HEALTH PROBIOTIC
MILK, COW'S, FLUID, ACIDOPHILUS, 1% FAT
MILK, COW'S, FLUID, ACIDOPHILUS, 2% FAT
NATREN'S MEGA DOPHILUS DAIRY CAPSULES
NATREN'S MEGA DOPHILUS DAIRY POWDER
NATROL ACIDOPHILUS PROBIOTIC
NATURAL FACTORS MULTI ACIDOPHILUS WITH FOS CAPSULES 4 BILLION ACTIVE CELLS
NATURE CITY TRUELIFE PB PROBIOTIC AND PREBIOTIC BLEND PROFESSIONAL STRENGTH! 30 BILLION LIVE 'GOOD BACTERIA' CELLS PER DOSE PROVIDES 6 HEALTH BOOSTING PROBIOTIC STRAINS
NATURE MADE ACIDOPHILUS 500 MILLION LIVE CELLS PER TABLET
NATURE MADE ACIDOPHILUS PROBIOTICS 1 BILLION LIVE CELLS PER SERVING
NATURE MADE TRIPLE PROBIOTIC
NATURE'S BLEND HIGH POTENCY CHEWABLE ACIDOPHILUS
NATURE'S BLEND PROBIOTIC BLEND
NATURE'S BOUNTY ACIDOPHILUS
NATURE'S BOUNTY ACIDOPHILUS PROBIOTIC 100 MILLION ORGANISMS LACTOBACILLUS ACIDOPHILUS 1 PER DAY TABLET
NATURE'S BOUNTY CHEWABLE PROBIOTIC ACIDOPHILUS 1 BILLION ORGANISMS WITH BIFIDUM CHEWABLE MILK FREE WAFER
NATURE'S BOUNTY PROBIOTIC ACIDOPHILUS
NATURE'S BOUNTY PROBIOTIC ACIDOPHILUS 100 MILLION ORGANISMS LACTOBACILLUS ACIDOPHILUS 1 PER DAY CAPSULE
NATURE'S BOUNTY PROBIOTIC ACIDOPHILUS CAPSULES
NATURE'S BOUNTY ULTRA STRENGTH ADVANCED PROBIOTIC 10 10 PROBIOTIC ORGANISMS WITH ACTIVE CULTURE SUPPORT 20 BILLION LIVE PROBIOTIC CULTURES
NATURE'S LIFE LACTOBACILLUS ACIDOPHILUS APPLE PECTIN LIVE, ACTIVE ORGANISMS PROBIOTIC CAPSULES
NATURE'S PLUS ADULT'S EAR, NOSE & THROAT LOZENGES WITH K12 PROBIOTICS

NATURE'S PLUS SOURCE OF LIFE ANIMAL PARADE ACIDOPHIKIDZ WITH PROBIOTICS, FOS & RHODODENDRON CHILDREN'S CHEWABLE WITH WHO
NATURE'S SUNSHINE BIFIDOPHILUS FLORA FORCE BIFIDOBACTERIUM LONGUM & LACTOBACILLUS ACIDOPHILUS 470 MG
NATURE'S SUNSHINE BIFIDOPHILUS FLORA FORCE PROBIOTIC 4 BILLION TOTAL MICROORGANISMS PER CAPSULE
NATURE'S SUNSHINE PROBIOTIC ELEVEN
NATURE'S WAY ONCE DAILY PRIMADOPHILUS
NATURE'S WAY ONCE DAILY PRIMADOPHILUS CHILDREN POWDER WITH FOS TRUE IDENTITY BIFIDOBACTERIA & LACTOBACILLI TRUE POTENCY
NATURE'S WAY ONCE DAILY PRIMADOPHILUS CHILDREN POWDER WITH SCFOS TRUE IDENTITY BIFIDOBACTERIA & LACTOBACILLI TRUE POTENCY 3 BILLION CFU
NATURE'S WAY ONCE DAILY PRIMADOPHILUS FOR CHILDREN
NATURE'S WAY ONCE DAILY PRIMADOPHILUS JUNIOR TRUE IDENTITY LACTOBACILLI & BIFIDOBACTERIA TRUE POTENCY 3 BILLION CFU TRUE
NATURE'S WAY ONCE DAILY PRIMADOPHILUS KIDS TRUE POTENCY 3 BILLION CFU TRUE IDENTITY LACTOBACILLI & BIFIDOBACTERIA ORANGE
NATURE'S WAY ONCE DAILY PRIMADOPHILUS KIDS TRUE POTENCY 3 BILLION CFU TRUE IDENTITY LACTOBACILLI & BIFIDOBACTERIA ORANGE AGES 2-12
NATURE'S WAY PRIMADOPHILUS BIFIDUS ONCE DAILY TRUE POTENCY 5 BILLION CFU TRUE IDENTITY BIFIDOBACTERIA & LACTOBACILLI TRU
NATURE'S WAY PRIMADOPHILUS ORIGINAL ONCE DAILY TRUE POTENCY 5 BILLION CFU TRUE IDENTITY ACIDOPHILUS & RHAMNOSUS TRUE RELEASE TARGETED DELIVERY ENTERIC-COATED FOR ALL AGES
NATURE'S WAY PRIMADOPHILUS REUTERI SUPERIOR PROBIOTIC MULTI STRAIN PLUS SCFOS TRUE POTENCY 5 BILLION CFU ENTERIC-COATED ONCE DAILY
NEW CHAPTER EVERY MAN II WHOLE-FOOD PROBIOTIC MULTI-NUTRIENT, MINERAL & HERBAL COMPLEX
NEW CHAPTER EVERY WOMAN WHOLE-FOOD PROBIOTIC MULTI-VITAMIN, MINERAL & HERBAL COMPLEX
NEW CHAPTER EVERY WOMAN'S ONE DAILY WHOLE-FOOD PROBIOTIC MULTI-VITAMIN, MINERAL & HERBAL COMPLEX
NEW CHAPTER ORGANICS PROBIOTIC NUTRIENTS EVERY MAN II FOR MEN OVER 40 MADE WITH ORGANIC INGREDIENTS
NEW CHAPTER ORGANICS PROBIOTIC NUTRIENTS PERFECT PRENATAL
NEW CHAPTER VITAMIN B COMPLEX WHOLE-FOOD HERBAL PROBIOTIC NUTRIENT COMPLEX
NEW VITALITY RUBY REDS NEW & IMPROVED FORMULA POMEGRANATE, ACAI & MAQUI A DELICIOUS FRUIT AND VEGETABLE WITH POTENT VITAMINS, MINERALS, ENZYMES, HERBS, NUTRIENTS AND PROBIOTICS 100% DAILY VALUE 7 VITA
NOW BERRY DOPHILUS 4 PROBIOTIC STRAINS 2.5 BILLION INPUT NATURAL BERRY FLAVOR XYLITOL SWEETENED CHEWABLES

NSI NUTRACEUTICAL SCIENCES INSTITUTE PROBIOTIC 15-35 15 STRAINS / 35 BILLION MICROORGANISMS PER SERVING
NUTRI-HEALTH FLORA SOURCE MULTI-PROBIOTIC BLEND OF 16 STRAINS / 16 BILLION CELLS CAPSULES
NUTRICOLOGY PROGREENS WITH ADVANCED PROBIOTIC FORMULA
NUTRILITE INTSTIFLORA-7 STICK PACKS
NUTRITION NOW PB8 PRO-BIOTIC ACIDOPHILUS FOR LIFE ORIGINAL FORMULA 14 BILLION GOOD BACTERIA
NUTRITION NOW PROBIOTIC ACIDOPHILUS
NUTRITION NOW RHINO CHEWABLE FOS & ACIDOPHILUS CHEWABLE TABLETS 100% DAILY VALUE OF VITAMIN C
O'DONNELL FORMULAS, INC. FLORA-BALANCE BACILLUS LATEROPODUS BOD STRAIN CAPSULES
OPTIMUM FREEZE DRIED ACIDOPHILUS LACTOBACILLI
ORIGIN ACIDOPHILUS EASY TO SWALLOW
PB8 PRO-BIOTIC ACIDOPHILUS FOR LIFE
PHILLIPS' COLON HEALTH PROBIOTIC CAPS BAYER
PHILLIPS' COLON HEALTH PROBIOTIC CAPS DAILY PROBIOTIC ONE DAILY BAYER
PREMIER RESEARCH LABS GALLBLADDER-ND PROBIOTIC-DERIVED FORMULA FEATURING ND TECHNOLOGY
PRO-BIOTICS ACIDOPHILUS
PROBIOTIC PEARLS WITH L. ACIDOPHILUS AND B. LONGUM
PROBIOTICA LACTOBACILLUS REUTERI CHEWABLE TABLETS
PROCAPS LABORATORIES FIBERMUCIL U.S.P. PSYLLIUM BULK AND WATER SOLUBLE FIBER FRIENDLY FLORA LACTOBACILLUS
PROJOBA INTERNATIONAL PROBACILLUS PLUS
PURE RESEARCH PRODUCTS, LLC DEL-IMMUNE V LACTOBACILLUS RHAMNOSUS LYSED POWDER
PURITAN'S PRIDE ACIDOPHILUS
PURITAN'S PRIDE POTENT ACIDOPHILUS WITH PECTIN
PURITAN'S PRIDE VITA-FRESH LIFE'S GREENS WITH ADVANCED PROBIOTIC FORMULA 29,205 TOTAL ORAC VALUE VEGETARIAN
RAINBOW LIGHT 50+ MINI-TAB AGE-DEFENSE FORMULA FOOD-BASED MULTIVITAMIN WITH COQ10 1,000 IU VITAMIN D3 PROBIOTICS & DIGES
RAINBOW LIGHT ACTIVE HEALTH TEEN FOOD-BASED MULTIVITAMIN TARGETED TEEN NUTRITION 75 MILLION LIVE ACTIVE PROBIOTICS
RAINBOW LIGHT JUST 1 ONCE NATURALS WOMEN'S ONE MULTIVITAMIN/MINERAL NOW WITH PROBIOTICS
RAINBOW LIGHT KIDS ONE MULTISTARS CHEWABLE MULTIVITAMIN / MINERAL WHOLESOME VEGETABLE CONCENTRATES 15 MILLION ACTIVE PRO
RAINBOW LIGHT MEN'S ONE JUST 1 ONCE FOOD-BASED MULTIVITAMIN ENERGY B-COMPLEX & PROBIOTICS

RAINBOW LIGHT WOMEN'S ONE JUST 1 ONCE FOOD-BASED MULTIVITAMIN 800 IU VITAMIN D3 & PROBIOTICS
RBC DIGESTION FORMULA WITH ENZYMES, PROBIOTICS AND NANOCLUSTERS
RENEW LIFE FLORABEAR FOR KIDS
REPHRESH PRO-B PROBIOTIC FEMININE
REXALL NATURALS GESTAZYME MULTI-ENZYME PROBIOTIC FORMULA ACIDOPHILUS PROBIOTIC FORMULA 1 BILLION ACTIVE CULTURES PER SER
REXALL NATURALS ULTIMATE PROBIOTIC FORMULA ACIDOPHILUS MADE WITH BIO FLORA 2 BILLION ACTIVE CULTURES
RITE AID PHARMACY NATURAL ACIDOPHILUS PROBIOTIC COMPLEX MILK FREE 300 MG
RITE AID PHARMACY PROBIOTIC COLON CARE LACTOBACILLUS ACIDOPHILUS, BIFIDOBACTERIUM LONGUM, B. BIFIDUM PROBIOTIC ONE DAILY
SALUS FLORADIX CALCIUM LIQUID MINERAL NO ADDED SUGAR HERBAL
SAV-ON OSCO CENTRAL-VITE SELECT MULTIVITAMIN/MULTIMINERAL FOR MATURE ADULTS WITH FLORAGLO LUTEIN
SAV-ON OSCO ONE CHOICE THE MOST COMPLETE MULTIVITAMIN WITH HERBS WITH FLORAGLO LUTEIN
SCHIFF DIGESTIVE ADVANTAGE PROBIOTIC GUMMIES
SCHIFF SUSTENEX PROBIOTIC GUMMIES
SHAKLEE BIFIDUS & ACIDOPHILUS OPTIFLORA PROBIOTIC COMPLEX
SHAKLEE OPTIFLORA FOS, INULIN, & MORE PREBIOTIC DIETARY SUPPLEMENT
SHAKLEE OPTIFLORA PREBIOTIC COMPLEX FOS, INULIN, & MORE
SOLARAY MULTIDOPHILUS 12 OVER 20 BILLION MICROORGANISMS 12 PROBIOTIC STRAINS
SOLGAR ADVANCED 40+ ACIDOPHILUS NON-DAIRY LACTOSE FREE
SOLGAR ADVANCED ACIDOPHILUS PLUS 500 MILLION MICROORGANISMS PER USE
SPRING VALLEY 1 PER DAY DOSE ALL NATURAL CRANBERRY WITH VITAMIN C & PROBIOTICS NEW IMPROVED FORMULA
SPRING VALLEY ACIDOPHILUS
SPRING VALLEY PROBIOTIC ACIDOPHILUS 1 BILLION ACTIVE CULTURES
SPRING VALLEY PROBIOTIC MULTI-ENZYME DIGESTIVE FORMULA 1 BILLION ACTIVE CULTURES PER SERVING
SPRING VALLEY PROBIOTIC MULTI-ENZYME DIGESTIVE FORMULA WITH ACTIVE ACIDOPHILUS CULTURES
SPRING VALLEY SUPER STRENGTH PROBIOTIC ACIDOPHILUS 2 BILLION ACTIVE CULTURES
STOCKBRIDGE NATURALS TRI-DOPHILUS
SUNDOWN ACIDOPHILUS XTRA
SUNDOWN NATURALS CHEWABLE ACIDOPHILUS WITH BIFIDUM 1 BILLION ACTIVE CULTURES PER WAFER VEGETARIAN FORMULA
SUNDOWN NATURALS PROBIOTIC ACIDOPHILUS XTRA 40 MILLION LIVE CELLS PER SERVING VEGETARIAN FORMULA

SUNDOWN NATURALS ULTIMATE PROBIOTIC FORMULA ACIDOPHILUS 2 BILLION ACTIVE CULTURES VEGETARIAN FORMULA 1 PER DAY
SUSTENEX DAILY PROBIOTIC 2 BILLION CELLS OF GANEDENBC30
SWANSON ULTRA FLORAGLO LUTEIN 10 MG SOFTGELS
SWANSON ULTRA PROBIOTIC COMPLEX
THE HONEST CO. BABY & TODDLER MULTI POWDER COMPLETE MULTI-VITAMIN + MINERALS + SUPERFOODS + ANTIOXIDANTS + AMINO ACIDS + PROBIOTICS WHOLE-FOOD BASED ULTRA PURE GENTLE & ENRICHING HYPOALLERGENIC 100% N
THE VITAMIN SHOPPE ACIDOPHILUS WITH PECTIN
THE VITAMIN SHOPPE PSYLLIUM HUSK WITH ACIDOPHILUS
THE VITAMIN SHOPPE ULTIMATE '10' PROBIOTIC 13 BILLION ORGANISMS WITH FRUCTO-OLIGOSACCHARIDES ENTERIC COATED
THERALAC PROBIOTIC MASTER 5 HUMAN STRAINS 2 PREBIOTICS 5 + 2 30 BILLION CFU PER CAPSULE WITH LACTOSTIM
TOTAL PROBIOTICS NUTRI-WEST
TRADER JOE'S ACIDOPHILUS & PROBIOTIC COMPLEX 2 BILLION ORGANISMS PER TABLET
TRADER JOE'S ACTIVE 50+ ONCE DAILY MULTIVITAMIN & MINERAL PLUS PROBIOTICS 1000 IU VITAMIN D
TRUBIOTICS DAILY PROBIOTIC FROM THE MAKERS OF ONE A DAY BAYER
TRUNATURE DIGESTIVE PROBIOTIC 10 BILLION ACTIVE CULTURES
TWINLAB ALLERDOPHILUS ACIDOPHILUS CAPSULES HIGH POTENCY 182 MG
ULTIMATE FLORA CRITICAL CARE 50 BILLION EXTRA-STRENGTH PROBIOTIC 10 PROBIOTIC STRAINS RENEW LIFE
ULTIMATE FLORA EXTRA CARE DAILY PROBIOTIC 30 BILLION LIVE CULTURES PER CAPSULE 10 PROBIOTIC STRAINS ONE CAPSULE ONCE A DAY PROBIOTIC RENEW LIFE
ULTIMATE FLORA KIDS PROBIOTIC 3 BILLION LIVE CULTURES PER TABLET PROBIOTIC RENEW LIFE
VIBRANT HEALTH GREEN VIBRANCE 25 BILLION PROBIOTICS PER DOSE FROM 12 STRAINS ORGANIC GREENS & FREEZE DRIED GRASS JUICES
VITAMIN WORLD ACIDOPHILUS & PSYLLIUM HUSK 4 BILLION ACTIVE CULTURES 4,000 MG PSYLLIUM HUSK PER SERVING
VITAMIN WORLD CHEWABLE ACIDOPHILUS WITH BIFIDUS NATURAL STRAWBERRY FLAVOR
WALGREENS ACIDOPHILUS 10 MG
WALGREENS ACIDOPHILUS PLUS CITRUS PECTIN FREEZE DRIED
WINDMILL NATURAL ACIDOPHILUS PRO-BIOTIC BLEND WITH PECTIN
Y.S. ORGANIC BEE FARMS 100% PURE BEE POLLEN CAPSULES MULTI FLORAL, WILD CRAFTED
NEW CHAPTER ORGANICS PROBIOTIC NUTRIENTS CAL MAG BONE HEALTH CALCIUM AND SYNERGISTIC NUTRIENTS IN THEIR MOST ACTIVE FORM
WHOLE FOODS PREMIER FORMULA PROBIOTIC COMPLEX PLUS
NUTRITION NOW PB8 PRO-BIOTIC ACIDOPHILUS FOR LIFE
CVS/PHARMACY ADULT PROBIOTIC 3 BILLION LIVE BACTERIA CELLS PER CAPSULE WITH 8 NATURAL PROBIOTIC STRAINS, INCLUDING 2 BIFIDOBACTERIA STRAINS ONE-A-DAY

CULTURELLE PROBIOTIC NATURAL HEALTH & WELLNESS ONCE DAILY CAPSULES ALL NATURAL PROBIOTIC WITH LACTOBACILLUS GG
RAINBOW LIGHT ACTIVE SENIOR JUST 1 ONCE FOOD-BASED MULTIVITAMIN B-COMPLEX 1,000 IU VITAMIN D3 & PROBIOTICS
NEW CHAPTER ORGANICS PROBIOTIC ALL-FLORA WHOLE FOOD LIVE PROBIOTICS
GARDEN OF LIFE RAW PROBIOTICS ULTIMATE CARE 100 BILLION GUARANTEED! 34 PROBIOTIC STRAINS ONCE DAILY INCLUDES THE CLINICALLY STUDIED REPLENISH BLEND RAW WHOLE FOOD PROBIOTIC
DEFAULT CHILDREN'S PROBIOTIC PACKET
METABiotic PROBIOTIC WITH BIO-ACTIVE 12 PROBIOTIC
SPRING VALLEY PROBIOTIC ACIDOPHILUS 2 BILLION ACTIVE CULTURES
DEFAULT GUMMY ADULT PROBIOTIC
NATURE'S SUNSHINE L. ACIDOPHILUS MODIFIED RELEASE PROBIOTIC FORMULA
DAVINCI LABORATORIES OF VERMONT NONDAIRY PROBIOTIC 50 VEGETARIAN / GLUTEN FREE
NATURE MADE DIGESTIVE HEALTH PROBIOTIC 10 BILLION LIVE CELLS ONCE DAILY

Table S2. – Variables included in the adjusted models

	Variables included in all analyses	Variables included only in specific analysis
Model 1	Age, sex, ethnicity (Mexican American, other Hispanic, non-Hispanic white), annual family income (<\$25000, \$25000 to \$75000, >\$75000), and education (<9th grade, >= 9th grade)	
Model 2	Model 1 + alcohol intake, smoking status (never a smoker, current smoker, or former smoker), physical activity (low, intermediate, high), ingested kcal per day, ingested carbohydrates/kcal per day, ingested proteins/kcal per day, ingested fibres/kcal per day, and ingested polyunsaturated/saturated fatty acids ratio.	BMI (all analyses except obesity related analyses); Sodium intake per day (only in hypertension and blood pressure analyses)
Model 3 (supplementary)	Model 1 + alcohol intake, smoking status, physical activity, and DASH dietary pattern score*. * The DASH score is based on 9 target nutrients (sodium, total fat, saturated fat, protein, fiber, cholesterol, calcium, magnesium, and potassium). Individuals meeting the DASH goal were given a score of 1.0 for that nutrient and if they attain an intermediate goal were given a score of 0.5 for that nutrient. The DASH score is the sum of the score for each individual nutrient.	BMI (all analyses except obesity related analyses); Sodium intake per day (only in hypertension and blood pressure analyses)

Table S3. – Odds ratio of disease in subjects exposed to probiotics compared to non-exposed (model 3).

	Model 3, OR (95%CI)	P value
Obesity	0.86 (0.78-0.94)	0.002*
Diabetes	1.01 (0.84-1.21)	0.937
Hypertension	0.79 (0.71-0.88)	<0.001*
Dyslipidemia	1.00 (0.89-1.13)	0.957

Model 3: Model 1 + alcohol intake, smoking status, physical activity, and DASH dietary pattern score. Model 3 also includes BMI in all analyses except in the obesity analysis; and includes sodium intake per day only in the hypertension analysis. *statistically significant

Table S4. – Variation of cardiometabolic parameters in participants exposed to probiotics compared to non-exposed (model 3).

	Model 3	P value
BMI, kg/m ²	-0.33 (-0.59 to -0.06)	0.015*
HbA1c ^a , %	0.01 (-0.01 to 0.03)	0.416
Glucose ^a , mg/dL	-0.07 (-0.82 to 0.68)	0.862
Systolic BP ^b , mmHg	-1.54 (-2.38 to -0.70)	<0.001*
Diastolic BP ^b , mmHg	-0.89 (-1.49 to -0.29)	0.004*
LDL ^c , mg/dL	0.27 (-2.29 to 2.83)	0.836
HDL ^c , mg/dL	1.45 (0.71 to 2.18)	<0.001*
Triglycerides ^c , mg/dL	-9.62 (-16.42 to -2.82)	0.006*

Model 3: Model 1 + alcohol intake, smoking status, physical activity, and DASH dietary pattern score. Model 3 also includes BMI in all analyses except in the BMI analysis; and includes sodium intake per day only in the BP analyses.

^a Excluding participants treated with anti-hypertensive drugs. ^b Excluding participants treated with anti-dyslipidemic drugs. ^c Excluding participants treated with antidiabetic drugs. *statistically significant

6. DISCUSSION

In the present PhD dissertation it was sought to expand the knowledge about the role of gut microbiota-host clinical interaction in obesity and related metabolic disturbances. To comprehensively achieve this, four phases were defined according to the following:

- 1) **First phase:** Gut microbiota **profiling in obesity**, T2DM and NAFLD, as well as **therapeutic strategies** to modulate gut microbiota in metabolic diseases;
- 2) **Second phase:** Gut microbiota **characterisation in obesity driven by HFD**, and the **role of I-FABP** in modulating intestinal injury, inflammation and dysbiosis;
- 3) **Third phase:** Gut microbiota and **anthropometric, metabolic and inflammatory changes** in mild obese adults after metabolic surgery, specifically those related with T2DM improvement/resolution;
- 4) **Fourth phase: Probiotic ingestion** prevalence and association with **obesity** or other related metabolic disorders.

The above-mentioned phases were covered by the different PhD studies, using different methodologies as follows, respectively:

- 1) **Literature review and synthesis studies:** Two bibliographic reviews on gut microbiota composition in obesity, T2DM and NAFLD, with an additional focus in possible interventions targeting gut microbiota modulation; and one systematic review to assess gut microbiota association with NAFLD and metabolic disturbances;
- 2) **Basic research:** An experimental study in a HFD-induced obesity rat model, designed to consolidate knowledge on gut microbiota dysbiosis and inflammatory status of obesity, as well as on the role of I-FABP as a non-invasive marker of gut barrier dysfunction;
- 3) **Clinical research – Intervention study:** An open-label RCT, designed to assess gut microbiota and anthropometric, metabolic and inflammatory changes after metabolic surgery, compared to medical therapy, in mild obese patients with T2DM;
- 4) **Clinical research – Big data analysis:** A cross-sectional study using data from the NHANES (1999-2014) on a large and representative U.S. population, to evaluate the association of probiotic ingestion with obesity and metabolic related disorders.

At the end, this PhD project brought some important and comprehensive data on gut microbiota and its key importance on the regulation of obesity and related metabolic diseases. Noteworthy, this dissertation is composed of different and complementary research strategies and methodologies (from bench to bedside – basic, translational and clinical research), enhancing the research experience that came along with this body of work. Detailed discussion of PhD outcomes was done in each published manuscript, presented above. In the next sections, a focus will be putted in the major findings and in some global discussion for general integration of the outcomes.

6.1 Major findings

The main results of the original research studies included in this PhD thesis showed that:

- a) Mice under HFD-induced obesity had lower Bacteroidetes ($\log_{10}$ rRNA gene copies/20ng of DNA: 5.45 in HFD vs. 6.43 in standard diet group, $p<0.05$) and higher Firmicutes-to-Bacteroidetes ratio (1.20 in HFD vs. 1.03 in standard diet group, $p<0.05$), which might indicate an increased capacity of harvesting energy from food. HFD-induced obesity model were also associated with insulin resistance and increased fecal levels of LPS, TLR4 expression, and increased plasma proinflammatory cytokines, which reflects a proinflammatory status. This study also showed that HFD may have also up-regulated the expression of I-FABP and, simultaneously, may have increased GLP-2 to improve mucosal barrier integrity and blunt inflammatory tone.
- b) In the first RCT that simultaneously studied clinical and microbiota changes in diabetic patients with class-1 obesity after Roux-en-Y gastric bypass (RYGB) versus standardized medical therapy, both surgical and medical arms improved anthropometric outcomes at 1 year of follow-up. However, surgical arm patients had significantly higher improvements: at month (M) 12, the percentage of weight loss was 25.5% vs. 4.9% ($p<0.001$), and hemoglobin A1c (HbA1c) was 6.2% vs. 7.7% ($p<0.001$). Only RYGB patients achieved diabetes improvement/remission ($n=7$, 87.5%). It was also observed a continuous increase of genus richness after RYGB up until M12, while in the medical arm genus richness ended-up being significantly lower at that time. Composition analysis indicated significant changes of the overall microbial ecosystem (permanova $p=0.004$, $[R^2=0.17]$) during the follow-up period after RYGB. There was also a strong association between improvement of anthropometric/metabolic/inflammatory biomarkers and increase in microbial richness and Proteobacterial lineages.
- c) In a big data cross-sectional analysis from a large and representative U.S. population sample from NHANES ($n=38,802$ adults), 13.1% reported probiotic ingestion. Probiotic ingestion was associated with 17% lower prevalence of obesity and 21% lower prevalence of hypertension. Accordingly, even after analytic adjustments, BMI was significantly lower in the probiotic group, as well as systolic and diastolic blood pressure (BP). Participants with probiotic ingestion reported lower odds of having

diabetes; however, when accounting for potential confounders, differences between groups were no longer significant. Furthermore, there were no differences in prevalence of dyslipidemia; however, HDL was higher and triglycerides were significantly lower in the probiotic group on adjusted model.

6.2 General discussion

The obesity burden has been increasing in the last decades. The traditional theory of weight gain, almost exclusively focused on an imbalance between energy expenditure and consumption, turned out to be very simplistic to explain all the factors related with this disease. Among the potential environmental factors that contribute to the onset and maintenance of obesity and associated metabolic diseases, unequivocal evidence has revealed the role of a 'novel' partner – the gut microbiota ³⁴.

The human gastrointestinal tract gut is composed by a complex microorganism community that is dominated by bacteria belonging to Firmicutes and Bacteroidetes phyla ^{41,42}. Collectively, the number of intestinal microbial cells is estimated to be 10 times higher than the number of human body cells. This intestinal ecosystem performs essential functions, including protective, structural and metabolic roles, partially responsible for maintaining human health ⁴³. Intestinal dysbiosis is a state characterized by change in the composition of the microbiota or a change in bacterial metabolic activity ³³. Thus, microbial ecology can be an important regulator of energy homeostasis, namely in obesity physiopathology.

Gut microbiota profile in obesity and related metabolic disturbances

Obesity and related metabolic disturbances, namely, T2DM and NAFLD, are associated with compositional changes of gut microbiota. These changes may be due to deviations in the relative abundance of some phyla or by genus richness variations.

Firmicutes-to-Bacteroidetes ratio

Ley *et al* performed the first few studies in this field. They have firstly analyzed gut microbiota of genetically obese ob/ob mice and lean ob/+, showing that ob/ob animals had 50% reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes ⁴⁴. Furthermore, it was demonstrated that trait was transmissible: colonization of germ-free mice with an 'obese microbiota' resulted in a significantly greater increase in total body fat (20% increase) and insulin resistance than colonization with a 'lean microbiota' ³⁵. Hepatic fat accumulation and, consequently, NAFLD were also been associated with increased Firmicutes-to-Bacteroidetes ratio ⁴⁵. Our data from HFD-induced obesity rat model with increased insulin resistance were consistent with this division-wide, showing that obese

animals had lower Bacteroidetes ($\log_{10}$ rRNA gene copies/20ng of DNA: 5.45 in HFD vs. 6.43 in standard diet group, $p < 0.05$) and higher Firmicutes-to-Bacteroidetes ratio (1.20 in HFD vs. 1.03 in standard diet group, $p < 0.05$). The Firmicutes bacteria are more efficiently than Bacteroidetes in extracting calories from carbohydrates by fermentation of otherwise indigestible food components to short-chain fatty acids ^{46,47}. Our results reinforce that obesity and insulin resistance were linked to a specific gut microbiota profile.

In obese humans, the relative proportion of Bacteroidetes was also found to be decreased by comparison with lean people ³⁸. Moreover, again Ley *et al* showed the occurrence of a shift toward higher relative abundance of Bacteroidetes and decreased number of Firmicutes in obese patients that lost weight by ingestion of a low-calorie diet ³⁸. However, other studies contradict this finding and were not able to demonstrate differences in the relative abundance of Firmicutes and/or Bacteroidetes in obese subjects ^{39,48}. In the same line, T2DM has been linked to a decrease in Firmicutes and an increase in bacteria belonging to Bacteroidetes and Proteobacteria, when compared to obese patients ⁴⁹. In our RCT, the anthropometric and metabolic improvement after metabolic surgery was not accompanied by significant differences on the Firmicutes-to-Bacteroidetes ratio. These data emphasize that the differential adaptation of human gut microbiota that accompanies metabolic improvement after RYGB cannot be sufficiently explained at the phylum level. In fact, the huge variability of gut microbiota in humans may mask these large-scale differences and higher taxonomic levels may not necessarily reflect specific bacteria changes.

Genus richness

Genus richness is a simple description of the number of taxa present in a specific ecosystem among dominant bacteria. This definition is used to describe the microbial diversity ⁵⁰.

A healthy microbial ecosystem is usually characterized by an elevated level of microbial richness ^{51,52}. Indeed, it was previously demonstrated that bacterial diversity differed in humans from different countries, being lower in populations with higher prevalence of obesity and other metabolic-related diseases ⁵⁰. One of the key features of obesity is a low level of microbial richness, which was correlated with metabolic disorders, such as low-grade inflammation, insulin resistance, and adipocyte size ^{51,53}. In fact, two different perspectives confirmed the importance of genus richness in obese patients: 1) low bacterial

richness was linked to an increase in overall adiposity, 2) obese adults with lower bacterial richness had gain more weight over a 9-year follow-up period ⁵². NAFLD has also been associated with reduced microbial diversity ⁴⁵. In T2DM, reduction of gut microbial diversity also led to an increase in pathogenic bacteria, gut inflammation and deterioration of glucose status ⁵⁴.

In our RCT, which included mild obese patients with T2DM, it was observed a continuous increase of genus richness after RYGB during the 12 months of follow-up (from 61.375 in M0 to 76.375 in M12). Our results confirmed previous observations that RYGB increased microbiome richness, not only in morbidly obese patients, but also in a broader BMI range, including patients with a BMI 30-35 Kg/m² ⁵⁵. The causal mechanisms remain unclear, but dietary modifications after RYGB can probably account for it; RYGB induces drastic reduction in nutrients supply, including a reduction in fat content ⁵⁶. In populations characterized by decreased fat intake in favor of polysaccharides, the overall gut diversity is huger compared to the consumers of western diets ⁵⁰. We had also found a strong association between increase in microbial richness and improvement of clinical phenotype, including anthropometric, metabolic (waist circumference, diastolic BP, HbA1c), and inflammatory (high-sensitivity c-reactive protein [hsCRP]) biomarkers. Dao *et al* previous results were consistent with our data on the importance of microbial richness in regulation of energy balance; in a cohort of overweight and obese subjects under a dietary intervention for weight loss, it was observed an increase in microbial gene count, which occurred in parallel with improvement of metabolic clinical parameters, such adiposity ⁵³. Putting this together, our results showed that systemic and anatomical changes induced by RYGB restored a putative loss of microbial richness with an improvement of metabolic status.

Host-gut microbiota interactions in obesity and related metabolic disturbances

The storage hypothesis

The gut microbiota has been proposed to have a key role in energy metabolism, modulating energy homeostasis ³⁵. The hypothesis that obesity-associated gut microbiome has an increased capacity for energy harvest derived from several observations.

Adult germ-free conventionalization with a normal microbiota collected from the cecum of conventionally raised mice, led to 60% increase in body fat and insulin resistance, despite reduced chow consumption ³⁴. In line with these findings, microbiota transplantation from the cecum of obese donor (ob/ob) to adult germ-free mice resulted in a greater relative abundance of Firmicutes and a significantly increase in body fat over two weeks, compared to mice colonized with a 'lean microbiota' ($1.3\text{g} \pm 0.2$ vs. $0.86\text{g} \pm 0.1$ of fat), despite no differences in food intake ³⁵. Thus, the 'obese microbiota' has increased energy-harvesting capacity, leading to a state where energy from diet was obtained in a more efficient way. This can be explained by: 1) an improved capacity to decompose indigestible polysaccharides by the host, which leads to high monosaccharide and short-chain fatty acids production, and 2) the regulation of genes expression involved in lipogenesis, including acetyl-CoA carboxylase and fatty acid synthase ^{34,35,57}. Furthermore, conventionalization of germ-free mice resulted in a two-fold increase of the capillaries density from villus epithelium of the small intestine, increasing the gut absorptive capacity ⁵⁸.

In our experiment, rat model fed with HFD, compared to those with standard diet, gained more weight (205.5 ± 37.8 vs. 134.7 ± 22.5 g, $p < 0.05$) and had a proportional body fat mass increase (206.3 ± 21.03 vs. 166.8 ± 21.26 , $p < 0.05$), during the 12 weeks of the study. Firmicutes-to-Bacteroidetes ratio was positively correlated with weight gain ($r = 0.829$, $p < 0.05$), while Bacteroidetes were negatively correlated with weight gain ($r = -0.800$, $p < 0.05$). These findings are consistent with the fact that Firmicutes are butyrate producers, which can account for lower calories lost in faeces ^{35,59}. Thus, we might assume that the dysbiosis pattern observed in our obese rat model might have led to an increased capacity of harvesting energy from food, contributing to fat gain and insulin resistance.

In our RCT, we also found that genus richness and some bacteria belonging to Proteobacteria phylum were inversely associated with anthropometric parameters, specifically body fat distribution (visceral fat and total body fat mass), as well as with metabolic parameters (HbA1c, fasting glucose and insulin blood levels). Remarkably, Kong *et al* have also observed increased microbial richness after RYGB and a significant association between gut microbiota and the white adipose tissue genes expression and clinical phenotype ⁶⁰. These data highlight and support the role of gut microbiota composition in the regulation of adipose tissue remodelling and storage capacity.

The metainflammatory hypothesis

Other possible explanation linking gut microbiota dysbiosis to regulation of fat storage is the metabolic endotoxemia, which associates gut microbiota changes with a low-grade inflammatory state – the ‘metainflammatory hypothesis’.

Intestinal mucosa has an important role not only in the absorption of vital nutrients, but also in the regulation of barrier functions, thus preventing bacterial translocation. In normal conditions, the gut barrier function is highly efficient due to complex multifaceted mechanisms (e.g., tight-junction proteins, thickness and composition of the mucus layer, presence of antimicrobial factors or secretion of immunoglobulin A) ⁶¹. HFD dramatically increase intestinal permeability, by a mechanism associated with reduced expression of epithelial tight-junction proteins, including zonulin and occludin ⁶². LPS is a component of the cell wall of gram-negative bacteria, which in combination with CD14 serves as ligand for TLR4 at the surface of innate immune cells ⁶³. Moreover, LPS is physiologically absorbed and carried into the blood through a mechanism involving chylomicron formation, a process stimulated by HFD ⁶⁴. It was shown that high-fat feeding augmented plasma LPS-containing microbiota, which induced a proinflammatory response with an increase in body weight and insulin resistance ⁶⁵.

In our rat model, we observed that in HFD fed rats, intestinal dysbiosis might have triggered intestinal inflammation since faecal levels of LPS and TLR4 expression were increased in those animals. Consequently, these animals also had increased plasma levels of proinflammatory cytokines and developed insulin resistance. These results support the causative role of gut microbiota changes in the development of weight gain and insulin resistance in rats under

HFD, by a mechanism dependent on inflammatory response, which was also previously shown by other authors ^{66,67}.

In this context, the gut barrier has been recognized as having an important role in the prevention of luminal LPS dissemination into the blood stream ⁶⁸. Furthermore, obese subjects with chronic hyperglycemia had increased I-FABP levels ⁶⁹. I-FABP is a protein expressed in mature enterocytes of the small and large intestines ⁷⁰, and its plasma levels were associated with gut epithelial cell damage ⁷¹. It was therefore speculated that the increased I-FABP levels in chronic hyperglycemia was due to increased enterocyte loss, which promoted endotoxin-induced low-grade inflammation. Interestingly, in our study we found that the relative expression of I-FABP tended to be increased in HFD rats, and plasma I-FABP levels were reduced. As intestinal absorption capacity can be adapted to the dietary fat content, we hypothesized that HFD may have up-regulated several genes known to play an important part in long-chain fatty acids uptake such as I-FABP ⁷². In consonance, we also found that animals fed with HFD had increased GLP-2 levels. GLP-2 is produced by intestinal L-cells and is involved in gut barrier function, by tight junction proteins regulation ⁷³. As suggested by other authors, GLP-2 may increase after HFD to further promote fat absorption in the intestine ⁷⁴. Additionally, the inflammatory state induced by microbiota changes after HFD exposure might have increased GLP-2 production in order to improve the mucosal barrier integrity and, therefore, blunt the inflammatory stress.

In the RCT conducted in this PhD project, RYGB resulted in striking weight reduction and impressive improvements of glycemic control and inflammatory status in mild obese patients with T2DM. The levels of hsCRP were significantly reduced (-1.22 mg//L, $p=0.035$) at M12 after RYGB, along with metabolic and clinical phenotype improvement. It was also observed a strong inverse association between genus richness and Proteobacteria lineages and hsCRP. One possible explanation to the favourable metabolic adaptation seen after RYGB was the improvement of the low-grade inflammatory state linked to gut microbiota changes induced by this procedure.

Taken together, these results reinforce the hypothesis that gut microbiota regulates weight loss and fat-mass reduction via modulation of the systemic low-grade inflammation, which is a hallmark of obesity and associated metabolic disorders.

New therapeutic targets into gut microbiota modulation in obesity and related metabolic disturbances

The new insights into the role of gut microbiota on obesity and metabolic-related diseases have motivated interest in developing innovative therapeutic targets to metabolic diseases. Furthermore, understanding the metabolic impact of these interventions, which modulate gut microbiota, will further expand the knowledge on the crosstalk between gut microbiota-host and metabolic disturbances.

As it was reviewed, metabolic surgery and probiotics are two of the most promising therapeutic approaches into shaping gut microbiota composition.

Metabolic surgery

RYGB is a restrictive and malabsorptive surgery, consisting in the creation of a small stomach pouch connected to the proximal jejunum and bypassing food to the small intestine. Physiological and anatomical rearrangements include changes in stomach size, biliary flow, colon pH, vagal modulation, enteric or adipose tissue hormones and nutrient flow ⁶¹.

RYGB is a highly effective surgery for severely obese patients [BMI $\geq 35\text{Kg/m}^2$] with great effects not only on weight reduction, but also on T2DM improvement or resolution ^{75,76}. Such encouraging results led to further research using the same approach into patients with mild obesity (BMI 30-35 kg/m²); the outcomes also proved the efficacy of this surgery in this subgroup of obese patients ⁷⁷. The metabolic improvement, particularly the one of the glucose metabolism in patients with T2DM, occurs rapidly after RYGB; however, this was not yet been completely understood. Recent studies have shown that gut microbiota modulation contributes to beneficial metabolic effects ^{60,78}. Thus, it seemed that RYGB was an effective model to study gut microbiota profile and to clarify the metabolic adaptations underlying the beneficial effects of this procedure.

In this PhD project, we developed the first RCT that simultaneously studied clinical and microbiota changes in diabetic patients with class-1 obesity after RYGB vs. standardized medical therapy. In line with previous observations, we found that both RYGB and standardized medical therapy groups improved anthropometric outcomes at 1 year of follow-up, with RYGB patients having significantly higher improvements ⁷⁶. Only RYGB patients achieved diabetes improvement/remission (n=7, 87.5%), and significantly improved

anthropometric and glycemic profiles, independently and progressively, during the first year of follow-up. These changes occurred simultaneously with gut microbiota changes. During the 12 months of follow-up after RYGB, it was observed a continuous increase in genus richness, despite a trend of lower baseline values in the surgical arm. Thus, these results suggest that surgery induced anatomical and environmental changes, which might have affected gut microbiota composition ⁶¹. On the contrary, genus richness did not change in the standardized medical therapy arm and ended-up being significantly lower at M12, with no differences in glycemic profile, comparing to baseline. Hence, standardized medical therapy optimization did not have an important role on gut microbiota modulation, which reinforces the hypothesis that modulation of gut microbiota by pre- or probiotics could be a complementary strategy for improving glycemic status in this context.

Yet, in our RCT, there were no significant differences in genus richness or in clinical variables at baseline across enterotypes. We hypothesize that as the sequencing depth was relatively low in this study, it provided a low number of observed genera, which significantly influenced the enterotyping outcome. Finally, the number of individuals in each group (four for the baseline K4 medical therapy, and five for the baseline K4 surgery) decreased the statistical power, making the interpretation difficult. One year after surgery, we observed a significant decrease in three bacterial taxa belonging to the Firmicutes phylum (*Ruminococcus*, *Lachnospiraceae_unclassified* and *Faecalibacterium*). Specifically, these bacteria were recognized for their anti-inflammatory properties with beneficial impact on metabolic health ^{55,78}, which may indicate that RYGB did not have the ability to completely restore a healthy microbiome composition, despite the improvement of metabolic status; a highly dysbiotic microbiome state at baseline may contributed for these final results. We also observed an increase in abundance of bacteria of the phylum Proteobacteria and of Proteobacteria/Firmicutes ratio, a common finding after bariatric surgery which was linked to an improvement of metabolic and inflammatory parameters, as it was observed in other studies ^{79–82}. The phylum Proteobacteria is composed of facultative anaerobes and, consequently, after RYGB, an environment with higher oxygen exposure, as well as higher pH could be responsible for the increase of these bacteria and related impact on metabolic health ⁸³.

Our study, highlighted the importance of RYGB in inducing specific changes in gut microbiota composition that contribute to the post-operative metabolic improvements.

Probiotics

Probiotics are food components or supplements with living microorganisms that confer health advantages to the host ⁸⁴. Specific strains have been increasingly studied as potential therapeutic approaches to shape gut microbiota composition, with possible benefits for weight control and diabetes management ^{85–88}.

We have conducted a cross-sectional analysis on a large and representative U.S. population sample (n=38,802 adults), and found that 13.1% reported the use of probiotic supplements or yogurt ingestion. There was a lack of large cross-sectional studies to objectively assess population-based prevalence of probiotic intake and metabolic differences in those exposed and not exposed to probiotics. To our knowledge, this was the first large cross-sectional analysis aiming to assess the association of probiotic ingestion with metabolic disturbances.

In our study, probiotic ingestion was associated with 17% lower prevalence of obesity. Also, subjects exposed to probiotics had significant lower BMI regardless of higher consumption of carbohydrates, fibers or proteins. These results support our hypothesis of the beneficial impact of probiotic ingestion *per se* on metabolic health, namely its effects on the regulation of body weight. In agreement with our study, a recent meta-analysis of RCTs showed that probiotics administration significantly reduced body weight by -0.60 Kg and BMI by -0.27 kg/m² ⁸⁹. Probiotics modulate gut microbial communities exerting metabolic beneficial effects through the regulation of multitudinous physiological metabolic pathways. Among several molecular mechanisms, probiotics may improve gut barrier function, with reduction of metabolic endotoxemia, modulation of host immune system, regulation of adipogenesis and stimulation of insulin signalling. These are some of the suggested key players in the crosstalk between probiotics and metabolic disorders ⁹⁰.

Obesity is associated with metabolic diseases, including T2DM, hypertension or dyslipidemia. In our big data analysis we did not find differences in probiotic ingestion regarding diabetes prevalence, when adjusted for individual characteristics and confounders. T2DM is largely determined by individual demographic characteristics, such as age, ethnicity and, mainly, BMI ⁹¹. Interestingly, probiotic supplementation was more effective in reducing HbA1c in diabetic patients with higher baseline BMI ⁹². In other study, probiotic exposure showed only a trend towards a glucose-lowering effect in subjects without diabetes ⁸⁷; in this study, it was observed that probiotic usage was associated with greater benefits for

T2DM patients with higher fasting glucose levels ⁸⁷. Taken together, these specific points may mask the metabolic benefit of probiotic supplements, particularly on glucose metabolism and may, in part, justify our results when diabetes prevalence was adjusted for potential confounders.

We also did not find differences in the odds of dyslipidemia, according to probiotic ingestion. However HDL was significantly increased and triglycerides were significantly decreased in the probiotic group, even after adjustment. Our results are in line with a study by Fu *et al*, which showed that probiotic usage was associated to a 4.5% of variance in BMI, 6% in blood levels of triglycerides, and 4% in HDL, but had little effect on LDL or total cholesterol ⁹³. A recent meta-analysis including 32 RCTs concluded, however, that probiotics significantly reduced serum total cholesterol (mean change of -13.27 mg/dL) ⁶¹. The heterogeneity of the studies and differences on the baseline levels of cholesterol may explain divergent results. There are several mechanisms proposed for cholesterol lowering effects of probiotics. Probiotics may adhere to cholesterol molecules in the intestinal lumen and promote their excretion. They may also interfere with the rate-limiting enzyme in hepatic cholesterol synthesis and some bacteria can deconjugate bile acids through bile salt hydrolase activity ⁶¹. Given the clinical correlation between obesity and dyslipidemia, it is possible that the observed associations between probiotics exposure and lipid levels can be also mediated, in part, through the effects on BMI.

Regarding hypertension, probiotic ingestion was associated with a 21% reduction of its prevalence. A meta-analysis of RCTs supports our results, showing that probiotic consumption significantly reduced systolic BP by -3.56 mmHg and diastolic BP by -2.38 mmHg, compared to control groups ⁹⁴. In our study, for the adjusted-model, probiotics ingestion was associated with a lower systolic BP by -1.48mmHg and a lower diastolic BP by -0.86 mmHg. The modulation of BP by probiotics may be linked to several mechanisms, including the probiotic capacity to improve lipids profile, to reduce BMI, to improve gut barrier function, to modulate metabolic endotoxemia and to produce bioactive peptides with angiotensin-converting-enzyme inhibitory activity ⁹⁴⁻⁹⁷.

Our research supports the beneficial impact of gut microbiota modulation, either by metabolic surgery or through probiotic ingestion, on metabolic health. Further understanding of this complex crosstalk will benefit the development of more tailored and targeted therapies to improve or even cure these prevalent metabolic diseases.

6.3 Final remarks and future directions

The increase prevalence of obesity and related metabolic disorders, including T2DM and NAFLD, has become a worldwide serious public health concern.

This PhD thesis brought comprehensive data on the clinical effects of the interaction between host-gut microbiota in obesity and metabolic-related diseases. It was demonstrated that gut microbiota is intrinsically involved with the host for energy homeostasis regulation, including fat mass gain and glucose metabolism. In the obesity rat model, intestinal dysbiosis was characterized by reduced Bacteroidetes levels and higher Firmicutes-to-Bacteroidetes ratio. Moreover, these bacterial changes triggered a proinflammatory response, a core feature in obesity and related metabolic disturbances. Bacterial diversity was also shown to be of great relevance. Metabolic surgery, specifically RYGB, was used as a research model to understand the pathophysiological mechanisms underlying the beneficial metabolic effects after the surgery. Improvement in anthropometric and metabolic parameters occurred simultaneously with an increase in genus richness, during the 12 months after surgery. Finally, probiotics ingestion resulted in 17% lower prevalence of obesity and a 21% lower prevalence of hypertension, which indicated that gut microbiota modulation, through probiotics supplementation, may be an attractive target to prevent obesity or hypertension. These outcomes support that specific bacterial molecular targets research is crucial to better understand the current obesity epidemic and, also, eventually minimize and reverse this disease.

Driven by the last advances in high throughput sequencing and bioinformatic, the impact of gut microbiota on obesity and metabolic health emerged as an important focus on biomedical research. Nevertheless, important questions are yet to be addressed. What compositional or functional changes in the gut microbiota precede the onset of obesity and its complications? What features of host–microbiota crosstalk are critical for maintaining a healthy stable microbiota and gut homeostasis, and how can these be manipulated to restore equilibrium under conditions of intestinal dysbiosis? Which individuals benefit the most from the selective modulation of microbiota? Long-term studies of such microbiota–host metabolic crosstalk may also considerably improve the knowledge on pathogenic processes in obesity and metabolic health.

The wide comprehension of the implications of host-gut microbiota crosstalk presents significant challenges. Thus, multidisciplinary and integrated collaborations among microbiologists, endocrinologists, nutritionists, and cell-to-molecule computational and systems biologists are crucial at both the preclinical and clinical level of research. This is an exciting area of research that will continue to generate new data on obesity and metabolic health processes. These discoveries will possibly allow the development of new preventive and therapeutic strategies for improvement of human metabolic health.

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8. APPENDICES

8.1 PhD Tasks

1) PhD Planning and Setup

Dates: January 2012 - October 2015

Work completed:

- a) Regular meetings with supervisor and co-supervisors;
 - b) Definition of PhD project tasks;
 - c) Strategies of dissemination of results;
 - d) Presentation of PhD project in a scientific meeting;
 - e) Progression report of PhD project.
-

2) Bibliographic review

Dates: January 2012 - December 2015

Work completed:

- a) Bibliographic review of relevant published evidence focused on:
 - i. Obesity burden
 - ii. Metagenome hypothesis
 - iii. Characterisation of gut microbiota in obesity and metabolic disturbances
 - iv. Gut microbiota modulation approaches
-

3) The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity

Dates: January 2015 - April 2016

Work completed:

- a) Definition of study methodology;
 - b) Implementation of the experimental protocol;
 - c) Completion of laboratory analysis;
 - d) Data analysis and interpretation;
 - e) Writing of manuscript drafts;
 - f) Publication of Paper 4;
 - g) Presentation in congress.
-

4) Gut microbiota changes after metabolic surgery in mild obese diabetic adults: a randomized controlled trial.

Dates: January 2012 - June 2018

Work completed:

- a) Study setup;
- b) Submission to the Ethical Committee of University of Porto;
- c) Registration of the randomized clinical trial
- d) Participants enrolment and screening;
- e) Randomization of participants;
- f) Participants intervention, including completion of standard operational procedures;
- g) Completion of follow-up;
- h) Completion of laboratory analysis;
- i) Data analysis and interpretation;
- j) Writing of manuscript drafts;
- k) Presentation of preliminary results in Portuguese Congress of Endocrinology, Diabetes and Metabolism.

Work to be completed:

- a) Dissemination (paper publication) of the results within the aims of the study (submitted, under review) – Paper 5

5) Probiotic ingestion, obesity, and metabolic-related disorders: results from NHANES, 1999-2014

Dates: April 2017 – June 2019

Work completed:

- a) Definition of study methodology;
 - b) Data analysis and interpretation
 - c) Writing of manuscript drafts;
 - d) Presentation of preliminary results in Portuguese Congress of Endocrinology, Diabetes and Metabolism;
 - e) Publication of Paper 6
-

6) PhD thesis completion

Dates: January 2018 – July 2019

Work completed:

- a) Draft of the final thesis of PhD;

Work to be completed:

- a) Public presentation and discussion of PhD thesis.

8.2 PhD Outputs

PEER-REVIEW SCIENTIFIC PUBLICATIONS

1

["Beyond gut microbiota: understanding obesity and type 2 diabetes"](#)

Full-paper published in *Hormones* [[Impact Factor 1.643 of *Journal Citation Reports®*, *ISI Web of Knowledge*; 16.9th percentile of 'Endocrinology and Metabolism ' (journal n.121th/145)]

Eva Lau, Davide Carvalho, Cidália Pina-Vaz C, Adelino Barbosa, Paula Freitas. **Beyond gut microbiota: understanding obesity and type 2 diabetes.** *Hormones* 2015;14(3):358-69

2

["Gut microbiota: association with NAFLD and metabolic disturbances"](#)

Full-paper published in *Biomed Research International* [Impact factor 2.197 of *Journal Citation Reports®*, *ISI Web of Knowledge*; 39.3th percentile of 'Medicine, Research and Experimental' (journal n. 83th/136)]

Eva Lau, Davide Carvalho, Paula Freitas. **Gut microbiota: association with NAFLD and metabolic disturbances.** *Biomed Res Int* 2015;2015:979515.

3

["Disbiose e microbioma na obesidade, diabetes tipo 2 e esteatose hepática não alcoólica"](#)

Full-chapter published in Book *Microbioma, disbiose, probióticos e bacterioterapia*

Eva Lau, Davide Carvalho, Paula Freitas. **Disbiose e microbioma na obesidade, diabetes tipo 2 e esteatose hepática não alcoólica.** Capítulo 25 - livro *Microbioma, disbiose, probióticos e bacterioterapia*, editora manole ltd 2017. www.ufrgs.br/bibicbs/livros-novos/microbioma-disbiose-probioticos-e-bacterioterapia

4

"The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity"

Full-paper published in *Nutrition & Metabolism* [Impact factor 3.599 of *Journal Citation Reports®*, ISI Web of Knowledge; 71.5th percentile of 'Nutrition and Dietetics' (journal n. 25th/86)]

Eva Lau, Cláudia Marques, Diogo Pestana, Mariana Santoalha, Davide Carvalho, Paula Freitas, Conceição Calhau. **The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity.** *Nutr Metab* 2016;13(31):1-7.

5

"Gut microbiota changes after metabolic surgery in adult diabetic patients with mild obesity: a randomised controlled trial"

Full-paper submitted, under review

Eva Lau, Eugeni Belda, Paul Picq, Davide Carvalho, Manuel Ferreira-Magalhães, Isaac Barroso, Flora Correia, Isabel Miranda, Cidália Pina Vaz, Eduardo Lima Costa, Adelino Barbosa, Karine Clément, Joel Doré, Paula Freitas, Edi Prifti. **Gut microbiota changes after metabolic surgery in adult diabetic patients with mild obesity: a randomized controlled trial.** [Full-paper; Submitted]

6

"Probiotic ingestion, obesity, and metabolic-related disorders: results from NHANES, 1999-2014"

Full-paper published in *Nutrients* [Impact factor 4.171 of *Journal Citation Reports®*, ISI Web of Knowledge; 82th percentile of 'Nutrition and Dietetics' (journal n. 16th/86)]

Eva Lau, João Sérgio Neves, Manuel Ferreira-Magalhães, Davide Carvalho, Paula Freitas. **Probiotic ingestion, obesity, and metabolic-related disorders: results from NHANES, 1999-2014.** *Nutrients* 2019, 11(7):1-12

ORAL PRESENTATIONS AND POSTERS

7

["Metabolic surgery versus medical therapy in mild obese adults with type 2 diabetes: a randomised controlled trial"](#)

Poster presented at Portuguese Congress of Endocrinology, Diabetes and Metabolism
Abstract published in *Revista Portuguesa de Endocrinologia, Diabetes e Metabolismo*

*Eva Lau, Edi Prifti, Eugeni Belda, Paul Picq, Davide Carvalho, Manuel Ferreira-Magalhães, Isaac Barroso, Flora Correia, Isabel Miranda, Cidália Pina Vaz, Eduardo Lima Costa, Adelino Barbosa, Karine Clément, Joel Doré, Paula Freitas. **Gut microbiota changes after metabolic surgery in mild obese diabetic adults: a randomised controlled trial.** Revista Portuguesa de Endocrinologia, Diabetes e Metabolismo 2019: 14(1); p3-4.*

8

["Gut microbiota changes after metabolic surgery in mild obese diabetic adults: a randomized controlled trial"](#)

Poster presented at Portuguese Congress of Endocrinology, Diabetes and Metabolism
Abstract published in *Revista Portuguesa de Endocrinologia, Diabetes e Metabolismo*

*Eva Lau, Edi Prifti, Eugeni Belda, Paul Picq, Davide Carvalho, Manuel Ferreira-Magalhães, Isaac Barroso, Flora Correia, Isabel Miranda, Cidália Pina Vaz, Eduardo Lima Costa, Adelino Barbosa, Karine Clément, Joel Doré, Paula Freitas. **Gut microbiota changes after metabolic surgery in mild obese diabetic adults: a randomized controlled trial.** Revista Portuguesa de Endocrinologia, Diabetes e Metabolismo 2019: 14(1); p5-6.*

9

["Probiotic ingestion, obesity, and metabolic-related disorders: results from NHANES, 1999-2014"](#)

Poster presented at Portuguese Congress of Endocrinology, Diabetes and Metabolism
Abstract published in *Revista Portuguesa de Endocrinologia, Diabetes e Metabolismo*

*Eva Lau, João Sérgio Neves, Manuel Ferreira-Magalhães, Davide Carvalho, Paula Freitas. **Gut microbiota changes after metabolic surgery in mild obese diabetic adults: a randomized controlled trial.** Revista Portuguesa de Endocrinologia, Diabetes e Metabolismo 2019: 14(1); p22.*

10

"The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in a high fat-diet induced obesity rat model"

Poster presented at 7º Simpósio de Metabolismo – Abordagem terapêutica na Síndrome Metabólica, Porto, 2015

Eva Lau, Cláudia Marques, Diogo Pestana, Mariana Santoalha, Davide Carvalho, Paula Freitas, Conceição Calhau. **The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in a high fat-diet induced obesity rat model.** *Revista Portuguesa de Diabetes* 2015; 10(4); p188-189.

DISSEMINATION ACTIVITIES TO THE SCIENTIFIC COMMUNITY

11

"Gut microbiota changes after metabolic surgery in mild obese diabetic adults: a randomized controlled trial"

Public session - "Seminário de Bioquímica", in Department of Biochemistry, Faculty of Medicine of University of Porto, 2019

12

"The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in a high fat-diet induced obesity rat model"

Public session - "Seminário de Bioquímica", in Department of Biochemistry, Faculty of Medicine of University of Porto, 2016

8.3 Awards

1

Competitive grant from Sociedade Portuguesa de Endocrinologia, Diabetes e Metabolismo

Project "Gut microbiota changes and type 2 diabetes resolution in mild obesity after metabolic surgery".

