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Modulation of gut microbiota from healthy-weight and obese individuals by pectin, by-products of tropical fruits and probiotic strains

Fernanda Bianchi

Double PhD thesis presented to Food and Nutrition Post-graduation, Brazil and to the Food Science Post-graduation, Denmark to obtain the title of PhD in Food and Nutrition and, Food Science, respectively.

Concentration area: Nutritional Science

Supervisors: Profª. Drª. Katia Sivieri (Brazil); Profª. Drª Lene Jespersen (Denmark)

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Preface

This PhD thesis was carried out at the Department of Food and Nutrition, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Araraquara, State of São paulo, Brazil, during the period from May 2015 to February 2019. This period included a one-year stay at the Department of Food Science, Faculty of Science, University of Copenhagen, Denmark, where the rRNA sequencing and other experiments were conducted. This PhD project was partnered by Chr. Hansen (Denmark) and CP Kelco (Denmark).

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Abstract

Several by-products of tropical fruits, which are often discarded by the food industry, have high fibre content and bioactive compounds. These compounds, as well as certain probiotic strains and some pectins present in the by-products, have the potential to modulate the human gut microbiota, promoting several health benefits, including the attenuation of obesity parameters. The aim of this work was to evaluate the effects of lemon pectin, dried by-products of tropical fruits (acerola and camu-camu), as well as of different probiotic strains (*Bifidobacterium longum* BB-46, *Lactobacillus acidophilus* LA-5 and *L. paracasei* L-431) on the gut microbiota from healthy-weight and obese individuals using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). Six articles were developed in order to meet the proposed aims. The first article is a mini-review and the other five are original articles.

In the first article, we summarized the principal findings on obesity-related microbiota composition and reviewed new strategies for gut microbiota modulation in favour of obesity treatment. We showed that the composition of the microbiota is essential for understanding the mechanisms involved in the aetiology of obesity and, that several strategies, such as consumption of probiotics and prebiotics, as well as moderate and regular physical activity, can modulate the gut microbiota in favour of obesity treatment.

In the second article, the chemical composition, the total phenolic compounds and the *in vitro* antioxidant capacity of the camu-camu by-product were evaluated. An *in vitro* gastrointestinal survival essay was also performed using the three studied probiotic strains along with the camu-camu by-product. This study indicated that although the camu-camu by-product might be useful for the development of new food products, increasing its nutritional value, it can have a negative effect on the survival rate of determined probiotic strains during the gastrointestinal transit.

In the third article, the impact of a lemon pectin on the survival rate of *B. longum* BB-4, *L. acidophilus* LA-5 and *L. paracasei* L-431 was evaluated using an *in vitro* gastrointestinal survival essay. The citric pectin was shown to have a positive impact on the survival of *B. longum* BB-46, but had no positive effects on *L. acidophilus* LA-5 and *L. paracasei* L-431 survival. Therefore, *B. longum* BB-46 with lemon pectin was one of the combinations selected for further experiments in the SHIME® model.

In the fourth article, we evaluated the chemical composition, the total phenolic compounds and the *in vitro* antioxidant capacity of the acerola by-product. We also evaluated the survival of all studied probiotic strains (LA-5, L-431 and BB-46) combined with acerola by-product using an *in vitro* gastrointestinal survival essay. This article also includes the effects of *Bifidobacterium longum* BB-46 in combination with the acerola by-product on the intestinal microbiota of healthy-weight individuals using the SHIME®. The acerola by-product showed an excellent chemical composition and high antioxidant activity when compared to other dried by-products. Moreover, the by-product could improve the survival of *B. longum* BB-46 during the *in vitro* gastrointestinal assay and was, therefore, the second combination selected for

the experiments in SHIME® model. The results suggested that *B. longum* BB-46 with acerola by-product has a positive effect on the gut microbiota metabolism and might be used in new studies about functional product development.

In the fifth article, the effects of *Bifidobacterium longum* BB-46 in combination with the citric pectin on the intestinal microbiota of healthy-weight individuals were evaluated using the SHIME® and 16S rRNA gene sequencing. We observed that although each treatment (*B. longum* BB-46 and *B. longum* BB-46 combined with lemon pectin) could modulate the microbiota, the combination was more effective in decreasing intestinal NH_4^+ levels and in increasing butyric acid-producing bacteria. These findings indicate that *B. longum* BB-46, especially when combined with lemon pectin, might have a beneficial impact on human health.

In the sixth article, the effects of *Bifidobacterium longum* BB-46 and lemon pectin, combined or not, on the intestinal microbiota of obese individuals were evaluated using the SHIME® and the 16S rRNA gene sequencing. The results indicated that both *B. longum* BB-46 and pectin can modulate the obesity-related microbiota; however, when the pectin is combined with BB-46, the predominant effect of the pectin can be observed. Treatments with pectin and pectin combined with *B. longum* BB-46 showed a high increase in butyric acid-producer bacteria as well as in bacteria with potential anti-inflammatory effects and a decrease in the *Lachnospiraceae* family, recently associated with the development of obesity. These findings indicate that the studied pectin can probably have a protective role in obesity.

Finally, with the results reported in this study, we could conclude that both the acerola and camu-camu by-products might be useful in the development of new functional foods due to their excellent physico-chemical characteristics. However, we could demonstrate that the by-product of camu-camu may be harmful to the survival of certain probiotic strains. Moreover, this study allowed us to conclude that *B. longum* BB-46 in combination with the acerola by-product can positively alter intestinal metabolites and that *B. longum* BB-46 in combination with the citric pectin has the ability to modulate the microbiota of both obese and healthy-weight individuals, but in different ways. This study also enabled us to observe that the stimulation or inhibition of certain bacterial families or genera are also dependent on the initial composition of the microbiota. Although this study indicates that *B. longum* BB-46 in combination with the citric pectin may be useful in preventing obesity-related symptoms, clinical studies including blood parameters and weight control are required to demonstrate the *in vitro* observed effects.

Keywords: Gut microbiota; Obesity; Pectin; By-product of fruits; Probiotics; 16S rRNA.

Portuguese abstract

Diversos subprodutos de frutas tropicais, os quais são frequentemente descartados pelas indústrias alimentícias, apresentam elevado conteúdo de fibras e de compostos bioativos. Estes compostos, assim como determinadas cepas probióticas e algumas pectinas presentes nos subprodutos, têm o potencial de modular a microbiota intestinal humana, promovendo diversos benefícios à saúde, tais como a atenuação de parâmetros relacionados à obesidade. O objetivo deste trabalho foi avaliar os efeitos da pectina do limão, de subprodutos secos de frutas tropicais (acerola e camu-camu) e de diferentes cepas probióticas (*Bifidobacterium longum* BB-46, *Lactobacillus acidophilus* LA-5 and *L. paracasei* L-431) na microbiota intestinal de indivíduos eutróficos e obesos utilizando o Simulador do Ecossistema Microbiano Humano (SEMH®). Seis artigos foram desenvolvidos a fim de se responder os objetivos propostos. O primeiro artigo trata-se de uma mini-revisão e, os cinco restantes, artigos originais.

No primeiro artigo, sumarizou-se os principais achados sobre a composição da microbiota intestinal de obesos e, revisou-se as novas estratégias de modulação da microbiota intestinal em favor do tratamento da obesidade. Foi possível mostrar que a composição da microbiota intestinal é essencial para o entendimento de mecanismos envolvidos na etiologia da obesidade e que, várias estratégias, tais como, o consumo de prebióticos e probióticos, bem como a prática de atividade física moderada e regular, podem modular a microbiota em favor do tratamento da obesidade.

No segundo artigo, foram avaliados a composição centesimal, o teor de compostos fenólicos totais e a capacidade antioxidante *in vitro* do subproduto do camu-camu. Nesta etapa foi também realizado um teste *in vitro* de viabilidade gastrointestinal utilizando os três probióticos em estudo, juntamente com o subproduto do camu-camu. Os resultados indicaram que embora o subproduto do camu-camu possa ser útil no desenvolvimento de novos produtos alimentícios, aumentando seus respectivos valores nutricionais, tal subproduto pode ter um efeito negativo na sobrevivência de determinados probióticos durante a passagem gastrointestinal.

No terceiro artigo, foi avaliado o impacto da pectina do limão na taxa de sobrevivência das três cepas probióticas estudadas utilizando um ensaio *in vitro* de sobrevivência gastrointestinal. A pectina cítrica mostrou ter um impacto positivo na sobrevivência do *B. longum* BB-46, porém nenhum efeito positivo foi observado para as cepas *L. acidophilus* LA-5 e *L. paracasei* L-431. Desta forma, o probiótico BB-46 juntamente com a pectina do limão foi uma das combinações selecionadas para os futuros experimentos no SEMH®.

No quarto artigo, foram avaliados a composição centesimal, o teor de compostos fenólicos totais e a capacidade antioxidante *in vitro* do subproduto da acerola. Foi também avaliada a taxa de sobrevivência de todas as cepas probióticas estudadas em combinação com o subproduto da acerola utilizando ensaio *in vitro* de sobrevivência gastrointestinal. Este artigo incluiu também os efeitos do probiótico *B. longum* BB-46 em combinação com o subproduto da acerola na microbiota de indivíduos eutróficos utilizando o SEMH®. O subproduto da acerola mostrou uma excelente composição centesimal e

elevada atividade antioxidante quando compado a outros subprodutos secos. Além disso, o referido subproduto foi capaz de melhorar a taxa de sobrevivência do probiótico durante a simulação gastrointestinal e, portanto, foi a segunda combinação selecionada para os experimentos no SEMH®. Os resultados sugeriram que a combinação tem um efeito positivo no metabolismo da microbiota intestinal e pode ser uma opção no desenvolvimento de produtos funcionais.

No quinto artigo, foram avaliados os efeitos da combinação entre *Bifidobacterium longum* BB-46 e pectina cítrica na microbiota intestinal de indivíduos eutróficos utilizando o SEMH® e sequenciamento do gene 16S rRNA. Observou-se que embora cada tratamento (BB-46 e BB-46 combinado com pectina de limão) tenha sido capaz de modular a microbiota intestinal, a combinação entre ambos foi mais eficaz em reduzir os níveis de NH_4^+ intestinal e em aumentar bactérias produtoras de acido butírico. Estes achados indicam que o probiótico *B. longum* BB-46, especialmente quando combinado com a pectina cítrica, pode proporcionar efeitos benéficos à saúde.

No sexto artigo, foram avaliados os efeitos do probiótico *Bifidobacterium longum* BB-46 e da pectina cítrica, em combinação ou não, na microbiota intestinal de indivíduos obesos utilizando o SEMH® e sequenciamento do gene 16S rRNA. Os resultados indicaram que tanto o probiótico quanto a pectina são capazes de modular a microbiota de obesos. No entanto, quando combinados, observa-se um efeito predominante da pectina. Os tratamentos com a pectina e pectina em combinação com *B. longum* BB-46 proporcionaram um elevado aumento de bactérias produtoras de ácido butírico, bem como de bactérias com potenciais efeitos anti-inflamatórios e redução de membros pertencentes à família *Lachnospiraceae*, recentemente associada ao desenvolvimento de obesidade. Esses achados indicam que a pectina estudada pode exercer um provável papel protetor na obesidade.

Finalmente, por meio dos resultados reportados neste estudo, pudemos concluir que tanto o subproduto da acerola quanto o do camu-camu podem ser úteis no desenvolvimento de novos alimentos funcionais devido às suas excelentes características físico-químicas. Observou-se, no entanto, que o subproduto de camu-camu pode ser prejudicial à sobrevivência de certas cepas probióticas. Este estudo mostrou também, que a combinação entre *B.longum* BB-46 e subproduto de acerola é capaz de alterar os metabólitos intestinais de forma positiva e que, a combinação entre *B. longum* BB-46 e a pectina cítrica tem a capacidade de modular a microbiota tanto de indivíduos eutróficos quanto de a obesos, porém de formas distintas. Observou-se ainda, que o estímulo ou a inibição de determinadas famílias ou gêneros bacterianos dependem também, da composição inicial da microbiota. Apesar deste estudo indicar que a combinação entre *B. longum* BB-46 e pectina pode ser útil na prevenção dos sintomas relacionados à obesidade, estudos clínicos incluindo parâmetros sanguíneos e controle de peso são necessários para comprovar os efeitos observados *in vitro*.

Palavras-chave: Microbiota intestinal; Obesidade; Pectina; Subproduto de frutas; Probióticos; 16S rRNA.

Dansk abstract

Flere biprodukter fra tropiske frugter, som ofte kasseres af fødevareindustrien, har højt fiberindhold og bioaktive forbindelser. Disse forbindelser, såvel som visse probiotiske stammer og nogle pektiner, der er til stede i biprodukterne, har potentialet til at modulere den humane tarmmikrobiota, der fremmer flere sundhedsmæssige fordele, herunder dæmpning af fedmeparametre. Formålet med dette arbejde var at evaluere virkningerne af citronpektin, tørrede biprodukter fra tropiske frugter (acerola og camu-camu), samt forskellige probiotiske stammer (*Bifidobacterium longum* BB-46, *Lactobacillus acidophilus* LA-5 og *L. paracasei* L-431) på tarmmikrobioten fra raske og overvægtige individer, under anvendelsen af den humane intestinale mikrobielle økosystem simulator (SHIME®). Seks artikler blev udviklet for at opfylde de foreslåede mål. Den første artikel er en mini-anmeldelse, og de andre fem er originale artikler.

I den første artikel opsummerede vi de vigtigste fund om fedme-relateret mikrobiota sammensætning og gennemgik nye strategier for tarmmikrobiota modulering til fordel for fedmebehandling. Vi viste, at mikrobiotas sammensætning er afgørende for forståelsen af mekanismerne i fedmens etiologi, og at flere strategier, såsom forbrug af probiotika og præbiotika, samt moderat og regelmæssig fysisk aktivitet kan modulere tarmmikrobiotaten til fordel for fedme behandling.

I det anden artikel blev den kemiske sammensætning, de samlede phenolforbindelser og *in vitro* antioxidantkapaciteten af camu-camu biproduktet evalueret. En *in vitro* gastrointestinal overlevelsesopgave blev også udført under anvendelse af tre probiotiske stammer (*Bifidobacterium longum* BB-46, *Lactobacillus acidophilus* LA-5 og *L. paracasei* L-431) sammen med camu-camu biproduktet. Denne undersøgelse viste, at selv om camu-camu-biproduktet kunne være nyttigt til udvikling af nye fødevarer, hvilket øger næringsværdien, kan det have en negativ indvirkning på overlevelsesraten for bestemte probiotiske stammer under den gastrointestinale passage.

I den tredje artikel blev virkningen af et citronpektin på overlevelseshastigheden af *B. longum* BB-4, *L. acidophilus* LA-5 og *L. paracasei* L-431 evalueret under anvendelse af en *in vitro*-mavetarm-overlevelsesopgave. Citronpektin viste sig at have en positiv indvirkning på overlevelsen af *B. longum* BB-46, men havde ingen positive virkninger på *L. acidacilus* LA-5 og *L. paracasei* L-431 overlevelse. Derfor var *B. longum* BB-46 med citronpektin en af kombinationerne valgt til yderligere forsøg i SHIME®-modellen.

I den fjerde artikel evaluerede vi den kemiske sammensætning, de samlede phenolforbindelser og in vitro antioxidantkapaciteten af acerola biproduktet. Vi vurderede også overlevelsen af alle undersøgte probiotiske stammer (LA-5, L-431 og BB-46) kombineret med acerola biprodukt ved anvendelse af en in vitro-gastrointestinal overlevelsesopgave. Denne artikel indbefatter også virkningerne af *Bifidobacterium longum* BB-46 i kombination med acerola biproduktet på tarmmikrobioten af sunde individer, der bruger SHIME®. Acerola biproduktet viste en fremragende kemisk sammensætning og høj antioxidantaktivitet sammenlignet med andre tørrede biprodukter.

Endvidere kunne biproduktet forbedre overlevelsen af *B. longum* BB-46 under in vitro-gastrointestinaltest og var derfor den anden kombination valgt til forsøgene i SHIME®-modellen. Resultaterne antyder, at *B. longum* BB-46 med acerola biprodukt har en positiv effekt på tarmmikrobiota metabolisme og kan anvendes i nye undersøgelser om funktionel produktudvikling.

I den femte artikel blev virkningerne af *Bifidobacterium longum* BB-46 i kombination med citruspektin på intestinale mikrobiotaten af sunde-vægt individer evalueret under anvendelse af SHIME®- og 16S-rRNA-gen-sekventeringen. Vi observerede, at selvom hver behandling (*B. longum* BB-46 og *B. longum* BB-46 kombineret med citronpektin) kunne modulere mikrobiotaten, var kombinationen mere effektiv i formindskelse af intestinale NH_4+ -niveauer og i stigende smørsyreproducerende bakterier. Disse resultater viser, at *B. longum* BB 46, især i kombination med citronpektin, kan have en gavnlig indvirkning på menneskers sundhed.

I den sjette artikel blev virkningerne af *Bifidobacterium longum* BB-46 og citronpektin, kombineret eller ej, på de intestinal mikrobiota af overvægtige personer evalueret ved anvendelse af SHIME® og 16S rRNA-gen-sekventeringen. Resultaterne viste, at både *B. longum* BB-46 og pectin kan modulere den fedme-relaterede mikrobiota; Imidlertid, når pektinet kombineres med BB-46, kan den overvejende virkning af pektinet observeres. Behandlinger med pectin og pectin kombineret med *B. longum* BB-46 viste en høj stigning i smørsyreproducentbakterier såvel som hos bakterier med potentielle antiinflammatoriske effekter og et fald i familien Lachnospiraceae, der for nylig var forbundet med udviklingen af fedme. Disse fund viser, at det undersøgte pektin sandsynligvis kan have en beskyttende rolle i fedme.

Endelig kan vi med de resultater, der blev rapporteret i dette studie, konkludere, at både acerola og camu-camu biprodukter kan være nyttige til udvikling af nye funktionelle fødevarer på grund af deres fremragende fysisk-kemiske egenskaber. Vi kunne dog godtgøre, at biproduktet af camu-camu kan være skadeligt for overlevelsen af visse probiotiske stammer. Derudover har denne undersøgelse givet os mulighed for at konkludere, at *B. longum* BB-46 i kombination med acerola biproduktet positivt kan ændre intestinale metabolitter, og at *B. longum* BB-46 i kombination med citruspektin har evnen til at modulere mikrobiota af både overvægtige og sunde individer, men på forskellige måder. Denne undersøgelse gjorde det også muligt for os at observere, at stimulering eller hæmning af visse bakteriefamilier eller slægter også afhænger af mikrobiotas oprindelige sammensætning. Selv om denne undersøgelse indikerer, at *B. longum* BB-46 i kombination med citruspektin kan være nyttigt til forebyggelse af fedme-relaterede symptomer, kræves kliniske undersøgelser, herunder blodparametre og vægtkontrol, for at demonstrere de in vitro-observerede virkninger.

Nøgleord: Tarm mikrobiota; Fedme; Pektin; Biprodukt af frugter; Probiotika; 16S rRNA.

Abbreviations and acronyms

AD: Alzheimer's disease
BB-46: *Bifidobacterium longum* BB-46
BIM-25 agar: *Bifidobacterium* iodoacetate medium 25
BMI: Body Mass Index
bp: base pair
BSH: bile salt hydrolases
C: control period
Caco-2: human adenocarcinoma cells
cAMP: Cyclic adenosine MonoPhosphate
CD: Coeliac disease
CD14: cluster of differentiation
cDNA: complementary deoxyribonucleic acid
CFU: Colony Forming Units
CYP7A1: cholesterol 7α-hydroxylase
DCA: deoxycholic acid
DGGE: Denaturing gradient gel electrophoresis
DIDGI: Dynamic Gastrointestinal Digester
DM: degree of methyl esterification
DNA: Deoxyribonucleic acid
DP: Parkinson's disease
DPPH: sequestration of radical 2.2-difenyl-1-picrylhydrazyl
ENA: European Nucleotide Archive
ESIN: Engineered Stomach and Small Intestine
FA: Food alergies
FC: Functional constipation
FGF15/19: fibroblast growth factor 15/19
FISH: Fluorescence *in situ* hybridization
FOS: fructooligosaccharides
FRAP: Ferric Reducing Ability of Plasma
FXR: farnesoid X receptor
GAE: gallic acid equivalent
GBA: gut-brain axis
GF: germ free
GLP-1: glucagon-like peptide-1
GOS: galactooligosaccharides
HDL: High-density lipoprotein
HFD: high fat diet
HM: high-methylated pectin
HMOs: human milk oligosaccharides
HT-29: mucus-producing goblet cell
IBS: Irritable bowl syndrome
IL-1β: Interleukin 1 beta
IL-6: interleukin-6
ISAPP: International Scientific Association for Probiotics and Prebiotics
IviDiS: *In vitro* Digestive System

L-431: *Lactobacillus paracasei* L-431
LA-5: *Lactobacillus acidophilus* LA-5
LCA: lithocholic acid
LDL: Low-density lipoprotein
Lm: low-methylated
LPS: lipopolysaccharides
MetS: Metabolic syndome
MRS: Man Rogosa Sharpe
OUT: Operational Taxonomic Units
PYY: peptide YY
QIIME: Quantitative Insight Into Microbial Ecology
qPCR: quantitative polymerase chain reaction
rRNA: Ribosomal ribonucleic acid
SCFAs: short chain fatty acids
SHIME: Simulator of the Human Intestinal Microbial Ecosystem
T1: Treatment with *B. longum* BB-46
t10,c12-CLA: conjugated linoleic acid isomer
T2: Treatment with *B. longum* BB-46 and pectin
T2DM: Type II Diabetes Mellitus
T3: Treatment with pectin
TAE: Tris-acetate-EDTA
TCA: taurocholate
TDCA: taurodeoxycholate
TE: Trolox equivalents
TG: triglycerides
TGR5: G protein-coupled receptor
TIM-1 and TIM-2: TNO Gastro-Intestinal Model 1 and 2
TLR4: Toll-like receptor 4
TNF: tumor necrosis factor
TPTZ: 2,4,6-Tri(2-pyridyl)-s-triazine
T-RFLP: Terminal restriction fragment length polymorphism
TβMCA: tauro-β-muricholic acid
V3: Vessel 3 (Ascending colon)
V4: Vessel 4 (Transverse colon)
V5: Vessel 5 (Descending colon)
W: washout period
XOS: xylooligosaccharide

Introduction

1. The human gut microbiota

1.1. Gastrointestinal tract structure and composition

The human gastrointestinal tract hosts at least 10^{14} microorganisms, which is approximately 10 times the total number of human body cells (1,2). This population encodes 3 to 4 million genes, exceeding the number of human genome by approximately 150 times (3,4).

The colonization of the digestive tract starts at birth, with reduced diversity and high instability. Different factors such as birth type (caesarean section or vaginal delivery), mother's intrauterine microbiota, method of postnatal feeding (formula or breast) and genetic factors, such as intestinal immunity, biliary secretion, mucosal barrier composition, digestive enzymes, and intestinal motility, can affect the composition of bacteria in the gut's child (5–7). Although the microbiota of adults' individuals is relatively stable, some changes can occur due to the lifestyle, type of diet, environmental influences, place of residence and antibiotic use (8).

The gastrointestinal microbiota distribution varies according to its location in the digestive tube (3,9). As shown in Fig. 1, a low bacterial density is found in the stomach and duodenum regions. This fact occurs because of the presence of acidic gastric juice and pancreatic enzymes (3,10). On the other hand, a gradual increase of bacterial density is reported in the distal small intestine, reaching its highest concentration in the colon (3,10), where the

intestinal transit time, availability of nutrients and pH are favourable (11), totalling 1-2 kg of our body weight (12).

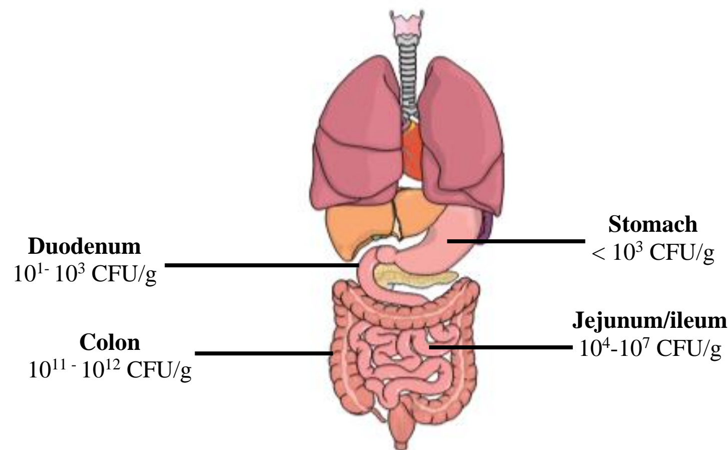


Figure 1. Total number (CFU/g) of microorganisms in the gastrointestinal tract

In the literature, the gut microbiota is referred to as a "separate organ" (13) or "forgotten organ" (10,14) because of the exorbitant number of microorganisms inhabiting this locale. The gut microbiota is composed of both anaerobic and aerobic microbial communities although the vast majority are strictly anaerobic and difficult to be cultured *in vitro* (3). The gut microbiota consists of more than 1500 species distributed in at least seven different phyla (3,15). The most dominant bacterial phyla found in the human gut are *Firmicutes* (including for example the genera *Lactobacillus, Ruminococcus, Clostridium* and *Eubacteria*) and *Bacteroidetes (e.g.* genera *Bacteroides, Porphyromonas, Prevotella* etc.), followed by *Proteobacteria* (e.g. *Klebsiella, Enterobacter, Succinivibrio* genera) and *Actinobacteria* (including, for example, *Bifidobacterium* and *Coreobacteria* genera). Other phyla can be found in minor proportions such as *Fusobacteria, Verrucomicrobia,*

Tenericutes, Cyanobacteria, Spirochaetes, and *Synergistetes* with less than 2% (9,16,17). All these microorganisms interact with each other and with their host, exerting influence on the physiology and health of the human (3).

Despite the inter-individual variability, determined by place of residence, lifestyle, disease conditions, hygiene, sex or age, which will determine the microbiota composition, it has been proposed that the human gut microbiome can be classified in two main specific microbial enterotypes. The identification of each enterotype is based on a relatively high abundance of a single microbial genus: *Bacteroides* (enterotype 1) or *Prevotella* (enterotype 2) (18–20). Each enterotype is linked with long-term diets. *Bacteroides* enterotype is associated with a high fat or protein diet while *Prevotella* are linked with a high-carbohydrate and high dietary fibre diet (19).

1.2. Gut microbiota function and the relationship with diet

Several studies have shown the influence of diet on the microbiota composition and, consequently, on health (21–25). According to the cited authors, a diet high in fat, especially in trans and saturated fat, is related with gut dysbiosis, leading to obesity and metabolic syndrome. Pendyala et al. (21), for example, observed that a high-fat Western diet increases the concentration of endotoxin in plasma (endotoxemia), probably due to failure in intestinal barrier function, leading to an inflammatory process. According to Willson and Situ (25), diets with low fat and high in fruits, complex fibres, vegetables, and supplemented with probiotics are key to a healthy gut microbiota.

The gut microbiota uses ingested dietary components not absorbed in the small intestine, such as carbohydrates, lipids and proteins, by means of fermentation leading to the production of several metabolites, which can directly or indirectly affect the human metabolism and health (26). Short-chain fatty acids (SCFAs), especially propionic, acetic and butyric acids, are one of the main metabolites produced by the microbiota in the presence of non-digestible carbohydrates (27,28). Besides the SCFAs, the gut microbiota can produce other metabolites like choline, bile acid metabolites (29), indole and phenolic derivatives (26–28).

The SCFAs are the main source of energy for the colon epithelium. Moreover, they stimulate the proliferation of epithelium cells, the visceral blood flow and enhance the absorption of sodium and water (30). Studies have shown that besides nourishing microorganisms and enterocytes, the SCFAs, including acetic, butyric and propionic acids, have other refined functions (31–33). They are considered important regulators of immunity, energy metabolism and adipose tissue expansion and can prevent the growth of bacterial pathogens due to the acidification of the colonic lumen (31,32). According to Ji et al. (33), butyric acid is able to decrease the expression of pro-inflammatory cytokine of tumour necrosis factor (TNF-α and TNF-β), interleukin-6 (IL-6) and interleukin-1β (IL-1β), alleviating thus the inflammatory process, and to inhibit the genotoxic capacity of nitrosamines, reducing the risk of colon cancer (34). Moreover, it has been proposed that butyric acid has the ability to reduce food intake, moderating weight gain. Propionate and acetate were also reported to have an action on appetite regulation and, consequently,

on body weight. The mechanism connecting the production of intestinal SFCAs to food intake regulation includes the stimulation of anorectic gut hormones' release, such as the peptide YY (PYY) and the glucagon-like peptide-1 (GLP-1), which can inhibit appetite, partly by regulating soluble leptin receptors. (31,32,35–37).

Although the carbohydrates represent the main substrates leading to the production of SCFAs, fatty acids with an aliphatic chain (C1-C5) have also been shown to be able to produce acetate, propionate, butyrate, as well as isobutyrate, valerate, and isovalerate (29).

Besides SCFA production, the gut microbiota performs many other functions like the supply of vitamin K and several B vitamins (26,27), biotransformation of polyphenols into metabolites, prevention of pathogenic bacteria colonization by means of competition for essential resources and ecological niche, and exerts a significant influence over immunological processes (11,38,39). Moreover, the gut microbiota attends to the function of the gut-brain axis (GBA). GBA is a bidirectional communication between the central system and the enteric nervous. This communication links peripheral intestinal functions with the emotional and cognitive centres of the brain (40). Some of the functions of the gut microbiota are represented in Fig. 2.

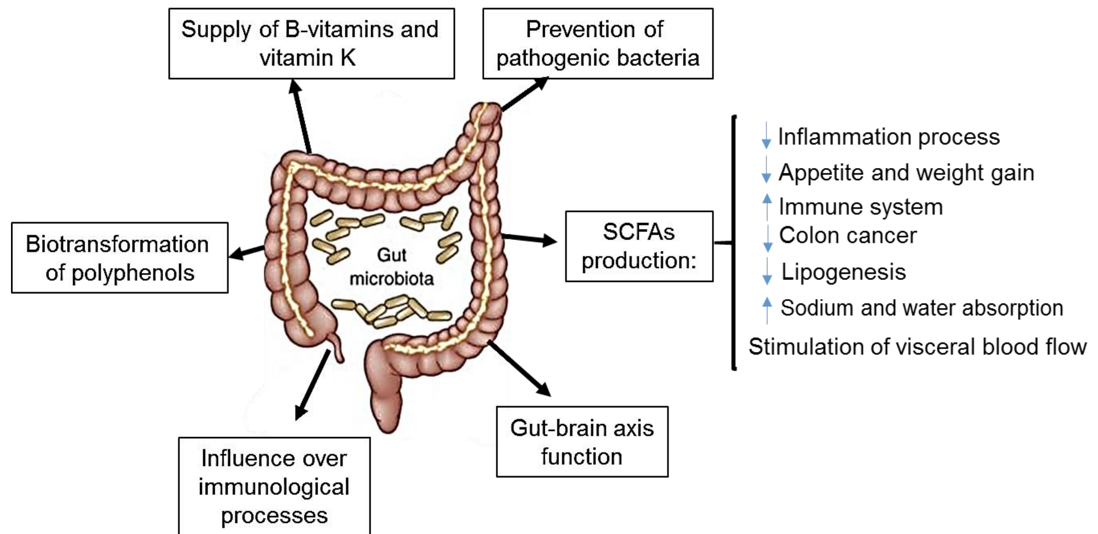


Figure 2. Different functions of gut microbiota

It is also important to highlight that the anaerobic metabolism of the gut bacteria can include proteolytic fermentation, which will produce nitrogenous derivatives like amines and ammonium, some of which have carcinogenic effects (41).

Several factors such as lifestyle and diet can affect the composition and activity of gut microbiota and, consequently, interfere in the various already-cited functions of the microbiota, leading to the improvement of health or to the development of various diseases such as obesity, as illustrated in Fig. 3.

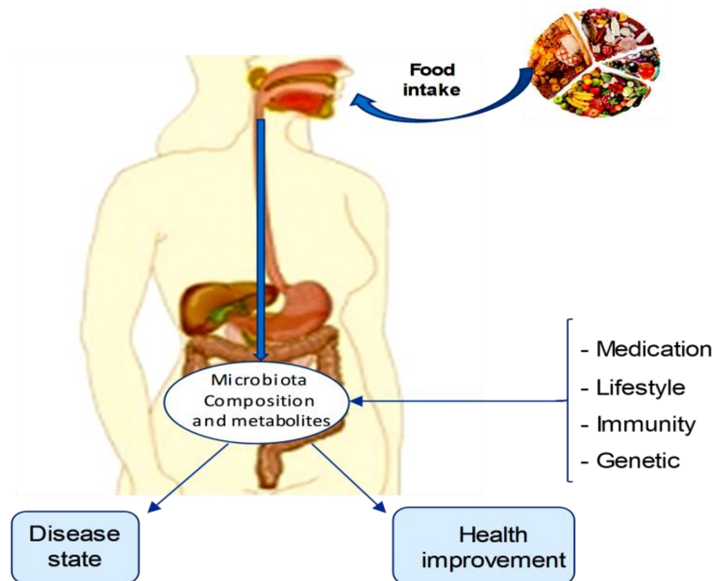


Figure 3. Interaction between diet, gut microbiota and host.

1.3. Gut microbiota and diseases

Several studies have demonstrated the link between changes in the gut microbiota composition and various metabolic disorders, such as diabetes mellitus, liver cirrhosis, hypertension, atherosclerosis and clinical depression (42–49), as shown in Table 1.

Table 1. Different metabolic disorders and their relationship with the gut microbiota

Disease	Subjects	Observed changes in the disease-related microbiota compared to control	Authors
Subclinical Diabetes type II	36 stool samples from Korean adults with sub-clinical state of diabetes type II	- Decrease in *Akkermansia muciniphila* - Negative correlation between *Akkermansia muciniphila* and body mass index	(42)
Hypertension	Faecal samples of hypertension rats (SHR) and a small cohort of human hypertension patients compared with control (patients with normal systolic blood pressure.	- Lower microbial richness, diversity and evenness - Decreased production of acetate and butyrate. - Higher quantities of *Streptococcus* and *Turicibacter* and lower of *Coprococcus* and *Pseudobutyrivibrio* in the SHR	(43)
Diabetes type I	Stoll sample of 16 children with type 1 diabetes and 16 healthy Caucasian children	- Increase in the number of *Clostridium*, *Bacteroides* and V*eillonella* - Decrease in the number of *Lactobacillus, Bifidobacterium, Blautia* - Negative correlation between the plasma glucose level and *Lactobacillus*, *Bifidobacterium* - Positive correlation between the plasma glucose level and *Clostridium.*	(44)
Diabetes type II	Stool samples from 18 adults with T2DM and from 18 non-diabetic individuals	- High levels of *Lactobacillus* and lower of *Bifidobacterium*	(45)
Atherosclerosis	Stool samples from 12 patients with symptomatic atherosclerotic plaques and 13 health patients.	- *Collinsella* genus was enriched in patients with symptomatic atherosclerosis. - *Roseburia* and *Eubacterium* were enriched in healthy controls. - Increase peptidoglycan from bacteria showed positive correlation with triglycerides levels	(46)
Liver cirrhosis	Faecal microbiota from 36 patients with liver cirrhosis and 24 healthy controls	↓ *Bacteroidetes* ↑ *Fusobacteria and Proteobacteria* ↑ *Enterobacteriaceae, Veillonelaceae and Streptococcaceae families* ↑ *Lachnospiraceae family*	(47)
Liver cirrhosis	21 patients with fatty liver-related cirrhosis (NFLD) and hepatocellular carcinoma (HCC), 20 patients with NAFLD-related cirrhosis without HCC and 20 healthy controls.	↑ *Enterobacteriaceae* and *Streptococcus* ↓ *Akkermansia* genus Increased *Bacteroides* and *Ruminococcaceae* and reduced *Bifidobacterium* in the nonalcoholic fatty liver-related cirrhosis and hepatocellular carcinoma group	(48)
Depressive disorder	Faecal samples from 46 patients with depression and 30 health controls	↑ levels of *Bacteroidetes, Proteobacteria,* and *Actinobacteria* and reduced *Firmicutes* - Increased levels of *Enterobacteriaceae* and *Alistipes* - Reduced levels of *Faecalibacterium* - Negative correlation between *Faecalibacterium* and the severity of depressive symptoms	(49)

The studies have also demonstrated a relationship between the intestinal microbiota in the etiopathogenesis of many gastroenterological diseases, such as irritable bowel syndrome, cancer, constipation, coeliac disease and food allergies (50–56), as shown in Table 2.

Table 2. Different gastroenterological diseases and their relationship with the gut microbiota

Disease	Subjects	Observed changes in the disease-related microbiota compared to control	Authors
Irritable bowel syndrome (IBS)	Faecal sample of 23 patients with irritable bowel syndrome and 23 samples from healthy controls	↓ *Bacterial richness* ↑ *Enterobacteriaceae* ↑ *Proteobacteria* ↓ *Faecalibacterium*	(50)
Irritable bowel syndrome (IBS)	Faecal sample and structural brain images from 29 adult IBS and 23 healthy control subjects	- Gut microbial composition correlated with structural measures of brain regions	(51)
Colorectal cancer	Faecal sample from 15 patients with colorectal cancer and 21 health patients	- Increased microbial diversity - Enrichment of several taxa including *Fusobacterium, Selenomonas* and *Peptostreptococcus*	(52)
Functional constipation (FC)	Stool samples from 68 FC individuals and 79 healthy subjects	- Depletion of genera *Bacteroides*, *Roseburia* and *Coprococcus*	(53)
Coeliac disease (CD)	Duodenal biopsies and faeces of CD patients (n = 23), FDRs (n = 27) and control subjects (DC, n= 24)	- Genera *Acinetobacter, Lactobacillaceae, Corynebacter*, and one OTU *Prevoella* and *Pseudomoans* were abundant in duodenal biopsies - *Corynebacterium, Commamonas* and *Novosphingobium* were differentiating genera in faeces of celiac patients.	(54)
Coeliac disease (CD)	48 faecal samples from CD subjects, 32 biopsy samples from CD subjects and 30 faecal and 8 biopsy samples from control	↑ *Bacteroides, E coli, Staphylococcus* and *Clostridium leptum* in faeces and biopsies ↓ *Bifidobacterium* in faeces and biopsies	(55)
Food allergies (FA)	Faeces of 34 infants with FA and 45 healthy controls.	↓ *Bacteroidetes, Proteobacteria*, and *Actinobacteria* phyla ↑ *Firmicutes* phylum - Increased levels of *Clostridium sensu stricto* and *Anaerobacte*r and, decreased levels *of Bacteroides* and *Clostridium* XVIII. - Positive correlation between *Clostridium sensu stricto* and serum-specific IgE	(56)

Changes in intestinal microbiota have also been associated with neurological conditions like Alzheimer's, Parkinson's and autism spectrum disorder (57–61) (Table 3).

Table 3. Different neurological conditions and their relationship with the gut microbiota

Disease	Subjects	Observed changes in the disease-related microbiota compared to control	Authors
Alzheimer's disease (AD)	Faecal samples of 25 elderly subjects with AD and 25 elderly without AD	- Decreased microbial diversity - Reduced levels of SCFAs - High levels of *Bacteroidetes*, especially *Bacteroides* genus - Decreased levels of *Dialister, Blautia Bifidobacterium* genus	(57)
Autism	Faecal samples of 40 subjects with clinical diagnosis of autism and 40 age and sex-matched neurotypical healthy subjects	↓ of genera *Alistipes, Bilophila, Dialister, Parabacteroides*, and *Veillonella* ↑ of genera *Collinsella, Corynebacterium*, *Dorea*, and *Lactobacillus.*	(58)
Autism	Duodenal microbiome analysed in biopsies from 21 autistic subjects and 19 children without autism.	- Positive correlation was found between the abundance of *Clostridium* species and disaccharidase activity in autistic individuals - Increased abundance *of Burkholderia* and reduced of *Neisseria* genus	(59)
Parkinson's disease (DP)	Faecal microbiomes of 72 Parkinson's disease patients and 72 control subjects	- Abundance of *Prevotellaceae* reduced by 77.6% - Relative abundance of *Enterobacteriaceae* positively associated with the severity of postural instability and gait difficulty.	(60)
Parkinson's disease (DP)	Faecal samples of 75 patients with Parkinson's disease and 45 age-matched controls	- Increases in the abundance of four bacterial families and decreases in the abundance of seventeen bacterial families - *Lachnospiraceae*, in particular, was reduced by 42.9% in patients with PD, and *Bifidobacteriaceae* significantly enriched	(61)

Trying to understand the link between the gut microbiota and certain diseases, some studies showed that alterations in the microbiota composition

and/or function can result in alterations of intestinal permeability and motility, immune response and visceral sensitivity, which can lead to a pro-inflammatory state (15,62). According to Sanz et al. (63) and Chassaing et al. (64), the gut microbiota is involved in the regulation of antimicrobial peptides, mucin gene expression and maturation of the B cells and T cells, important for the regulation and development of the host defence system, and, consequently, human health (63–65).

Besides the aforementioned diseases, several studies have also linked the obesity condition to gut microbiota composition and metabolism (66–69).

1.4. Relationship between gut microbiota and obesity

Obesity has been one of the main public health concerns of the 21st century. According to Swinburn et al. (70), more than 500 million people across the world are considered obese, which highlights the severity of the disease. Many factors such as lifestyle, host genetics, diet and metabolism as well as the side-effects of drugs are known to contribute to the progression or development of obesity (71,72). Beyond these factors, some evidence suggests that the gut microbiota composition also has a high influence on obesity onset. (69,73–78).

The first evidence linking the gut microbiota to obesity came from Bäckhed et al. (79) study. These authors transplanted the caecal microbiota from conventionally-raised mice to germ-free (GF) mice. They observed that the GF mice gained more fat pad mass and body weight despite the reduction

of food consumption, which led to insulin resistance along with higher glucose and leptin levels in the blood. Other studies have also shown that transplantation of caecal or faecal microbiota from obese to lean mice significantly increased body fat mass, insulin resistance, adiposity and obesity-associated metabolic phenotypes compared to controls (80–83). On the other hand, clinical trials showed that obese volunteers receiving faecal microbiota from lean donors had an improvement of serum insulin sensitivity over a 6-week period (78,84). These findings suggest that the obese phenotype is transmissible from one microbiota to another, and that the microbiota composition can interfere with the host's health (85).

Several hypotheses exist linking the gut microbiota composition/metabolites to obesity. Among these hypotheses, we can highlight the role of lipopolysaccharides (LPS), the bile acid production and the short-chain fatty acids (SCFAs) produced by microbiota (86–88).

1.4.1. Role of lipopolysaccharides (LPS), bile acid production and short-chain fatty acids (SCFAs) in linking obesity to gut microbiota.

Aiming to investigate the relationship between gut microbiota and obesity, several studies have demonstrated that lipopolysaccharides (LPS) and short-chain fatty acids (SCFAs) play a role in linking obesity to gut microbiota. The studies have also shown that the gut microbiota has an influence on bile acid production and on lipid metabolism (86–88).

LPS is an endotoxin found in the cell wall of Gram-negative bacteria (89). It is normally found at low and high concentrations in the blood of healthy and obese individuals, respectively (90). High concentrations of plasma LPS have been associated with increased levels of cluster of differentiation 14 (CD14, receptor for the LPS found in the surface of monocytes and macrophage) and IL-6, which are markers of inflammation (91).

Studies suggest that the ingestion of a high fat diet (HFD) alters the gut microbiota composition, which consequently increases the luminal levels of bacterial LPS. The LPS in contact with Toll-like receptor 4 (TLR4, receptor for the LPS found in the surface of monocytes and macrophage) triggers inflammatory signalling pathways and pro-inflammatory cytokines secretion in the intestine. This inflammatory state can subsequently induce deficiencies in the secretion, production and thickness of the mucus layer and can act by reducing the expression of genes that code the intestinal tight junction proteins such as ZO-1 and occludin. Such a reduction will consequently favour the increase of intestinal epithelium permeability, allowing thus the passage of bacterial components, such as LPS from the intestinal lumen to the adipose tissue and circulation, where it can promote systemic inflammation, hyperglycaemia, insulin resistance and adipogenesis (92,93) as shown in Fig. 4.

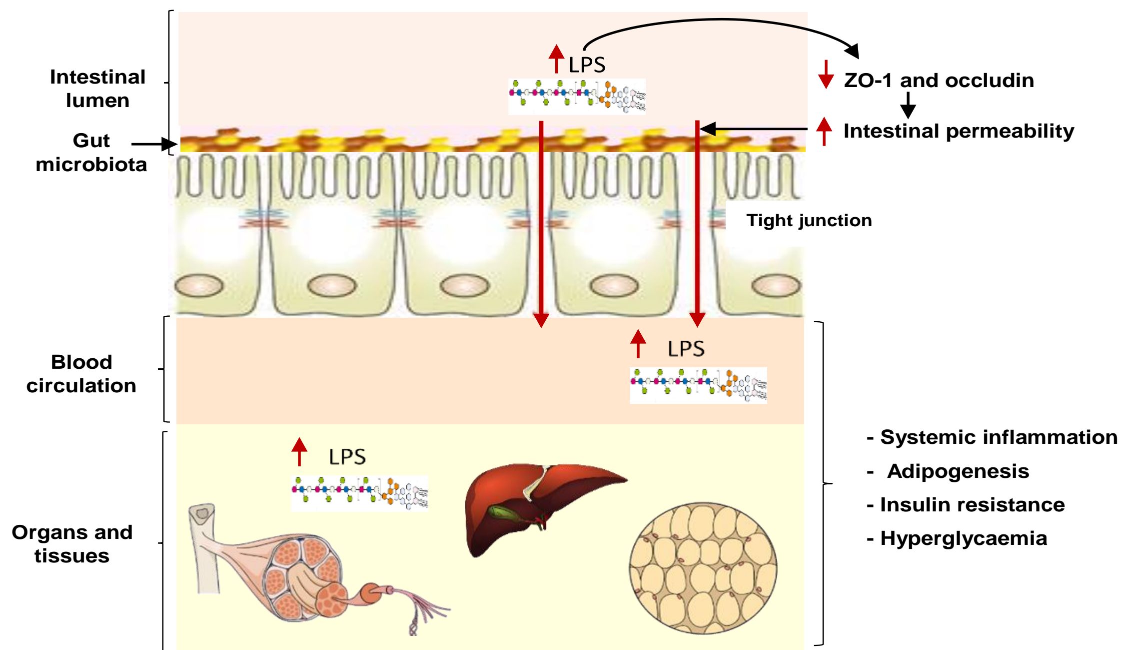


Figure 4. Role of lipopolysaccharides (LPS) in microbiota profile and consequent metabolic complications.

According to Graham et al. (91), the main source of LPS is the *Prevotellaceae* family (Gram-negative bacterial subgroup belonging to *Bacteroidetes* phylum), which are found in great abundance in the gut microbiota of obese people (91) .

The gut microbiota composition also has an influence on bile acid production and on the lipogenesis process, which can have an impact on obesity. Swann et al. (86) demonstrated that mice with a distinct gut microbial structure have a divergent energy metabolism due to different bile acid metabolites in their organs.

Bile acids are usually classified into primary and secondary bile acids. The gut microbiota plays a role in the secondary production of bile acids. In

the colon, taurocholate (TCA) and taurodeoxycholate (TDCA), primary conjugated bile acids produced in the liver, are de-conjugated by bacterial bile salt hydrolases (BSH), and dehydroxylated by bacterial 7α-dehydroxylases to form the secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA), respectively (94).

The gut microbiota modulates bile acid metabolism by influencing nuclear bile acid receptor farnesoid X receptor (FXR)/G protein-coupled receptor (TGR5) signalling, which can indirectly contribute to obesity development (72). FXR is a receptor that negatively regulates the expression of the gene cholesterol 7α-hydroxylase (CYP7A1), which encodes the enzyme cholesterol 7α-hydroxylase. The enzyme cholesterol 7α-hydroxylase catalyses the initial step of cholesterol catabolism and bile acid synthesis in liver (95). In the intestine, FXR induces fibroblast growth factor 15/19 (FGF15/FGF19), which will indirectly signal to inhibit CYP7A1 gene transcription and, consequently, the initial step of cholesterol catabolism and bile acid synthesis in liver. Decreasing BSH activity in gut microbiota increases tauro-β-muricholic acid (TβMCA), which antagonizes FXR activity, reducing FGF15/19 and stimulating CYP7A1, which will consequently stimulate bile acid synthesis in hepatocytes (Fig. 5) (94). The G protein-coupled receptor (TGR5) is a membrane receptor sensitive to the presence of bile acids expressed in the ileum and colon. This receptor promotes an intracellular elevation of cyclic Adenosine MonoPhosphate (cAMP). When cAMP is elevated in the adipose and muscle tissue cells, it triggers mechanisms of energy expenditure and stimulates insulin secretion in pancreatic β cells to improve insulin sensitivity. (Fig. 5)

(94,96,97). This way, it is possible to state that the gut-liver axis has an important role in metabolic homeostasis regulation and bile acid synthesis (94).

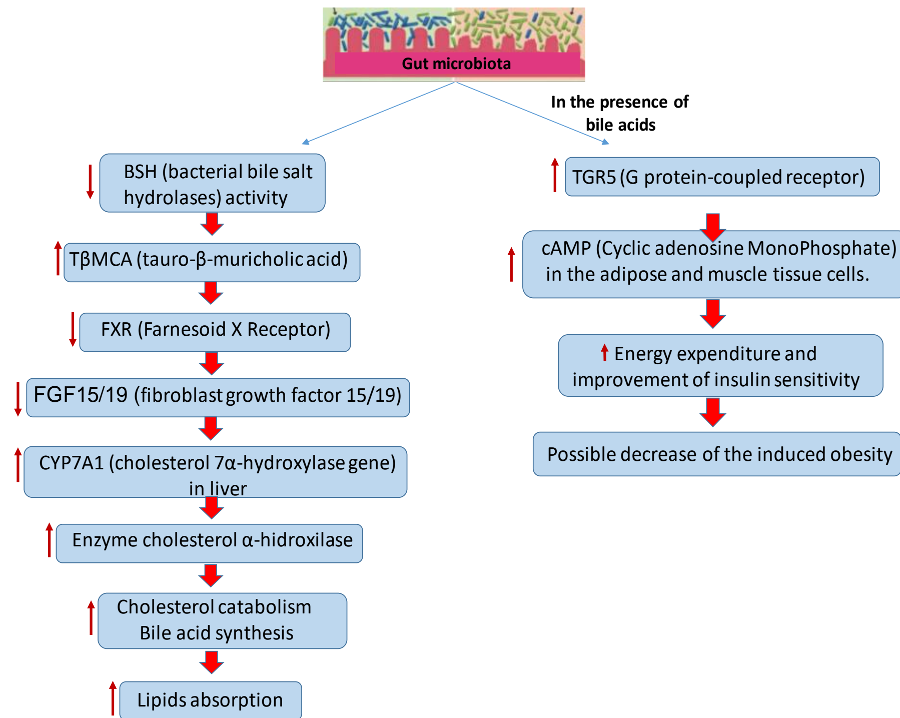


Figure 5. Influence of gut microbiota on bile acid production and lipid metabolism

Regarding SCFAs, evidence that specific metabolites, such as acetic, propionic and butyric acids, have important physiological functions has been growing (88). Although some studies have linked SCFAs with obesity — hypothesizing that the SCFAs can contribute to additional calories through fermentation, causing an imbalance in energy regulation and thus contributing to obesity (82,98,99) — according to Chakraborti (88) and Morrison and Preston (100), this hypothesis is not supported by the evidence that high fibre diets, which normally cause an increase in SCFA production, are able to

protect against weight gain. Moreover, according to Chakraborti (88), Canfora et al. (101) and Morrison and Preston (100), SCFAs, especially butyrate and propionate, are considered predominantly anti-obesogenic metabolites. In agreement, Brahe et al. (102) suggested that reduced levels of butyrate produced by the gut bacteria might be associated with metabolic risk in humans.

Butyrate has been found to improve insulin sensitivity (103), to increase leptin gene expression, thus regulating food intake and body weight (104) and to possess obesity-related anti-inflammatory action by regulation of tight junction gene expression, increasing, consequently, intestinal barrier function (102,103). Propionic acid also has a favourable effect on leptin gene expression reducing the intake of food and regulating weight gain (105,106). Additionally, propionate is able to reduce cholesterol synthesis (104). Although acetate serves as a substrate for synthesis of cholesterol and has an influence on the synthesis of lipids in liver (107), it has been demonstrated that acetic acid also has the ability to inhibit weight gain (36,106).

In face of all these cited factors, it is clear that the composition of and metabolites produced by gut microbiota play a role in obesity and metabolic diseases, although some mechanisms have not been fully elucidated. In this way, the key for positive microbial changes is certainly the consumption of an adequate diet.

1.5. By-products of tropical fruits/pectin and their effects on gut microbiota

Although Brazil is considered the largest producer of tropical fruits in the world (108), 30-40% of agro industrial residues are generated during the production of juices and pulps, which can contribute to the increase of organic waste, causing serious environmental problems, as well as economic losses for the industry (109,110).

Studies have shown that, in addition to diverse nutrients, tropical fruits have a high content of fibre and other bioactive compounds, which are mainly concentrated on the peel and seeds (111,112). Several bioactive compounds, such as polyphenols and some types of fibres, have the ability to modulate the composition or activity of the gut microbiota (113). Studies highlight acerola, camu-camu and other Brazilian tropical fruits as potent functional foods because of their high polyphenol content and antioxidant capacity (114,115). According to Tuohy et al. (116), both fibre and polyphenols present in the peel and seeds can be metabolized by the gut microbiota, resulting in an increase in SCFA. The hydrolysis of polyphenols by colonic microbiota can increase bioavailability, and possibly the biological activity of polyphenols that reach the colon region. According to Tuohy et al. (116), the intestinal microbiota has extensive hydrolytic activity and the ability to break down many complex polyphenols into smaller phenolic acids, which can be absorbed across the intestinal mucosa, thus increasing the production of SCFA. The same occurs

with fibres. According to Shen et al. (117), a population with a higher intake of dietary fibre tends to have higher concentrations of SCFA in faeces.

The pectins, also found in by-products of tropical fruit, are soluble dietary fibres that arrive intact in the colon, where they are fermented by the intestinal bacteria and may have prebiotic properties. In addition, pectin exerts a physiological effect on the gastrointestinal tract, such as delaying gastric emptying and reducing glucose absorption (118). According to Min et al. (119), certain pectins reveal great prospects in the development of new functional products. Several studies have shown that different pectins are able to modulate the gut microbiota, increasing the abundance of beneficial bacteria like *Lactobacillus* and members of *Ruminococacceae* family, as well as the production of SCFA and improvement of the gut barrier function (120–122). According to Wicker and Kim (123) and Gómez et al. (121), some pectins have the potential to stimulate specifics groups of bacteria, such as *Faecalibacterium prausnitzii* and *Roseburia intestinalis,* considered butyric acid producers. Moreover, according to Larsen et al. (124), pectins are able to protect different probiotic bacteria through the gastro-intestinal transit.

2. Study of intestinal microbial community using *in vitro* models

The action of probiotics and prebiotics as well as other components in the intestine can be evaluated by *in vivo* or *in vitro* models. *In vivo* models can be studied using human volunteers or animal models. Although human interventions are the best way to accurately study the ingestion and food

effects, several barriers such as high cost, technical difficulty and ethical standard restrictions exist. Moreover, to study the duodenum and colon microbiota in humans, invasive techniques are usually necessary, and, therefore, the majority of studies end up using only faecal samples to study the microbiota (125,126).

Because of these and other limiting factors, the most common *in vivo* models available are animal models. The first decision to be made in an animal study is to choose the type of animal, whether it is small or large. Small animal models include rodents, such as rats, mice and rabbits, and large animal models include dogs, goats, sheep, pigs and horses. The choice will depend on the target of study (127). Animal models, however, also present some limitations, such as delay in obtaining results and difficulty in finding an optimal dose for different species. Moreover, although many scientists try very hard to make comparisons between animal models and humans, the results obtained in animal models cannot be always extrapolated to humans because of wide physiological differences. In addition, animal safety and well-being requirements will consequently limit some types of experiments (125,128).

The *in vitro* models allow simplifying the reality and are able to separately study the metabolism of the native and added microbiota, in the presence of specific substrates (129). Moreover, they can faithfully simulate the small intestine and the different compartments of the colon beyond being cheaper, more flexible and having controlled parameters. Another positive aspect of these kinds of models is that there are no ethical aspects to consider (125,126).

The *in vitro* models include the static and dynamic systems. In the static models, the samples are maintained immobile during the entire experimental period while in the dynamic models, there is movement seeking to imitate the changing conditions of the human gastrointestinal tract. The static models are usually used to study the enzymatic and chemical conditions of digestibility and for intestinal absorption studies. Within the absorption studies, the human adenocarcinoma cells (Caco-2) and the mucus-producing goblet cell (HT-29) are the most popular and used static models (130,131). The dynamic models are usually used to study the gastrointestinal tract. These models (semi-continuous and continuous system) are suitable for studies of different intestinal functions (132,133).

The *in vitro* fermentation models represent a technological and innovative platform that allows the investigation of both the existence of intestinal microbial species and their respective functionalities and metabolite production. *In vitro* fermentation models are characterized by the inoculation of a single or multiple reactors with faecal material and operating under retention time, temperature, and pH similar to those found in humans and under anaerobic conditions (134,135). The most common and complex dynamic models reported in the literature are the TNO Gastro-Intestinal Model (TIM-1 and TIM-2), Engineered Stomach and Small Intestine (ESIN), The dynamic Gastrointestinal Digester (DIDGI), the *In vitro* Digestive System (IViDiS) and the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) (126). All these cited models present controlled pH, anaerobic conditions and are computer-controlled. None of them are added with

microbiota in the small intestine. Each of them has, however, their own peculiarity, which will differentiate them, as shown in Table 4. These differences will determine the most appropriate model for the type of study.

Table 4. Differences between dynamic *in vitro* models available on the market

Model Name	Year	Simulated compartments	Intestine absorption simulation	Mean goals of the models	Authors
TIM	1990	- Stomach - Duodenum - Jejunum - Ileum	Yes	- Widely used to study food digestion and the availability of nutrients for absorption - Nowadays used within the pharmaceutical industry for release and bioaccessibility	(136)
SHIME	1993	- Stomach - Duodenum - Ascending colon - Transverse colon - Descending colon	No	- Widely used to study food digestion and to evaluate their impact on the intestinal microbial community composition and metabolites.	(137)
IviDiS	2005	- Stomach -Upper duodenum - Lower duodenum	Yes	- Used to assess the survival of probiotic bacteria in different food matrixes	(138)
DiDGI	2014	- Stomach - Duodenum - Ileum	No	- Used to determine the impact of food structures on nutrient bioavailability	(139)
ESIN	2016	- Meal supply - Salivary ampule - Stomach - Duodenum - Jejunum - Ileum	Yes	- Created for testing dissolution, swelling and erosion of pharmaceutical formulations	(140)

The dynamic intestinal models, such as the SHIME® model, have been used to investigate the metabolism and ecology of the intestinal microbiota, with emphasis on probiotics and prebiotics (141–143). The SHIME® model has a flexible setup, enabling the simulation of the gastrointestinal tract of different age groups as well as a variety of animals (144,145). In this study, the Simulator of Human Intestinal Microbial Ecosystem (SHIME®) was the model

used to analyse the effects of determined probiotic and food components on a healthy-weight and obesity-related microbiota.

3. Methods of intestinal microbial community characterization

The human gut microbiota houses a complex and abundant ecosystem where many interactions among the microbial community exist having both local and systemic impacts. This way, the characterization of the gut microbiota composition of health and unhealthy populations is essential to understand the influence of the microbiota on various gastrointestinal and nutritional diseases (3,146).

Bacteriological culture-based techniques were the unique means available to characterize the gut microbiota until the 1990s (146). This technique has, however, some drawbacks like the fact that it can distort the composition of the natural community due to the selective growth of some organisms. This technique is also limited since about 60% to 80% of gut microorganisms simply cannot be cultured by conventional *in vitro* techniques because the optimal growth conditions for these bacteria have not been discovered (146,147).

The more recent studies have enhanced understanding of the gut microbiota and its interaction with the host health by means of culture-independent techniques. These techniques are based on bacterial ribosomal RNA sequence analysis, in particular the 16S rRNA. 16S rRNA is an universal molecule present in all bacteria and has highly conserved domains flanking hypervariable sequences which permit the distinction among bacterial groups

(148). Molecular analyses can demonstrate the gut microbiota richness and diversity, qualitative and quantitative information about bacterial genera and species and changes in the microbiota composition and function due to certain diseases. Moreover, they are fast and have high throughput (146). Some examples of these techniques are quantitative polymerase chain reaction (qPCR), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescence *in situ* hybridization (FISH) and next-generation sequencing of the 16S rRNA gene or its amplicons. In this present study, the DGGE and next-generation sequencing of the 16S rRNA were used.

The DGGE technique is based on the separation of short-to medium-length DNA fragments of differing nucleotide sequences by means of a decreased electrophoretic mobility of partially melted double-stranded DNA amplicons in a polyacrylamide gel containing a linear gradient of DNA denaturants (149), as represented in Figure 6.

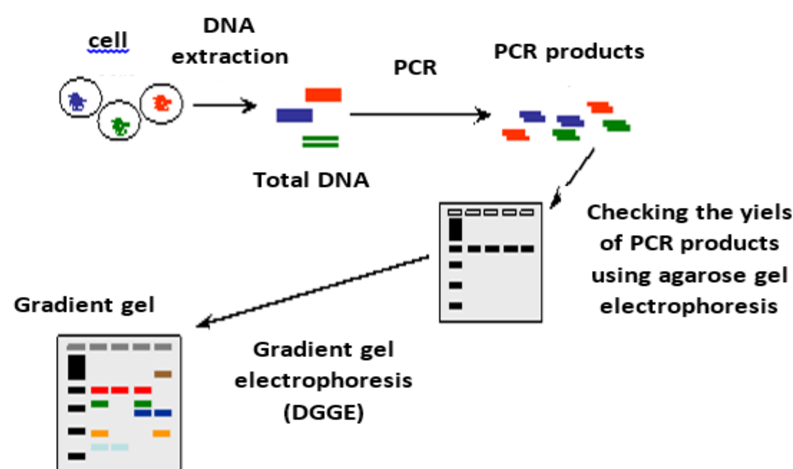


Figure 6. Denaturing gradient electrophoresis (DGGE) scheme.

Sourse: adapted from http://wiki.biomine.skelleftea.se/biomine/molecular/index_11.htm.

Initially, the DNA fragments will move according to their molecular weight alone, but with the increased exposure to denaturant, the DNA strands will begin to denature. The migration will halt when the fragments are almost fully denatured. As different microorganisms have different DNA sequences, the migration will halt at different positions in the denaturing gradient, which will result in separation of the segments and 'band' formation (146).

Next-generation sequencing, or high-throughput sequencing, is the term used to describe modern sequencing technologies including Illumina sequencing. It is described as massively parallel sequencing because a huge number of DNA templates can be sequenced at the same time and in the same reaction set-up. It can phylogenetically characterize the microbiota components and quantify the relative proportions of organisms present (146). The principle of sequencing is: the DNA or cDNA (from RNA) fragments are loaded onto a glass flow-cell containing specific adapters for DNA fragments to attach to. PCR is carried out to amplify each read in the Cluster Station. Many copies of the same read are separated into single strands to be sequenced. With the help of polymerase, fluorescence-labelled nucleotides are incorporated onto the strand. The 3′OH bonds of the nucleotides are inactivated to avoid incorporation of more than one base at one cycle. Images are taken to identify the corresponding base after the addition of each nucleotide. The fluorescent is then removed and the 3′-end is unblocked to enable the next incorporation. The process is repeated, adding one nucleotide at a time and imaging in between. Computers are then used to detect the base

at each site in each image and these are used to construct a sequence (150).

A brief outline of the sequencing process can be found in Figure 7.

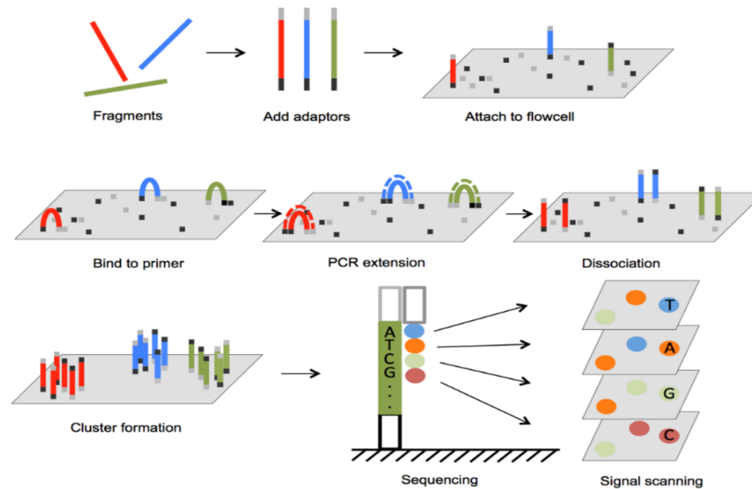


Figure 7. Illumina Sequencing Platform- Outline of Illumina genome analyser sequencing process. Adaptors are annealed to the ends of sequence fragments. Fragments bind to primer-loaded flow cell and bridge PCR reactions amplify each bound fragment to produce clusters of fragments. During each sequencing cycle, one fluorophore attached nucleotide is added to the growing strands. Laser excites the fluorophores in all the fragments that are being sequenced and an optic scanner collects the signals from each fragment cluster. Then, the sequencing terminator is removed and the next sequencing cycle starts.

Source: http://www.3402bioinformaticsgroup.com/service/.

Aims of the study

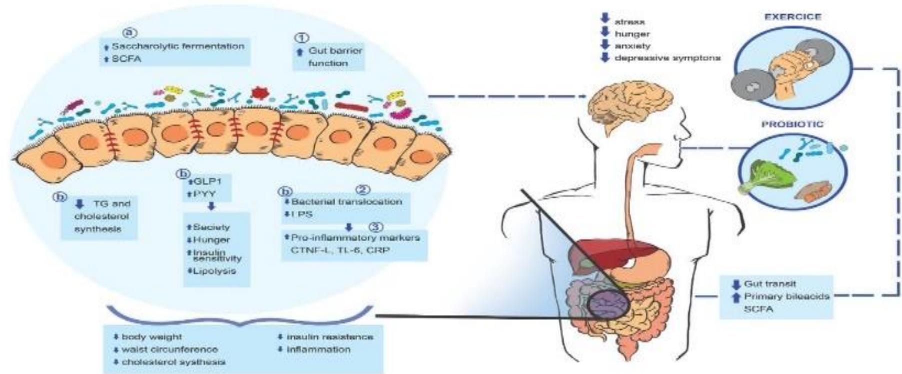
General aim

To evaluate the effects of lemon pectin, camu-camu and acerola by-products as well as of different probiotic strains on gut microbiota from healthy-weight and obese individuals using the Simulator of the Human Intestinal Microbial Ecosystem

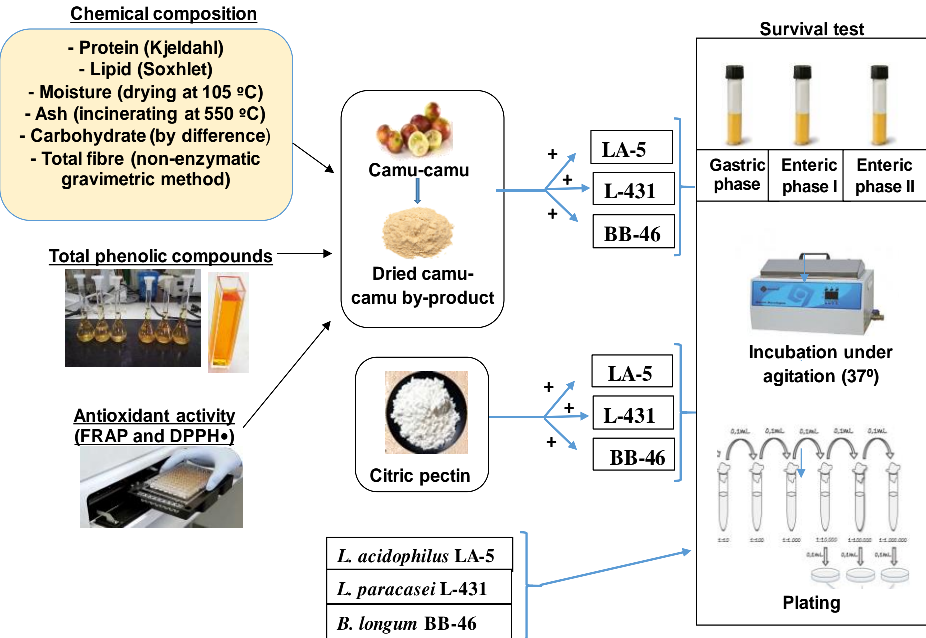
Specific aims

- To evaluate the chemical composition, total phenolic contents and the *in vitro* antioxidant capacity of the acerola and camu-camu by-products;
- To evaluate the survival of *L. acidophilus* LA-5, *L. paracasei* L-431 and *B. longum* BB-46 in combination with a citric pectin and two dried by-products of tropical fruits (acerola and camu-camu) using an *in vitro* gastrointestinal model, and to select the combinations with the best probiotic survival results;
- To evaluate the action of the selected combinations on the composition and activity of a healthy-weight related microbial population, and to define the best combination using the Simulator of Human Intestinal Microbial Ecosystem;
- To evaluate the action of the selected combination on the composition and activity of an obesity-related microbiota using the SHIME® model;
- To evaluate the microbiota composition during treatment with the selected combination (obese and healthy-weight individuals) by 16S rRNA gene sequencing using the Illumina NextSeq® Platform.

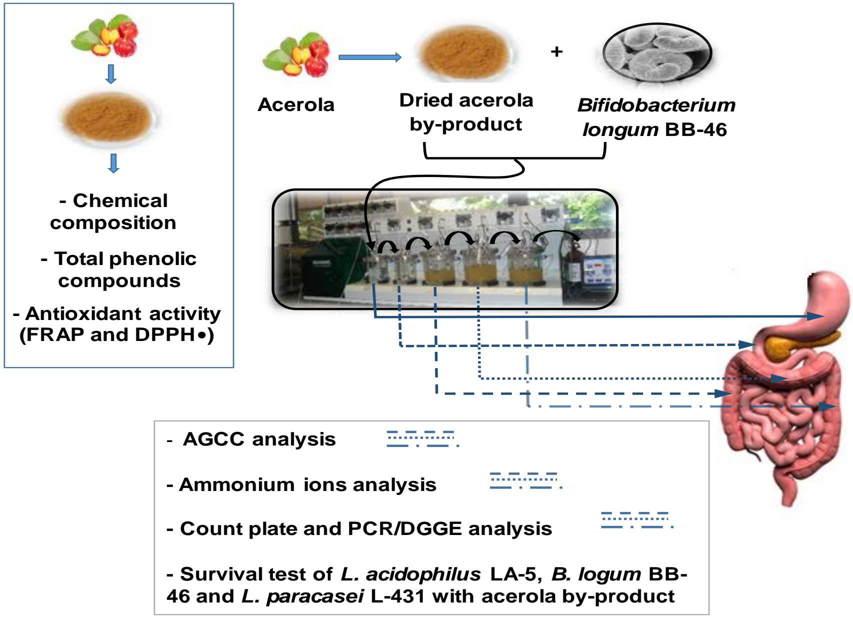
CHAPTER 1



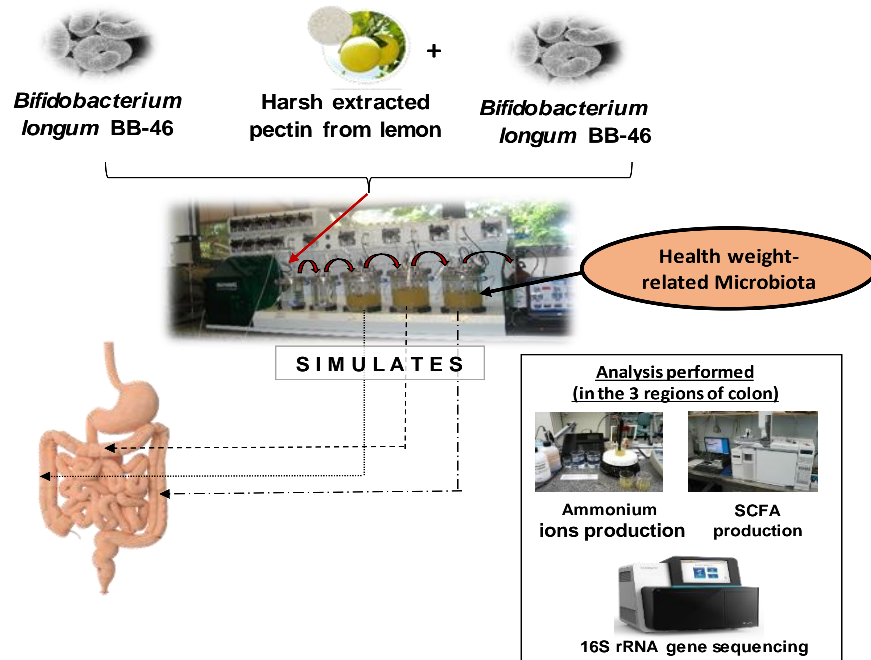
CHAPTER 2 and 3

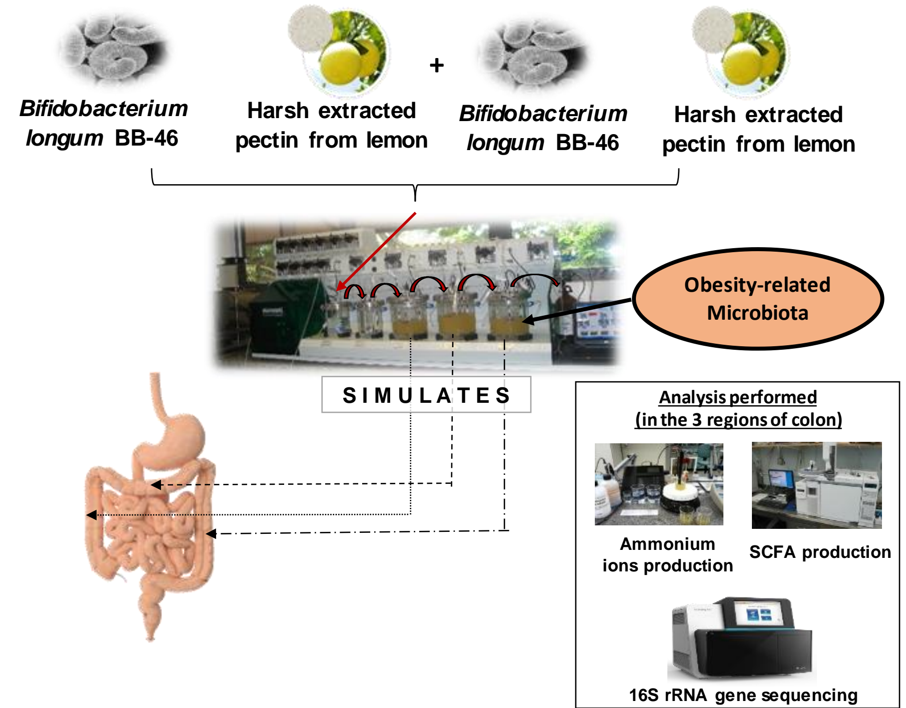


CHAPTER 4



Summary of the main objectives of chapters





Summary of the main objectives of chapters (Cont.)

Chapter 1.

Gut microbiome approaches to treat obesity in humans

Mini-review article published in the Journal “Applied Microbiology and Biotechnology”

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Gut microbiome approaches to treat obesity in humans

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Keywords gut microbiota • obesity • management of obesity

Abstract

The rising worldwide prevalence of obesity has become a major concern having many implications for the public health and the economy. It is well-known that many factors such as lifestyle, increased intake of foods high in fat and sugar and a host's genetic profile can lead to obesity. Besides these factors, recent studies have pointed to the gut microbiota composition as being responsible for the development of obesity. Since then, many efforts have been made to understand the link between the gut microbiota composition and obesity, as well as the role of food ingredients, such as pro and prebiotics, in the modulation of the gut microbiota. Studies involving the gut microbiota composition of obese individuals are however still controversial, making it difficult to treat obesity. In this sense, this mini-review deals with obesity and the relationship with gut microbiota, summarizing the principal findings on gut microbiome approaches for treating obesity in humans.

Introduction

Obesity is defined as "*abnormal or excessive fat accumulation that may impair health"* and is characterized by a Body Mass Index (BMI) above or equal to 30 kg/m^2 (Obesity level 1: BMI = 30-34.9; obesity level 2: BMI = 35-39.9 and obesity level 3: BMI > 40 kg/m^2) (World Health Organiazation 2018). According to the World Health Organization (WHO 2018), the worldwide prevalence of obesity practically tripled between 1975 and 2016. The European Association for the Study of Obesity (EASO 2013) and the WHO (2018) showed that overweight and obesity cause approximately 2.8 million deaths per year and are, therefore, considered one of the most important health problems we face.

The increased BMI of obese individuals raises the risk of hypertension, diabetes type 2, dyslipidaemia, cardiovascular diseases (mainly heart disease and stroke), obstructive sleep apnoea, musculoskeletal disorders (especially osteoarthritis), psychological problems, and some types of cancers, such as endometrial, breast, ovarian, prostate, liver, gallbladder, kidney, and colon cancer (Banack and Kaufman 2014; WHO 2018).

To address all the health problems caused by obesity, significant economic resources are required. According to Dobbs et al. (2014), the global economic impact of obesity was around 2.8% of the global gross domestic product (GDP) in 2014. Obesity has a direct and indirect economic burden on the individual, their family and nation (Dee et al. 2014). Direct economic expenses include the medical costs for treating obesity and its related diseases, while the indirect costs encompass problems related to lost productivity and foregone economic growth as a result of lost work days, physical limitations, lower productivity at work and low life expectancy (Dee et al. 2014).

Lifestyle, socioeconomic status, family influence and cultural aspects are some of the elements involved in the obesity aetiology (Dahiya et al. 2017; WHO 2018). Gut microbiota composition and diet have also been pointed out as being key to effective weight gain and, therefore, have been the target of several studies involving obesity (Ley et al. 2006; Bomhof and Reimer 2015; Bianchi et al. 2018).

Although many studies can be found exploring the composition of obesity-related microbiota compared to that in lean individuals, as well as potential strategies to modulate microbiota and treat obesity, the results, especially in terms of microbiota composition, are still controversial. Moreover, some mechanisms involving the different strategies concerning the modulation of gut microbiota are yet to be fully elucidated. This mini-review deals with obesity and the relationship with gut microbiota, summarizing the principal findings on obesity-related microbiota composition and reviewing new strategies for gut microbiota modulation in favour of obesity treatment.

Gut microbiota composition and obesity

Differences between the gut microbiota composition of lean and obese individuals have recently been observed, suggesting a relationship between obesity and microbiota composition (Bomhof and Reimer 2015; Kasai et al. 2015; Peters et al. 2018). Although the majority of studies have found a higher proportion of *Firmicutes* phylum and a lower amount of *Bacteroidetes* in obese microbiota when compared to the lean (Ley et al. 2006; Bervoets et al. 2013; Kasai et al. 2015; Koliada et al. 2017), considerable debate regarding the significance of *Firmicutes* and *Bacteroidetes* proportions in obesity and lean microbiota has taken place. According to Ley et al. (2006), when obese individuals lose weight after a one-year low-calorie diet, the proportion of *Firmicutes* decreases, while the *Bacteroidetes* proportion increases, more closely resembling the *Firmicutes/Bacteroidetes* proportions of lean individuals. However, Duncan et al. (2008), as well as Ismail et al. (2011) and Tims et al. (2013), found that weight loss did not change the relative proportions of *Bacteroidetes* or *Firmicutes* present in the human gut. Accordingly, Hu et al. (2015) studied the faecal samples from lean and obese Korean adolescents and observed no significant differences in the proportion of *Bacteroidetes* and *Firmicutes*. Schwiertz et al. (2010) studied the faecal microbiota of lean and obese volunteers and, in contrast to the studies cited above, observed that proportions of *Firmicutes* were greater in lean than in overweight and obese volunteers. These findings suggest that the

Firmicutes/*Bacteroidetes* ratio can change depending on the obese population under study and, therefore, a more detailed study of the intestinal microbiome, covering bacterial families, genera and species, is required for a better understanding of the relationship between obesity and the gut microbiota.

Another alternative for studying the relationship between gut microbiota composition and obesity, aside from the *Firmicutes*/*Bacteroidetes* ratio, is the classification of individuals into different enterotypes. Many recent studies have suggested the stratification of individuals according to two microbial enterotypes, the *Prevotella*: P-type or *Bacteroides*: B-type (Vieira-Silva et al. 2016; Christensen et al. 2018; Hjorth et al. 2018). The *Bacteroides* enterotype is reported to be predominant in individuals consuming more protein and animal fat (Western diet), whereas the *Prevotella* enterotype is predominant in subjects consuming more carbohydrates and fibres (Christensen et al. 2018). Thus, the enterotypes are characterized by biomarkers with digestive functions under specific dietary substrates. Consequently, the enterotype potentially affects the individual's ability to lose weight when following a specific diet (Vieira-Silva et al. 2016). The elegant study, conducted by Hjorth et al. (2018), evaluated the influence of an *ad libitum* New Nordic Diet (NND) high in fibre/whole grains on losing body fat of two enterotypes groups, with high and low *Prevotella*/*Bacteroides* ratios (*P*/*B* ratio). They found that subjects with a high *P/B* ratio seem to be more susceptible to losing body fat on diets high in fibre and whole grains than subjects with a low *P/B* ratio.

The microbiota composition is key to understanding the metabolic processes leading to obesity. Thus, some studies have focussed on demonstrating which specific bacterial family, genera or species may be linked to obesity. Peters et al. (2018) found an increased abundance of *Streptococcaceae* and *Lactobacillaceae* families, and a decreased abundance of *Christensenellaceae*, *Clostridiaceae*, and *Dehalobacteriaceae* in the microbiota of obese American adults. In addition, Ottosson et al. (2018), as well as Ravussin et al. (2012), found a positive correlation between body mass index, body weight gain and members of the *Lachnospiraceae* family.

In terms of genera, Cani et al. (2008) observed that when mice are treated with a high fat diet (HFD) during 4 weeks, a reduction in the abundance of the genera *Lactobacillus, Bifidobacterium* and *Bacteroides-Prevotella* can be observed when compared to mice fed with a standard diet. In accordance with these results, regarding *Bifidobacterium* spp., Reyes et al. (2016) and Ignacio et al. (2016) observed that *Bifidobacterium* is more abundant in lean subjects when compared to overweight groups. Accordingly, Aoki et al. (2017) found a positive correlation between *Bifidobacterium*, acetate levels and colonic glucagon-like peptide-1 (GLP-1, a peptide hormone able to enhance the secretion of insulin) and a negative correlation between *Bifidobacterium* and visceral fat.

Still, regarding bacterial genera and obesity, Li et al. (2012) and Nakayama et al. (2017) showed that, besides anti-inflammatory effects, the genus *Succinivibrio* has a negative correlation with total fat intake. Other bacterial genera were also linked with the obesity condition. Cui et al. (2017), for example, found a negative correlation between the bacterial genera *Hungatella*, *Oscillibacter*, *Odoribacter* and obesity genotypes and a positive correlation between the genera *Isosphaera* and *Methylotenera* and obesity genotypes. Lye et al. (2017) showed that the genus *Oscillospira* is negatively correlated with faecal cholesterol concentration. Regarding *Lactobacillus* genus, different studies have found anti-obesity effects (Arora et al. 2013; Kim et al. 2017).

In terms of bacterial species, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* were found to be negatively correlated with obesity (Louis et al. 2010; Schneeberger et al. 2015; Dao et al. 2016; Hippe et al. 2016; Remely et al. 2016), while *Bacteroides fragilis* was positively related with the pathology (Ignacio et al. 2016). *F. prausnitzii* was found in high abundance in healthy adults and, together with other members of *Ruminococcaceae* family, are considered butyric acid producers and consequently beneficial for health (Louis et al. 2010). Inconsistencies in the results were, however, found in different studies regarding obese human subjects and *F. prausnitzii*. Balamurugan et al. (2010), for example, found an increased population of the

referred specie in obese subjects, while Dao et al. (2016) and Hippe et al. (2016) found the opposite and Feng et al. (2014) non-significant results in their findings. Regarding *A. muciniphila,* only a negative correlation between this specie and obesity has been found, showing weight-lowering effects under its administration (Schneeberger et al. 2015; Dao et al. 2016; Remely et al. 2016).

Evidence that some dietary ingredients, especially probiotics and prebiotics, can modulate the human gut microbiota in a positive way has been growing, stimulating new studies focused on pre/probiotics and the management of obesity (Reyes et al. 2016). Besides diet, other strategies such as physical activity have been reported as a positive factor able to alter the microbiota and reduce obesity symptoms (Liu et al. 2015). Transplantation of faecal microbiota has also been an option to alter the gut microbiota and treat obesity (Dao and Clément 2017).

Strategies for modulating microbiota and treating obesity

The use of prebiotics, probiotics and synbiotics for the management of obesity

Probiotics are considered '*live microorganisms that, when administered in regular and adequate amounts, can confer beneficial effects to the host*' (Hill et al. 2014), while prebiotic is '*a substrate that is selectively utilized by host microorganisms conferring a health benefit'* (Gibson et al. 2017). When probiotics and prebiotics are administered together, the resulting food or supplement is considered a synbiotic product (Ohshima et al. 2016). Many studies have suggested prebiotics and probiotics, and their combination, as good options for the management of obesity as they can positively alter the gut microbiota in a safe and effective way (Reyes et al. 2016; Dahiya et al. 2017).

Different prebiotics, such as oligofructose, inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), arabinoxylan and resistant starch, have been reported to have a beneficial impact on obesity (Burokas et al. 2017; Nicolucci et al. 2017; Slizewska et al. 2017; Chen et al. 2018a; Singh et al.

2018). Although in most of these studies mentioned the prebiotic has a positive effect on the serum lipids, body weight, serum inflammatory markers — as for example interleukin-6 (IL-6) and lipopolysaccharides (LPS) (Table 1) — it is clear that both the metabolic effects and the impact on the microbiota composition will depend on the prebiotic type and daily dose, as proposed by Singh et al. (2018). The majority of the studies cited in Table 1 have shown, for example, an increase of the *Bifidobacterium* genus after prebiotic carbohydrate-based ingestion. However, Burokas et al. (2017) found a decrease of this genus after treatment with GOS, FOS, and a combination of FOS and GOS. Interestingly, Burokas et al. (2017) have also shown a link between the gut microbiota, prebiotics and anxiolytic and antidepressant-like effects, demonstrating the function of the gut-brain axis (GBA). GBA consists of bidirectional communication between the enteric nervous and the central system, linking cognitive and emotional centres of the brain with peripheral intestinal functions (Carabotti et al. 2015).

Different pectins have also shown diverse positive effects on obesity parameters (Table 1). In most of the studies listed in Table 1, beyond microbiota modulation, pectin stimulates short-chain fatty acid production, especially butyric and propionic acids. However, it is important to highlight that not all pectins can be classified as prebiotic. To be classified as prebiotic, the substrate needs to be broken down and selectively utilized by the gut microorganisms, conferring a health benefit to the host (Gibson et al., 2017). Thus, many studies have been focused on proving the prebiotic effects of different pectins, as shown in Table 1.

As described in the new definition of prebiotics reported by Gibson et al. (2017), besides carbohydrate-based substances, other substances such as polyphenols and polyunsaturated fatty acids can be considered as prebiotic, since their beneficial effects on health can be proven. In this sense, recent studies have shown the positive effects of different polyphenols as well as of polyunsaturated fatty acids on gut microbiota modulation (Table 2). Although the genera *Eubacterium*, *Roseburia* and *Faecalibacterium* have been reported as beneficial bacteria — able to produce butyric acid, which has beneficial

effects on the obesity symptoms (Louis et al. 2010; Ríos-Covián et al. 2016) — Zhang et al. (2018) have found a reduction of these genera during treatment with green tea polyphenols (Table 2). This reduction, however, did not affect the ability of the polyphenol to reduce the parameters positively related to obesity.

Different probiotic strains have also shown a beneficial impact on obesity (Table 3), especially strains of *Lactobacillus* spp. and *Bifidobacterium* spp. Besides gut microbiota modulation, many of the different probiotics mentioned in Table 3 have reported a reduction in plasma lipids and in pro-inflammatory genes, as well as an increase in SCFA production. However, it is possible to observe that both specific microbiota modulation and metabolic effects depend on the bacterial strain.

In addition, several studies have shown that a mix of different probiotics strains as well as combinations of probiotics and prebiotics have a better impact on human health compared to when used individually (Bianchi et al. 2014; Nyangale et al. 2014; Alard et al. 2016; Kim et al. 2017).

The possible relationship between prebiotics, probiotics, gut microbiota composition and reduction of obesity symptoms can be observed in Fig. 1. According to O'Connor et al. (2017), prebiotics can modulate the gut microbiota, which, by means of cascade metabolic processes, can culminate in improvement of lipid profiles (LDL, TG, HDL), reduction of blood pressure, improvement of glucose homeostasis, and reduction in inflammation, as observed in Fig. 1. Figure 1 also shows that modifications in lipid profiles, blood pressure, glucose homeostasis and inflammation processes can happen due to the action of different probiotics or the combination of pro and prebiotics. The prebiotic can directly stimulate the probiotic, which will have an impact on gut microbiota composition and/or on gut barrier function. The prebiotic can also indirectly stimulate the probiotic activity by means of cross-feeding between specific bacterial groups, stimulated by the prebiotic, and the probiotic strain (Ríos-Covián et al. 2016; Moens et al. 2017).

Physical activity and gut microbiota modulation for the management of obesity

It is well known that physical exercise has a positive impact on human health, helping to prevent excessive weight gain and triggering a cascade of events leading to the metabolic balance of the human body, preventing or treating several diseases such as diabetes, cancer, metabolic syndrome and heart disease (Pedersen and Saltin 2015; Weiss et al. 2017). Apart from the already known beneficial factors provided by exercise, several recent studies have reported that exercise has an impact on the composition and on the metabolic activity of the gut microbiota (Petriz et al. 2014; Costa et al. 2017; Monda et al. 2017; Chen et al. 2018b; Codella et al. 2018; Denou et al. 2018). According to Petriz et al. (2014), Monda et al. (2017) and Codella et al. (2018), physical exercise is able to improve the microbiota diversity, to stimulate different bacteria capable of improving the barrier function as well as the mucosal immunity, and to produce SCFAs under different nutritional contexts, therefore offering a therapeutic approach for obesity and other metabolic diseases.

According to the literature, the type of physical activity will have a different impact on the gut microbiota. Moderate-intensity exercise has been reported to reduce the gut transit time and increase the microbiota diversity (Evans et al., 2014, Campbell et al., 2016), while prolonged strenuous exercise can increase gut permeability resulting in bacterial translocation from the colon, diarrhoea and gastrointestinal bleeding and metabolic disorders (Martin 2011). In addition, exercise associated with extreme food restriction (anorexia) can decrease beneficial bacteria and increase the chance of gut mucosal barrier disorders (Queipo-Ortuño et al. 2013).

Some studies have analysed the impact of exercise on gut microbiota composition and metabolites. Matsumoto et al. (2008) were pioneers in this regard. They showed that voluntary physical exercise can stimulate butyrate-producing bacteria species and, consequently, SCFA production (n-butyrate) in the caecum of rats. Other, more recent animal and human studies have reported an increase in butyrate-producing bacteria and SCFA production, as well as in gut bacterial diversity after physical exercise (Evans et al. 2014;

Campbell et al. 2016; Estaki et al. 2016). The bacterial species *Akkermansia muciniphila* and/or *F. prausnitzii,* both considered important for intestinal health, were also related to the increased gut microbiota diversity of athletes (Clarke et al. 2014), of exercising rats (Kang et al. 2014), and of women regularly performing the minimum dose of exercise recommended by the WHO (Bressa et al. 2017).

Although the majority of studies report an increase of *Bacteroides* and a decrease of *Firmicutes* as a result of physical exercise (Evans et al. 2014; Mika et al. 2015; Denou et al. 2018), some studies have found the inverse (Clarke et al. 2014; Kang et al. 2014; Lambert et al. 2015), highlighting once again the controversy regarding the proportions of *Firmicutes* and *Bacteroidetes* and their impact on health and metabolic diseases.

Even though the mechanisms behind exercise and positive gut modulation remain undetermined (Codella et al. 2018), some hypotheses have been proposed and can be viewed in Fig. 1. Increases of short-chain fatty acid production and consequent alteration of intestinal pH, as well as reduction of gut transit time and increased excretion of primary bile acids (reported to have anti-microbial activity) are some of the suggested factors by which exercise may affect gut microbiota composition (Evans et al. 2014; Costa et al. 2017; Monda et al. 2017; Codella et al. 2018).

SCFA, especially butyric acid, have several beneficial effects on human health. They can, for example, improve gut integrity by increasing tight junction gene expression (Mika et al. 2015), improve glucose and lipid metabolism, control anorectic hormones, resulting in weight loss (Canfora et al. 2015; O´Connor et al. 2017) and induce mucin synthesis, which may serve as substrate for different gut bacteria, including *Akkermansia muciniphila* (Lewis et al. 2010; Johansson et al. 2011). Moreover, there is evidence that SCFAs produced by gut microorganisms can activate vagal afferents' receptors of the enteric nervous system, which will carry information to the Central Nervous System (Forsythe et al. 2014). In addition to this finding, it was recently postulated that exercise plays a role in the brain-gut-muscle axis, which affects the gut microbiota in a cycle-feedback (Codella et al. 2018). According to

Codella et al. (2018), as exercise is considered a stress, it can be considered as the first impulse in this cycle-feedback.

Faecal transplantation as a strategy for the management of obesity

Faecal microbiota transplantation (FMT), a procedure involving the transfer of faecal microbiota from healthy individuals to the intestinal tract of a recipient, is attracting increasing attention as a possible treatment for obesity and related diseases (Jayasinghe et al. 2016).

A large body of evidence demonstrates that FMT is highly effective in the treatment of recurrent *Clostridium difficile* infection (Carlucci et al. 2016; Cammarota et al. 2017; Zhou et al. 2017), and is a good model to study the causality of intestinal microbiota and metabolic disorders (Dao and Clément 2017). Nevertheless, the therapeutic potential of FMT-based intervention for obesity remains unclear.

Studies in animal models have demonstrated that FMT can modify animal phenotypes, including adiposity or metabolic complications. Ridaura et al. (2013) and Zhang et al. (2015) found that mice transplanted with faecal microbiota from obese animals showed significant increases in total body mass, adiposity, and obesity-associated metabolic phenotypes compared to mice receiving intestinal microbiota from lean animals. In addition, both studies reported that the caeca's concentrations of SCFAs, butyric acid and iso-butyric acid were significantly lower in the obese group, resulting in changes of intestinal microbiota activity. In accordance with the research cited above, several other studies evaluating the impact of FMT on obesity-related gut microbiota in animal models showed that FMT is able to modulate gut microbiota and to promote changes in host metabolism-related disorders, such as insulin resistance and non-alcoholic steatohepatitis (Bäckhed et al. 2004; Turnbaugh et al. 2006; Shafquat et al. 2014; Zhou et al. 2017). These studies also suggested FMT as having clinical applications through targeted manipulation of the intestinal microbiota.

In humans, a limited number of studies evaluating the efficacy of FMT for the treatment of obesity can be found. Vrieze et al. (2012), in a pioneering

work, conducted a pilot study to evaluate the transplantation of faecal microbiota from lean and healthy donors in patients with metabolic syndrome. The authors observed a significant increase in insulin sensitivity at 6 weeks post-FMT. This result was associated with changes in gut microbial diversity, including an increase in butyrate-producing bacteria, particularly *Roseburia intestinalis* and *Eubacterium hallii*. The authors attributed the observed therapeutic effect to the increased production of butyrate by the intestinal bacteria. A more recent study evaluated both the short- and long-term effect of FMT from lean donors in a group of obese individuals with metabolic syndrome (Kootte et al. 2017). The authors reported that alterations in the composition of the intestinal microbiota by FMT from lean and healthy donors had a short-term beneficial effect (6 weeks) on insulin sensitivity of obese patients with metabolic syndrome. On the other hand, no metabolic changes were observed at 18 weeks after FMT (long-term). Although the overall metabolic effects of FMT were modest and showed wide variability among patients, the authors concluded that changes in plasma metabolites, as a consequence of the altered composition of the intestinal microbiota, may be an explanation for the beneficial effects of FMT on insulin sensitivity (Kootte et al. 2017).

The clinical trials performed by Vrieze et al. (2012) and Kootte et al. (2017) suggest that FMT may be a therapy for metabolic syndrome due to the short-term benefits changing the composition of intestinal microbiota and insulin sensitivity. Moreover, according to different animal studies (Bäckhed et al. 2004; Ley et al. 2006; Ridaura et al. 2013; Zhang et al. 2015), FMT may offer good proof for a conceptual approach to study the relationship between microbiota and obesity. Although FMT can be considered a promising treatment modality for obesity, it is important to highlight that most of the studies performed to date are experimental and require greater standardization of procedure (Carlucci et al. 2016; Bouter et al. 2017). In addition, further clinical trials are needed to confirm the long-term results, efficacy, and safety of FMT in the treatment of obesity and related diseases.

Conclusions and future perspectives

This review showed that the composition of the microbiota is essential for understanding the mechanisms involved in the aetiology of obesity. There are several obesity control strategies, such as consumption of probiotics and prebiotics, as well as moderate and regular physical activity, which have a beneficial impact on the gut microbiota. Faecal microbiota transplantation is also an option, but it seems to be a more invasive strategy. Although the researches have greatly advanced in recent years, controversy regarding obese microbiota composition and the mechanisms involved still exists, and thus new studies concerning this important thematic are of great importance. In this way, in the not too distant future, a new area of nutritional science, known as personalized nutrition, is expected to emerge with the advances in the knowledge of the microbiota composition. Finally, the gut microbiota is key to modern disease management.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The article does not contain any studies with human participants or animals performed by any of the authors.

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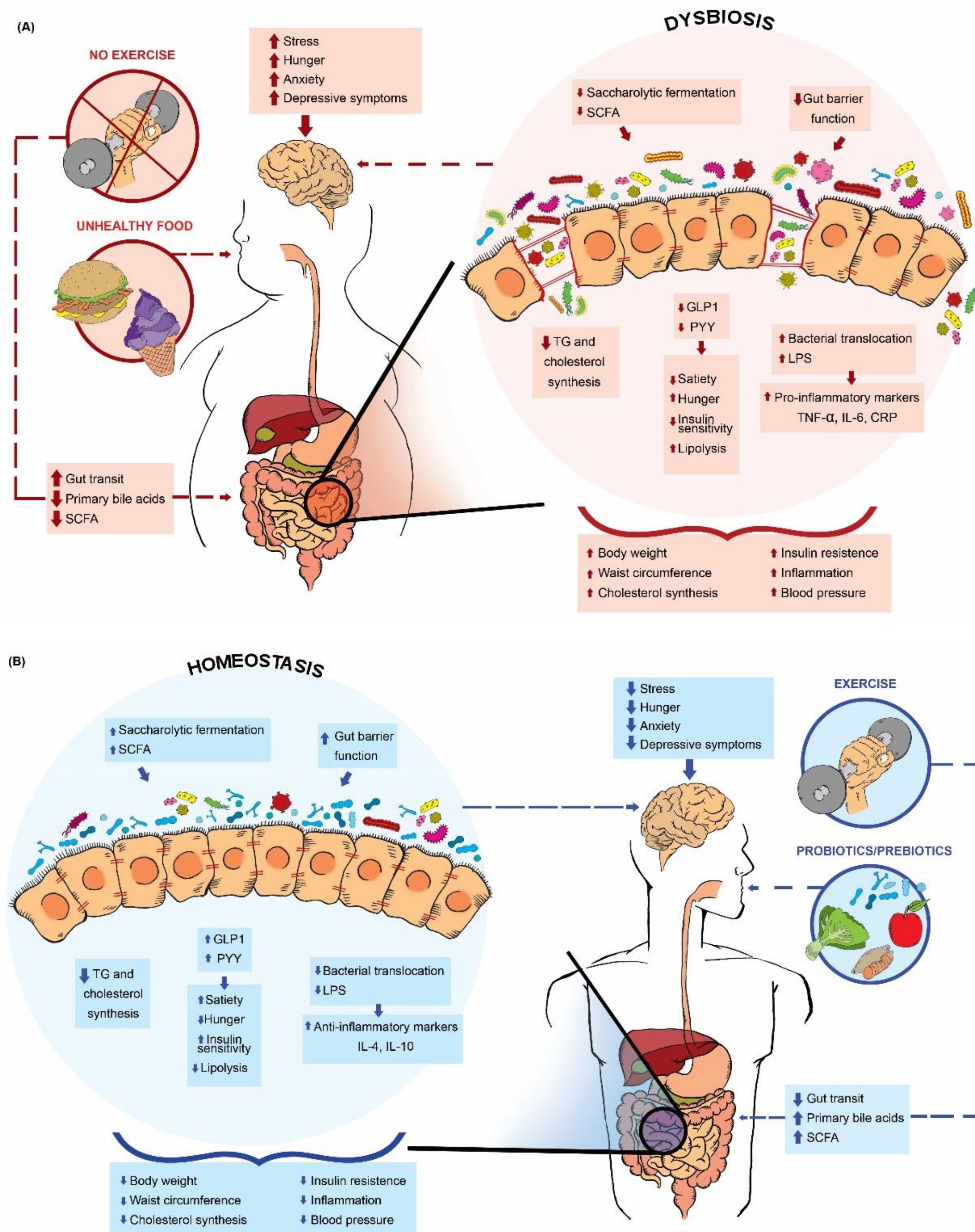

Fig. 1 The gut microbiota in homeostasis and dysbiosis promoted by the intensive cross-talk orchestrated by diet, practice or not of physical exercise, gut microbiota and gut-brain axis and the consequent impact on obesity development or prevention. (A) Impact of high-fat and sugar diet and absence of exercise on gut microbiota composition and consequent effects on obesity parameters; (B) Impact of prebiotics, probiotics and physical exercise on gut microbiota modulation and consequent effects on obesity prevention. The intake of pre and probiotics can positively modulate the gut microbiota, resulting in increased production of saccharolytic fermentation, short chain fatty acid (SCFA) and improvement of gut barrier function. The increase on SCFA is implicated in the release of anorectic gut hormones, the peptide YY (PYY) and glucagon-like peptide-1 (GLP-1), which have important impact on satiety, hunger, insulin sensitivity and lipolysis. SCFA can also act inhibiting triglycerides (TG) and cholesterol synthesis, as well as improving the gut barrier function. Increased gut barrier function may reduce the bacteria translocation and blood LPS, with consequent reduction of pro-inflammatory markers (e.g., interleukin-6 (IL-6), tumour necrosis factor (TNF) and C-reactive protein (CPR)) and increase of anti-inflammatory markers (e.g., interleukin-4 (IL4), interleukin-10 (IL-10)). Physical exercise is suggested to modulate the gut microbiota by means of increased gut transit and SCFA and reduction of primary bile acids. The metabolites/hormones produced by the microbiota modulated by exercise, pro and prebiotics have possible impact on brain function, decreasing hunger, stress, anxiety and depressive symptoms, which can consequently affect some obesity parameters, such as body weight and waist circumference. On the other hand, the consumption of foods high in fat and sugar as well as lack of exercise may provide reverse effects to those cited.

Table 1 Effects of prebiotics or candidate to prebiotics (carbohydrate-based) on obesity parameters.

Prebiotic/ prebiotic candidate	Subject	Dose/duration	Main findings after treatment with prebiotic/ prebiotic candidate	Authors
Oligofructose-enriched inulin	Overweight or obese 7–12 years old children.	8 g/day of oligofructose-enriched inulin once daily for 16 weeks	Compared to placebo: ↓ percent body fat ↓ body weight ↓ percent trunk fat ↓ level of interleukin 6 ↓ serum triglycerides ↑ *Bifidobacterium* spp. ↓ *Bacteroides vulgatus* ↓ *Faecalibacterium prausnitzii* and *Roseburia* spp.	Nicolucci et al. (2017)
Inulin	Male Sprague-Dawley rats randomized to six high-fat diet groups with different inulin concentration.	High-fat diets with 0% inulin, 2.5% inulin, 10% inulin, 25% inulin and 25% cellulose for 3 weeks	Compared to high-fat diets with 0%: ↓ body weight (inulin dose dependently) ↓ caloric intake and respiratory quotient (inulin dose dependent) ↑ glucose tolerance (inulin dose dependent) ↑ plasma peptide YY (inulin dose dependent) ↑ glucagon-like peptide-1 concentrations (inulin dose dependent) ↑ *Bacteroidetes* and *Bifidobacterium* spp. (inulin dose dependent) ↓ *Clostridium clusters* I and IV (inulin dose dependent)	Singh et al. (2018)
FOS+GOS, FOS, GOS	Male C57BL/6J mice fed with FOS, GOS, or a combination of FOS and GOS	0.3–0.4 g/mouse/day during 4 to 10 weeks	- No effect on body weight ↑ *Ruminococcaceae* members (by GOS) ↑ *Verrucomicrobiaceae* (by FOS+GOS) ↑ *Bacteroides* and *Parabacteroides* (by FOS+GOS, FOS /GOS) ↑ *Akkermansia* (by FOS+GOS) ↓ *Bifidobacterium, Ruminococcus, Desulfovibrio* (by FOS+GOS, FOS and GOS) ↓ *Lactobacillus* (by FOS+GOS, GOS) ↓ stress-induced hyperthermia (by FOS+GOS) ↓ stress-induced defecation (by FOS+GOS, GOS) ↓ pro-inflammatory cytokine levels (by GOS+FOS) ↓ L-tryptophan levels in plasma (by GOS+FOS) ↓ depression-like and anxiety-like behaviour (by GOS+FOS) ↑ serotonin levels in the prefrontal cortex (by GOS+FOS, FOS) ↑ propionate (by GOS+FOS, GOS) ↑ *acetate* (by GOS+FOS, FOS) ↓ *Actinobacteria*/*Proteobacteria* ratio after stress. Opposite ratio after treatment with FOS+GOS	Burokas et al. (2017)
Bamboo-shaving polysaccharide - BSP (arabinoxylan)	Male C57BL/6J mice with diet induced obesity	BSP at 200 mg/kg body weight (BW) and 400 mg/kg BW for 8 weeks	Compared to a High fat died group (control), BSP: ↓ TNF-α, IL-6 and IL-1β (400 mg/kg) ↑ intestinal barrier integrity ↑ insulin resistance ↓ weight and cell size of adipose tissue (400 mg/kg) ↓ LPS ↓ TC, T-CHO, LDLC serum (400 mg/kg) ↓ *Firmicutes* to *Bacteroidetes* ratio ↓ *Enterobacter* ↑ *Akkermansia muciniphila* (400 mg/kg) ↑ *Lactobacillus* (400 mg/kg)	Chen e al. (2018a)

Table 1 (Continued)

Prebiotic/ prebiotic candidate	Subject	Dose/duration	Main findings after treatment with prebiotic/ prebiotic candidate	Authors
Potato starch dextrin (PD)	Male Wistar rats with high-fat diet induced obesity	Diets with 5% PD for 12 weeks	↓ feed consumption compared to control (high fat diet group) ↑ *Bacteroidetes and Actinobacteria strains* ↓ *Firmicutes* strains ↑ *Bifidobacterium, Prevotella*, and *Bacteroides* strains compared to control (high fat diet) ↑ *SFCA,* especially propionate	Slizewska et al. (2017)
Apple-Derived Pectin	Male Sprague-Dawley rats with high-fat diet (HFD) induced obesity	HFD supplemented with pectin (5% wt/wt) for 4 weeks	Compared to *HFD group (Control), the pectin:* ↓ *weight gain* ↓ *serum total cholesterol level* ↑ *claudin 1 expression* ↓ *decreased Toll-like receptor 4 expression in ileal tissue* ↓ TNFa, IL-6 and metabolic endotoxemia	Jiang et al. (2016)
Citrus pectin	Male C57BL/6J mice divided into 2 groups: normal diet group and citrus pectin group.	*Normal diet added with 15% (w/w) (0.6 g/day) of citrus pectin for 4 weeks*	↓ weight gain ↓ epididymal adipose tissue weight ↑ glucose tolerance ↓ fasting levels of glycose ↑ propionic acid ↑ *Bacteroidetes*	Shtrike et al. (2017)
LM pectin from lemon	In vitro fermentation of faecal samples from human obese volunteers.	8g of pectin/day for 1 week. 1 week-control period without pectin	↑ *Ruminococaaceae* members ↓ *Lachnospiraceae* family ↑ *Succinivibrio, Holdemanella* and *Alteromonadaceae* ↓ *Clostridium, Bacteroides* and *Streptococcus* ↑ short chain fatty acids (SCFA), especially butyric acid ↓ intestinal ammonium ions	Bianchi et al. (2018)
-Pectic oligosaccharides from sugar beet pulp (SBPOS) -Pectic oligosaccharides from lemon peel wastes (LPOS) -Pectin from lemon peel wastes (LPW) -Pectin from sugar beet pulp (SBP) -Fructooligosaccharides (FOS)	In vitro fermentation of faecal samples from adult volunteers.	1% (w/v) of substrates fermented at 0, 5, 10, 18, 24, 32 and 48 h	↑ bifidobacteria and lactobacilli in LPOS, SBPOS and FOS ↑ *Faecalibacterium* and *Roseburia* in all the substrates, especially in LPOS fermentation after 24h ↑ increscent SCFA/time in all the substrates, especially in LPOS and SBPOS fermentation after 48h.	Gómez et al. (2018)

Table 2 Effects of prebiotics or candidate to prebiotics (non carbohydrate-based) on obesity parameters.

Prebiotic/ prebiotic candidate	Subject	Dose and Duration	Main findings after treatment with prebiotic/ prebiotic candidate	Authors
Grape polyphenols (GP)	Male C57BL/6J mice with diet–induced metabolic syndrome fed with grape polyphenols.	26.2 ± 1.7 mg polyphenols per day for 13 weeks	Relative to vehicle controls, GP: ↓ body weight gain ↓ adiposity ↓ serum inflammatory markers (tumour necrosis factor (TNFa), interleukin (IL-6), and lipopolysaccharide (LPS)) ↓ gene for glucose absorption (Glut2) ↑ occludin intestinal expression ↑ proglucagon intestinal expression ↑ bacterial specie *Akkermansia muciniphila* ↑ genus *Alistipes* ↓ several taxa within the order *Clostridiales* ↓ *Firmicutes* to *Bacteroidetes* ratio	Roopchand et al. (2015)
Blueberry polyphenol extract (PPE)	Male C57BL/6J mice with high-fat diet induced obesity. High-fat diet supplement with Orlistat = positive control	200 mg/kg body weight (bw)/day (equivalent to 16 mg/kg/day of PPE for adults) for 12 weeks	↓ body weight gain *compared to positive control* ↑ genera *Bifidobacterium, Desulfovibrio*, *Adlercreutzia,* and *Flexispira* compared to *positive control* ↓ genera *Adlercreutzia* and *Prevotella* compared to *positive control*	Jiao et al. (2018)
Green tea polyphenols (GTP)	Male C57BL/6J mice with high-fat diet induced obesity. High fat diet group (HFD) without GTP = control	High fat diet with tea polyphenols 0.1% (w/w) for 8 weeks	↓ body weight gain *compared to HFD group* ↓ liver weight gain *compared to HFD group* Alleviation of HFD-induced inflammation ↑ richness and diversity of faecal microbiota ↑ genera *Prevotella, Bacteroides, Megamonas* and *Sutterella* ↓ genera *Eubacterium, Clostridium, Roseburia, Faecalibacterium, Coprococcus, Ruminococcus* and *Phascolarctobacterium* ↓ *Firmicutes* to *Bacteroidetes* ratio	Zhang et al. (2018)
Omega-3 fatty acids	Males ICR mice with high-fat diet induced obesity. High fat diet group (HFD) without oil = control	HFD with: 600 μg/g fish oil; 600 μg/g krill oil; 600 μg/g mixture of fish oil and krill oil (1:1); 600 μg/g mixture of fish oil and krill oil (2:1); 600 μg/g mixture of fish oil and krill oil (2:1); 600 μg/g mixture of fish oil and krill oil (3:1) for 12 weeks	↑ body weight ↓ liver index, total cholesterol, triglyceride and low-density lipoprotein cholesterol compared to HFD group ↓ *Firmicutes*/*Bacteroidetes* ratio compared to HFD group ↓ *Hungatella, Oscillibacter, Odoribacter.* Negative correlation between these genera and obesity phenotypes ↑ *Isosphaera* and *Methylotenera.* Positive correlation between these genera and obesity phenotypes	Cui et al. (2017)

Table 3. Effects of different probiotic strains on obesity parameters.

Probiotic	Subject	Dose and duration	Main findings after treatment with probiotic	Authors
-*Lactobacillus fermentum* FTDC 8312 -*Lactobacillus fermentum* JCM 1173 (type strain control)	Males BALB/c mice with hypercholesterolemia induction consuming probiotic strain and high fat diet (HFD). Control consuming phosphate buffer saline (PBS) and HFD	HFD + 400 µL PBS containing *L. fermentum* FTDC 8312 (10^9 CFU/mL) or *Lactobacillus fermentum* JCM 1173 (10^9 CFU/mL) for 7 weeks	Comparing to control and type strain control, FTDC 8312: ↓ serum total cholesterol levels ↓ serum low-density lipoprotein cholesterol ↓ apoB100:apoA1 ↑ high-density lipoprotein cholesterol (HDL-C) levels ↑ faecal TC, TG, and total bile acid levels - Serum triglyceride levels of mice fed with FTDC 8312 and JCM 1173 comparable to control - No effects on body weight ↑ *Akkermansia, Oscillospira, Desulfovibrio* and *Lactobacillus* by FTDC 8312 and only *Lactobacillus* by JCM 1173 - negative correlation between faecal cholesterol concentration and *Oscillospira*	Lye et al. (2017)
-*Lactobacillus plantarum* HAC01 -*L. rhamnosus* GG (strain reference)	Males C57BL/6J fed with HFD and probiotics or phosphate buffer saline and HFD	1 × 10^8 CFU viable cells of *L. plantarum* HAC01 and *L. rhamnosus* GG suspended in 20 µL of PBS for 8 weeks	↓ mesenteric adipose depot ↓ body weight gain (HACO1 and GG) compared to the control group (HFD). ↓ *Ruminococcaceae* (HACO1) and increase of this family by GG compared to the control group (HFD). ↓ *Bacteroides* (HACO1)	Park et al. (2017)
-*Bifidobacterium animalis* ssp. *lactis* GCL2505 (BlaG) -*B. longum* ssp. *longum* JCM1217T (BloJ)	Males C57BL/6J mice with diet-induced-obesity. Control consuming HFD and normal standard diet	BlaG and BloJ strains (1 × 10^9 FCU/mL) for 7 weeks	Compared to HFD group: - No effect on weight gain and energy intake (by BlaG and BloJ) ↓ visceral fat accumulation and glucose tolerance (by BlaG) ↑ *Bifidobacterium* and *Lactobacillus* genera (by BlaG) ↓ *Clostridium* genus (by BlaG and BloJ) ↑ levels of propionate, acetate and glucagon-like peptide-1 (by BlaG) - negative correlation between caecal acetate and visceral fat and positive correlation between caecal acetate and plasma GLP-1 levels - positive correlation between *Bifidobacterium* and acetate levels and colonic GLP-1 levels and negative correlation between *Bifidobacterium* and visceral fat	Aoki et al. (2017)
-*Lactobacillus rhamnosus* LMG S-28148 -*Bifidobacterium animalis* subsp. *lactis* LMG P-28149 -Mix of both strains	Males C57BL/6J mice fed with low fat diet (LFD), LFD + probiotic Mix, High fat diet (HFD), HFD + probiotic Mix.	~10^9 CFU of each strain for 7 weeks	Compared to HFD group: - *B. animalis* subsp. *lactis* LMG P-28149 reduced body weight, epididymal adipose tissue (EWAT) and blood as efficiently as the probiotic mix. *L.rhamnosus* LMG S-28148 did not yield such effects. - both strains decreased the expression levels of Cd11c, Cd11b, F4/80 and Cd68 in the EWAT - only the mix and *B. animalis* increased the *A. muciniphila*, compared with HFD-fed mice ↑ butyrate and propionate production by the probiotic mix	Alard et al. (2016)
Kefir (a natural complex probiotic composed by more than 50 species of microorganisms, including lactic acid bacteria, acetic acid bacteria, and yeast)	Males C57BL/6 mice with diet-induced-obesity divided in two groups: the kefir group and control group	0.2 ml of kefir milk, twice daily for 12 weeks	Compared to control group: ↓ body weight, histopathological liver lesion score ↓ total-cholesterol - upregulation of genes related to fatty acid oxidation, (PPARα and AOX) ↓ plasma concentration of IL-6 ↑ *Lactobacillus*, *Lactococcus*, and *Candida* - no alterations of *Firmicutes- Bacteroidetes* ratio - negative correlations between the abundance of *Lactobacillus, Lactococcus*, and *Candida* and final body weight, epididymal adipose tissue weight, and plasma concentration of total cholesterol.	Kim et al. (2017)

Chapter 2.

Camu-camu (*Myrciaria dubia*) by-product characterization and impact on the gastrointestinal survival of different probiotic strains

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Camu-camu (*Myrciaria dubia*) by-product characterization and its impact on the gastrointestinal survival of different probiotic strains

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Abstract: *Backgound*: The survival during the gastrointestinal tract transit is essential for the success of a probiotic product. For this reason, studies have been evaluated the positive/negative impact of several food ingredients on the survival of different probiotic strains, as well as its impact on the nutritional quality of food products.

Objective: This study aimed to evaluate the chemical composition, the total phenolic compounds and the *in vitro* antioxidant capacity of a dried camu-camu by-product and to verify the effects of this by-product on the survival rate of different probiotic strains during *in vitro* simulated gastrointestinal conditions.

Methods: Contents of protein, lipid, moisture, ash, carbohydrate and total fibre were measured. The total phenolic compounds were determined by Folin-

Ciocalteu reagent and, the *in vitro* antioxidant activity by Ferric Reducing Ability of Plasma (FRAP) and sequestration of radical 2.2-difenyl-1-picrylhydrazyl (DPPH•). An *in vitro* gastrointestinal essay was used to evaluate the probiotic strains survival.

Results: The camu-camu by-product showed an excellent chemical composition, with high content of fibre and protein and reduced lipids. Moreover, it showed to be an excellent source of total phenolic compounds with high *in vitro* antioxidant activity. The high content of total phenolic compounds seemed, however, to be harmful for the survival of all studied strains during the *in vitro* gastrointestinal test.

Conclusion: Although the camu-camu by-product might be useful for the development of new food products, increasing its nutritional value, it can be disadvantageous for the probiotic survival during the gastrointestinal passage. More studies are welcome to evaluate the effects camu-camu by-product on different *Lactobacillus*/*Bifidobacterium* strains.

Keywords: Probiotics, camu-camu, by-product, phenolic compounds, antioxidant activity, *in vitro* gastrointestinal survival

1. INTRODUCTION

Probiotics are considered live microorganisms that when regularly administered in adequate amounts, is able to confer beneficial effects to the host health [1,2]. The most commonly microorganisms used as probiotics belong to the bacterial genera *Lactobacillus* and *Bifidobacterium* as well as to some yeasts, such as *Saccharomyces cerevisiae* [2–4]. The benefits provided by probiotics are strain-dependent and include the stimulation of commensals bacteria and inhibition of pathogens; alleviation of certain types of intolerances, such as lactose intolerance; enhancement of nutrients bioavailability; anticarcinogenic and hypocholesterolemic effects; stimulation of the immune

system; reduction of allergic manifestations and diseases associated with the gastrointestinal tract [5–8]. Strains of *Lactobacillus acidophilus*, *L. paracasei* and *Bifidobacterium longum* have been demostrated several beneficial effects on human health. Specific strains of *Lactobacillus acidophilus*, for example, showed to be able to reduce abdominal pain or discomfort in patients with irritable bowel syndrome [9] and to reduce in insulin resistance [10]. *L paracasei* strains were reported to treat ulcerative colitis and allergic rhinitis [11,12] and, *B. longum*, to improve the intestinal environment and defecation frequency [13] as well as to provide anti-allergy effects [14,15].

For the probiotic exerts its specific actions in the intestine, it must however, be capable of surviving the stomach acidic environment, the bile salts and the substrates of the small intestine, as well as having success in the competition with other intestinal microorganisms [16]. In this sense, many efforts have been made in order to find specific food components capable of increasing the probiotic survival under the gastrointestinal conditions. Among these components, the use of prebiotics and other fibres has been highlighted for their positive effect on the probiotic survival [17–19].

Prebiotic is defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” [20]. Inulin, resistant starch, fructooligosaccharides (FOS), galactooligosaccharides (GOS), some pectins, xylooligosaccharides, arabinoxylan, glucooligosaccharides, human milk oligosaccharides (HMOs), and arabinogalactan are some examples of prebiotics [21]. Other non-carbohydrate substances, such as polyphenols and polyunsaturated fatty acids can also be included as prebiotics [20].

There is a constant search to find new candidates for prebiotic ingredients. In this way, the camu-camu (*Myrciaria dubia*), a small Amazonian fruit with high vitamin C content and high *in vitro* antioxidant activity, might meet this purpose [22]. Besides being rich in many nutrients, this tropical fruit has been reported to have high contents of dietary fibres such as resistant starch and pectin and also of phenolic compounds, which are mostly concentrated in their skins and seeds, representing 40 % of the fruit weight [23,24]. Due to its nutritional composition and high contents of fibre and

phenolic compounds, by-products of tropical fruits (composed by skins, seeds, and pulp residues) can be an option for the improvement of probiotic survival under the adverse gastrointestinal conditions. Moreover, the by-products, usually discarded by food industries during processing of juices, can be useful on improving the nutritional value of food products [25]. As such, this study aimed to evaluate the chemical composition, the total phenolic compounds, the *in vitro* antioxidant capacity of the dried camu-camu by-product and to verify the effects of this by-product on the survival rate of different probiotic strains during simulated gastrointestinal conditions.

2. MATERIALS AND METHODS

2.1. Camu-camu (*Myrciaria dubia*) by-product obtaining and drying

The camu-camu by-product, consisted of peels, residual skins and of seeds (34.4 g and 65.6 g/100 g of by-produt, respectively), was provided by a fruit processing industry of Amazonas, Brazil. The industrial by-products were kept frozen until the drying process [24]. The previously thawed camu-camu by-products were firstly spreaded on a perforated tray and than the by-products were submitted to hot air drying process at 50 °C and air speed of 4 m/s (HAD50) [24]. Informations concerning the hot dryer equipment were previously described by Azevêdo *et al.* [26].

2.2. Chemical composition determination of camu-camu by-product

The protein, as well as the lipid, moisture and ash contents present in the camu-camu by-product were determined by micro-Kjeldahl method, Soxhlet method and by the methods of drying in an oven at 105 ºC and incinerating at 550 ºC, respectively. Non-enzymatic gravimetric method was used to obtain the total fibre content. All methods were in accordance with the official methods of analysis of the Association Official Analytical Chemist [27]. The carbohydrate content was calculated by the difference.

2.3. Phenolic compounds extraction

The phenolic compounds of the dried by-product were extracted in triplicates with 1 g of the by-product and 20 mL of aqueous methanol (methanol: water, 70: 30 v/v) for one hour at 25 ºC, using a magnetic stirrer. Next, they were centrifuged at 10000 rpm/15 min. at 10 ºC. Afterwards, the supernatant was kept and the pellets re-extracted with 20 mL of aqueous methanol (methanol: water, 70: 30 v/v) and mixed in an ultra turrax (Polytron-Kinematica GnbH- Luzers, 3 times for 1 min at speed 4). Finally, the solution of camu-camu by-product was again centrifuged at 10000 rpm for 15 min at 10 ºC, the supernatants added to the previous kept supernatant and the volume of each one completed to 50 mL with aqueous methanol (methanol: water, 70: 30 v/v) [28].

2.4. Total phenolic content and Antioxidant activity by FRAP (Ferric Reducing Ability of Plasma) and DPPH• (sequestration of radical 2.2-difenyl-1-picrylhydrazyl)

The total phenolic contents were analyzed according to the methodology proposed by Singleton *et al*. [29] with some modification [30]. Aliquots of 0.25 mL from the diluted extracts (extract:methanol dilution of 1:40) were mixed with 2 mL distilled water and 0.25 mL Folin-Ciocalteu reagent during 3 minutes at room temperature. Afterwards, 0.25 mL of a saturated sodium carbonate solution (Na_2CO_3) was added to the obtained solution and kept at 37 °C in a water bath for 30 minutes. The absorbance were measured through an Ultrospec 2000 spectrophotometer model (Amersham Biosciences, Cambridge, U.K.) at 750 nm. The result was expressed in gallic acid equivalent (GAE) in mg/g of sample on a dry basis.

The *in vitro* antioxidant activity of camu-camu by-product was determined by FRAP and by DPPH•. The Ferric Reducing Ability of Plasma was performed according to Benzie and Strain [31]. A volume of 20 uL of the diluted extracts (extract:methanol dilution of 1:100) were mixed with 150 uL of the FRAP reagent (sodium acetate buffer /TPTZ solution /HCl solution) and added to a 96 wells polystyrene microplate (Costar, Cambridge, MA). After five-minute

incubation at 37 °C, the absorbance was read at 593 nm with a Benchmark Plus Microplate Spectrophotometer (Synergy ™ H1, Biotek Instruments Inc., Vermont, USA). The results were carried out in quadruplicates and expressed in µmoles of Trolox equivalents (TE) per g of sample on a dry basis. The sequestration of radical 2.2-difenyl-1-picrylhydrazyl (DPPH•) was performed according to Brand-Williams *et al*. [32] with modifications proposed by Duarte-Almeida *et al*. [33]. A volume of 50 uL of the diluted extracts (extract:methanol dilutions of 1:200) were added to 150 uL of DPPH• solution (0.03080 mg/mL) on a microplate spectrophotometer. The absorbance were read at 517 nm after 20 minutes of reaction time, and converted to antioxidant activity percentage terms. The analyses were performed in quadruplicates and the results expressed in µmoles of Trolox equivalent/g of dried sample.

2.5. Survival of *L. paracasei* L-431, *L. acidophilus* LA-5, and *B. longum* BB-46 under *in vitro* simulated gastrointestinal conditions

2.5.1. Bacterial strains

Fresh cultures of the strains *L. acidophilus* LA-5, *L. paracasei* 431, and *B. longum* BB-46 were provided by Christian Hansen (Hoersholm, Denmark) and maintained at -80 ºC in MRS broth with glycerol. Strains of *B. longum* BB-46 were activated in MRS broth supplemented with L-cysteine (0.05%) at 37 °C/24 h under anaerobic conditions, and *L. paracasei* L-431 and *L. acidophilus* LA-5 in MRS broth (Acumedia, Baltimore, USA) at 37 °C/24 h under anaerobic and aerobic conditions, respectively. The strains were further centrifuged at 2600 rpm/10 min at 4°C and washed with saline solution (0.85 % w/v) to be used in the *in vitro* simulated gastrointestinal test.

2.5.2. The in vitro simulated gastrointestinal test

The survival of the probiotic strains (*Lactobacillus acidophilus* LA-5, *Lactobacillus paracasei* L-431, and *Bifidobacterium longum* BB-46) combined

or not with the by-product was evaluated according to the *in vitro* model described by Buriti *et al.* [34] with some modifications.

In this assay, we simulated three different gastrointestinal phases: gastric, enteric I and enteric II. 10 mL of each strain (10^8 CFU/mL in saline solution 0.85 %) along with or without the by-product (1% w/v) were distributed in sterile bottles simulating each phase. To simulate the gastric phase, the pH of aliquots (10 mL) were adjusted to 2.07 - 2.72 with 1M HCl solution (Merck). Next, lipase (Amano Lipase G, Sigma-Aldrich) and pepsin solutions (Pepsin from porcine gastric mucosa, Sigma-Aldrich) were added at sufficient amounts to achieve concentrations of 0.9 mg/L and 3 g/L in the final solution, respectively. Finally, the gastric samples were incubated in a water bath at 37 °C/2 h under constant stirring at 150 rpm. To simulate the enteric phase I, the pH of samples from the gastric phase were adjusted to 4.21 - 5.03 with the addition of a sterile alkaline solution (15% of 1N NaOH + 11.16 g/ L of NaH_2PO_4). In this step, bile (bile from bovine and ovine, Sigma-Aldrich) and pancreatin (Pancreatin from porcine pancreas, Sigma-Aldrich) solutions were added until the final solution reached 10 g/L and 1 g/L, respectively. The samples were than incubated at 37 °C/2 h under constant stirring (150 rpm). The last phase (enteric phase II) was performed by adjusting the pH to 6.12 - 6.97 with the addition of the sterile alkaline solution and by the addition of pancreatin and bile solutions at final solution concentration of 1 g/ L and 10 g/ L, respectively. To finalize the assay, a new incubation was perfomed at 37° C /2 h under constant agitation. Samples were collected after each phase (before the assay (0h), gastric phase (2h), enteric phase I (4), and enteric phase II (6h)) to count the viable cells.

Enumeration of *B. longum* BB-46 during each phase was performed using the Bifido Medium BIM-25 agar (Difco, France) with cultivation at 37 °C/72 h under anaerobic conditions (Anaerogen Anaerobic System, Probac do Brasil), while counts of *L. paracasei* 431 and *L. acidophilus* LA-5 were performed using acidified MRS agar (pH 5.4) with incubation at 37 °C/48 hours (anaerobic conditions) and MRS agar (Himedia, India) with incubation at 37

°C/48 hours (aerobic conditions), respectively [17]. The experiments were repeated twice, each one in triplicates.

2.6. Statistical analysis

The significance of the results was determined using a one-way ANOVA, and individual means were compared by means of Tukey test ($p < 0.05$), employing Biostat 5.0 software (IBM, Brazil).

3. RESULTS AND DISCUSSION

3.1. Chemical composition of the dried camu-camu by-product

The dried camu-camu by-product showed high levels of protein (5.73 ± 0.23 g/100 g) and carbohydrate (85.97 ± 0.12 g/100 g), among which 68.72 ± 0.18 g/100 g corresponded to fibre. Reduced levels of moisture (5.73 ± 0.23 g/100 g), ash (1.10 ± 0.00 g/100 g), and lipid (1.40 ± 0.18 g/100 g) were also found.

The high content of protein as well as the reduced content of lipids found in the camu-camu by-product can be confirmed by comparing the results with other dried by-products of Brazilian tropical fruits. Sousa *et al*. [35], for example, found higher lipid content in the by-products of guava (2.49 g /100 g), cupuaçu (3.69 g /100), acerola (3.59 g/100) and graviola (2.28 g /100 g) and lower contents of protein in the by-products of guava (2.82 g/100 g), cupuaçu (1.65 g/100 g), acerola (1.65 g/100 g) and graviola (1.09 g/100 g) when compared to the results of camu-camu by-product found in this study. Regarding total fibre content, the by-product of camu-camu showed similar results from passion fruit seeds (64.8%) [36] and Persian lime peel (66.7%) [37], but higher values were found in the camu-camu by-product when compared to acerola by-product (56.28%) [38] and mango pulp (44.7 %) [39]. Souza *et al*. [40] characterized a dried by-product of camu-camu and found lower values of fibre (24.6 g/100 g) and higher contents of lipids (2.4 g/100 g) and proteins (7.9 g/100 g) compared to the results found in the present study.

According to Silva *et al*. [41], variations in the chemical composition of fruits are common and may occur due to different geographic locations, cultivation practices, exposure to sunlight, rainfall patterns and fruit's maturation stage.

The chemical composition obtained in this study indicates that the camu-camu by-product may be a good option for increasing the nutritional value of food products, especially due to the high fibre and protein contents.

3.2. Total phenolic compounds and *in vitro* antioxidant activity of the dried camu-camu by-product.

Table 1 shows the total phenolic compounds and the *in vitro* antioxidant activity (by DPPH• scavenging and FRAP) found in the camu-camu by-product.

Several studies have confirmed the presence of phenolic compounds in the camu-camu fruit and in their by-products using Folin-Ciocalteu reagent [42–44]. Our results were similar to that found by Rufino *et al*. [44] (116 mg GAE/g) in the dried camu-camu fruit, but lower than that found by Myoda *et al*. [43] in the dried seeds (467 mg GAE/g) and peels (344 mg GAE/g) of camu-camu. These variations, according to Deng *et al*. [45] and Langley *et al*. [42], can be a consequence of the stage of fruit maturation, type of drying, cultivation practices, climate and extraction method. When compared to other by-products of tropical fruits, the total phenolic contents found in the camu-camu outweighed those found in the acerola by-product (53 mg/g) [38], pineapple (9.1 mg/ g) and passion fruit (41.2 mg/g) [46], as well as in the by-product of cajá-ambu (14.48 mg GAE/g) and jambolão (28.80 mg GAE/g) extracted with ethanol /water (70:30) using Folin's reagent [47]. Accordingly, Langley *et al*. [42] and Rodrigues *et al*. [48] have classified the camu-camu as one of the tropical fruits with higher levels of phenolic compounds.

As expected and showed by DPPH• scavenging and FRAP, the by-product of camu-camu contains constituent(s) with hydrogen donating capacity providing its antioxidant-activitiy. These results are in accordance with other studies evaluating the antioxidant activity of the dried camu-camu [35,40,42,46,49,50]. Moreover, several studies have described camu-camu as

the tropical fruit with the highest antioxidant activity compared to other tropical fruits like guava, pineaple and cupuaçu among others [35,40,42,46,49,50]. Although the acerola by-product has been reported as a by-product with high *in vitro* antioxidant activity (518 umols TE/g of dried sample by DPPH• scavenging and, 785 umols TE/g of dried sample by FRAP) [38], the camu-camu by-product evaluated in this study showed superior values. According to Langley *et al.* [42], the highest proportion of antioxidant components of tropical fruits are present in the seeds and peels and the anthocyanins cyanidin-3-glycosidic and delphinidin-3-glycosidic are the main phenolic compounds present in camu-camu [51].

3.3. Survival of *L. paracasei* L-431, *L. acidophilus LA-5*, and *B. longum* BB-46 in the presence and absence of camu-camu by-product under *in vitro* simulated gastrointestinal conditions

The strains *L. paracasei* L-431, *L. acidophilus* LA-5 and *B. longum* BB-46, in combination or not with the camu-camu by-product, were exposed to an *in vitro* gastrointestinal simulation.

All the studied bacterial strains without the by-product showed low survival results during the gastrointestinal assay. A reduction of 3 to 5 log cycles during the gastric and enteric phases was observed for the different strains (Fig. 1). This result is, however, in accordance with that found in the literature. Hansen *et al*. [52] and Amakiri and Thantsha [53] showed a reduction of 3–4 log and 2.34 log in *B. longum* BB46 and *B longum* LMG 13197, respectively, after 2 h of experiment (gastric phase). Moreover, Amakiri and Thantsha [53] reported a ~5-6 log reduction in *B. longum* after 6 h of experiments (enteric phase II). In addition, Santos et al. [54] showed a reduction in *L. acidophilus* La-5 of 6.2 log cycles during the gastric phase and of 7.4 log cycles during the enteric phase II. Because of the low survival of some bacterial strains under the gastrointestinal conditions, different studies have been conducted aiming to find substances capable of improving the survival of probiotics. In this sense, studies have shown that food containing

inulin fibre [17,55,56] and some polyphenols [38,57] are able to improve the survival rate of different *Lactobacillus* and *Bifidobacterium* strains.

Interestingly, we observed that the dried camu-camu by-product seemed to be harmful for the survival of the three studied probiotic strains (Fig. 1). Several studies have shown that although polyphenols are associated with a number of health benefits, such as prevention of cancer, diabetes, immune disorders and cardiovascular diseases [58,59], these compounds may inhibit the growth of certain microorganisms, including beneficial bacteria, depending on their concentration and the type of polyphenol [60]. The antibacterial mechanism of the polyphenols is still not fully elucidated, however, several mechanisms have been proposed. One of them is that polyphenols may bind to the cell membranes of bacteria causing a disturbance of membrane function and, consequently, the inhibition of cell growth [61]. Another mechanism is that flavonoids possess the ability to form strong complexes with heavy metals like metalloenzyme, present in some bacteria [62]. These complexes can cause metabolic disturbances, such as enzymatic inhibition, deficiency in ion channel function and in the levels of iron in the intestine, affecting, consequently, the most sensitive bacterial populations [63]. In this context, we can suppose that the camu-camu by-product have impaired the survival of the studied bacteria during the gastrointestinal simulation due to its high content of phenolic compounds. On the other hand, Bianchi *et al.* [38] found that the acerola by-product, which was reported to have a high content of phenolic compounds [38], improved the survival of *B. longum* BB-46 and *L. paracasei* L-431 during their passage through the gastric juice. However, the content of phenolic compounds found in the camu-camu by-product in our study was two times higher than that found in the acerola by-product reported by Bianchi *et al*. [38].

CONCLUSION

This study showed that the camu-camu by-product presents an excellent chemical composition, with high amount of fibre, proteins and phenolic compounds. Although the camu-camu have shown high phenolic compounds, and consequently, high *in vitro* antioxidant activity, the high phenolic

compounds seemed to be harmful to the survival of the three studied probiotic strains (*L. paracasei* L-431, *L. acidophilus* LA-5 and *B. longum* BB-46) during the *in vitro* gastrointestinal simulation. More studies are welcome to evaluate the effects of camu-camu by-product on different *Lactobacillus*/*Bifidobacterium* strains.

LIST OF ABBREVIATIONS

FRAP = Ferric Reducing Ability of Plasma

GAE = gallic acid equivalent

TE = Trolox equivalent

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Table 1. Total phenolic content and *in vitro* antioxidant activity of the dried camu-camu by-product

Total phenolic compound (Mean ± SD) (mg GAE/g)	**Antioxidant activity (Mean ± SD)**	
	DPPH• (umols TE/g)	FRAP (umols TE/g)
102.05 ± 2.35	517.97 ± 15.28	785.75 ± 33.79

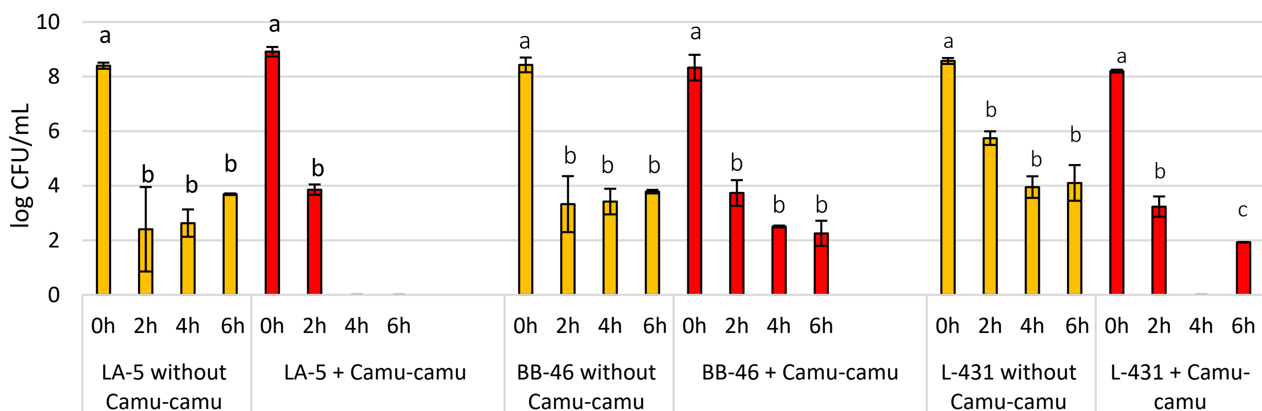

Fig. 1. Population (log CFU/mL) of *L. acidophilus* LA-5 (A) , *B. longum* BB-46 (B) and *L. paracasei* L-431 (C), in combination or not with the camu-camu by-product before the assay (0h) and during gastric phase (2h), enteric phase I (4), and enteric phase II (6h) simulation. Different letters represents statistical difference ($p < 0.05$) between the different phases for the same treatment.

Chapter 3.

Impact of lemon pectin on probiotic strains survival under *in vitro* gastrointestinal conditions

Impact of lemon pectin on probiotic strains survival under *in vitro* gastrointestinal conditions

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Abstract

The aim of this study was to evaluate the impact of a pectin extracted from lemon on the survival rate of different probiotic strains during simulated gastrointestinal conditions. An *in vitro* gastrointestinal assay was used to evaluate the probiotic strains survival during six hours. The probiotic with the best survival results was selected for scanning electron microscopy. The citric pectin showed a good impact on the survival rate of *B. longum* BB-46. The scanning electron microscopy showed the presence of pectin around the cells, providing a possible protective effect during the gastric phase. There was no positive impact of pectin on the survival rate of *L. acidophilus* LA-5 nor on *L. paracasei* L-431. More studies are welcome to evaluate the beneficial effects of pectins on different *Lactobacillus*/*Bifidobacterium* strains.

Keywords: Probiotics; Pectin; *In vitro* gastrointestinal survival

Introduction

Pectins are water-soluble dietary fibres present in the cell walls of plants. They contain predominantly galacturonic acid and have an important contribute to the integrity and rigidity of plant tissue (1,2). The pectins can be classified according to the degree of methylation (DM). If the pectin has less than 50% of the carboxyl groups methylated, it is called low-methylated (LM) pectins, and more than 50%, high-methylated pectins (HM) (3,4).

Pectins can be extracted from fruits by-products such as citrus peel and apple pomace and are mainly used as gelling, thickener and stabilizer agents (2). They are also used as an agglutinated in blood therapy and as filler in pharmaceutical preparation (5). Moreover, pectins have been reported to be able to to reduce serum cholesterol (6), to modulate the intestinal microbiota, culminating in increased production of short chain fatty acids (7,8) and to have anti-carcinogenic effects (9). It was also suggested that pectins can support the growth and improve the survival of probiotic strains in the gastrointestinal tract (10,11). All the effects provided by pectins are, however, dependent on the structure of the pectins (12).

Probiotics are '*live microorganisms that, when administered in regular and adequate amounts, can confer beneficial effects to the host*' (13). The mainly used and studied probiotic strains belong to the *Lactobacillus* and *Bifidobactrium* genera. Probiotics can confer several benefits for human health, which are strain specific, including the improvement of gut barrier function, immunomodulation, prevention or treatment of cancer, diarrhea associated with rotavirus, irritable bowel syndrome, food allergies, obesity and diabetes (14).

Besides resisting the manufacturing and storage process, the microorganism must resist the physico-chemical conditions of the gastrointestinal tract in order to be considered as a probiotic (15). In this sense, the search for food ingredients able to improve the probiotic survival through the gastrointestinal transit is constant and of great importance. Although some studies have evaluated the impact of specific pectins on determined probiotic survival, the effects are dependent on the bacterial strain and pectin struture. As such, this study aimed to evaluate the impact of a citrus LM pectin on the survival rate of different probiotic strains during *in vitro* simulated gastrointestinal conditions.

Materials and methods

Pectin origin and bacterial culture conditions

The pectin (harsh extracted low-methoxyl (LM) pectin from lemon) was provided by CP Kelco (Lille Skensved, DK) in powder form.

Strains of *Bifidobacterium longum* BB-46, *Lactobacillus paracasei* 431 and *L. acidophilus* LA-5 were provided by Christian Hansen (Hoersholm, Denmark) as fresh cultures and maintained at -80 ºC in MRS broth with glycerol. Both the *L. paracasei* L-431 and *L. acidophilus* LA-5 were individually reactivated in MRS broth (Acumedia, Baltimore, USA), while the *Bifidobacterium* BB-46 strain was activated in MRS broth supplemented with L-cysteine (0.05%). All strains were incubated at 37 °C for 24 hours. Afterward, the cells were centrifuged at 4 °C during 10 minutes at 2600 rpm and washed with saline solution (0.85 % w/v).

MRS agar (Himedia, India) with incubation at 37 °C/48 hours under aerobic conditions and, acidified MRS agar (pH 5.4) with incubation at 37 °C/48 hours under anaerobic conditions were the conditions used to count *L. acidophilus* LA-5 and *L. paracasei* 431, respectively. *Bifidobacterium longum* BB-46 enumeration was perfomed using the Bifido Medium BIM-25 agar (Difco, France) with cultivation at 37 °C/72 h under anaerobic conditions (Anaerogen Anaerobic System, Probac do Brasil) (16).

The *in vitro* simulated gastrointestinal test

The impact of the lemon pectin on the survival of *L. acidophilus* LA-5, *L. paracasei* L-431, and *B. longum* BB-46 was evaluated according to the model described by Buriti et al. (17) with some modifications.

The strains (10 mL each, in saline solution 0.85 %) were individualy added to 90 mL of 0.85 % saline solution along with the pectin (1% w/v) and homogenized in a Bag Mixer (Interscience, St Nom). To simulate the stomach region, aliquots of 10 mL (10^8 CFU. mL^{-1}) were transferred to sterile bottles and the pH adjusted to 2.07 - 2.72 with a 1M HCl solution (Merck). Pepsin

(Pepsin from porcine gastric mucosa, Sigma-Aldrich) and lipase solutions (Amano Lipase G, Sigma-Aldrich) were added at sufficient amounts to achieve concentrations of 3 g/L and 0.9 mg/L in the final solution, respectively. The bottles were next incubated in a water bath at 37 °C for two hours under constant stirring at 150 rpm. After this period, the pH was adjusted to 4.21 - 5.03 with the addition of a sterile alkaline solution (15% of 1N NaOH + 11.16 g/ L of NaH_2PO_4) to simulate the enteric phase I. Bile (bile from bovine and ovine, Sigma-Aldrich) and pancreatin (Pancreatin from porcine pancreas, Sigma-Aldrich) solution was added in this step at sufficient concentrations so that the final solution contained 10 g/L and 1 g/L, respectively. The samples were next incubated at 37 °C for more two hours under constant stirring at 150 rpm. To simulate the last phase (enteric phase II), the samples were added with pancreatin and bile solution (keeping the concentration at 1 g/ L and 10 g/ L of the final solution, respectively) and the pH adjusted to 6.12 - 6.97 with the addition of the sterile alkaline solution. A new incubation took place at 37° C for two hours under constant agitation. To count the viable cells, the samples were collected after each phase (control (0h), gastric phase, enteric phase I, and enteric phase II). The experiments were repeated twice, each one in triplicates.

Scanning electron microscopy

The combination with the best probiotic survival was selected for the scanning electron microscopy. Samples of each phase (gastric and enteric phases I and II), as well as of the microorganism (in saline solution at 0.85 % w/v) was used to verify morphological changes of the selected probiotic strain combined with the pectin under simulated gastrointestinal conditions. This part of the study was performed according to the methodology proposed by Matias et al. (18). The samples were centrifuged (6500 rpm/10 min.), the supernatants discarded and the pellet used in the next steps as described by Bianchi et al. (19).

Statistical analysis

The significance of the results was determined using a one-way ANOVA, and individual means were compared through the Tukey test ($p < 0.05$), employing Biostat 5.0 software (IBM, Brazil).

Results and Discussion

The strains *L. paracasei* L-431, *L. acidophilu*s LA-5 and *B. longum* BB-46, in combination or not with the pectin, were exposed to an *in vitro* gastrointestinal simulation. As showed in Figure 1, the LM pectin from lemon was able to improve the survival ($p < 0.05$) of *B. longum* BB-46 during the simulated passage through the stomach and was the combination selected for the scanning electron microscopy.

The process of pectin gelling in the stomach can probable explain the mentioned result in the gastric phase. According to Albuquerque (20), certain pectins can form stable gels in acid conditions. In this study, we could observe the formation of a viscous gel in the gastric phase, which provided a possible "protective effect" to the bacterial cells, explaining the good survival of *B. longum* BB-46 during this phase. In alkaline medium, however, a rapid demethylation and degradation of pectin starts by the breakdown of the β-glycosidic bond (C4) into a carboxylic ester group by the β-elimination mechanism (21), explaining thus the reduction of probiotic survival during the enteric phase (basic pH). The electron microscopy of *B. longum* BB-46 combined with the pectin during the different gastrointestinal phases (Fig. 2) corroborate with this hypothesis. In this figure, we can observe the possible gelling of pectin involving the bacterial cells during the gastric phase (Fig. 2B). On the other hand, we can still visualize some fibres around the cells during the enteric phase II, which we supposed to be the degraded pectin (Fig 2D).

The survival of *L. acidophilus* LA-5 and *L. paracasei* L-431 were not improved by the LM pectin (Fig. 1). According to Klu and Chen (22), the survival of probiotic cells under simulated gastric and enteric conditions depends on the probiotic genera, species and strain variability. Still according

to these authors, *Bifidobacterium* strains usually have higher survival rates than *Lactobacillus* strains. Bedani et al. (23), reported that *L. acidophilus* LA-5 was more sensitive than *Bifidobacterium animalis* Bb-12 to the simulated gastrointestinal conditions.

Larsen et al. (10) studied the effects of different pectins on the survival rate of different *Lactobacillus* (*L. fermentum PCC, L. reuteri RC-14, L. rhamnosus LGG* and *L. paracasei F-19*) and concluded that different pectins, including the studied LM pectin of this study, have the potential to protect different *Lactobacillus* strains through the gastrointestinal transit. Accordingly, Nazzaro et al. (24) showed that pectin was able to improve the survival rate of *Lactobacillus acidophilus* DSM 20079 during stress conditions of stomach and intestine. However, Sen et al. (11) observed that different pectins could not provide any protection for *Lactobacillus acidophilus* MTCC 10307 against the acidic pH of stomach. These findings suggest that the effects provided by pectins is dependent on the bacterial species/strains. Still according to Larsen et al. (10), the protective effect of pectins during the gastrintestinal conditions might be linked to the electrostatic interactions between bacteria and pectins provived by the complex variations of surface charges, which can propably explain the positive and negative observed effects of the pectin on the different bacterial strains in our study.

In this study we also observed that the microorganism *B. longum* BB-46 combined with pectin obtained similar survival results compared to *B. longum* BB-46 without the pectin during the enteric phase II, different from the other probiotic microorganisms (*L. acidophilus* LA-5 and *L. paracasei* L-431), which showed inferior survival rates when combined with the pectin during the enteric phase II, demonstrating the probable positive electrostatic interactions between the pectin and *B. longum* BB-46.

Conclusions

This study indicated that the action of the lemon pectin on the survival rate is specie/strain specific, once different probiotics showed different survival results in the presence of the pectin. We also showed that the studied pectin

had a good impact on *B. longum* BB-46 survival rate during the gastric phase, but had no large effects on enteric phase. More studies are welcome to evaluate the effects of different pectins on different *Lactobacillus*/*Bifidobacterium* strains.

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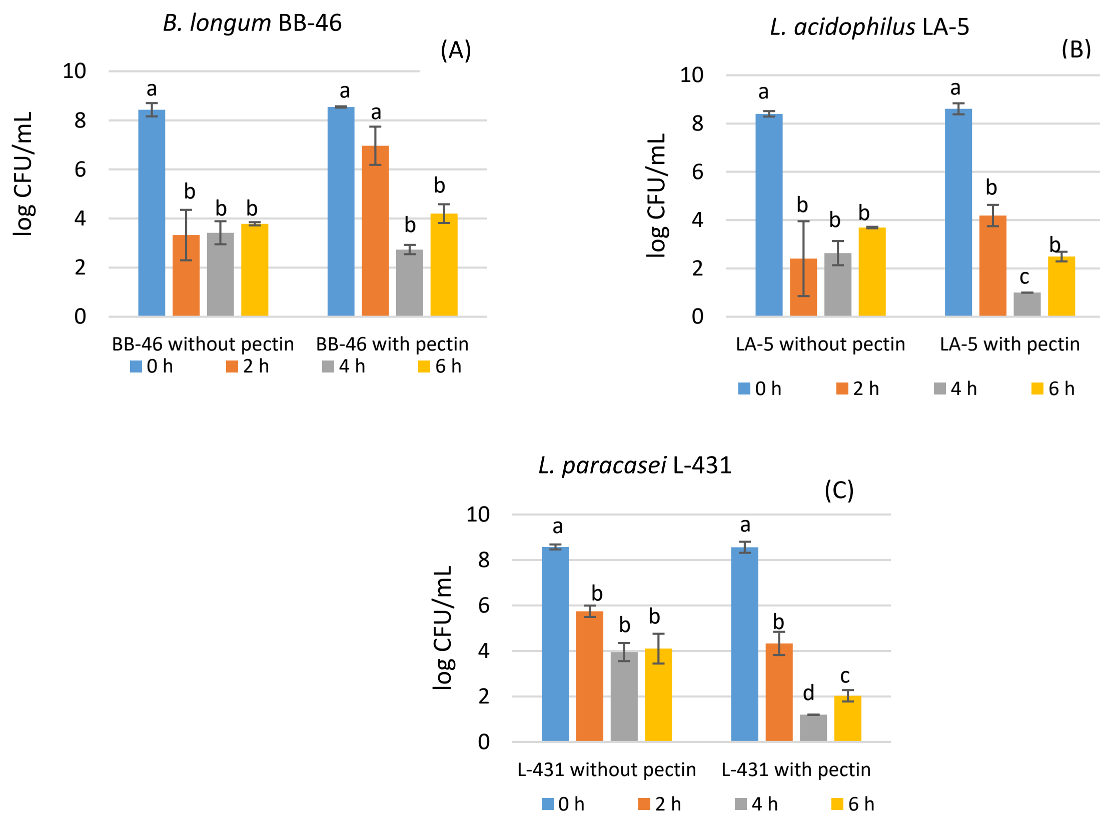

Figure 1. Population (log CFU/mL) of *B. longum BB-46* (A), *L. acidophilus LA-5* (B) and *L. paracasei* L-431 (C), in combination or not with the pectin before the assay (0 h) and during gastric phase (2 h), enteric phase I (4 h), and enteric phase II (6 h) simulation. Different letters represents statistical difference ($p < 0.05$) between the different phases for the same treatment.

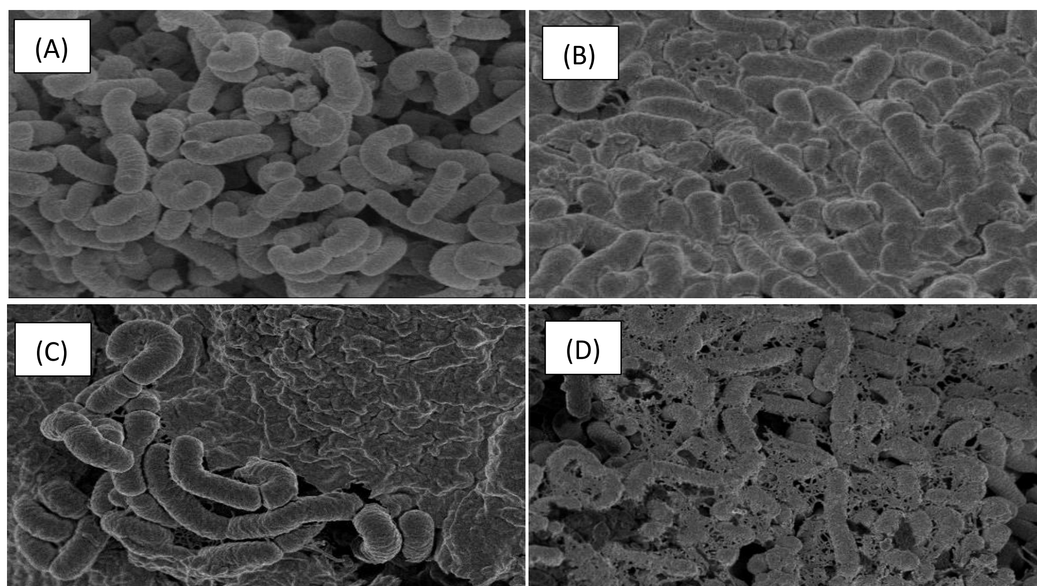

Figure 2. Electron microscopy (X 10,000) of *Bifidobacterium longum* BB-46 under simulated gastrointestinal conditions (A): BB-46 without the pectin, (B): BB-46+pectin in the gastric phase, (C): BB-46+pectin in the enteric phase I, (D): BB-46+pectin in the enteric phase II

Chapter 4.

Impact of combining acerola by-product with a probiotic strain on a gut microbiome model

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Impact of combining acerola by-product with a probiotic strain on a gut microbiome model

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ABSTRACT

In this study, we firstly investigated the survival of three probiotic strains, individually and combined with acerola by-product during simulated gastrointestinal conditions. Next, we investigated the effects of acerola by-product combined with *Bifidobacterium longum* BB-46 on a gut microbiota model (SHIME®). Chemical composition, total phenolic compounds, antioxidant activity of the acerola by-product and microbial counts, denaturing gradient gel electrophoresis (DGGE), ammonium ions (NH_4^+), and short-chain fatty acids (SCFAs) analysis of the SHIME® samples were performed. Acerola

by-product revealed high protein and fibre, reduced lipid contents, and showed to be an excellent source of total phenolic compounds with high *in vitro* antioxidant activity. A decreased amount of NH_4^+ in the ascending colon and an increase ($p < 0.05$) in SCFAs were observed in the three regions of colon during treatment with BB-46 and acerola by-product. BB-46 combined with acerola by-product showed positive effects on the gut microbiota metabolism in SHIME® model.

Keywords: probiotic; SHIME®; acerola by-product; phenolic compounds; human gut microbiota.

Introduction

It is estimated that 10^{14} intestinal microorganisms belonging to over 1000 different species are distributed along the human gastrointestinal tract, with the largest population being in the colon (Payne et al. 2012). Alterations in the intestinal microbiota composition, due to lifestyle, age, diet, and immunological factors may bring several health problems, like intestinal and gastric disorders, as well as susceptibility to other diseases. The consumption of products containing probiotic microorganisms, fibres, and/or many other bioactive compounds has been an option to achieve a favourable composition of the intestinal microbiota, thereby ensuring better quality of life (Woodmansey 2007).

Some probiotic strains are reported to confer health benefits to the consumer, such as increased resistance to diarrheal-type diseases, risk reduction of some types of cancers, lactose digestion improvement, and overall enhancement in cell-mediated immunity (Hoover 2014). The *Bifidobacterium longum* genome sequence determination has revealed several physiological traits that could explain the successful adaptation of these bacteria to the human colon in terms of metabolic and immunomodulatory activities, and adhesion ability (Kavanaugh et al. 2013)

In addition to probiotics, there are several bioactive compounds, such as polyphenols, as well as fibres, that besides having the ability to improve the probiotic survival through the gatrointestinal conditions (Sendra et al. 2016;

Valero-Cases et al. 2017), can modulate the colon microbiota composition or activity, thus either increasing or reducing the growth rate of certain intestinal bacteria (Duda-Chodak 2012).

A wide number of polyphenols are found in tropical fruits, which are largely produced in Brazil (IBRAF 2009). Although Brazil is considered the world's largest producer of tropical fruits, about 30-40% of agricultural by-products are generated during the production of juices and pulps (IBRAF 2009; Sousa et al. 2011). According to Junior et al. (2005), about 40 % of acerola by-products are generated during the process of acerola juice. These by-products may contribute to increased production of organic waste, causing serious environmental problems as well as economic losses to the industry (Sousa et al. 2011).

Studies have shown that, besides being rich in many nutrients, tropical fruits have high contents of fibres, and other bioactive compounds which are mostly concentrated in their skins and seeds (Gorinstein et al. 2011). Acerola (*Malpighia emarginata* D.C.) is a tropical fruit rich in phytochemicals with proven antioxidant activities, and it is commonly known as an excellent vitamin C source (Mezadri et al. 2008). Many reports are found in literature describing the advantageous chemical composition of acerola fruit, as well as its antioxidant activity (Oliveira et al. 2009; Rufino et al. 2010; Silva et al. 2014). Realini et al. (2015) have characterized a dried acerola by-product, and concluded that it could be reused for various purposes as a source of bioactive compounds, thereby avoiding its disposal into the environment. The acerola by-product can be used as flour or be added to different flours, improving the nutritional value of several food products, as for example breads and cookies (Marques et al. 2013).

Therefore, this study aimed to select a combination between the acerola by-product and a probiotic strain, among *Lactobacillus acidophilus* LA-5, *Lactobacillus paracasei* L-431 or *Bifidobacterium longum* BB-46, based on the highest probiotic survival results during simulated gastrointestinal conditions and to investigate the effects of the selected combination on a gut microbiota model (SHIME®). The evaluation and evidences of positive effects of the

acerola by-product in combination with the probiotic strain on the intestinal microbiota can encourage the development of new functional products, generating an added value to the acerola by-product, often discarded by the food processing industries.

Materials and methods

Acerola by-product preparation and characterization

Preparation of the acerola by-product

Ripe acerola (*M. emarginata* D.C.), cabocla variety, São Paulo region cultivation, was purchased from a frozen distributor (Pura Polpa, Araraquara, Brazil). The dried acerola by-product (dried skin, seeds and residues of pulp) was produced in laboratorial scale. For this purpose, sanitization and bleaching of the fresh fruits was conducted, followed by crushing in a sieve (0.5 mm mesh) and drying the by-product in an oven at 60 ºC for 24 h. Subsequently, the dry by-product was treated in a blender (1 min.) and sieved (0.5 mm mesh) in order to obtain a powder. The concentration of the by-product (2.5% (w/v)) was based on the minimum level established by the Brazilian Health Regulatory Agency **(**Anvisa, 2012) for food to be considered as source of fibre.

Chemical composition determination

The acerola by-product was analyzed for lipid content (Soxhlet method), protein content (micro-Kjeldahl method), and for moisture content and ash content, respectively, by the methods of drying in an oven at 105 ºC, and incinerating at 550 ºC. The total fibre content was obtained by the non-enzymatic gravimetric method. All methods were in accordance with the AOAC (2010). The total carbohydrate content of the acerola by-product was calculated by the difference.

Phenolic compounds extraction

Samples (1 g) of the dried acerola by-product were extracted in triplicates with 20 mL of aqueous methanol (methanol: water, 70: 30 v/v), for one hour at 25 ºC, with a magnetic stirrer. Afterwards, it was centrifuged (at 10000 rpm) for 15 min. at 10 ºC. Next, the supernatant was kept and pellet re-extraction was performed by adding 20 mL of aqueous methanol (methanol: water, 70: 30 v/v) and mixing it in an ultra turrax (Polytron-Kinematica GnbH- Luzers, 3 times for 1 min at speed 4). After this process, the solution was again centrifuged (10000 rpm for 15 min at 10 ºC), the supernatant was added to the kept one and their volume completed to 50 mL with aqueous methanol (methanol: water, 70: 30 v/v) (Nóbrega et al. 2015).

Total phenolic content

The analysis was performed according to the methodology proposed by Singleton et al. (1999) with some modifications (Genovese et al. 2003). An aliquot (0.25 mL) of extract was mixed with 0.25 mL Folin-Ciocalteu reagent and 2 mL distilled water. After 3 minutes at room temperature, 0.25 mL of a saturated sodium carbonate solution (Na_2CO_3) was added and the mixture was kept at 37 °C in a water bath for 30 minutes. The absorbance was measured at 750 nm through an Ultrospec 2000 spectrophotometer model (Amersham Biosciences, Cambridge, U.K.). Gallic acid was used as reference standard. Results were expressed in gallic acid equivalent (GAE) in mg/g of sample on a dry basis.

Antioxidant activity by FRAP (Ferric Reducing Ability of Plasma) and DPPH• (sequestration of radical 2.2-difenyl-1-picrylhydrazyl)

The antioxidant activity was determined by FRAP, according to Benzie & Strain (1996). Briefly, a volume of 20 uL of the diluted extract and 150 uL of the FRAP reagent (sodium acetate buffer/TPTZ solution/HCl solution) were added to a 96 wells polystyrene microplate (Costar, Cambridge, MA). After 5 minutes of incubation at 37 °C, the absorbance was read at 593 nm with a

Benchmark Plus Microplate Spectrophotometer (Synergy ™ H1, Biotek Instruments Inc., Vermont, USA). The results were expressed in µmoles of Trolox equivalents (TE) per g of sample (d.b.) and carried out in triplicates.

The antioxidant activity was also determined by DPPH•, according to Brand-Williams et al. (1995) with modifications proposed by Duarte-Almeida et al. (2006). After 20 minutes of reaction time, the absorbance was read at 517 nm on a microplate spectrophotometer and converted to antioxidant activity percentage terms. The analyses were carried out in triplicates and the results expressed in µmoles of Trolox equivalent/g of sample.

Bacterial culture conditions and plate counts

Strains of *Lactobacillus paracasei* L-431, *L. acidophilus* LA-5, and *Bifidobacterium longum* BB-46 were provided as fresh cultures by Christian Hansen (Hoersholm, Denmark) and maintained at -80 ºC in MRS broth with glycerol.

Lactobacillus L-431 and LA-5 strains were individually reactivated in MRS broth (Acumedia, Baltimore, USA). The *Bifidobacterium* BB-46 strain was activated in MRS broth supplemented with L-cysteine (0.05%). The three strains were cultured at 37 °C for 24 hours. The cells were centrifuged at 2600 rpm/10 minutes at 4 °C and washed with a saline solution (0.85 %).

MRS agar (Himedia, India) and acidified MRS agar (pH 5.4) with incubation at 37 °C/48 hours under aerobic and anaerobic conditions were used for counting, respectively, *L. acidophilus* LA-5 and *L. paracasei* L-431. For *Bifidobacterium longum* BB-46 enumeration, the Bifido Medium BIM-25 agar (Difco, France) was used, with cultivation at 37 °C/72 h under anaerobic conditions (Anaerogen Anaerobic System, Probac do Brasil), as previously described by Bianchi et al. (2014).

Survival of L. paracasei L-431, L. acidophilus LA-5, and B. longum BB-46 under in vitro simulated gastrointestinal conditions

The resistance of the probiotic strains in combination with the acerola by-product was determined according to the model described by Buriti et al. (2010) with some modifications. A volume of 10 mL of each strain suspension along with the by-product were added to 90 mL of 0.85 % saline solution (w/v), and homogenized in a Bag Mixer (Interscience, St Nom). Aliquots of 10 mL (10^8 CFU. mL^{-1}) were transferred to sterile bottles and the pH of the solution was adjusted to 2.07 - 2.72 with a 1M HCl solution (Merck). Pepsin (Pepsin from porcine gastric mucosa, Sigma-Aldrich) and lipase solution (Amano Lipase G, Sigma-Aldrich) were added at sufficient quantities to achieve concentrations of 3 g/L and 0.9 mg/L in the final solution, respectively. Next, the bottles were incubated in a water bath (CT232, Cientec, Belo Horizonte, BR) at 37 °C under constant stirring at 150 rpm for two hours (gastric phase). After 2 hours of incubation, pH was adjusted to 4.21 - 5.03 with the addition of a sterile alkaline solution (150 mL of 1N NaOH + 11.16 g/L of NaH_2PO_4). In the next step, a bile (bile from bovine and ovine, Sigma-Aldrich) and pancreatin (Pancreatin from porcine pancreas, Sigma-Aldrich) solution was added at sufficient concentrations so that the final solution contained 10 g/L and 1 g/L, respectively. The samples were again incubated at 37 °C for two hours (Enteric Phase I). At the end of the enteric Phase I, the pH was adjusted to 6.12 - 6.97, and samples were added to the Pancreatin and Bile solution (keeping the concentration at 10 g/L and 1 g/L of the final solution, respectively), and a new incubation took place at 37° C for two hours under constant agitation (enteric phase II), totalling 6 hours of experiment. For each phase of the assessment (0, 2, 4, and 6 hours), samples were collected to conduct the selected strains enumeration.

Scanning electron microscopy

To verify morphological changes of the selected probiotic with the acerola by-product under simulated gastrointestinal conditions (using the gastrointestinal

in vitro model described by Buriti et al. (2010)), a scanning electron microscopy was performed. This part of the study was performed according to the methodology proposed by Matias et al. (2016). Samples of each phase (gastric and enteric), as well as a sample of the microorganism (in saline solution at 0.85 % w/v) without passing through any simulated gastrointestinal juices were centrifuged (6500 rpm/10 min.) and the supernatants were discarded. The pellet was suspended in a NaCl (0.9 % w/v) solution until a final concentration of 5 log CFU mL^{-1} was reached. Aliquots of 1 mL of cell suspension were fixed for 2 h in 2% glutaraldehyde (v/v), followed by fixation in osmium tetroxide for 2 h. Next, the membranes were washed 3 times in purified water and dehydrated, with a solution of 70 % (15 minutes, twice), 95 % (15 minutes, twice) and 100% ethanol (15 minutes, four times), and then dried to a critical point of CO_2. The dried membranes were transferred to an aluminium base by a sputtering machine, coated with gold and analyzsed using scanning electron microscopy at 2.5 Kv. (JEOL JSM-7401F, JEOL, Tokyo, Japan) (Matias et al. 2016).

Action of selected combination on a gut microbiota model (SHIME®).

The action of the selected combination (acerola by product and probiotic strain) under simulated gut microbiota conditions was determined by using the SHIME® system. SHIME® is a dynamic model (from Ghent University and ProDigest) which simulates the human intestinal microbial ecosystem. The SHIME® is composed by five double-jacketed vessels simulating the stomach (vessel 1), the duodenum (vessel 2), and the ascending (colon vessel 3), transverse (colon vessel 4), and descending (colon vessel 5) colons. This dynamic system is connected with a software, which controls the pH, residence time, and temperature of each vessel as described by Molly et al. (1994) and Possemiers et al. (2004).

The passage of the combination through the stomach was simulated by the vessel 1 with HCl for the regulation of pH, along with a feeding medium [carbohydrate-based medium that allows the adaptation to specific environmental conditions of the ascending, transverse, and descending colons

in terms of pH range, retention time, and available carbon sources (Molly et al. 1994). The feeding medium composition was previously described by Possemiers et al. (2004).

The passage of the combination (acerola by-product with BB-46) through the duodenum was simulated by the vessel 2 with 60 mL of artificial pancreatic juice (12.5 g/L of $NaHCO_3$, 6 g/L of Oxgall, and 0.9/L g of pancreatin) at a rate of 4 mL/min. for 15 minutes (Molly et al. 1994; Possemiers et al. 2004).

In order to maintain the homogeneity in each vessel, a magnetic stirrer was used. The system was maintained in anaerobic conditions through daily N_2 flushing for 30 min. The pH of colon vessels 3, 4, and 5 was automatically adjusted by the addition of NaOH 0.5 M or HCl 0.1 M. For the stomach region, the pH was automatically adjusted by the addition of NaOH 0.5 M and HCl 1 M (Molly et al. 1994; Possemiers et al. 2004).

Faecal inoculum

Before starting the experiments, the colon vessels (V3, V4, and V5) were inoculated with bacteria from a stool sample of 3 healthy adults (BMI between 18.5- 24.9 kg/m^2 and waist circumference < 80 cm) which had not consumed probiotic products over the past 3 to 6 months and with no history of antibiotic treatment within a period of six months prior to the study.

As the production of methane gas is directly related to an increase of acetic acid and, consequently, the increase in total short-chain fatty acids, the volunteers classified as methane producers were excluded (Wolever et al. 1991). For this classification, the breath methane dosage of four lean volunteers was performed through a Quintron digital Breathtracker Microlyzer at the Pediatric Gastroenterology Department of the São Paulo Federal University.

40 g of faeces (~ 13.5 g of each donor) belonging to the selected donors (methane values < 3 ppm), were colected and diluted in phosphate buffer (200 mL) containing 0.1% of Na-thioglicolate, 0.05 mol/L NaH_2PO_4 and 0.05 mol/L of Na_2HPO_4 (pH = 6.5). The diluted samples were than stirred in a homogenizer (Stirrer model 130, Nortecientífica, Brazil) for 10 minutes and

centrifuged for 15 min. at 3000 rpm. From the supernatant, 40 mL were added to vessels 3, 4, and 5, which were already filled with a feeding medium at specific volumes, thus allowing the adjustment and stabilization of the microbial community (Molly et al. 1994).

Study protocol of combinations in a SHIME® reactor

The study started with 2 weeks of control period, where 200 mL of the feeding medium entered through the system twice a day. This two weeks are important to stabilise the microbial community and to allow the adaptation of the microbial community to physicochemical and nutritional conditions prevailing in different parts of the colon (Molly et al. 1994; Possemiers et al. 2004). After this period, the treatment with the selected combination was started to continue for the next two weeks. The selected combination was added along with the feeding medium (200 mL), twice a day, with 10^8 CFU. mL^{-1} of the selected probiotic and 2.5 % (w/v) of the acerola by-product. After this period, the washout period was initiated, in which 200 mL of the feeding medium entered the simulator twice a day for two weeks.

Probiotic survival in the stomach and duodenum using the SHIME® model

The survival of the probiotic microorganism in combination with the acerola by-product, after the passage through the stomach and duodenum regions, was performed weekly. Samples (5 mL) of the stomach and the duodenum were collected from the SHIME®, both after 2 hours of entrance into these compartments. Each sample (1 mL) was suspended in 9 ml of peptone water. Serial dilutions were performed and the inoculation carried out in suitable culture media.

Microbiological analysis

Samples from the 3 colon vessels throughout the experimental period (2 weeks of control, 2 weeks of treatment and of washout) were weekly colected for the plate counts.

The intestinal microbiota composition analysis was based on the enumeration of total anaerobic bacteria, *Lactobacillus* spp., *Bifidobacterium* spp., *Clostridium* spp., and total coliforms, according to Bianchi et al. (2014). Total anaerobic bacteria amounts were determined by plating on Standard Methods agar and anaerobic incubation (Anaerogen Anaerobic System, Probac do Brasil) at 37 °C/48 h. MRS agar with anaerobic incubation (Anaerogen Anaerobic System, Probac do Brasil) at 37 °C/48 h was used to determine the population of lactobacilli. BIM-25 agar with anaerobic incubation (Anaerogen Anaerobic System, Probac do Brasil) at 37 °C/72 h was used to determine the population of *Bifidobacterium* spp. *Clostridium* spp. was enumerated anaerobically by using Reinforced Clostridial Agar at 37 °C/48 h. Petrifilm™ EC (3M) with aerobic incubation at 37 ºC/48 h was used to determine the population of total coliforms.

Molecular methods (PCR–DGGE) were used to analyse the behaviour of total bacteria throughout the experimental period. “Isolation of DNA from Stool for Human DNA” (Qiagen, Hilden, Germany) was the protocol used in this study with some modifications according to Bianchi et al. (2014). QIAamp DNA Stool Mini Kit (Qiagen, Germany) was employed to extract DNA from each reactor vessel at each period of study. DNA yield was quantified by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The primers used to amplify DNA were 968FGC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and 1401R (5′-CGG TGT GTA CAA GAC CC-3′) (Nübel et al. 1996). DNA polymerization was performed by using a GoTaq® Green Master Mix (Promega, USA). Samples were amplified in a Veriti® 96-well thermal cycler (Applied Biosystems, USA) by using the following setup: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing of primer

at 56 °C for 40 s, elongation at 72 °C for 1 min and extension at 72 °C for 5 min, followed by final cooling to 4 °C.

Electrophoresis was carried out as previously described by Heilig et al. (2002), i.e. in an 8 % polyacrylamide gel with a denaturant gradient of 45–65 % for 16 h at 85 V in a 0.5× TAE buffer at constant temperature of 60 °C. Gels were stained with ethidium bromide, scanned at 400 d.p.i., and further analysed by the BioNumerics 6.0 software (Applied Maths, Kortrijk, Belgium). BioNumerics 6.0 software (Applied Maths, Kortrijk, Belgium) was used to analyse the distance matrices of each DGGE based on the Pearson similarity.

Ammonium (NH_4^+) and short-chain fatty acids (SCFAs) analyses

Samples from the three colon vessels were collected weekly for NH_4^+ and SCFA analyses throughout the experimental period (control, treatment, and washout).

NH_4^+ amounts were determined by a selective ion meter (HI 4101 model, Hanna Instruments) coupled with an ammonia selective-ion electrode (Orion 95–12). The samples (25 mL) were added to 0.5 mL of an ammonia pH ionic strength adjusting solution (Orion). The analyses were performed in triplicates.

For SCFA analysis, the samples (2 mL) were centrifuged at 14000 rpm for 5 min. Afterwards, 100 µL of the supernatant were added in 1900 µL of ultrapure water containing 1 g of NaCl, 100 µL of crotonic acid, 70 µL of isobutanol, and 200 µL of 2 M H_2SO_4. The SCFAs were analysed by using a 2010-Model gas chromatograph (Shimadzu, Japan) equipped with a split/splitless injector, a flame ionization detector, and a CombiPAL automated sampler for headspace analysis. The SCFAs were separated by using a HP-INNOWAX column (30 m× 0.25 mm× 0.25 µm) (Agilent Technologies, USA). The carrier gas was hydrogen and the flow rate was set at 1.45 mL/min. The temperature of both the injector and the detector was 240 °C (Adorno et al. 2014).

Statistical analysis

The significance of the results was investigated with a one-way ANOVA. Individual means were compared by Tukey's test ($p < 0.05$) with the BioEstat 5.0 software (IBM, Brazil).

Results

Chemical composition, total phenolic content and in vitro antioxidant activity of the dried acerola by-product.

The acerola by-product showed high levels of protein (9.50 ± 1.10 g/100 g) and carbohydrate (81.69 ± 0.10 g/100 g), among which 56.28 ± 0.19 g/100 g corresponded to fibre. Reduced levels of lipid (1.31 ± 0.22 g/100 g), ash (2.33 ± 0.11 g/100 g), and moisture (5.17 ± 0.05 g/100 g), as well as high values of total phenolic compounds (average of 52.50 ± 1.25 mg GAE/g of dried by-product) were found. The studied dried acerola by-product showed an antioxidant activity of 226.86 ± 4.84 umols TE/g (DPPH•) and of 351.00 ± 16.91 umols TE/g (FRAP).

Survival of L. paracasei L-431, L. acidophilus LA-5, and B. longum BB-46 in the presence and absence of acerola by-product under in vitro simulated gastrointestinal conditions and using SHIME® model.

L. paracasei L-431, *L. acidophilus* LA-5, and *B. longum* BB-46, either in combination with the acerola by-product or not, were exposed to *in vitro* simulated gastrointestinal conditions, and the results are shown in Figure 1.

The by-product improved ($p < 0.05$) the survival of L-431 and of BB-46 during their passage through the gastric juice. However, a high decrease in all strains survival, either with or without the acerola by-product, was observed in the enteric phase (4 h and 6 h of survival test). Among the studied bacterial strains, *L. acidophilus* LA-5 was the one with the worst resistance in all phases.

The acerola by-product provided a higher increase in the BB-46 survival (of 5 log cycles) compared to L-431 (2 log cycles) during the gastric phase in

comparison with these probiotic strains without it. For this reason and by considering the best survival adaptation strategies of *Bifidobacterium* strains, BB-46 in combination with the acerola by-product was selected to be tested in the Simulator of Human Intestinal Microbial Ecosystem (SHIME®).

As in the *in vitro* simulated gastrointestinal test cited above, it was observed that the acerola by-product provided a high survival rate for BB-46 during the gastrointestinal simulation in the SHIME® model (Figure 2).

Scanning electron microscopy

As can be seen in the electron microscopy (Figure 3), there was a reduction in the population densities of microorganisms, as well as changes in the structure of BB-46 during the enteric phase I (4 h of gastrointestinal test), and a recovery of the population and the bacterial structures during the enteric phase II (6 h of the gastrointestinal test).

In Figure 3(B), the presence of acerola fibres between the cells in the gastric phase can be observed. After 4 hours of experiment, the appearance of certain substances among the cells was observed, making it difficult to observe the microorganisms. In this phase, it was also observed that a few cells lose their form and appear to clew due to adverse conditions (Figure 3C). After 6 hours of experiment, there was a restructuring of cells.

Microbial changes during passage through the SHIME® reactor

Table 1 shows the microbial population of samples taken from each colon vessels (V3, V4, and V5), which simulated the ascending, transverse, and descending colons during the experimental period.

There was no significant alteration in the *Lactobacillus* spp. and *Bifidobacterium* spp. populations during the treatment period with the acerola by-product and BB-46, except for colon vessel 4. However, during the same period, a reduction ($p < 0.05$) of one log cycle of *Clostridium* spp. and of total anaerobes was observed in colon vessels 3, 4, and 5. After the washout period, the bacterial counts returned to their initial condition (control period).

There was no significant difference regarding total coliforms during the treatment period ($p>0.05$).

The DGGE analysis was used to monitor qualitative changes in the composition and structure of total bacteria in the three colon vessels. A cluster analysis of the DGGE profiles for colon vessel 3 resulted in two separated groups, showing great similarity (78%) between control and washout period. The treatment period, however, also showed a great similarity (64 %) with the control and the washout period in this region of the colon (Figure 4). In the colon vessel 4, one cluster grouping the treatment period with the washout period was observed, showing that changes in the total bacterial population in the colon 4 vessel was maintained after the treatment period. In the colon vessel 5, a cluster analysis of the DGGE profile showed a single group with the control and the treatment period, which bears great similarity (71%) with the washout period, thus showing low effects on total bacteria population in the vessel 5.

Effects on microbial metabolism in SHIME®: NH_4^+ and SCFA

Table 2 shows a reduction ($p < 0.05$) of NH_4^+ during the treatment period with the acerola by-product in combination with BB-46, but only in colon vessel 3, which simulates the ascending colon. In the other compartments there was no difference ($p < 0.05$), except for the washout period in reactors 4 and 5 (Table 2), which showed high values of NH_4^+ ($p < 0.05$).

An increase ($p < 0.05$) in short chain fatty acids, particularly butyric acid, was observed during the treatment with the acerola by-product and BB-46 combination in the three colon vessels (Figure 5). During the treatment period, an increase of 3.19, 2.60 and 4.93 folds in butyric acid and 1.25, 1.06 and 2.54 folds in propionic acid was observed, respectively, in colon vessels 3, 4, and 5. Acetic acid increased 100 % in all regions of colon.

Discussion

In this study, we first analysed the chemical composition, total phenolic content and the *in vitro* antioxidant activity of the dried acerola by-product. Next, we

assessed the survival of three different bacterial strains (*Lactobacillus acidophilus* LA-5 and *Lactobacillus paracasei* L-431 and *Bifidobacterium longum* BB-46) combined with acerola by-product under *in vitro* gastrointestinal conditions and, finally we investigated the effects of the combination with the best probiotic survival results on human gut microbiota using SHIME® model.

The dried acerola by-product showed an excellent chemical composition, which were consistent with previous reported by Marques et al. (2013). On the other hand, Abud & Narain (2009) as well as Sousa et al. (2011) found different composition results. According to Marques et al. (2013), fibre, fat, ash, and protein contents of acerola by-product can vary from 15.33 to 70.60 g/100 g, 0.57 to 5.62 g/100 g, 0.65 to 6.08 g/100 g, and 0.56 to 9.572 g/100g, respectively. Acerola composition may vary due to different geographical locations, cultivation practices, rainfall patterns, exposure to sunshine, genetic traits, and especially its maturation stage (Silva et al. 2016).

The high content of phenolic compounds and antioxidant activity of acerola by-product have also been reported by several studies (using Folin-Ciocalteu reagent, DPPH• scavenging and FRAP) (Oliveira et al. 2009; Rufino et al. 2010; Sousa et al. 2011; Silva et al. 2014), showing that the acerola by-product presents higher phenolic compounds and antioxidant activity when compared to other by-products of fruits like guava, pineapple, papaya and cupuaçu among others (Oliveira et al. 2009; Rufino et al. 2010; Sousa et al. 2011; Silva et al. 2014).

Several food ingredients as proteins, fibres and other carbohydrates can enhance the probiotic survival (Ranadheera et al. 2012; Sendra et al. 2016). The acerola by-product chemical composition showed high contents of protein, carbohydrates and fibres, which could have contributed to the improvement of the *Lactobacillus* L-431 and *Bifibibacterium longum* BB-46 survival during the gastrointestinal conditions (2 h of survival test).

In addition, the great content of phenolic compounds found in the acerola by-product can also have contributed to the probiotics survival during the gastrointestinal conditions. According to Valero-Cases et al. (2017), some

phenolic compounds can provide high survival to lactic acid bacteria after *in vitro* digestions, especially for *Bifidobacterium* strains, which present higher metabolism of phenolic compounds (Valero-Cases et al. 2017).

The low bacterial survival rate during the enteric phase (4 h and 6 h of survival test), of all studied strains, can be attributed to the presence of bile salts. According to Saarela et al. (2000), bile salts are toxic to cells because they disrupt the cellular membrane structure and, therefore, bile salt tolerance is considered one of the required characteristics for the survival of lactic acid bacteria in the duodenum. Although intrinsic bile tolerance appears to be strain dependent, both lactobacilli and bifidobacteria can progressively adapt to the presence of bile salts (Ruiz et al. 2013). Despite the low survival rate of BB-46 found in the plate counts during the end of the enteric phase (after 6 h.), the electron microscopy revealed morphological restructuring and high microbial density after this period.

L. acidophilus LA-5 showed the worst survival rate in all phases. According to Klu & Chen (2015) the survival of probiotic cells under simulated gastric and enteric conditions depends on the probiotic species/strain variability. Still according to these authors, *Bifidobacterium* strains usually have higher survival rates than *Lactobacillus strains*. Bedani et al. (2013) reported that LA-5 was more sensitive than Bb-12 to the simulated gastrointestinal conditions. In this mentioned study, *Bifidobacterium animalis* Bb-12 maintained a mean population of about 8 log CFU/g, while *L. acidophilus* La-5 could only maintain a mean population of less than 5 log CFU/g during the gastrointestinal test.

As the *Bifidobacterium* BB-46 showed, in this study, the best survival results during the *in vitro* gastrointestinal test, this bacteria was selected to be tested in SHIME® model along with the acerola by-product. During the treatment with the *Bifidibacterium* BB-46 and acerola by-product in SHIME® model, a reduction in *Clostridium* spp. and total anaerobics was observed. According to Macfarlane & Cummings (1991), some strains of *Bifidobacterium* produce mainly lactic and acetic acids that decrease the pH of the colon, inhibiting the growth of many pathogenic bacteria such as some species of *Clostridium.* Although some species of the genus *Clostridium* are associated

with the production of short-chain fatty acids, which are beneficial to health (Possemiers et al. 2010), some species may be harmful due to their metabolic activity and pathogenic character (Montesi et al. 2005). The reduction of *Clostridium* spp. and total anaerobes may also have been a reflex of a hypothetical increase of specific bacterial groups (stimulated by the acerola by-product components), generating a competitive environment.

Although the DGGE results suggest no effects of the combination on total bacteria in SHIME® model, significant changes in the gut microbiota metabolism (SCFA and NH_4^+) were noticed.

A decrease in NH_4^+ was observed in the ascending colon during the treatment with the acerola by-product and BB-46. According to Smith & MacFarlane (1998), the levels of NH_4^+ are directly affected by the presence of specific carbohydrates. This fact occurs due to the preference of the intestinal microorganisms to carbohydrates, inhibing the amino acids fermentation in favor of carbohydrate (Ito et al. 1993). Thus, in the present study, the high fibre or/and other carbohydrate content present in acerola by-product may have contributed to these results. Moreover, as already mentioned, the *Bifidobacterium* spp. produce lactic and acetic acids that decrease the colon pH, inhibiting the growth of some pathogenic bacteria such as some species of *Clostridium*, which use amino acids as source of nitrogen, carbon, and energy, generating NH_4^+ as a metabolite (Macfarlane & Cummings 1991).

The reduction of NH_4^+, regardless of which colon portion it takes place, is considered beneficial since NH_4^+ corresponds to one of the products of protein degradation which is generated by the gut bacteria, which is considered a metabolite that negatively affects the intestinal health (Davila et al. 2013).

A significant increase ($p < 0.05$) in SCFA was also observed during the treatment with the combination BB-46 and acerola by-product in the three regions of colon. According to Bedani & Rossi (2009), the production of SCFA depends on the available substrates and the microorganisms present in the gastrointestinal tract. As previously shown, the acerola by-product has high polyphenol and fibre content. In this context, according to Tuohy et al. (2012), the production of SCFA may occur due to the use of fibre or polyphenols

present in food, either in combination or not. According to these authors, *in vitro* studies have shown that both can be metabolized by the gut microbiota, resulting in a SCFA increase. The hydrolysis of polyphenols by colonic microbiota can increase the bioavailability, and possibly the biological activity of polyphenols that reach the colon region. According to Tuohy et al. (2012), the intestinal microbiota has an extensive hydrolytic activity and the ability to break down many complex polyphenols into smaller phenolic acids, which can be absorbed across the intestinal mucosa, thus increasing the production of SCFA. The same occurs with fibres. According to Shen et al. (2012), a population with a higher intake of dietary fibre tends to have higher concentrations of SCFA in faeces.

Although a large number of amino acid fermenters are present in the colon region, the majority of the gut microorganisms have predominantly saccharolytic metabolism and as a consequence, carbohydrate availability becomes an important nutritional factor, controlling the composition and metabolic activity of the intestinal microbiota (Davila et al. 2013). Thus, the presence of fibres and other carbohydrates present in acerola by-product may have been a stimulating factor of bacteria with saccharolytic metabolism generating SCFA.

The presence of *Bifidobacterium longum* BB-46 may also have had a contribution to the increased production of SCFA. Several studies have shown that lactate and/or acetate produced by bifidobacteria is used by some bacterial genus as *Roseburia*, *Eubacterium* and *Anaeroestipes*, which convert these metabolites into SCFA (Belenguer et al. 2006; Flint et al. 2015).

In this study, among the SCFAs, the butyric acid was the one with the highest increase. According to Chakraborti (2015), butyric acid has been found to increase mitochondrial activity, prevent metabolic endotoxemia, improve insulin sensitivity, exhibit colon cancer-preventive effects, increase intestinal barrier function and protect against diet-induced obesity without causing hypophagia.

Conclusion

The current study showed that *Bifidobacterium longum* BB-46 exhibited better gastrointestinal resistance then *Lactobacillus acidophilus* LA-5 and *Lactobacillus paracasei* L-431 when combined with the acerola by-product. *B. longum* BB-46 with acerola by-product reduced ammonium ions (in ascending colon) and provided an increase in acetic, butyric, and propionic acids concentrations in all colon vessels. Moreover, this study was able to show the excellent chemical composition and the high antioxidant activity of the acerola by-product. The results suggest that BB-46 with acerola by-product has a positive effect on the gut microbiota metabolism and might be used in new studies about functional products development, additionally generating an added value to the acerola by-product, often discarded by industries. However, new studies using more robust methods to analyse changes in intestinal microbiota, such as 16 S sequencing, are necessary to understand the action of *Bifidobacterium longum* BB-46 and acerola by-product, combined or not, on the intestinal microbiota composition, and consequently, better understand the microbial metabolism.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Table 1. Microbial counts (log CFU/mL) of different bacterial groups in different vessels that simulate the ascending (colon vessel 3), transverse (colon vessel 4), and descending (colon vessel 5) colons as a result of treatment with the acerola by product + *Bifidobacterium longum* BB-46.

	Colon vessel 3		
	Control	**Treatment**	**Washout**
Lactobacillus spp.	8.59 ±0.13 [a]	8.13 ± 0.74 [a]	8.79 ± 0.01 [a]
Bifidobacterium spp.	8.72 ± 0.04 [a]	8.10 ± 0.61 [a]	8.88 ± 0.03 [a]
Clostridium spp.	8.65 ± 0.08 [a]	7.60 ± 0.05 [b]	8.85 ± 0.00 [a]
Total anaerobes	8.70 ± 0.05 [b]	7.49 ± 0.07 [c]	8.87 ± 0.05 [a]
Coliforms	7.01 ± 0.43 [a]	6.64± 1.20 [a]	7.48 ± 0.02 [a]
	Colon vessel 4		
Lactobacillus spp.	8.21 ± 0.17 [a]	7.54 ± 0.37 [b]	8.12 ± 0.04 [ab]
Bifidobacterium spp.	8.29± 0.28 [a]	7.50 ± 0.28 [b]	8.16 ± 0.02 [ab]
Clostridium spp.	8.35± 0.14 [a]	7.25 ± 0.01 [b]	8.20± 0.05 [a]
Total anaerobes	8.44 ± 0.30 [a]	7.43 ± 0.02 [b]	8.33 ± 0.01 [a]
Coliforms	7.19 ± 0.62 [a]	6.78± 1.48 [a]	7.07 ± 0.02 [a]
	Colon vessel 5		
Lactobacillus spp.	7.74 ± 0.32 [a]	7.35 ± 0.53 [a]	7.40 ± 0.03 [a]
Bifidobacterium spp.	8.07 ± 0.05 [a]	7.30 ± 0.53 [a]	7.38± 0.11 [a]
Clostridium spp.	8.38 ± 0.13 [a]	7.12 ± 0.11 [b]	8.09 ± 0.07 [a]
Total anaerobes	8.3 ± 0.03 [a]	7.20 ± 0.09 [b]	8.19 ± 0.15 [a]
Coliforms	7.95 ± 0.16 [a]	7.85 ± 1.44 [a]	7.41 ±0.01 [a]

Different letters on the same line represent a statistically significant difference ($p < 0.05$) between different periods of the experiment for the same microorganism group

Table 2. NH_4^+ production (mmol/L) in different vessels that simulate the ascending (colon vessel 3), transverse (colon vessel 4), and descending (colon vessel 5) colons as a result of treatment with the acerola by product + *Bifidobacterium longum* BB-46.

	Ascending colon	**Transverse colon**	**Descending colon**
Control period	25.16 ±0.88 [A]	27.39 ±1.69 [A]	24.77 ±0.75 [A]
Treatment period	16.40 ±4.80 [B]	25.82 ±2.00 [A]	25.77 ±1.49 [A]
Washout period	23.70 ±9.40 [AB]	38.17 ±5.82 [B]	40.33 ±2.07 [B]

Different letters in the same column represent a significant difference ($p < 0.05$) between different periods of the experiment for the same vessel.

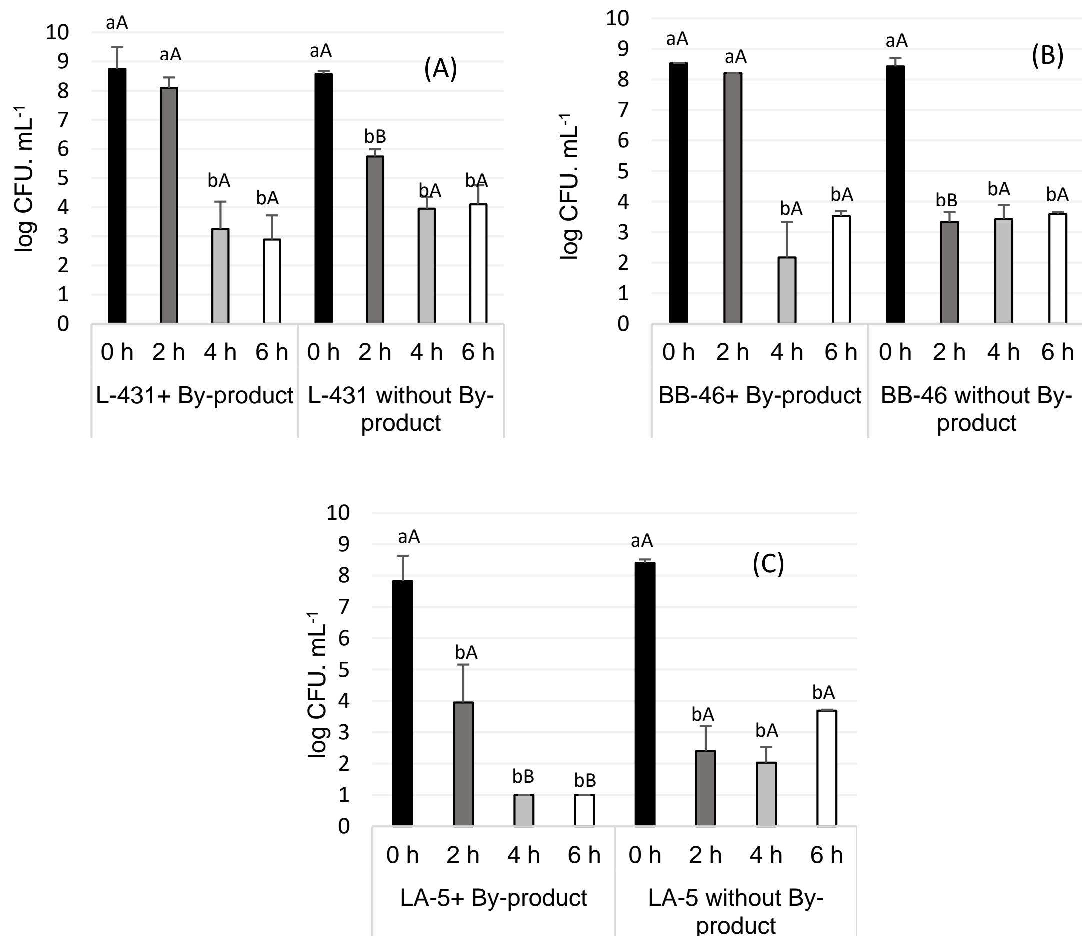

Figure 1. Population (log CFU. mL^{-1}) of *Lactobacillus paracasei* L-431 (A), *B. longum* BB-46 (B), and *L. acidophilus* LA-5 (C) either in combination or not with the acerola by-product after the simulated gastrointestinal conditions using the *in vitro* test. Different uppercase letters represent the difference ($p < 0.05$) between different treatments (probiotic with the acerola by-product and without it) whereas different lowercase letters represent the difference ($p < 0.05$) between phases (0, 2, 4, and 6 h).

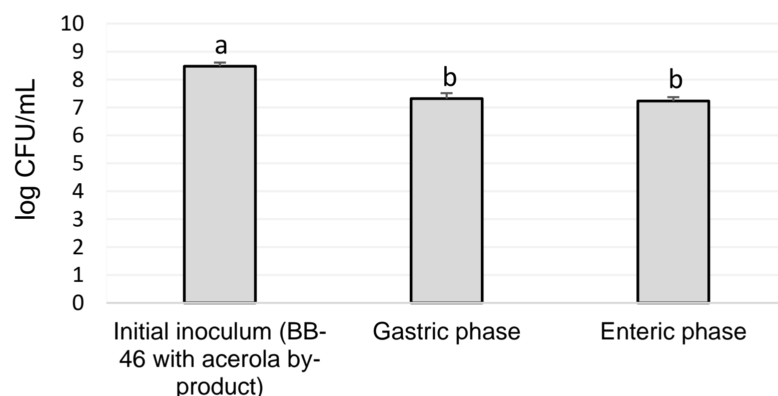

Figure 2. Population (log CFU/mL) of *B. longum* BB-46 with acerola by-product after the simulated gastrointestinal conditions using the SHIME® model. Different letters represent a statistically significant difference ($p < 0.05$) between the different phases.

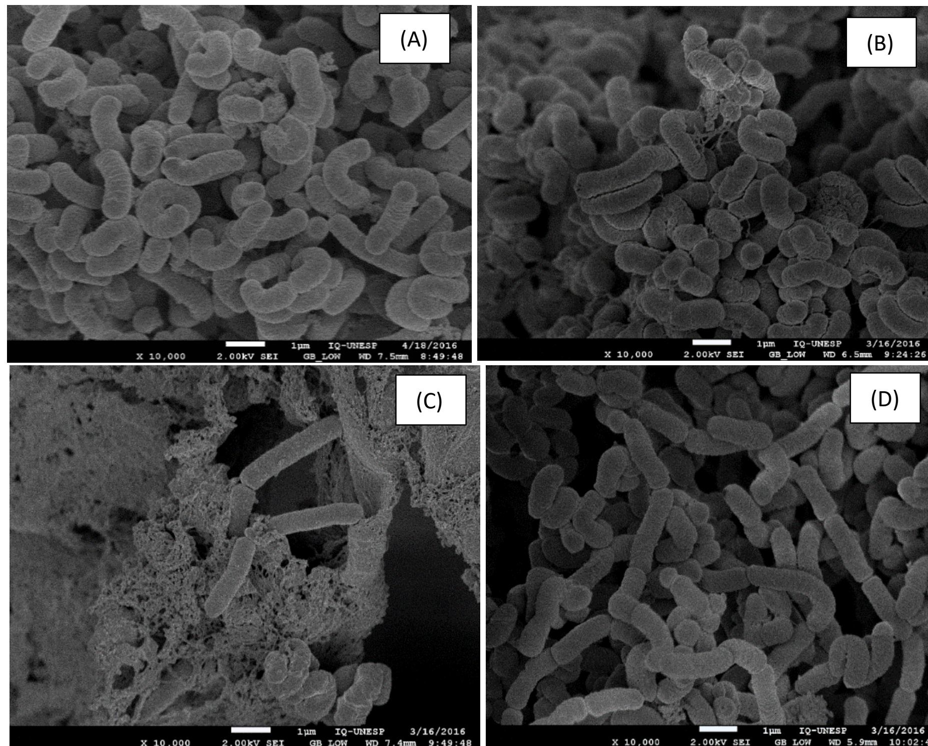

Figure 3. Electron microscopy of *Bifidobacterium longum* BB-46 under simulated gastrointestinal conditions (A): BB-46 without the acerola by-product, (B): BB-46+acerola by-product in the gastric phase, (C): BB-46+acerola by-product in the enteric phase I, (D): BB-46+acerola by-product in the enteric phase II.

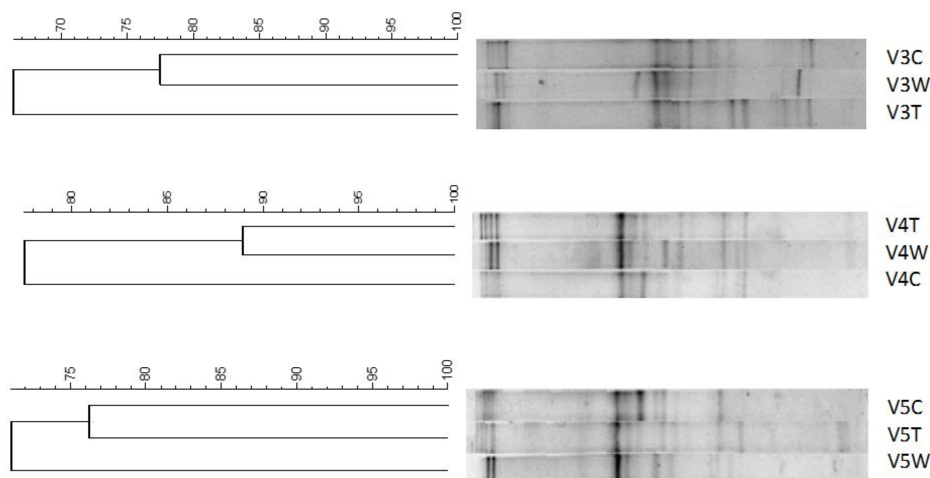

Figure 4. Cluster analysis of the DGGE profiles of total bacteria in different vessels that simulate the ascending (colon vessel 3), transverse (colon vessel 4), and descending (colon vessel 5) colons as a result of treatment with the acerola by product + *Bifidobacterium longum* BB-46. T: treatment period, C: Control period. W: Washout period

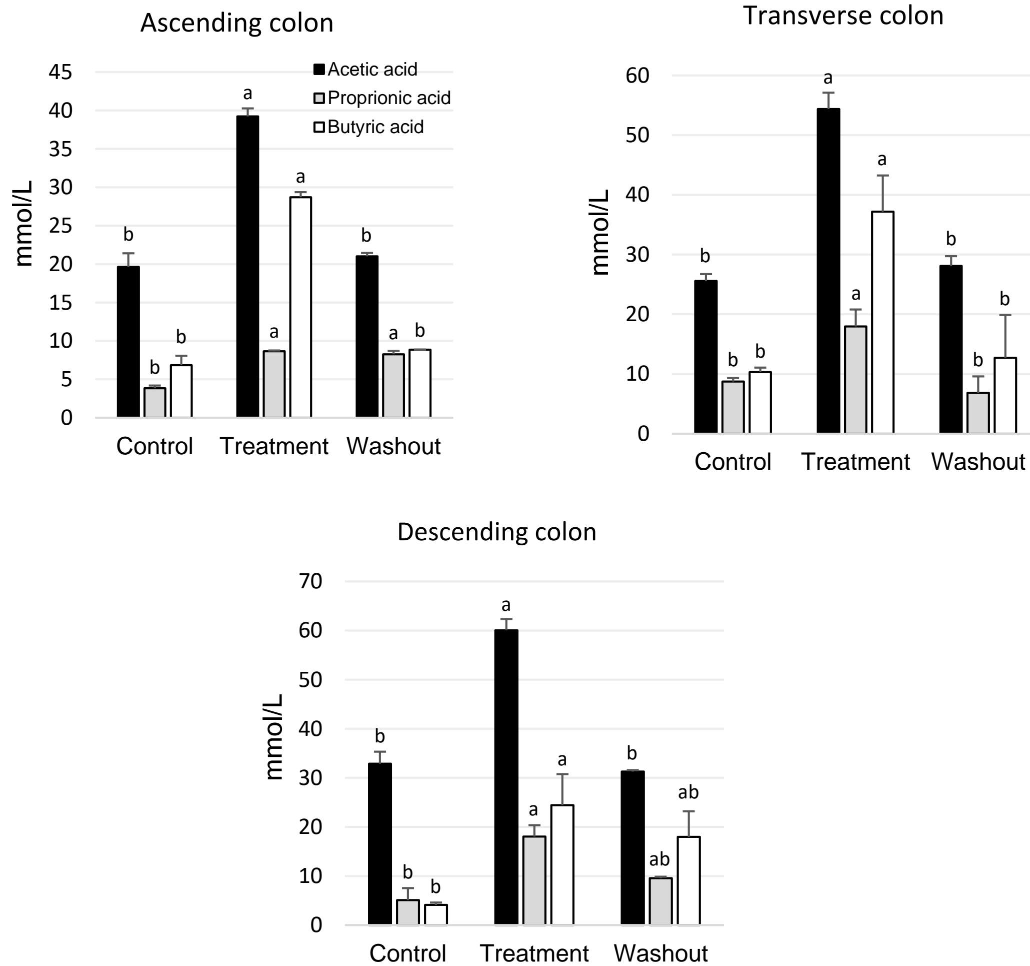

Figure 5. Metabolic activity of short-chain fatty acid (SCFA): acetic, propionic, and butyric acids in different vessels that simulate the ascending (colon vessel 3), transverse (colon vessel 4), and descending (colon vessel 5) colons as a result of treatment with the acerola by product + *Bifidobacterium longum* BB-46. Different letters represent a significant difference ($p < 0.05$) between different phases (control, treatment, and washout) for the same SCFA.

Chapter 5.

In vitro modulation of human gut microbiota composition and metabolites by *Bifidobacterium longum* BB-46 and a citric pectin

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In vitro modulation of human gut microbiota composition and metabolites by _Bifidobacterium longum_ BB-46 and a citric pectin

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Abstract

The gut microbiota composition and its metabolites have high impact on human health. Exploitation of prebiotics and probiotics for modulation of gut microbiota can lead to promising outcomes. This study aimed to evaluate the effects of the probiotic strain *Bifidobacterium longum* BB-46 alone and in combination with a citric pectin from lemon on the gut microbiota from healthy adults using the Simulator of Human Intestinal Microbial Ecosystem (SHIME®). Changes in microbiota composition and in metabolic activity were assessed by the 16S rRNA gene sequencing and by analyses of short-chain fatty acids (SCFAs) and ammonium ions (NH_4^+). An increase in the relative abundances

of *Firmicutes* (especially the members of *Lachnospiraceae* and *Lactobacillaceae* families) and *Bacteroidetes* was observed during treatment with *B. longum* BB-46 alone in all compartments of the colon. Treatment with *B. longum* BB-46 and pectin stimulated an increase in the proportions of genera *Faecalibacterium, Eubacterium* and *Lactobacillus*, as well as in the *Ruminococcaceae* family in the transverse and descending colons. Concurrently, the butyrate levels increased in these two compartments. Additionally, the combination of *B. longum* BB-46 and pectin reduced the abundance of proteolytic bacteria *Bacteroides*, *Clostridium, Peptoniphilus*, and *Streptococcus*, along with decreased NH_4^+ production. No significant changes could be observed on NH_4^+ production by treatment with *B. longum* BB-46, nor did it increase the amount of SCFAs. In this study, we observed that although each treatment was able to modulate the microbiota, the combination of *B. longum* BB-46 and pectin was more efficient in decreasing the intestinal NH_4^+ levels and in increasing butyric acid-producing bacteria. These findings indicate that *B. longum* BB-46, especially when combined with the specific citric pectin, might have beneficial impact on human health.

Keywords: human gut microbiota, pectin, *Bifidobacterium longum* BB-46, metabolites, 16S rRNA gene sequencing, SHIME®.

1. Introduction

It is estimated that the human gut harbours 3 to 4 million microbial genes, which equates to approximately 150 times more than the number found in the human genome (Ehrlich, 2010; Lozupone, Stomabaugh, Gordon, Jansson, & Knight, 2012). The gut microorganisms play an important role in the host's health, contributing to homoeostasis of the immune system, conversion of food into useful nutrients and protection against invasion by pathogenic microorganisms (Ehrlich, 2010; Lozupone et al., 2012). However, changes in the microbiota composition, caused by many factors such as place of residence, environmental influences, lifestyle, antibiotic use and diet (Lozupone et al., 2012), have been linked with several diseases (Alonso &

Guarner, 2013). As diet is considered an important factor affecting the gut microbiota composition and therefore the human health (Ramakrishna, 2013), interest in food strategies to improve the human gut microbial ecosystem has been growing. Among different strategies, the use of prebiotics and other fibres, as well as probiotics and combinations of pre- and probiotics has been highlighted (Alonso & Guarner, 2013).

The use of probiotic microorganisms has been extensively investigated in gut disorders. Probiotics are able to improve the immune system, the epithelial barrier function and to produce antibacterial factors (Sherman, Ossa, & Johnson-henry, 2009). Several positive effects of probiotic *Bifidobacterium longum* strains, such as anti-allergy effects (Dev et al., 2008), stimulation of gut immune system bacteria (Makioka, Tsukahara, Ijichi, & Inoue, 2018), and improvements in the intestinal environment and defecation frequency (Yaeshima et al., 1997) have been demonstrated in animal and human intervention studies. Besides hypocholesterolaemic (Abd El-Gawad et al., 2005) and anti-pathogenic effects (Pavlović, Hardi, Slačanac, Halt, & Kocevski, 2006; Silva et al., 2004), strains of *B. longum* BB-46 have also showed good survival rate alone and combined with different by-products of fruits under *in vitro* simulated gastrointestinal test (Bianchi et al., 2018a, Vieira, Badeni, Albuquerque, Biscola, & Saad, 2017), being considered as a probiotic strain (Havas, Kun, Perger-Mészáros, Rezessy-Szabó, & Nguyen, 2015).

Pectins are important water-soluble dietary fibres present in the cell walls of fruits and vegetables. They have been defined as emerging prebiotics demonstrating ability to modulate the gut microbiota, increasing *Bifidobacterium* spp. and butyric acid-producing bacteria, as for example *Faecalibacterium* and other members of *Ruminococcaceae* family (Bianchi et al., 2018b; Gómez, Gullón, Yáñez, Schols, & Alonso, 2016; Gullón et al., 2013; Henningsson, Margareta, Nyman, & Björck, 2002; Jiang et al., 2016; Tian et al., 2017). Moreover, pectins can increase probiotic bacteria survival, slow gastric transit and decrease glycemic index (Larsen, Cahú, Saad, Blennow, & Jespersen, 2018; Olano-Martin, Gibson, & Rastall, 2002). Pectins can be classified as high-methylated (HM) pectin, with degree of methyl esterification

(DM) > 50 %, and low-methylated (LM) pectin, with DM < 50 % (Onumpai, Kolida, Bonnin, & Rastall, 2011; Sila et al., 2009). These structural variations can lead to different effects on the colonic microbiota (Onumpai et al., 2011).

Dynamic colonic models have been used to study potential beneficial effects of pre- and probiotics in the intestinal microbiota (Bianchi et al., 2014; Bianchi et al., 2018b; Pham & Mohajeri, 2018). *In vitro* models, such as the Simulator of Human Intestinal Microbial Ecosystem (SHIME®), simulate the human gastrointestinal tract allowing investigation of the intestinal microbial composition and its metabolites production and functionalities (Molly, Woestyne, Smet, & Verstraete, 1994), avoiding the use of both invasive techniques in humans and animal ethical management (Parvova, Danchev, & Hristov, 2011). The influence of *Bifidobacterium* spp. and a few types of pectin on the gut microbial community has been demonstrated in several *in vitro* and *in vivo* preclinical and clinical studies (Ji et al., 2016; Maldonado-Gómez et al., 2016; Medina, De Palma, Ribes-Koninckx, Calabuig, & Sanz, 2008; Moya-Pérez, Neef, & Sanz, 2015; Tian et al., 2017). However, as far as we know, changes in the gut microbiota of healthy humans promoted by combination between *Bifidobacterium longum* and citric pectin have not been described. As such, the aim of this study was to evaluate the effects of the probiotic *Bifidobacterium longum* BB-46 combined with a LM pectin from lemon on the gut microbiota modulation using the SHIME® model.

2. Materials and Methods

2.1. Bacterial strain and pectin

The probiotic strain *Bifidobacterium longum* BB-46 was provided by Chr. Hansen A/S (Hoersholm, Denmark) and maintained in de Man Rogosa Sharpe (MRS) broth with 20 % (w/w) glycerol at -80 ºC. Before the experiments, the strain was grown in MRS broth supplemented with L-cysteine (0.05 %) at 37 ºC for 24 hours. Bacterial cells were collected by centrifugation (3,000 × g for 10 min), washed and resuspended in saline solution (NaCl 0.85 % (w/v)). The pectin used in this study was a harsh-extracted LM lemon pectin provided by CP Kelco ApS (Lille Skensved, Denmark).

2.2. *The Simulator of Human Intestinal Microbial Ecosystem (SHIME®)*

The SHIME® (registered trade name from Ghent University and ProDigest) is a simulator of human intestinal microbial ecosystem in which environmental conditions (pH, residence time and temperature) are controlled by a software (Molly et al., 1994). It consists of five double-jacketed vessels simulating the stomach, duodenum and ascending, transverse and descending colons. The five vessels were continuously stirred with a magnetic stirrer and the temperature was kept at 37 °C. The system was maintained anaerobically through a daily N_2 flushing of 30 min. The pH in the ascending (pH between 5.6–5.9), transverse (pH between 6.1–6.9) and descending colon (pH between 6.6–6.9) was automatically adjusted by addition of NaOH 1 M or HCl 0.1 M (Molly et al., 1994; Possemiers, Verthé, Uyttendaele, & Verstraete, 2004). Each compartment of the colon was filled with the SHIME® feed (carbohydrate-based medium that allows adaptation of microorganisms to specific environmental conditions of the colon in terms of pH range, retention time and available carbon sources) in specific volumes as previously described (Bianchi et al., 2014; Molly et al., 1994). The stomach conditions as well as the use and preparation of pancreatic juice (composed by Oxgall 6.0 g/L, $NaHCO_3$ 12.5 g/L and pancreatin 0.9 g/L) were based on the studies conducted by Bianchi et al. (2014) and Bianchi et al. (2018a).

2.3. *Experimental protocol using the SHIME® model*

By using the SHIME® model, we evaluated the impact of the probiotic bacterium *Bifidobacterium longum* BB-46 alone (T1) and combined with lemon pectin (T2) on the intestinal microbiota. Before starting the experiments, the colon vessels (simulating the ascending, transverse and descending colons) were inoculated with faecal microbiota from 3 healthy adults (BMI between 18.5 and 25 kg/m^2 and waist circumference < 80 cm). The faeces samples (≈ 13.5 g from each donor) were collected, diluted in 200 mL of phosphate buffer containing Na_2HPO_4 0.05 mol/L, NaH_2PO_4 0.05 mol/L and Na-thioglicolate 0.1 % (pH 6.5), stirred for 10 min in an homogenizer (Stirrer model 130, Norte Científica, São Paulo, BR.) and centrifuged

(3,000 × g for 15 min). The supernatants were subsequently added (40 mL) to the three colon vessels (Bianchi et al., 2014; Molly et al., 1994). All donors had no history of antibiotic treatment (at least 6 months prior to this study) and had not consumed probiotic products over the past 3 to 6 months.

The experimental protocol included a two-week control period (without intervention) after inoculation of the stool sample in the three colon vessels. This period allows the microbial community to adapt to the prevailing nutritional and physicochemical conditions in each region of the colon and is necessary for microbiota stabilization (Molly et al., 1994). During the control period, 200 mL of the SHIME® feed entered through the system two times a day for two weeks. After this period, the first vessel (stomach) was fed with *B. longum* BB-46 (final stomach concentration of 10^8 CFU/mL) (T1) during one week, followed by one more week of feeding the stomach with *B. longum* BB-46 combined with 2 % (w/v) of lemon pectin (T2). Both additions were carried out along with 200 mL of the SHIME® feed twice a day, i.e. 8 g of pectin per day. The stomach contents were later automatically and sequentially transferred to duodenum, ascending, transverse and descending colons. After the T2 period, one-week post-treatment (PT) was performed (200 mL of the SHIME® feed through the system twice a day).

2.4. Microbiota composition using 16S rRNA gene sequencing

Microbiota profiles of each experimental period (control, treatments and post-treatment) from the three colon vessels of the SHIME® model (two technical replicates per treatment) were determined using tag-encoded 16S rRNA gene sequencing NextSeq (Illumina, San Diego, USA).

The microbiota samples from SHIME® (4 mL) were centrifuged (10,000 × g for 5 min) and the pellet freeze-dried. Isolation of total bacterial DNA was performed using PowerLyzer@PowerSoil DNA Isolation Kit (Qiagen, Valencia, USA) according to its manual. The DNA library for amplicon sequencing was prepared as described by Williams et al. (2017). Briefly, the V3 region (≈190 bp) of the 16S rRNA gene was PCR amplified using primers compatible with the Nextera Index Kit (Illumina) (Primers NXt_388 and

NXt_518). The PCR I was performed using 12 µL of AccuPrime SuperMix II (Life Technologies, Camarillo, USA), 5 µL of genomic DNA (≈20 ng/µL), 0.5 µL of each primer (10 µM) and nuclease-free water to a total volume of 20 µL. The DNA was amplified using the following setup: initial denaturation at 95 °C for 2 min, 33 cycles of denaturation at 95 °C for 15 s, annealing of primer at 55 °C for 15 s and elongation at 68 °C for 30 s, followed by an extension at 68 °C for 4 min and final cooling to 4 °C. To incorporate primers with adapters and indexes, a second PCR was performed (PCR II) using 12 µL of Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific,Tewksbury, USA) and 2 µL of primers P5 and P7 (Nextera Index Kit). PCR reactions contained 2 µL PCR I product and nuclease-free water for a total volume of 25 µL. The DNA was amplified using the following setup: initial denaturation at 98 °C for 1 min, 13 cycles of denaturation at 98 °C for 10 s, annealing of primer at 55 °C for 20 s and elongation at 72 °C for 20 s, followed by an extension at 72 °C for 5 min and final cooling to 4 °C. After PCR II, the amplified fragments with adapters and tags were purified using AMPure XP beads (Beckman Coulter Genomic, Indianapolis, USA), providing a size selection step and removing short library fragments (Williams et al., 2017).

The sequencing was performed on the Illumina NextSeq instrument as a part of a flowcell using a 2 × 150 cycles MID output kit V2 (Illumina, San Diego, USA). The raw dataset containing pair-ended reads with corresponding quality scores were merged and trimmed using settings as described by Williams et al. (2017). Quantitative Insight Into Microbial Ecology (QIIME) open source software package (1.7.0 and 1.8.0) was used for subsequent analysis steps (Caporaso et al., 2011). The UPARSE pipeline was employed to purge the dataset of chimeric reads and to construct de novo Operational Taxonomic Units (OTU). As a reference database, the Green Genes 16S rRNA gene collection was used (McDonald et al., 2012). Aiming to normalize different depths of sequencing samples, the matrices' abundance of taxonomic units, of each sample, was divided by the total number of pairings. By means of the alpha rarefaction workflow, the alpha diversity measures, expressed as

observed species (sequence similarity of 97 % OTUs) values, were computed for rarefied OTU tables (23,000 reads/sample) (Williams et al., 2017).

2.5. Metabolite production: short-chain fatty acids (SCFAs) and ammonium ions (NH_4^+)

Samples (50 mL) were collected weekly from each compartment (ascending, transverse and descending colons) during the control, treatments T1 and T2 and post-treatment period for SCFAs and NH_4^+ analyses. The analyses were carried out in triplicates.

The determination of SCFAs was previously described by Adorno, Hirasawa, & Varesche (2014). For this purpose, we used a gas chromatograph equipped with a flame-ionization gas detector, a capillary split/splitless injector and an HP-INNOWAX column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, La Jolla, USA). Hydrogen was the carrier gas at a flow rate of 1.45 mL/min. The temperature of both the detector and injector was 240 °C (Adorno et al., 2014).

NH_4^+ levels were determined by a selective ion meter (HI 4101 model, Hanna Instruments, Leighton Buzzard, UK) coupled with an ammonium ion-selective electrode (Orion 95–12). The samples (10 mL) collected from each simulated region of the colon were added to 0.2 mL of ammonia pH-adjusting ionic strength adjuster (ISA) solution (Orion, Thermo Fisher, Millersburg, USA) and then the NH_4^+ levels (mmol/L) were computed.

2.6. Statistical analysis

One-way ANOVA followed by a Tukey post-hoc test ($p < 0.05$) was applied to test significant differences between results (composition of microbiota, production of SCFAs and NH_4^+) using Biostat 5.0 software (IBM, Belém, BR) (Ayres, Ayres, Ayres, & Santos, 2007). A simple correspondence analysis was used to observe correlation between the different treatments and the microbiota composition using Minitab Software (State College, USA) (Minitab, 2010). Correlations between short-chain fatty acids, NH_4^+ production and

specific bacterial genera were determined by Spearman correlation test ($p < 0.05$) using the open-source RStudio software program (RStudio, 2017).

3. Results

3.1. Richness and diversity of the gut microbiota

A total of more than 1.7 million high-quality reads (> 200 bp) were obtained from 24 microbiota samples collected during control periods, treatments with *Bifidobacterium longum* BB-46 alone and in combination with the pectin and post-treatments using the Nextseq Illumina sequencing. After normalizing the data, 552,000 sequences were produced, resulting in 23,000 sequences per sample. For each sample, a rarefaction curve was constructed to evaluate sequencing depth and species richness (Fig. S1). The curves showed that the sequencing depth was enough to cover most of the bacteria in the SHIME® samples.

Supplementary Fig. S2 shows alpha diversity within microbiota samples expressed by indices Chao1 (species richness) and Shannon (diversity). The treatment with *B. longum* BB-46 (T1) showed low richness in all colon vessels (average Chao1 of 242) but high diversity (average Shannon of 5.50) compared to controls (averages of 298 and 4.8 for Chao1 and Shannon, respectively). Treatment with *B. longum* BB-46 with pectin (T2) showed relatively low richness and diversity (averages of 216 and 3.92).

3.2. Changes in the gut microbiota during the treatments with B. longum BB-46 and B. longum BB-46 combined with citric pectin using 16S rRNA gene sequencing

Fig. 1 shows the relative abundance of the main bacterial families in different regions of the SHIME® model: ascending (V3), transverse (V4) and descending (V5) colon vessels. High relative abundance of *Bifidobacteriaceae* (V3 = 35 %, V4 = 38 % and V5 = 32 %) and *Coreobacteraceae* families (V3 = 14 %, V4 = 7 % and V5 = 5 %), both belonging to *Actinobacteria* phylum, as well as *Burkholderiaceae* (V3 = 10 %, V4 = 17 % and V5 = 17 %)

and *Enterobacteraceae* families (V3 = 17 %, V4 = 11 % and V5 = 10 %), both belonging to *Proteobacteria* phylum, were found during the control period.

An increase in the abundance of *Bacteroidaceae* (*Bacteroidetes* phylum), *Lachnospiraceae* (*Firmicutes* phylum), *Enterobacteriaceae* (*Proteobacteria* phylum) and *Lactobacillaceae* (*Firmicutes* phylum) families, as well as a reduction in the abundance of *Bifidobacteriaceae* were found in all colon vessels during the treatment with *B. longum* BB-46 (T1) (Fig.1). An increase in the relative abundance of *Lactobacillaceae* (from 0.28 % to 37.51 %) and *Enterobacteriaceae* (from 16 % to 33 %) was observed during the treatment using *B. longum* BB-46 with pectin (T2) in V3, while a high increase of *Ruminococcaceae* (from 3 % to 73 % in V4 and from 10 % to 43 % in V5) and a reduction of *Bacteroidaceae* and *Enterobacteriaceae* families was observed in the same experimental period (T2) in V4 and V5. The one-week post-treatment (PT) revealed similar results to treatment T2 in V3 and V5, indicating that the modifications obtained during T2 were maintained (Fig. 1).

A simple correspondence analysis was performed to investigate the association between the treatments and the microbiota composition (Fig. 2). The correspondence analysis was sufficient to interpret the results as seen from the explained variance (Component 1 = 36.3 % and Component 2 = 29.8 %). The control period of ascending (V3), transverse (V4) and descending (V5) colons were clustered according to the high abundance of *Bifidobacteriaceae*, *Burkholderiaceae, Streptococcaceae, Erysipelotrichaceae* and *Coreobacteriaceae* families. The high proportion of *Lachnospiraceae*, *Bacteroidaceae* and *Clostridiaceae* families found after treatment T1 accounted for the clustering of samples during this treatment. Grouping of the treatment T2 and PT from V3 was due to the high abundance of *Enterobacteriaceae* and *Lactobacillaceae* families. The *Ruminococcaceae*, *Rikenellaceae* and *Eubacteriaceae* families were associated with treatment T2 and PT from V4 and V5.

Changes in abundances of various bacterial taxa after treatments T1 and T2 were significantly different ($p < 0.05$) from controls (Fig. 3). The treatment with *B. longum* BB-46 (T1) stimulated proliferation of genera *Lactobacillus* and

Dorea (Lachnospiraceae family), *Bacteroides*, *Enterobacter*, as well as an unclassified genus of *Lachnospiraceae* and a reduction in *Streptococcus* in all three colon vessels. Genus *Eubacterium* was also stimulated during T1 but only in V5. The treatment with *B. longum* BB-46 combined with pectin (T2) significantly stimulated the genera *Lactobacillus, Veilonella*, *Enterobacter*, *Klebsiella* and *Erwinia* in V3, and significantly increased *Faecalibacterium, Eubacterium* and unclassified genus of *Ruminococcaceae* in V4 and V5. Furthermore, a depletion of *Enterobacter*, *Klebsiella* and *Erwinia,* as well as *Bacteroides, Peptinophilus* and *Streptococcus* was found during treatment T2 in V4 and V5, whereas genus *Lactobacillus* was stimulated in V5.

3.3. Metabolic activity

Levels of acetic and butyric acids had a significant decrease ($p < 0.05$) during the treatments with *B. longum* BB-46 (T1) and *B. longum* BB-46 with pectin (T2) in the ascending colon, whereas no visible changes were noted for propionic acid (Fig. 4). On the other hand, a significant increase ($p < 0.05$) in butyric acid was observed during the treatment T2 in the transverse and descending colons. There were no significant visible changes of propionic and acetic acids in the transverse and descending colons during treatments T1 and T2, except for a reduction of acetic acid in the transverse colon during treatment T1.

A significant decrease in NH_4^+ production was observed during treatment T2 in all colon vessels compared to the other periods of study (Table 1). Apparently, treatment T1 did not affect the NH_4^+ production. The intestinal levels of NH_4^+ during PT were higher ($p < 0.05$) than those found during treatment T2, but lower than those found in the control period ($p < 0.05$), thus showing some residual effects of treatment T2 (Table 1).

3.4. Correlation between metabolite production and bacterial genera during the treatments

We assessed the correlation between the relative abundance of bacterial genera, SCFAs and NH_4^+ to identify the genera that might contribute to the

production of SCFA and NH_4^+ (Fig. 5). Genera *Eubacterium, Dorea* and *Faecalibacterium* positively correlated (*r*-value = 0.60 – 0.80, $p < 0.05$) with butyric acid levels, while an unclassified genus of *Ruminococcaceae* had a positive correlation ($p < 0.05$) with acetic (*r*-value = 0.66) and butyric acids (*r*-value = 0.95) production. The relative abundance of genera *Klebsiella*, *Enterobacter*, *Erwinea* and an unclassified genus of *Enterobacteriaceae* showed negative correlation (*r*-value = -0.52 – -0.95, $p < 0.05$) with acetic, propionic and butyric acids. Numbers of *Streptococcus*, *Bacteroide*s, *Clostridium* and *Peptoniphilus* positively correlated with NH_4^+ production (*r*-value between 0.60 and 0.73, $p < 0.05$) (Fig. 5).

4. Discussion

The effects of *B. longum* BB-46 in combination with lemon pectin (T2) on microbiota composition and activity were evaluated using a gut microbiome model (SHIME®). We also evaluated the effects of *B. longum* BB-46 alone (T1) on the intestinal microbiota. The treatment T1 stimulated genera of *Firmicutes* phylum, such as *Lactobacillus* (*Lactobacillaceae* family), an unclassified genus of the *Lachnospiraceae* and *Dorea (Lachnospiraceae* family). The increased abundance of *Lachnospiraceae* members during T1 can most likely be explained by the production of lactate and/or acetate by the *B. longum* BB-46, which can be used by *Lachnospiraceae* members, stimulating their proliferation (Belenguer et al., 2006; Falony, Vlachou, Verbrugghe, & De Vuyst, 2006; Flint, Duncan, Scott, & Louis, 2015). During the same treatment (T1), an increase in the abundance of genus *Bacteroides* was observed. Bifidobacteria have been reported to modulate the microbiota in favour of saccharolytic bacteria, such as *Bacteroides,* possibly by cross-feeding (Turroni et al., 2016), which could probably explain our result. Increased abundances of *Bacteroides* in this study can be also due to production of exopolysaccharides by some strains of *B. longum,* which can be consumed by *Bacteroides* species, increasing their abundance (Ríos-Covián et al., 2016a).

B. longum BB-46 combined with pectin (T2) greatly stimulated an unclassified genus of the *Ruminococcaceae* family in the transverse and

descending colons. The correlation analysis revealed positive correlation between the unclassified genus of the *Ruminococcaceae* family and levels of butyric acid (Fig. 5). These results are in accordance with previous findings showing that family *Ruminoccocaeae* includes the major butyrate-producing species, having capacity to degrade pectin (Lopez-Siles et al., 2012; Louis, Young, Holtrop, & Flint, 2010). Tian et al. (2017), Gómez et al. (2016) and Jiang et al. (2016) studied the effects of different pectins on the gut microbiota and also demonstrated an increase in the abundance of *Ruminococcacea* members, such as *Faecalibacterium* and, consequently, in butyric acid production.

Other butyrate-producing bacteria, such as *Eubacterium* and *Faecalibacterium* (Louis et al., 2010; Moens, Verce, & De Vuyst, 2017), were similarly enriched during treatment T2 in the transverse and descending colons, and positively correlated with butyric acid. The increase in butyric acid during treatment T2 can also be attributed, at least to a certain extent, to the cross-feeding interactions between the probiotic strain *B. longum* BB-46 and other bacteria, such as *Faecalibacterium* and *Eubacterium,* stimulated by the presence of pectin. This assumption is based on previous studies showing that the formation of butyrate by *Faecalibacterium prausnitzii* is enhanced in the presence of bifidobacteria, demonstrating the cross-feeding between *Bifidobacterium* and *F. prausnitzii* (Ríos-Covián et al., 2016b). Moreover, Moens et al. (2017) demonstrated that some bacterial strains, such as *Eubacterium,* are able to consume lactate generated by other bacteria, such as *Bifidobacterium* spp., converting the lactate into butyric acid. These findings suggest an indirect interaction between the citric pectin and *B. longum* BB-46, resulting in stimulation of different bacterial genera and increase of butyric acid. High production of butyrate is commonly considered as beneficial since this metabolite can have a positive effect on the human health, such as the ability to increase the intestinal barrier function (Brahe, Astrup, & Larsen, 2016) and protect against colon carcinoma (Hijova & Chmelarova, 2007).

Production of propionic and acetic acids in the transverse and descending colons seemed to be unaffected by treatments T1 and T2 in this study, except

for a reduction in acetic acid levels in the transverse colon during treatment T1. Although a positive correlation was found between acetic acid production and the unclassified genus of *Ruminococcaceae*, which was increased during treatment T2, no significant increase in acetic acid was observed during this treatment. Consistent levels of propionic and acetic acids can be related to bacterial cross-feeding and complex interactions among gut microorganisms (Duncan, Louis, & Flint, 2004; Ríos-Covián et al., 2016b). Duncan et al. (2004), for example, evidenced an *in vitro* conversion of acetate to butyrate by *Faecalibacterium* spp., which can probably explain the increase in butyric acid and no alteration in acetic acid found in our study.

Reduction of acetic and butyric acids in the ascending colon during treatments T1 and T2 was associated with the high levels of *Enterobacteriaceae* in this region, which correlated negatively with butyric and acetic acids. Likewise, Van Der Wielen, Biesterveld, Hofstra, Urlings, & Van Knapen (2000) found a negative correlation between SCFAs and numbers of *Enterobacteriaceae,* which can be explained by the competition between the members of *Enterobacteriaceae* and butyrate/acetate-producing bacteria (Van Der Wielen et al., 2000).

Curiously, the highest amount of *Klebsiella* and other bacterial genera belonging to *Enterobacteriaceae* family was found in the ascending colon during treatment T2, while a significant reduction in these genera was observed in the transverse and descending colons. These results indicate that the ascending colon conditions (pH between 5.6 – 5.9 and available carbohydrates from the pectin and the feed medium) were preferable for growth of *Enterobacteriaceae* members, such as *Klebsiella*. Specifically, the pH of the ascending colon, corresponding to the optimum pH of polygalacturonase (a pectin-degrading enzyme found in some species of *Klebsiella*), might favour pectin utilization and consequent growth of *Klebsiella,* usually considered as pathogenic bacteria (Yuan et al., 2012).

The reduction of NH_4^+ during treatment T2 could be attributed to the decrease in the relative abundance of *Streptococcus, Peptoniphilus, Bacteroides* and *Clostridium* spp., which are classified as proteolytic bacteria

(Dai, Wu, & Zhu, 2011). Proteolytic bacteria use amino acids as sources of nitrogen and carbon, generating NH_4^+ as one of the intermediate or final metabolites (Macfarlane & Cummings, 1991). The simple addition of a carbohydrate (pectin) during the intervention could also have contributed to the decrease in NH_4^+, once inhibition of amino acids fermentation in favour of carbohydrate fermentation can occur due to the preference of gut microorganisms to carbohydrates (Ito, Kimura, Deguchi, Yajima, & Kan, 1993). Although an increase in *Bacteroides* and *Clostridium* spp. was observed in the transverse and descending colons during treatment T1, no significant visible changes on NH_4^+ production were found. This result can be explained by the balance between an increase in some proteolytic bacteria and a decrease in others, such as *Streptococcus*, or the use of NH_4^+ by other groups of bacteria. The decrease in NH_4^+ levels in the colon is considered beneficial to the host's health since high amounts of NH_4^+ can promote colon carcinogenesis by increasing DNA synthesis, culminating in changes in the morphology and intermediary metabolism of intestinal cells (Davila et al., 2013; Ichikawa & Sakata, 1998).

5. Conclusions

This study provided novel insights on the synbiotic potential of *Bifidobacterium longum* BB-46 with lemon pectin (T2) to modulate the gut microbiota. We observed that each treatment had a different effect on gut microbiota. Treatment with *B. longum* BB-46 alone (T1) mainly stimulated members of *Lachnospiraceae* and *Bacteroidaceae* families, whereas the combination of *B. longum* BB-46 with pectin stimulated specific bacterial groups able to degrade pectin and to increase the production of butyric acid. Using the intestinal model SHIME®, this study indicated that the combination (T2) could positively modulate the composition and metabolic activity of the gut microbiota. Although in this study we could deduce that the increase of butyric acid as well as of specific bacterial genera, such as *Faecalibacterium*, occurred partly due to an interaction between the probiotic *B. longum* BB-46 and the pectin, new studies using the *B. longum* BB-46 and pectin alone as

well as different types of pectins are welcome to confirm the beneficial effects of *B. longum* BB-46 combined or not with citric pectins on human health.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1

NH_4^+ production (mmol/L) by the microbiota in SHIME® colon vessels.[a]

	Control	**Treatment with BB-46**	**Treatment with BB-46 and pectin**	**Post-treatment**
Ascending colon	16.63±0.11^{A}	15.90±0.16^{A}	4.23±0.27^{C}	7.84±0.19^{B}
Transverse colon	20.51±0.03^{A}	20.87±0.38^{A}	6.31±0.11^{C}	17.21±0.15^{B}
Descending colon	22.73±0.38^{A}	21.20±0.54^{A}	8.58±0.85^{C}	17.37±0.07^{B}

[a] Different letters represent statistical difference ($p < 0.05$) between treatments for the same vessel (One-way ANOVA and Tukey post-hoc test).

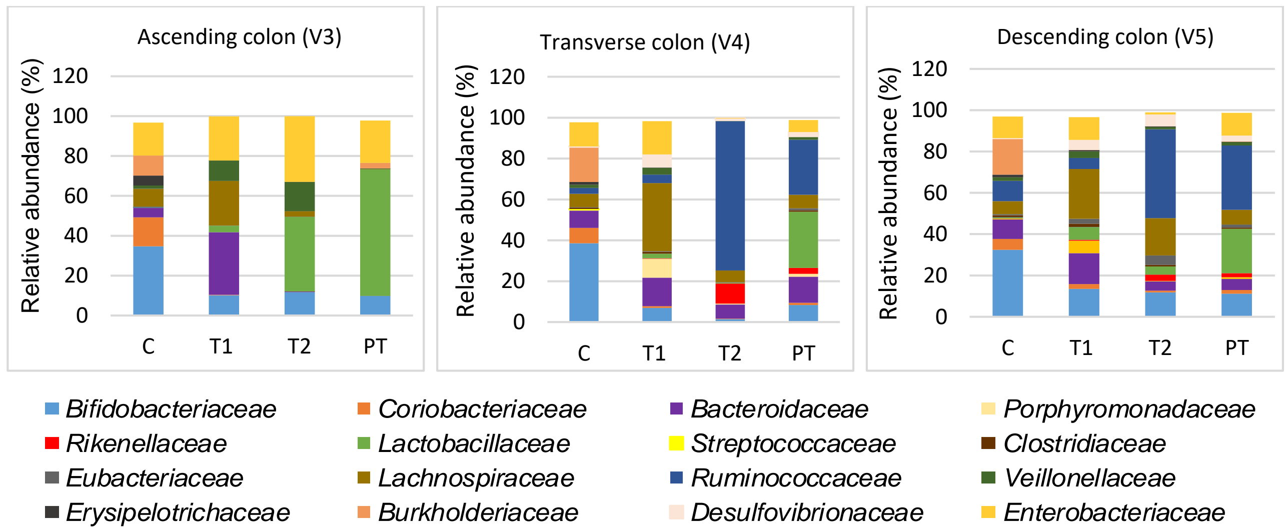

Fig. 1. Relative abundance of bacterial families in ascending (V3), transverse (V4) and descending (V5) colon vessels of SHIME®. C= control period; T1= treatment with *Bifidobacterium longum* BB-46; T2= treatment with BB-46 and pectin; PT= post-treatment.

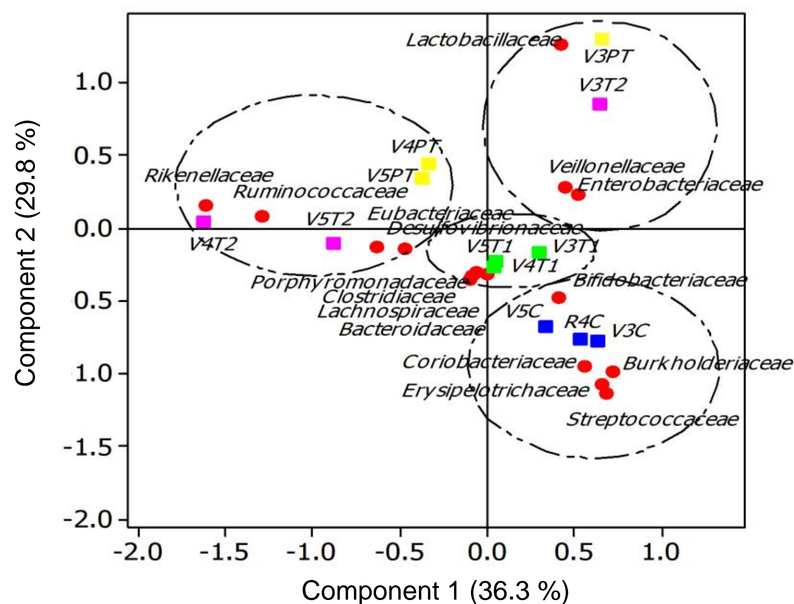

Fig. 2. Simple correspondence analysis showing relationship between the treatments and bacterial families in the three vessels of SHIME® model. C= control period; T1= treatment with *Bifidobacterium longum* BB-46; T2= treatment with BB-46 and pectin; PT= post-treatment; V3= ascending colon; V4= transverse colon; V5= descending colon.

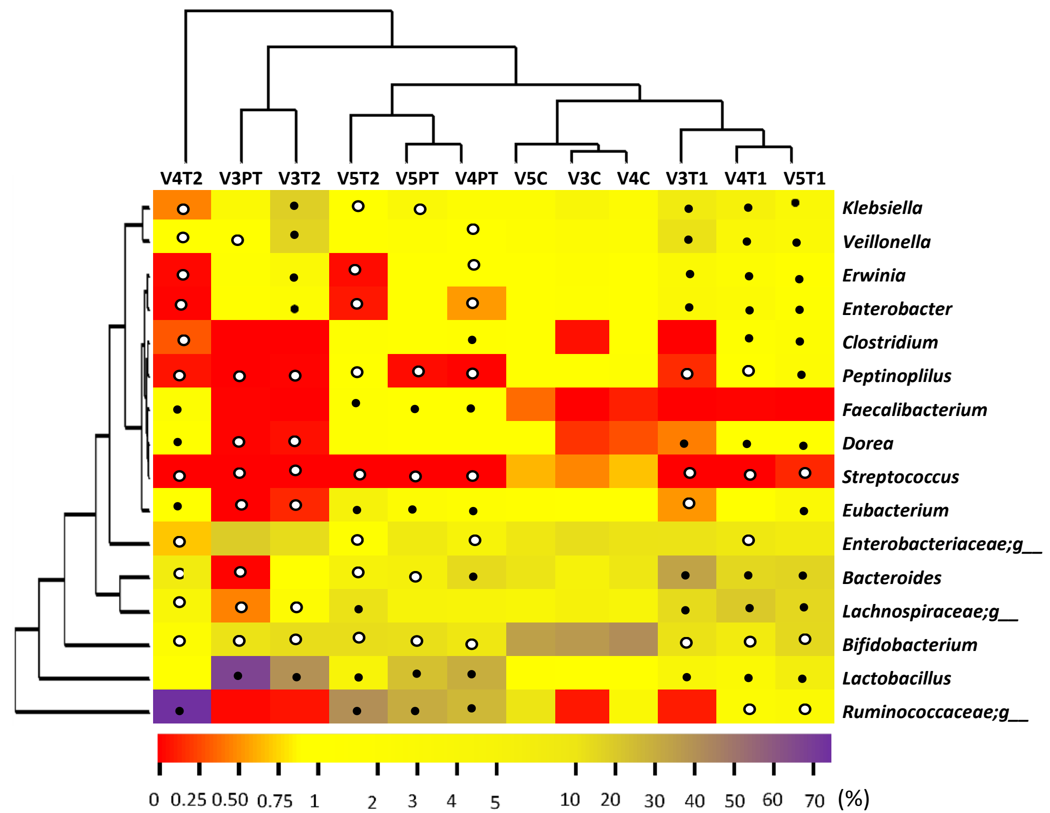

Fig. 3. Cluster analysis of the relative abundance of bacterial genera significantly changed during treatments in SHIME® colon vessels. Significant increase (p < 0.05) compared to control is represented by "●" and significant reduction (p < 0.05) compared to control is represented by "○" (One-way ANOVA and Tukey post-hoc test). C= control period; T1= treatment with *Bifidobacterium longum* BB-46; T2= treatment with BB-46 and pectin; PT= post-treatment. V3= ascending colon; V4= transverse colon; V5= descending colon. Unclassified genera are represented by "g_".

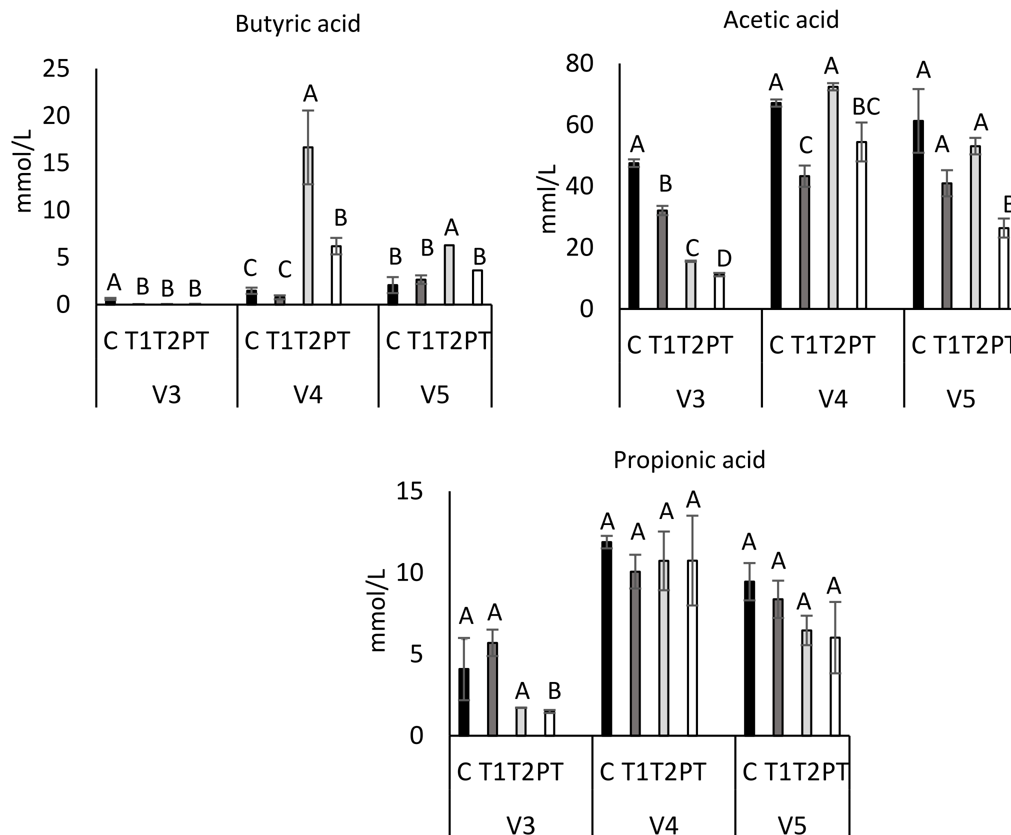

Fig. 4. Production of butyric, acetic and propionic acids by the microbiota in SHIME® colon vessels. Different letters denote statistical differences ($p < 0.05$) between the treatments for the same vessel (One-way ANOVA and Tukey post-hoc test). C= control period; T1= treatment with *Bifidobacterium longum* BB-46; T2= treatment with BB-46 and pectin; PT= post-treatment; V3= ascending colon; V4= transverse colon; V5= descending colon.

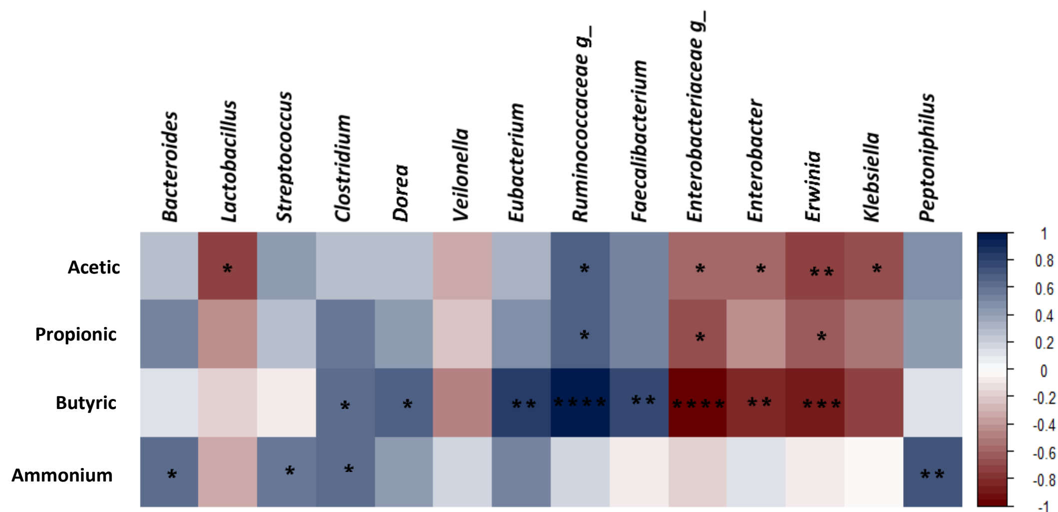

Fig. 5. Correlation between SCFA production, ammonium ions, and bacterial genera. Positive correlations are represented by blue color and negative correlations by red color. Color intensity are proportional to the correlation coefficients (Spearman correlation). Significant correlations are indicated by *($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). Unclassified genera are represented by "g_ "

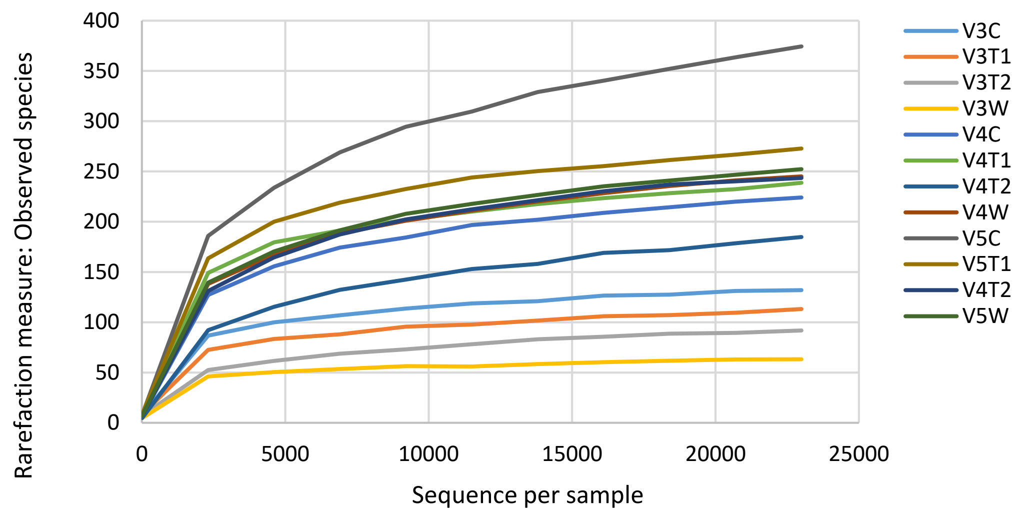

Fig. S1. Rarefaction curves of the observed species in the microbiota in SHIME® model. C= control period; T1= treatment with Bifidobacterium longum BB46; T2= treatment with BB46 and pectin; PT= post-treatment; V3= ascending colon; V4= transverse colon; V5= descending colon

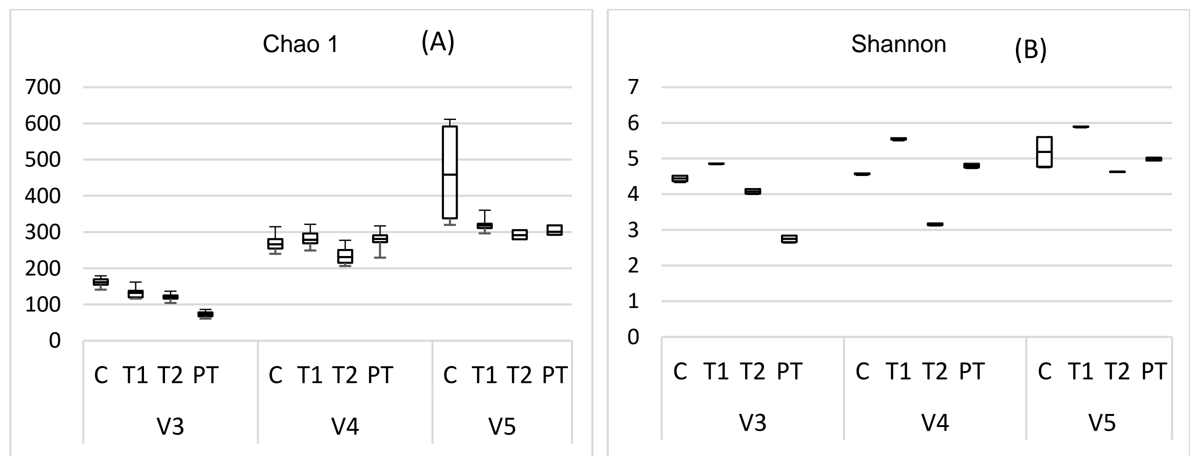

Fig. S2. Alpha diversity of colon microbiota in SHIME® model presented by (A) Chao1 index and (B) Shannon index based on 23,000 sequences per sample. C= control period; T1= treatment with *Bifidobacterium longum* BB-46; T2= treatment with BB-46 and pectin; PT= post-treatment; V3= ascending colon; V4= transverse colon; V5= descending colon.

Chapter 6.

Modulation of gut microbiota from obese individuals by in-vitro fermentation of citrus pectin in combination with *Bifidobacterium longum* BB-46

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Modulation of gut microbiota from obese individuals by in vitro fermentation of citrus pectin in combination with *Bifidobacterium longum* BB-46

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Keywords *Bifidobacterium longum* BB-46 • obese microbiota • pectin • SHIME® model • 16S rRNA sequencing.

Abstract

This study aimed to evaluate the effects of three treatments, i. e., *Bifidobacterium longum* BB-46 (T1), *B. longum* BB-46 combined with the pectin (T2), and harsh extracted pectin from lemon (T3) on obesity-related microbiota using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). The effects of the treatments were assessed by the analysis of the intestinal microbial composition (using 16S rRNA gene amplicon sequencing) and the levels of short chain fatty acids (SCFAs) and ammonium ions (NH_4^+).

Treatments T2 and T3 stimulated members of the *Ruminococcaceae* and *Succinivibrionaceae* families, which were positively correlated with an increase in butyric and acetic acids. Proteolytic bacteria were reduced by the two treatments, concurrently with a decrease in NH_4^+. Treatment T1 stimulated the production of butyric acid in the simulated transverse and descending colon, reduction of NH_4^+ as well as the growth of genera *Lactobacillus*, *Megamona*s and members of *Lachnospiracea.* The results indicate that both *B. longum* BB-46 and pectin can modulate the obesity-related microbiota; however, when the pectin is combined with *B. longum* BB-46, the predominant effect of the pectin can be observed. This study showed that the citric pectin is able to stimulate butyrate-producing bacteria as well as genera related with anti-inflammatory effects. However, prospects clinical studies are necessary to evaluate the anti/pro-obesogenic and inflammatory effects of this pectin for future prevention of obesity.

Introduction

Obesity is a global public health concern and can result in many health complications like insulin resistance, type II diabetes, dyslipidaemia, hepatosteatosis, and hypertension (Flegal et al. 2015). The metabolic syndrome, characterized by the association of risk factors for cardiovascular disease, peripheral vascular disease and diabetes, is highly prevalent in obese individuals and is directly related to a sedentary lifestyle, along with unhealthy eating behaviour (Kushner and Choi 2010).

The recent studies have demonstrated the interplay between the composition of intestinal microbiota and pro-inflammatory response, metabolic disturbances, gut barrier, and energy balance (Ley et al. 2006; Fleissner et al. 2010; Bomhof and Reimer 2015), showing that the human gut microbiota has a crucial role in the onset and establishment of obesity (Rosenbaum et al. 2015). The precise role of the gut microbiota in obesity is still being investigated, but it is known that changes in the composition of the gut microbiota as a consequence of the ingestion of high-fat diets may lead to

lipogenesis (Bäckhed et al. 2007), increased gut permeability of lipopolysaccharides (LPS), and chronic inflammation (Cani et al. 2008).

The intake of dietary fibre can modulate the microbiota, protecting against several health complications such as large bowel and stomach carcinoma, type 2-diabetes, metabolic syndrome, and cardiovascular disease (Trepel 2004; Anderson et al. 2009). The term ‘‘dietary fibre’’ includes a number of polymeric plant materials (polysaccharides, oligosaccharides, lignin, and compounds associated with the plant cell wall) which have beneficial physiological effects, including laxation as well as attenuation of blood glucose and cholesterol concentrations (DeVries et al. 2001).

Pectins are complex hetero-polysaccharides (Voragen et al. 2009), currently acknowledged as emerging prebiotics which are able to modulate the microbiota, including increases in bacterial species like *Faecalibacterium prausnitzii* or *Roseburia intestinalis* (Gómez et al. 2016). Furthermore, pectins slow the gastric transit, moderate the glycaemic index, help to control energy intake, and, in some cases, they may have the potential to reduce the risk of colon cancer and cardiovascular diseases (Olano-Martin et al. 2002). According to Wicker and Kim (2015), pectin is fermented by colonic bacteria generating short-chain fatty acids. Although some studies have linked short chain fatty acids (SCFAs) to obesity, showing higher total amount of SCFA in the obese microbiota, especially regarding acetic acid (Turnbaugh et al. 2006; Schwiertz et al. 2010; Rahat-Rozenbloom et al. 2014; Perry et al., 2016), there is a strong indication that acetate, propionate and butyrate have a protective action against weight gain, being considered predominantly anti-obesogenic (Chakraborti 2015; Lu et al. 2016; Morrison and Preston 2016). According Wren and Bloom (2007) and Zhou et al. (2008), the formation of SCFA has been associated with increased expression and production of hormones related to anorexigenic effects.

The SCFA production can also be increased by some probiotic strains as *Bifidobacterium adolescentis, B. longum* and *B. pseudocatenulatum,* which are able to modulate the composition of the microbiota, increasing the production of intestinal metabolites including SCFA (Duncan et al. 2004; Falony et al.

2006; An et al. 2011). Several studies have shown that lactate and /or acetate produced by bifidobacteria is used by some bacterial genera such as *Roseburia*, *Eubacterium*, and *Anaeroestipes*, which convert these metabolites into SCFA (Duncan et al. 2004; Falony et al. 2006). Furthermore, according to An et al. (2011), some specific strains of bifidobacteria are related to lipid-lowing effects and reduced body weight and therefore, may be potential therapeutic candidates for obesity management. Although many authors have been associating different bifidobacteria as well as pectin and other fibres to gut microbial and metabolite composition, few studies exist associating the synbiotic effect of *B. longum* BB-46 and pectin, especially on obesity-related microbiota.

The interactions between the intestinal microbiota community and determined probiotic, prebiotic and other fibres can be evaluated by both in vivo and in vitro systems. The in vivo models present some limitations such as high cost, invasive research methods and, in case of animal studies, the impossibility to extrapolate the obtained results to human reality (Parvova et al. 2011). Therefore, many studies have been using in vitro systems, as for example the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®), to study such interactions (Macfarlane and Macfarlane 2007). The SHIME® is a validated in vitro system able to simulate the different parts of the gastrointestinal tract, proven to be very useful in analysing the human intestinal microbial community (Molly et al. 1994; Kontula et al. 2002).

Therefore, the aim of this study was to investigate the impact of a pectin extracted from lemon and the probiotic strain *B.longum* BB-46, in combination or alone, on faecal microbiota collected from obese adults with the use of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®).

Materials and Methods

Bacterial culture conditions and pectin origin

B. longum BB-46 was provided by Christian Hansen (Hoersholm, DK) as fresh cultures and maintained at -80 ºC in MRS broth with glycerol. The strain was activated in MRS broth supplemented with L-cysteine (0.05%) and cultured at

37 ºC for 24 hours. The cells were centrifuged (2600 rpm/10 min., 4 °C) and washed with saline solution (0.85% (w/v) Nacl). The harsh extracted LM pectin from lemon was provided by CP Kelco (Lille Skensved, DK).

Microbiota fermentations in the SHIME®

Microbiota fermentations were performed in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). The SHIME® was developed by researchers from the University of Ghent (Ghent, Belgium) and validated by Molly et al. (1994). It is a system that enables the mimicking and maintenance of the human gastrointestinal microbial diversity in vitro for several months (Molly et al. 1993). In this system, the pH, residence time, and temperature are controlled by a software (Possemiers et al. 2004). The SHIME® is composed by five double-jacketed vessels. These vessels simulate the stomach, the duodenum, and the ascending, transverse, and descending colon.

The pH of the stomach was automatically adjusted by the addition of NaOH 1 M or HCl 1 M. The duodenum was simulated with 60 mL of artificial pancreatic juice (per liter: 12.5 g of $NaHCO_3$, 6 g of Oxgall, and 0.9 g of pancreatin) at a rate of 4 mL/min. for 15 min (Molly et al. 1994; Possemiers et al. 2004). The pH culture of the colon vessels 3 (V3), 4 (V4), and 5 (V5) was automatically adjusted by the addition of NaOH 0.5 M or HCl 0.1 M (Molly et al. 1994; Possemiers et al. 2004).

A magnetic stirrer provided the five vessels to be continuously stirred whereas the temperature was maintained at 37 °C. Continuous anaerobic conditions were provided through daily N_2 flushing for 30 min.

Faecal inoculum

At the beginning of the experiment, the colon vessels (V3, V4, and V5) were inoculated with bacteria from a mixed stool sample of 3 obese adults (BMI between 30 and 39.9 Kg/m^2) and waist circumference >80 cm). All donors had not consumed probiotic products over the past 3 to 6 months and had no history of antibiotic treatment within a period of six months prior to the study.

From the selected donors, 40 g of faeces (~ 13.5 g of each donor) were collected and diluted in phosphate buffer (200 mL) containing 0.05 mol/ L of Na_2HPO_4, 0.05 mol/ L NaH_2PO_4 and 0.1 % of Na-thioglicolate (pH = 6.5). Subsequently, after being stirred in a homogenizer (Stirrer model 130, Norte científica, São Paulo, BR) for 10 minutes, the diluted sample was centrifuged for 15 min. at 3000 rpm. From the supernatant, 40 mL were added to vessels 3, 4, and 5, which were already filled with the SHIME® feed [carbohydrate-based medium that allows the adaptation to specific environmental conditions of the ascending, transverse, and descending colon in terms of pH range, retention time, and available carbon sources] at specific volumes, allowing for the adjustment and stabilization of the microbial community (Molly et al. 1994). SHIME® feed is composed of starch (4.0 g/L (Maizena, São Paulo, BR)), mucin (4.0 g/L (Sigma, Saint Loiuz, USA)), yeast extract (3.0 g/L (Kasvi, São José dos Pinhais, BR)), arabinogalactan (1.0 g/L (Sigma, Saint Loiuz, USA)), xylan (1.0 g/L (Sigma, Saint Loiuz, USA)), peptone (1.0 g/L (Kasvi, São José dos Pinhais, BR)), cysteine (0.5 g/L (Sigma, Saint Loiuz, USA)) and glucose (0.4 g/L) (Synth, Diandema, BR).

Experimental protocol in SHIME® model

The experimental protocol included a 2-week control period after the stool sample inoculation to allow the adaptation of the microbial community to physicochemical and nutritional conditions prevailing in different parts of the colon (Molly et al. 1994) and also to stabilize the microbial community (Possemiers et al. 2004). During this period, 200 mL of the SHIME® feed entered through the system three times a day. After two weeks of stabilization (period where no more changes are observed in the microbiota composition and metabolites production.), the protocol was followed by one week of treatment with *B. longum* BB-46 (T1), one week of treatment with *B. longum* BB-46 and pectin (T2), one week of washout period (W) and one week of treatment with pectin (T3). *B. longum* BB-46, as well as pectin combined with BB-46 and pectin alone were applied together with the SHIME® feed (200 mL)

twice a day. *B. longum* was added at 10^8 CFU mL^{-1}, and the pectin at 2% (w/v). The complete protocol is shown in Fig. 1.

Microbiological analysis employing 16S rRNA gene sequencing

Samples from each compartment of the colon were weekly collected for 16S rRNA gene sequencing. Microbiota profiles of each compartment of the SHIME® were determined using tag-encoded 16S rRNA gene fragment amplicon sequencing. Two technical replicates were performed per treatment.

The DNA isolation of each sample was performed using the "PowerLyzer@PowerSoil DNA Isolation Kit" (Qiagen, Valencia, USA). Samples in a volume of 4.0 mL each were centrifuged (rpm), and the pellet freeze-dried. To start the DNA isolation, 700 uL of bead solution was added to the freeze-dried sample, and the next steps were performed according to the kit's manual.

After the DNA isolation, the Polymerase Chain Reaction I (PCR I) was conducted. The V3 region (~190 bp) of the 16S rRNA gene was amplified using primers compatible with Nextera Index Kit (Illumina) (NXt_388_F:5'TC GTCGGCAGCGTCAGATGTGTATAAGAGACAGACWCCTACGGGWGGCA GCA 3′ and NXt_518_R:5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGATTACCGCGGCTGCTGG-3'). The PCR was performed using 12 µL of AccuPrime SuperMix II (Life Technologies, Camarillo, USA), 5 µL of genomic DNA (~20 ng/ ul), 0.5 µL of each primer (10 µM). Nuclease-free water was added to complete the volume to 20 µL. The following setup was used: 95 °C for 2 min. of initial denaturation, followed by 33 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 15 s, followed by elongation at 68 °C for 30 s, final extension at 68 °C for 4 min. and final cooling to 4 °C (Williams et al. 2017).

To incorporate primers with adapters and indexes, a new PCR was performed (PCR II). PCR II reactions were performed using 2.0 µL of primers P5 and P7 (Nextera Index Kit), 12 µL Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Tewksbury, USA), 2 µL PCR I product and nuclease-free water for a total volume of 25 µL. The following setup was used:

initial denaturation at 98 °C for 1 min., followed by 13 cycles of 98 °C for 10 s, annealing at 55 °C for 20 s and elongation at 72 °C for 20 s, final extension at 72 °C for 5 min. and cooling to 4 °C. After PCR II, the purification of the amplified fragments, along with adapters and tags, was conducted through AMPure XP beads (Beckman Coulter Genomic, Indianapolis, USA) (Williams et al. 2017).

The sequencing was performed on the Illumina NextSeq instrument as a part of a flowcell using a 2x150 cycles MID output kit V2 (Illumina, San Diego, USA). The raw dataset of pair-ended reads and respective quality scores were merged and trimmed with settings, according to Williams et al. (2017). Subsequent analysis steps were conducted using the Quantitative Insight Into Microbial Ecology (QIIME) open source software package (1.7.0 and 1.8.0) (Caporaso et al. 2011). The UPARSE pipeline was employed in order to purge the dataset of chimeric reads, as well as to construct de novo Operational Taxonomic Units (OTU). As a reference database, the green genes (13.8) 16S rRNA gene collection was used, as previously described by McDonald et al. (2012). To normalize different depths of sequencing samples, the matrices abundance of taxonomic units of each sample were divided by the total number of pairings after cutting. For rarefied OTU tables (23,000 reads/sample), alpha diversity measures expressed with an observed species (sequence similarity 97% OTUs) value were computed. For this purpose, the alpha rarefaction workflow was employed.

Short chain fatty acids (SCFAs) and ammonium ions (NH_4^+) analyses

Samples were collected weekly from the vessels V3 (ascending colon), V4 (transverse colon), and V5 (descending colon) for SCFA and NH_4^+ analyses throughout the experimental period (control, treatments, and washout). For the determination of SCFA, 2 mL of the samples were centrifuged (14000 rpm for 5 min.), followed by the dilution of 100 µL of the supernatant in 1900 µL of ultrapure water. Next, NaCl (1 g) and crotonic acid (100 µL) were added, as well as isobutanol (70 µL) and 2 M H_2SO_4 (200 µL). The SCFAs analysis was conducted using a 2010-Model gas chromatograph (Shimadzu, Gifu, JP)

equipped with a split/splitless injector, a flame ionization detector, and a CombiPAL automated sampler for headspace analysis. Separation of the SCFAs took place through a HP-INNOWAX column (30 m× 0.25 mm× 0.25 μm) (Agilent Technologies, La Jolla, USA). Hydrogen was used as the carrier gas, the flow rate was set at 1.45 mL/min., and the temperature of the injector and the detector was maintained at 240 °C (Adorno et al. 2014).

NH_4^+ amounts were determined through a selective ion meter (HI 4101 model, Hanna Instruments, Leighton Buzzard, UK) coupled with an ammonia selective-ion electrode (Orion 95–12). Samples collected from the colon vessels (10 mL) were transferred to 0.2 mL of an ammonia pH ionic strength adjusting solution (Orion, Thermo Fisher, Millersburg, USA). The analyses were performed in duplicates.

Statistical analysis

The significance of the results was determined using a one-way ANOVA, and individual means were compared through the Tukey test ($p < 0.05$), employing Biostat 5.0 software (IBM, Belém, BR) (Ayres et al. 2007). A simple correspondence analysis was used to test the correlation between the treatments and the microbiota composition using the Minitab Software (State College, USA) (Minitab 2010). Correlation analyses were made to correlate the SCFA production and ammonium ions with specific groups of bacteria using the Spearman correlation test. A value of $p < 0.05$ was considered statistically significant. The Spearman correlation test was conducted using the open-source RStudio software program (RStudio 2017). This program was also used to create a heatmap based on the relative abundances of different genera.

Accession number

The sequences have been deposited at European Nucleotide Archive (ENA) under the accession number PRJEB23969.

Results

Sequencing characteristics

The sequencing yielded a total of 2,614,738 reads from 30 microbiota samples collected during treatment with *B. longum* BB-46, *B. longum* BB-46 combined with pectin and pectin alone. These sequencing reads were merged and clustered into operational taxonomic units (OTUs). After normalizing the data, a total of 690,000 sequences were produced, generating 23,000 sequences per sample. On average 406 OTUs were obtained per sample (ranking from 232 to 616). Rarefaction curves were constructed to evaluate the sequencing depth and the species richness. The curves suggested that sequencing depth was enough to cover most of the bacteria in the SHIME® samples (Supplemental Fig. S1). As Figure S2 shows, alpha diversity measurement suggested variations in species richness (Chao1) and diversity (Shanon index) between samples. Treatments with pectin (T3) and pectin with *B. longum* BB-46 (T2) showed the lowest richness (index of 271 to 487 during treatment T2 and 309 to 530 during treatment T3) and diversity (index of 3.72 to 4.88 during treatment T2 and 3.93 to 4.75 during treatment T3).

Microbiota composition

Fig. 2 shows the main bacterial phyla determined in the microbiota from obese individuals during all the experiment in SHIME® model. A high relative abundance of *Firmicutes* phylum (73%, 61%, and 51% for the ascending, transverse, and descending colon, respectively), followed by *Bacteroidetes* (19%, 27%, and 34% for the ascending, transverse, and descending vessel, respectively), and *Actinobacteria* (8%, 11%, and 13% for the ascending, transverse, and descending vessel, respectively) was observed during the control period. The effects of treatments were similar in the three regions of the colon vessels, with minor differences in abundance proportions. A high increase in *Firmicutes* as well as a decrease in *Bacteroidetes* were observed during the treatment with *B. longum* BB-46 (T1). Treatments T2 (pectin with BB-46) and T3 (pectin alone) stimulated the increase in *Proteobacteria* as well

as the reduction in *Bacteroidetes* phylum (Fig. 2). An increase in the abundance of *Firmicutes* was also observed during the treatment with pectin alone (T3).

As observed in Fig. 3A, the increase in *Firmicutes* phylum during the treatment with *B. longum* (T1) was mostly due to the abundance of the *Lachnospiraceae* and *Veillonellaceae* families and a small contribution of *Lactobacillaceae*, while during T3 (pectin alone) this increase was mostly attributed to the high abundance of *Ruminococcaceae*. A correspondence analysis was performed to test the correlation between the different treatments and the microbiota composition in terms of family (Fig. 3B). The two first axes of the correspondence analysis aggregated 61.43 % of the total variance, which is sufficient to explain the results. The impact of treatments (T1, T2 and T3) was similar in the three regions of the colon (V3, V4 and V5) and we could clearly see three distinct groups. One group was composed of the control period (C) and the washout period (W), whereas another was composed of the treatment with *B.longum* BB-46 (T1), and the final one contained the treatments T2 (pectin combined with *B. longum* BB-46) and T3 (pectin). These groups were clustered based on the microbiota composition similarity in terms of family. We could observe a relationship between T2 and T3 and the families *Succinivibrionaceae*, *Ruminococcaceae* and *Erysipelotrichaceae,* as well as between T1 and *Lachnospiraceae*, *Veillonellaceae*, *Lactobacillaceae* and *Synergistaceae* (Fig. 3B). As Fig. 3B shows, when *B. longum* was combined with pectin, only the pectin effects could be observed, and as a consequence, treatments T2 (pectin with BB-46) and T3 (pectin) were clustered together due to the similarity of the microbiota composition.

Fig. 4 shows the relative abundance of *Lachnospiraceae* family during the fermentation with *B. longum* BB-46 (T1), *B. longum* BB-46 and pectin (T2) and pectin (T3) in SHIME® model. Similar effects were observed in the three regions of the simulated colons. A significant increase in the *Lachnospiraceae* family ($p < 0.01$) was observed during the treatment T1 when compared to the control period, whereas a significant decrease was noticed during the

treatments T2 and T3. No significant difference was observed between the last two treatments.

Fig. 5 shows the relative abundance of bacterial genera in the obese microbiota during the different fermentations in SHIME® model. The control and washout periods showed similar bacterial genera composition, as well as treatments T2 (pectin and BB-46) and T3 (pectin). Treatment T1 (with BB-46) showed different genera composition, but closer to the control and washout periods. These results reinforce the idea that both pectin and *B. longum* BB-46 can modulate the obese microbiota in different ways, but when combined in a presented setup, the predominant effect of the pectin can be observed. The most dominant genera found during T2 and T3 were the *Succinivibrio* (relative abundance of 28 - 37 %) and an unclassified genera of the *Ruminococcaceae* family (relative abundance of 33 - 53 %). *Blautia* (relative abundance of 14-19 %) and *Megamonas* (relative abundance of 19 - 32 %) were the most dominant genera found during treatment T1 (Fig. 5).

Table 1 shows the relative abundance of bacterial genera with significant changes during the three treatments (BB-46 (T1), BB-46 with pectin (T2), and pectin (T3)) in SHIME® model. A significant increase ($p < 0.05$) in *Succinivibrio, Holdemanella*, *Alteromonadaceae,* unclassified genera of *Ruminococcaceae* (OTUs 1077, 1194, 1027, 1153, 1037, 601 and 576) and *Catenibacterium* genera was found during treatments T2 and/ or T3, when compared to the control period. During treatment T1, a significant increase ($p < 0.05$) in *Blautia, Megamonas, Succinivibio, Holdemanella, Lactobacillus* (only ascending and descending colon)*, Dorea,* unclassified genera of *Lachnospiracea*e family, *Catenibacterium*, and *Bacteroides* (only descending colon) was found.

Metabolic activity

Fig. 6 shows the results obtained for SCFA. *B. longum* BB-46 (T1) had no effect on SCFA production, except for an increase ($p < 0.05$) in butyric acid in the transverse and descending colon. However, a high and significant increase ($p < 0.05$) in acetic and butyric acid was observed during the treatments with the *B. longum* BB-46 and pectin (T2) and just pectin (T3), with a higher

increase in butyric acid, especially during the treatment with pectin (T3) in the three regions of the colon (increase by 7, 4.5 and 12 folds in the ascending, transverse and descending colon, respectively, comparing to control period). There were no significant changes in propionic acid contents during the different treatments in all colon regions.

As Fig. 7 shows, a significant decrease in NH_4^+ production in all compartments of the colon vessels was observed ($p < 0.05$) during the three different treatments (BB-46, BB-46 with pectin, and pectin) (Fig. 7). However, the largest reduction of NH_4^+ occurred during the treatments with BB-46 combined with pectin (T2), and pectin (T3). There was no statistical difference in ammonium levels between treatments T2 and T3 for all the colon regions evaluated.

Correlation analysis was performed to identify the genera related to production of SCFA or NH_4^+ (Fig. 8). The relative abundance of *Succinivibrio* and seven unclassified genera of *Ruminococcaceae* (OTUs 1077, 1037, 601, 576, 1197, 1153, and 1027) had positive correlations with production of butyric and acetic acid. *Holdemanella* (*Erysipelotrichaceae* family), and an unclassified genera of *Alteromonodaceae* also showed a positive correlation with butyric and acetic acid and a negative correlation with ammonium ions. The relative abundance of *Streptococcus*, *Bacteroide*s, and *Clostridium* positively correlated with the levels of ammonium ions. *Succinivibrio* and three unclassified genera of *Ruminococcaceae* (OTUs 1077, 601, and 1194) showed negative correlation with NH_4^+ (Fig. 8).

Discussion

In this study, we evaluated the effects of three treatments, i. e., *B. longum* BB-46 (T1), *B. longum* BB-46 combined with the pectin (T2), and harsh extracted pectin from lemon (T3), on obesity-related microbiota using a Simulator of the Human Intestinal Microbial Ecosystem. A high increase in *Ruminococcaceae* (mainly OTUs 1077, 1194, and 1027 - unclassified genera of *Ruminococcaceae)* and *Succinivibrionaceae* members (mainly *Succinivibrio* genus) was observed during the treatments T2 (pectin with BB-46) and T3

(pectin). Both families are able to degrade pectin as well as other carbohydrates such as starch (Duncan et al. 2007; Santos and Thompson 2014; Tian et al. 2017). The pectin probably stimulated the increase of *Ruminococcaceae* and *Succinivibrionaceae* members during the treatments T2 and T3, inhibiting the growth of several bacterial species due to a competitive advantage, which might explain the low bacterial diversity (in all colon vessels) during these two treatments.

The increase in *Succinivibrionaceae* and *Ruminococcaceae* members is however, considered as beneficial, since both families are associated with several health benefits (Louis et al. 2010; Li et al. 2012; Nakayama et al. 2017). Members of the *Succinivibrionaceae* family (*Proteobacteria* phylum) have a protective role against gut inflammation and are able to efficiently transport molecules implicated in immune recovery (Li et al. 2012). Moreover, investigating the impact of dietary habits on the gut microbiota Nakayama et al. (2017) showed that the genus *Succinivibrio* had a negative correlation with total fat intake. *Ruminococcaceae,* the other dominant family in this study (during T2 and T3), include members with potential specialization in ecological niches, such as the ability to generate energy from fermentable substrates available in the colon using different routes (Arumugam et al. 2011). According to Louis et al. (2010), members of this family have been associated with the maintenance of the gut health and the production of butyric acid. In addition, by investigating the correlation between changes in the body weight over time and the gut microbiome composition, Menni et al. (2017) showed that the family *Ruminococcaceae* was nominally protective against weight gain. Such findings are interesting for the present study, since we used faecal samples from obese people. Tian et al. (2017) as well as Gómez et al. (2016), also reported an increase in *Ruminococcaceae* members during fermentation of citrus pectin, but none of them observed changes in *Succinivibrionaceae* members. These studies were however, performed with faecal samples from piglets and lean individuals, respectively.

In this study, we observed an increase ($p < 0.05$) in acetic and butyric acid during the treatments with *B.longum* BB-46 and pectin (T2) and pectin

alone (T3). According to Santos and Thompson (2014), members of the *Succinivibrionaceae* family can ferment carbohydrates to succinate and acetate, which may explain the high increase in acetic acid contents during treatments T2 and T3. Correlation analysis between the abundances of the gut microbiome and SCFAs revealed that *Succinivibrio* correlated positively with acetic acid.

The family *Ruminococcaceae* includes the major butyrate-producing species (Louis et al. 2010), which may explain the high increase in the butyric acid production, especially during treatment T3 (pectin) in vessel 3. According to Gómez et al. (2016), some pectins and oligosaccharides derived from pectin have been identified as emerging prebiotics due to their intestinal microbiota modulation ability, including the increase in some bacteria from the *Ruminococcaceae* family such as *F. prausnitzii.* In this study, there was no increase in *F. prausnitzii* during the treatments but rather an increase in the OTUs 1194, 1153, 1077, 1037, 1027, 601, and 576 (unclassified genera of *Ruminococcaceae*) was observed during treatments T2 and/ or T3. Correlation analysis between the abundance of the gut microbiome and SCFAs revealed that the different unclassified genera of the *Ruminococcaceae* family correlated positively with butyric acid levels, confirming the relationship between *Ruminococcaceae* members and butyric acid production.

In this study, we also observed a positive correlation between the levels of butyric acid and two bacterial genera: an unclassified genera of *Alteromonadaceae* family and *Eubacterium biforme*, reclassified as *Holdemanella biformes* (De Maesschalck et al. 2014). *H. biforme* is considered butyrate producers (Schwiertz et al. 2010), which can explain the result. On the other hand, there is no scientific evidence that members of *Alteromonadaceae* family are butyric producer, however, a high correlation between butyric acid levels and members of this family was found in this study. Members of *Alteromonadaceae* family are often associated to nutrient-rich environments with the ability to degrade several complex polysaccharides such as agar, chitin, cellulose, β-glucan, laminarin, pectin, pullulan, starch, and xylan (López-Pérez and Rodriguez-Valera 2014). This way, the results

indicate that members of *Alteromonadaceae* probably used the pectin as a substrat to generate SCFA.

Once the acetic and butyric acid seems to be predominantly anti-obesogenic (Chakraborti 2015; Morrison and Preston 2016), their increase in the colon region, especially from obese people, are desirable. Butyrate has been found to increase mitochondrial activity, prevent metabolic endotoxemia, improve insulin sensitivity, possess anti-inflammatory potential, increase the intestinal barrier function, and protect against diet-induced obesity without causing hypophagia (Chakraborti 2015), while acetate appears to stimulate leptin secretion in adipocytes (Zaibi et al. 2010). Although there were no significant changes in propionic acid contents during the different treatments, propionate have also been found to be involved in obesity, inhibiting the cholesterol synthesis and regulating the body weight through a stimulatory effect on anorexigenic gut hormones (Chakraborti 2015) .

Despite the significant increase in the *Lachnospiraceae* members (related to butyric and propionic acids production (Louis et al. 2010)), as well as *Lactobacillus* (related to acetic acid production (Tachedjian et al. 2017)) during treatment T1, only an increase in butyric acid (transverse and descending colon) was observed, whereas the levels of acetic and propionic acids were maintained. We believe that certain SCFA, such as acetic acid produced during treatment T1, have probably been used by specific groups of bacteria, which converted them into other metabolites. The gut harbours a complex microbial community, where many interactions exist, including bacterial cross-feeding interactions. According to Ríos-Covián et al. (2016), bacterial cross-feeding has a large impact on the final balance of SCFA production since some groups of microorganisms can utilize the end products from the metabolism of another bacterial group, like for example, the conversion of acetic acid produced by specific bacteria into butyrate for other groups (Louis et al. 2010).

In this study, a significant decrease in ammonium ions was observed during all treatments (T1, T2, and T3), especially T2 and T3. The concentration of ammonium ions in the intestine mainly results from amino acid deamination

and urea hydrolysis by intestinal bacteria (Davila et al. 2013). According to Smith and MacFarlane (1998), the addition of fermentable carbohydrates to microbial populations is directly linked to the reduction of NH_4^+ in the intestine. This relationship, according to Ito et al. (1993), can be explained by the inhibition of the peptides and amino acid fermentation in favour of carbohydrate fermentation by the intestinal microbiota. In this study, however, the reduction of NH_4^+ seems to be more related to the decrease in proteolytic bacteria than to the switch of substrate, since the levels of ammonium ions was positively correlated with the proteolitic bacteria *Clostridium, Streptococcus* and *Bacteroides.* The proteolytic activity in the large intestine is mainly attributed to the *Bacteroides*, *Clostridium*, *Propionibacterium*, *Streptococcus*, and *Lactobacillus* genera, which use amino acids as sources of nitrogen, carbon, and energy, generating NH_4^+ as one of the intermediate or final metabolites (Macfarlane and Cummings 1991). A significant reduction in *Clostridium* spp. was observed during all treatments ($p < 0.05$) as well as *Streptococcus* and *Bacteroides* during treatments T2 and T3 (Table 1), reducing thus, the levels of ammonion ions.

Together with considerable decrease in proteolytic bacteria, treatment T1 (with *B. longum* BB-46) resulted in an increase of *Bacteroides* spp. (vessel 5) and *Lactobacillus* spp. (Table 1). This fact probably explains the lower reduction in ammonium ions production during the treatment T1 compared to treatments T2 and T3.

The reduction in NH_4^+ in the colon is considered to be beneficial as these ions may alter the morphology and intermediate metabolism of the intestinal cells, increasing DNA synthesis and promoting the development of tumours (Ichikawa and Sakata 1998; Davila et al. 2013). In addition, Hughes et al. (2008) demonstrated that NH_4^+ can increase cell permeability in the colonocytes, causing several host diseases.

In this study, it was also observed that pectin (T3) and pectin in combination with BB-46 (T2) inhibited the growth of *Lachnospiraceae.* Some studies have shown an association between obesity and the *Lachnospiraceae* family. Kameyama and Itoh (2014) identified a specific *Lachnospiraceae*

bacterium (strain AJ110941) involved in metabolic disorders. They concluded that intestinal colonization by a *Lachnospiraceae* contributes to the development of diabetes in obese mice. According to Ravussin et al. (2012), rats fed with a high fat diet, present high populations of *Lachnospiraceae* members, and a reduction is observed after weight loss. Changes in bacterial populations depend on many variables such as competition between bacteria for substrates, synthesis of antimicrobial agents, and bacterial metabolism (Mao et al. 2012). We suppose that the decrease on *Lachnospiraceae* members can probably be related to the *Ruminococcaceae* increase. Both families have members able to produce butyric acid (Onrust et al. 2015) and can probably need similar substrates to survive, thus creating a competitive environment.

In summary, the dynamic view of the microbiome and microbial metabolites, exhibited by *B. longum* BB-46, pectin and the combination of *B. longum* BB-46 and pectin, provided interesting insights into the interplay of probiotic and pectin with the microbiota. The results indicate that *B. longum* BB-46 and pectin have a different impact on obesity-related microbiota, but when combined, the predominant effect of the pectin can be observed. Treatments with pectin and pectin combined with *B. longum* BB-46 showed a high increase in bacteria with potential anti-inflammatory effects (*Succinivibrionaceae* members), an increase in SCFA, and a decrease in the *Lachnospiraceae* family. Based on literature, these findings indicate that the studied pectin can probably have a protective role on obesity. However, further clinical studies are necessary to evaluate the anti/ pro-obesogenic and inflammatory effects of this pectin.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Studies using faecal donations from humans volunteers do not require medical ethical committee approval in Brazil since they are considered as noninvasive.

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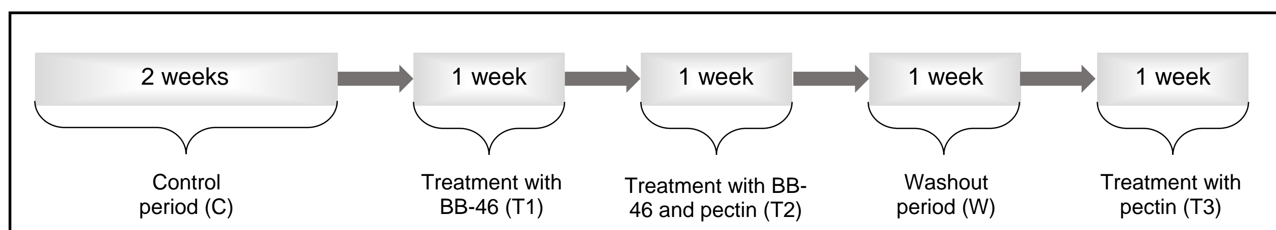

Fig. 1 Experimental SHIME® protocol employed in the treatments with *Bifidobacterium longum* BB-46, pectin and *Bifidobacterium longum* BB-46 combined with pectin.

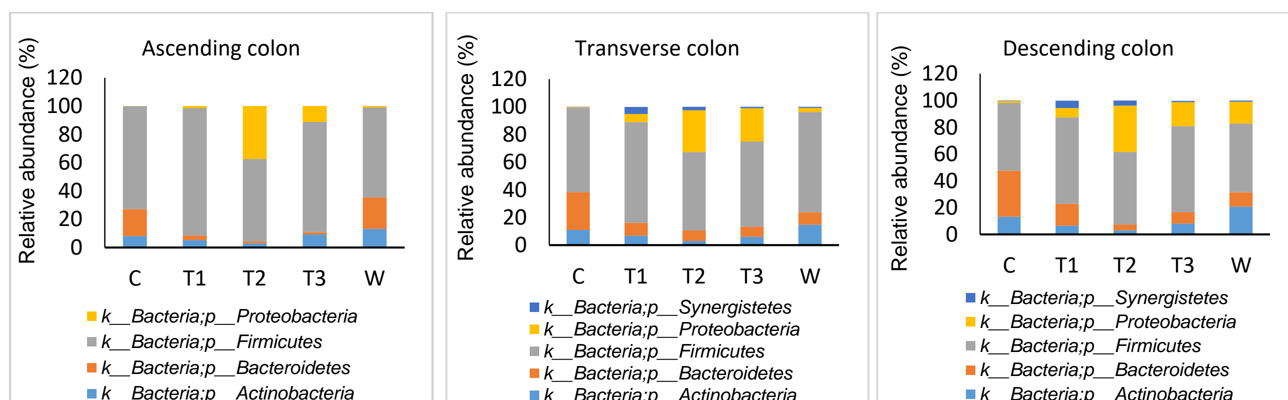

Fig. 2 The main bacterial phyla determined in the microbiota from obese individuals during all experiments in SHIME® colon vessels. C = control period; T1= treatment with *Bifidobacterium longum* (BB-46); T2 = treatment with BB-46 and pectin; T3= treatment with pectin; W= washout period.

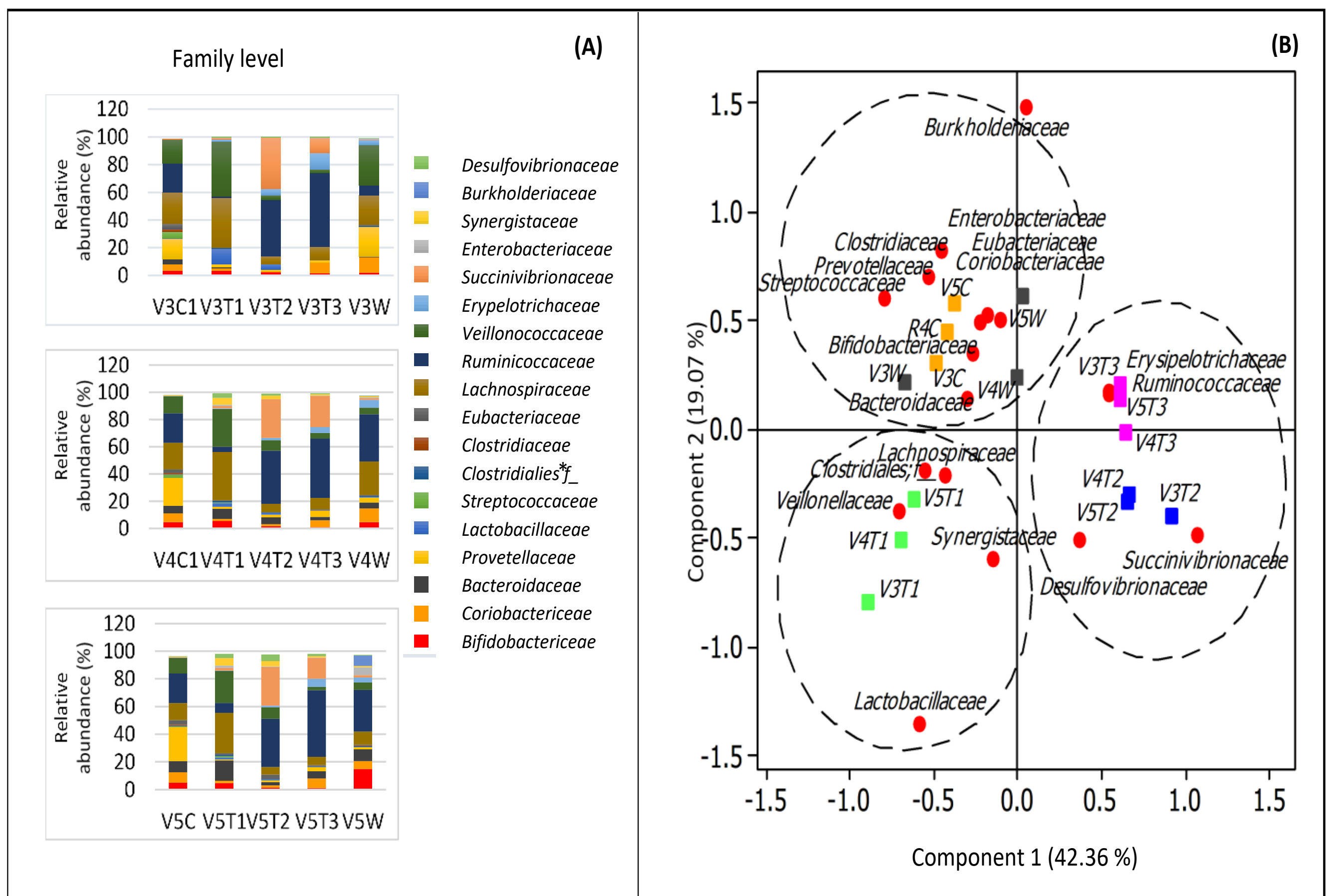

Fig. 3 Microbiota composition at a family level during all experiments in SHIME® colon vessels. (**A**) The main bacterial family determined during the experiments in the three simulated colons; (**B**) simple correspondence analysis showing relationship between the treatments and bacterial families in the three regions of SHIME® model. C = control period; T1= treatment with *Bifidobacterium longum* (BB-46); T2 = treatment with BB-46 and pectin; T3= treatment with pectin; W= washout period; V3= ascending colon; V4= transverse colon; V5= descending colon. The different families are represented by red dots and the treatments by colorful square. Control period is represented by *orange* color and T1, T2, W and T3 by *green, blue, black* and *purple*, respectively.
* Unclassified family of *Clostridiales* order.

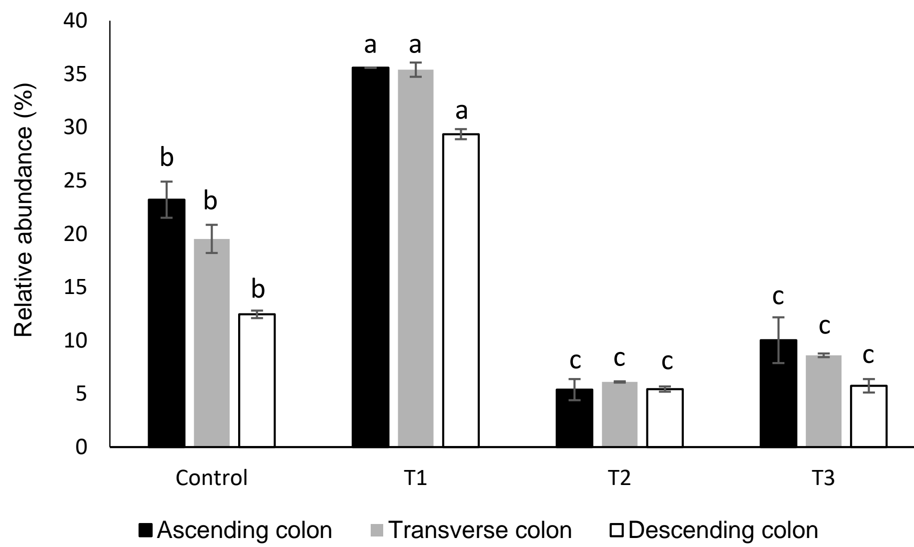

Fig. 4 Relative abundance (%) of the *Lachnospiraceae* family in the microbiota from obese individuals during all experiments in SHIME® colon vessels. C = control period; T1= treatment with *Bifidobacterium longum* (BB-46); T2 = treatment with BB-46 and pectin; T3= treatment with pectin. Different letters represent statistical difference ($p < 0.05$) between the treatments for the same vessel (One-way ANOVA and Tukey post-hoc test).

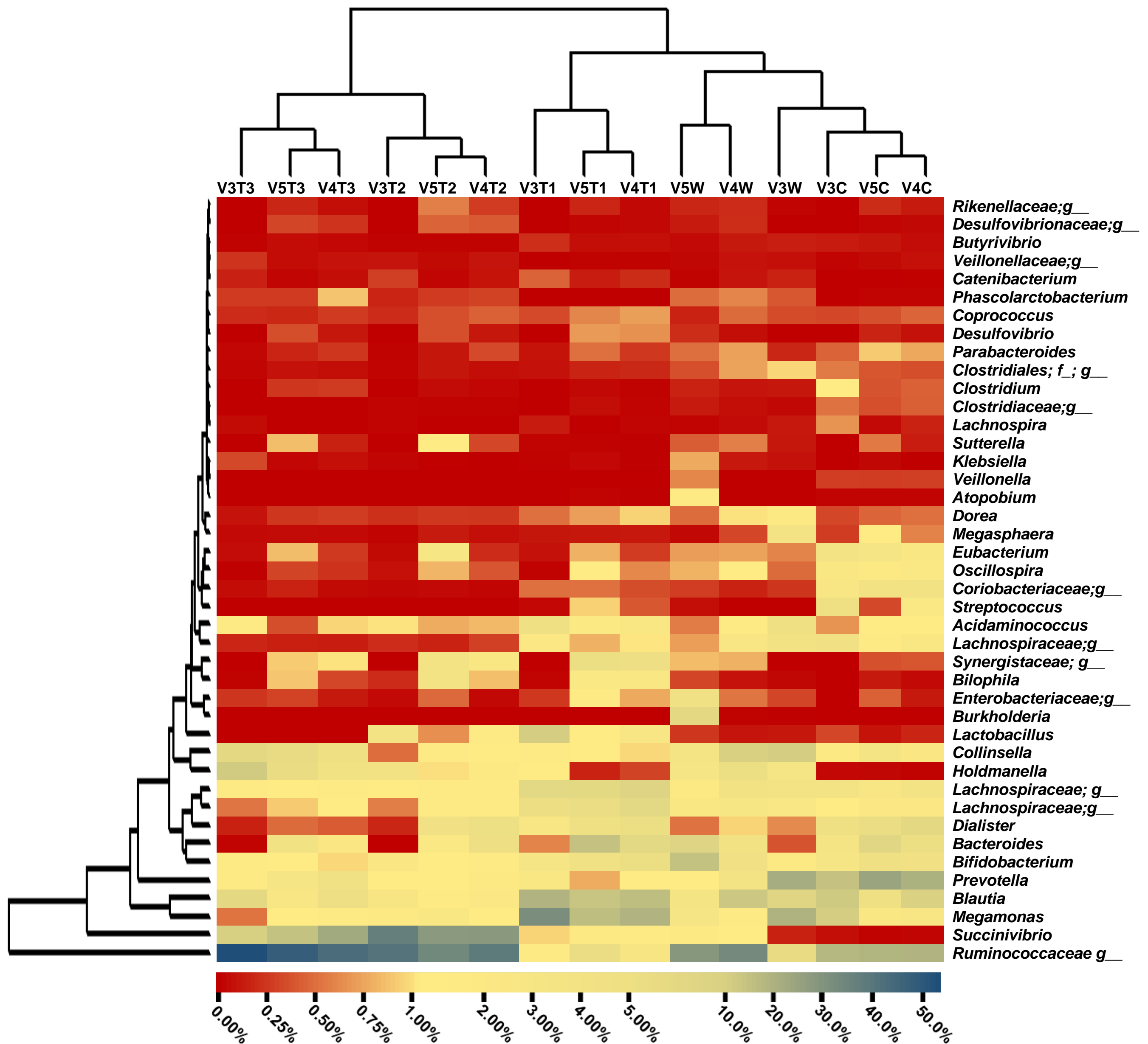

Fig. 5 Relative abundance of bacterial genera (%) in the obese microbiota during all experiments in SHIME® model. C= control period; T1= treatment with *Bifidobacterium longum* (BB-46); T2= treatment with BB-46 and pectin; T3= treatment with pectin; W= washout period. V3= ascending colon; V4= transverse colon; V5= descending colon. Unclassified genera are represented by “g_ “.

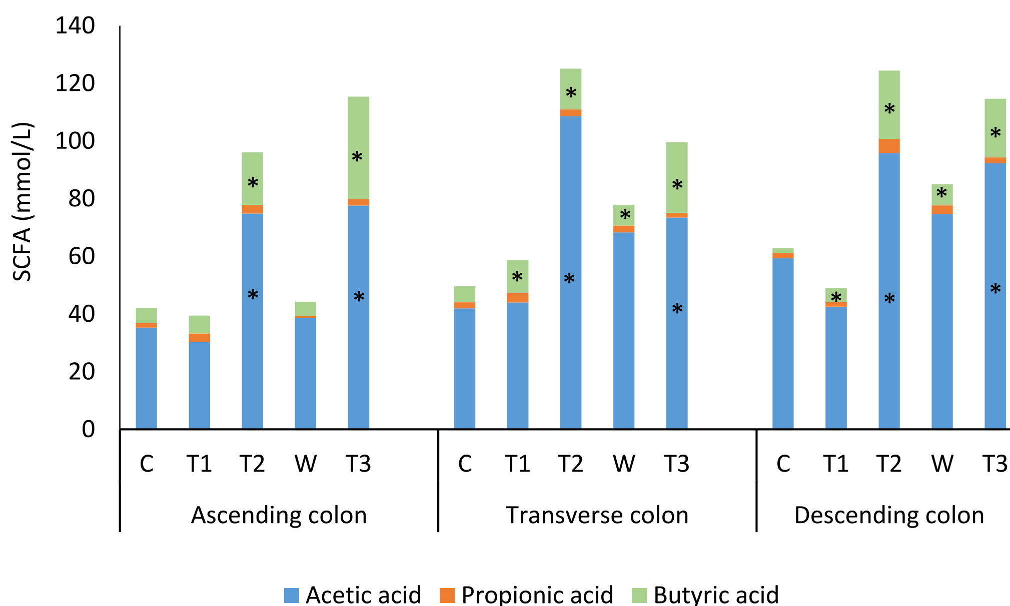

Fig. 6 Production of acetic, propionic, and butyric acids by microbiota from obese individuals during all experiments in SHIME® colon vessels. Significant increase compared to the control are indicated by * (*p* < 0.05) (One-way ANOVA and Tukey post-hoc test). C= control period; T1= treatment with *Bifidobacterium longum* (BB-46); T2= treatment with BB-46 and pectin; T3= treatment with pectin; W= washout period.

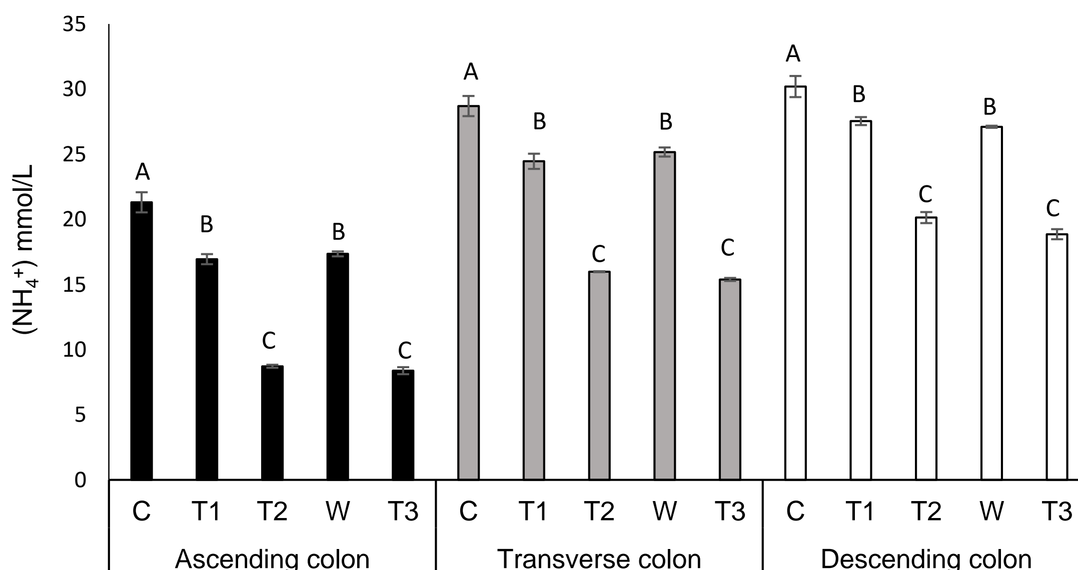

Fig. 7 NH_4^+ production (mmol/L) by microbiota from obese individuals during all the experiments in SHIME® colon vessels. C= control period; T1= treatment with *Bifidobacterium longum* (BB-46); T2= treatment with BB-46 and pectin; T3= treatment with pectin; W= washout period. Different letters represent statistical difference ($p < 0.05$) between the treatments for the same vessel (One-way ANOVA and Tukey post-hoc test

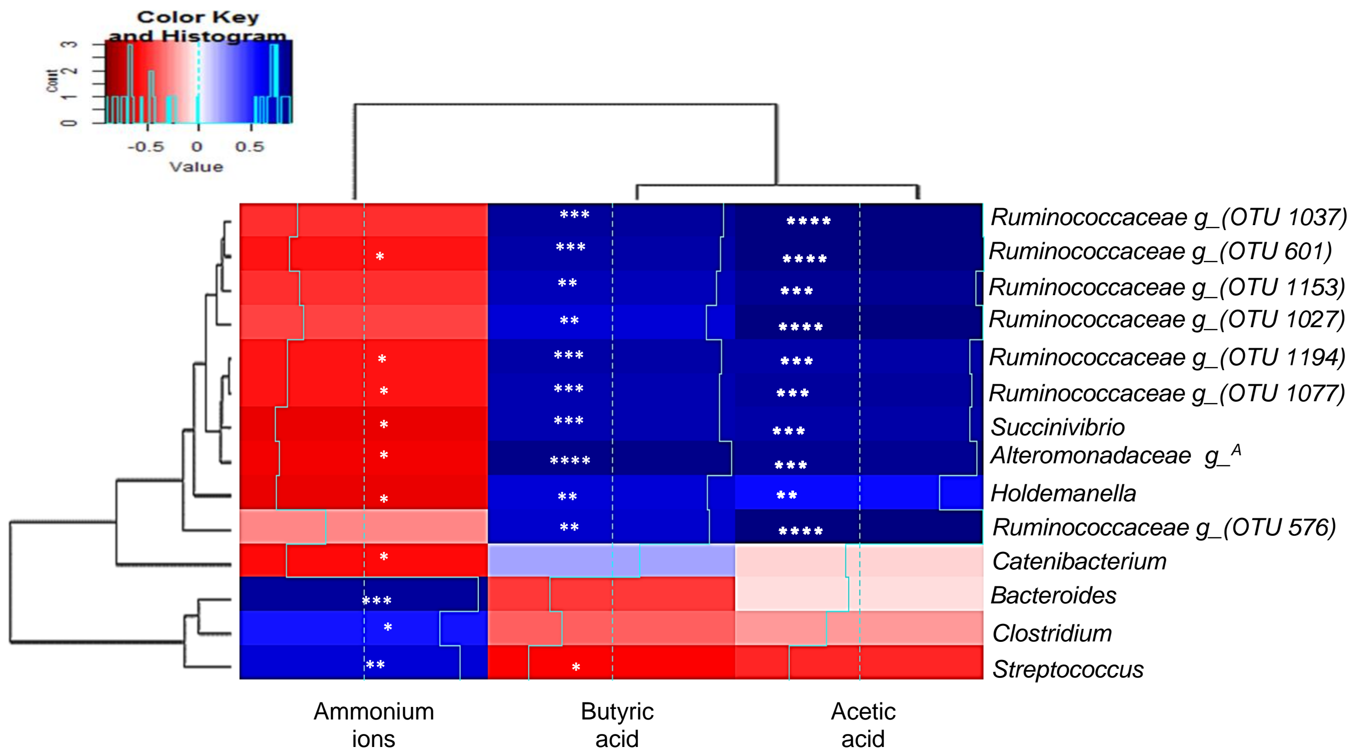

Fig. 8 Correlation between SCFA production (butyric and acetic acids), ammonium ions and bacterial genera. Significant correlations are indicated by *($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$) (Spearman correlation). [A], unclassified genera of *Alteromonadaceae* family.

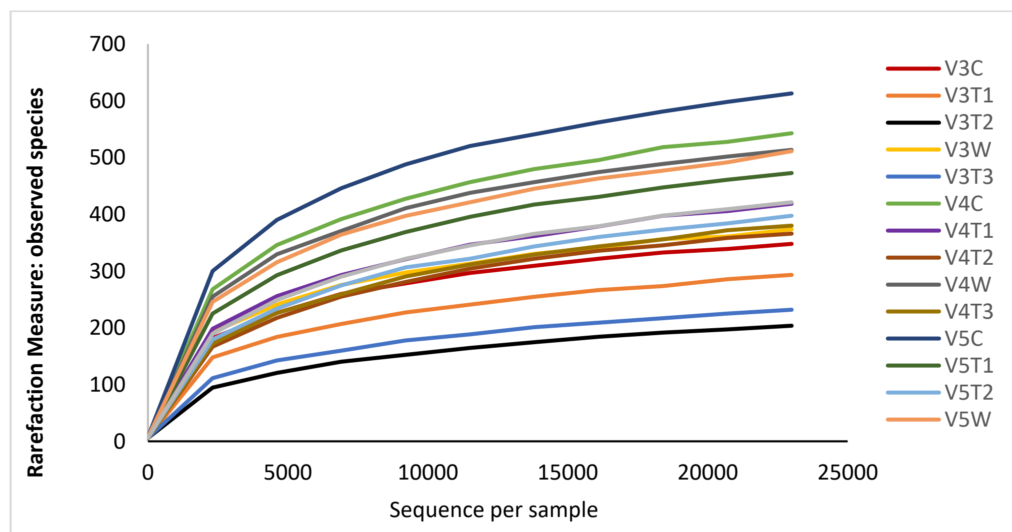

Fig. S1: Rarefaction measure: observed OTUS in the microbiota from obese subjects during fermentation with pectin and/or the probiotic *Bifidobacterium longum* BB46 in SHIME® colon vessels**.** C= control period; T1= treatment with *Bifidobacterium longum* (BB46); T2= treatment with BB46 and pectin; T3= treatment with pectin; W= washout period; V3= ascending colon; V4= transverse colon; V5= descending colon.

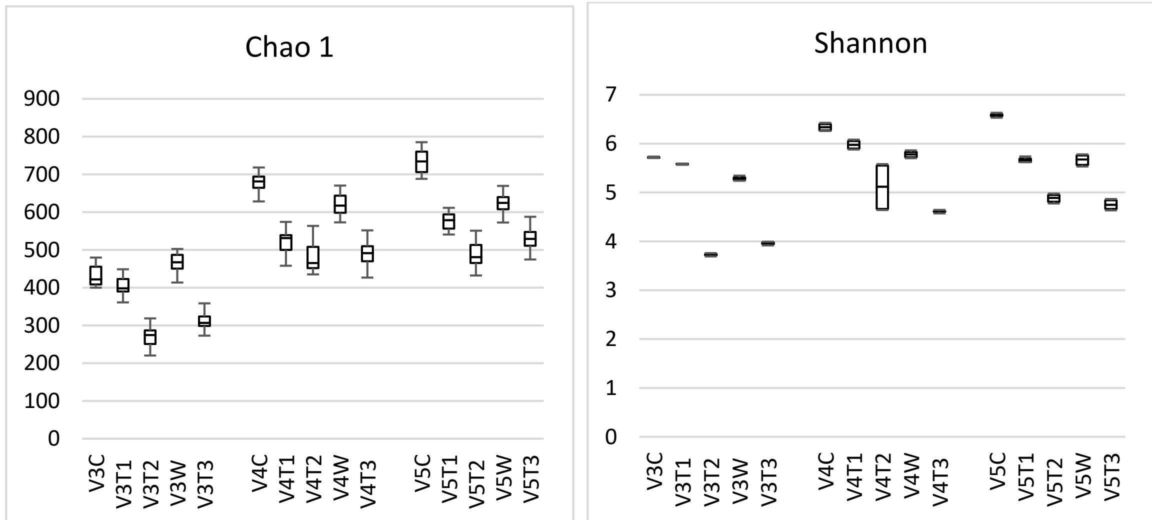

Fig. S2: Alpha diversity of microbiota from obese individuals during fermentation with pectin and/or the probiotic *Bifidobacterium longum* BB-46 in SHIME® colon vessels. (**A**) represents the species richness (Chao1 index) and (**B**) the diversity of the microbiota (Shannon index) based on 23000 sequence per sample. C= control period; T1= treatment with *Bifidobacterium longum* BB-46; T2= treatment with BB-46 and pectin; T3= treatment with pectin; W= washout; V3= ascending colon; V4= transverse colon; V5= descending colon.

Table 1 Relative abundance (mean ± SD) of bacterial genera with significant changes during all experiments in SHIME® colon vessels.

	Ascending colon					Transverse colon					Descending colon				
Genus	Control	Treatment T1	Treatment T2	Washout	Treatment T3	Control	Treatment T1	Treatment T2	Washout	Treatment T3	Control	Treatment T1	Treatment T2	Washout	Treatment T3
Ruminococcaceae g_ (OTU 1077)	8.11 ± 0.49	0.43 ± 0.00*	18.78 ± 0.94*	2.70± 0.04*	25.41 ± 0.96*	7.48 ± 1.03	0.89 ±0.23*	17.77 ± 2.14*	14.29 ± 0.40*	20.45 ± 0.09*	7.55 ± 0.63	1.59 ±0.03*	14.75 ± 3.26*	11.82 9.66	22.18 ±1.22*
Ruminococcaceae g_ (OTU 1194)	7.13 ± 0.51	0.37 ± 0.01*	17.93 ± 0.04*	2.41 ± 0.10*	23.48 ± 0.82*	7.08 ± 0.84	0.81 ± 0.17*	16.08 ± 1.55*	12.62 ± 0.08*	18.03 ± 0.52*	6.84 ± 0.74	1.40 ± 0.08*	13.59 ± 2.81	10.49 ± 8.61	19.90 ± 1.27*
Ruminococcaceae g_ (OTU 1027)	0.08 ± 0.00	0.01 ± 0.00*	1.22 ± 0.10*	0.03 ± 0.01	0.59 ±0.04*	0.10 ± 0.02	0.03 ± 0.01	0.86 ± 0.07*	0.21 ± 0.02	0.76 ± 0.06*	0.10 ± 0.00	0.05 ± 0.01*	0.80 ± 0.02*	0.20 ± 0.141	0.76 ± 0.09*
Ruminococcaceae g_ (OTU 576)	0.08 ±0.01	0.01 ± 0.00*	0.24 ± 0.10	0.06 ± 0.00	0.28 ±0.01*	0.16 ± 0.02	0.05 ± 0.05	0.36 ± 0.03*	0.22 ± 0.01	0.36 ±0.02*	0.21 ± 0.01	0.08 ± 0.00*	0.46 ± 0.02*	0.34 ± 0.06	0.46 ±0.10
Ruminococcaceae g_ (OTU 1153)	0.16 ± 0.00	0.03 ± 0.00*	0.36 ± 0.05*	0.07 ± 0.02*	0.38 ± 0.00*	0.20 ± 0.02	0.04 ± 0.02*	0.39 ± 0.00*	0.29 ± 0.01*	0.44 ± 0.06*	0.19 ± 0.06	0.06 ± 0.00	0.32 ± 0.07	0.30 ± 0.17	0.39 ± 0.01*
Ruminococcaceae g_ (OTU 1037)	0.12 ± 0.02	0.00 ± 0.00*	0.25 ± 0.04	0.06 ± 0.01	0.32 ± 0.00*	0.13 ± 0.04	0.03 ± 0.00	0.30 ± 0.07	0.23 ± 0.01	0.28 ± 0.00*	0.12 ± 0.00	0.02 ± 0.01*	0.32 ± 0.01*	0.178 ± 0.14	0.30 ± 0.05*
Ruminococcaceae g_ (OTU 601)	0.00 ± 0.00	0.00 ± 0.00	0.44 ± 0.03*	0.00 ± 0.00	0.26 ± 0.01*	0.00 ± 0.00	0.00 ± 0.00	0.25 ± 0.04*	0.01 ± 0.01	0.20 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.23 ± 0.01*	0.04 ± 0.03	0.23 ± 0.04*
Succinivibrio	0.06 ± 0.01	0.96 ± 0.03*	37.01 ± 2.61*	0.15 ± 0.06	10.45 ± 0.56*	0.03 ± 0.02	1.62 ± 0.02*	28.27 ± 3.33*	1.14 ± 0.08*	22.86 ± 3.33*	0.01 ± 0.01	1.88 ± 0.20*	28.08 ± 3.97*	1.74 ± 1.58	14.94 ± 0.14*
Holdemanella	0.03 ± 0.01	1.16 ± 0.01*	4.26 ± 1.80*	3.21 ± 0.99*	11.75 ± 1.46*	0.02 ± 0.00	0.30 ± 0.08*	1.77 ± 0.42*	5.65 ± 0.39*	4.34 ± 0.27*	0.03 ± 0.02	0.15 ± 0.01*	1.00 ± 0.31*	3.15 ± 2.98	6.05 ± 0.10*
Lactobacillus	0.32 ± 0.11	11.00 ± 0.01*	3.87 ± 2.23	0.10 ± 0.02	0.00 ± 0.00	0.16 ± 0.00	2.85 ± 1.63	1.55± 0.88	0.09 ± 0.00*	0.00 ± 0.00*	0.09 ± 0.00	1.34 ± 0.12*	0.65 ± 0.28	0.24 ± 0.27	0.00 ± 0.00*
Ateromonadaceae	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.05*	0.00 ± 0.00	0.14 ± 0.01*	0.00 ± 0.00	0.00 ± 0.00	0.12 ± 0.00*	0.01 ± 0.00	0.14 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.02*	0.03 ± 0.03	0.14 ± 0.02*
Catenibacterium	0.00 ± 0.01	0.45 ± 0.00*	0.28 ± 0.02*	0.15 ± 0.02*	0.15 ± 0.00*	0.01 ± 0.00	0.20 ± 0.07*	0.09 ± 0.01*	0.09 ± 0.01*	0.07 ± 0.02*	0.00 ± 0.00	0.13 ± 0.04*	0.02 ± 0.01	0.01 ± 0.00	0.03 ± 0.00*
Blautia	12.24 ± 1.17	19.24 ± 0.01*	3.08 ± 0.50*	8.42 ± 0.11*	7.48 ± 1.72	9.78 ± 1.03	15.98 ± 0.69*	2.22 ± 0.03*	13.07 ± 0.20*	5.22 ± 0.04*	5.00 ± 0.34	13.88 ± 0.85*	1.64 ± 0.03*	3.54 ± 2.89	2.91 ± 0.48*
Megamonas	11.05 ± 0.67	31.80 ± 0.00*	1.98 ± 2.39*	19.69 ± 2.14*	0.53 ± 0.12*	2.88 ± 1.11	19.33 ± 3.02*	1.07 ± 0.69	1.39 ± 0.09	1.71 ± 0.00	2.66 ± 0.02	16.08 ± 2.46*	2.66 ± 1.44	3.32 ± 3.78	1.14 ± 0.13*
Dorea	0.32 ± 0.00	0.51 ± 0.00*	0.22 ± 0.10	1.76 ± 0.45*	0.09 ± 0.02*	0.51 ± 0.02	0.95 ± 0.04*	0.25 ± 0.02*	1.02 ± 0.15*	0.27 ± 0.02*	0.45 ± 0.02	0.72 ± 0.00*	0.25 ± 0.01*	0.48 ± 0.15	0.25 ± 0.03*
Bacteroides	3.66 ± 0.72	0.59 ± 0.00*	0.00 ± 0.00*	0.37 ± 0.03*	0.01 ± 0.01*	5.61 ± 0.65	7.60 ± 1.02	5.04± 1.03	4.15 ± 0.55	2.41± 0.24*	8.16 ± 0.15	14.67 ± 0.64*	2.30 ± 0.61*	8.75 ± 0.92	5.10 ± 0.80*
*Lachnospiraceae g_* [A]	1.11 ± 0.02	5.28 ± 0.03*	0.57 ± 0.14*	2.68 ± 0.30*	0.54 ± 0.12*	2.06 ± 0.02	7.67 ± 0.97*	1.48 ± 0.07*	2.96 ± 0.01*	1.06 ± 0.05*	2.13 ± 0.01	5.94 ± 0.48*	1.52 ± 0.27*	2.53 ± 0.80	0.91 ± 0.06*
Lachnospiraceae g_ other [B]	3.92 ± 0.10	7.73 ± 0.01*	1.09 ± 0.15*	3.88 ± 0.52	1.49 ± 0.27*	3.87 ± 0.37	8.92 ± 0.53*	1.42 ± 0.03*	4.05 ± 0.03	1.62 ± 0.11*	2.82 ± 0.01	7.33 ± 0.04*	1.49 ± 0.07	1.98 ± 0.02*	1.30 ± 0.16*
Clostridium	1.16 ± 0.09	0.06 ± 0.00*	0.00 ± 0.00*	0.10± 0.02*	0.00 ± 0.00*	0.43 ± 0.03	0.01 ± 0.00*	0.03 ± 0.00*	0.09 ± 0.00*	0.26 ± 0.04	0.38 ± 0.01	0.03 ± 0.01*	0.05 ± 0.01*	0.15 ± 0.15	0.24 ± 0.03*
Streptococcus	4.69 ± 0.56	0.035 ± 0.03*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	2.25 ± 0.55	0.39 ± 0.21*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.33 ± 0.05	0.94 ± 0.00*	0.00 ± 0.00*	0.06 ± 0.08	0.00 ± 0.00*
Bifidobacterium	3.36 ± 0.47	3.20 ± 0.00	2.38 ± 0.15	1.84 ± 0.05*	1.63 ± 0.11*	4.38 ± 0.11	5.47 ± 1.47	1.66 ± 0.32*	4.47 ± 0.30	0.97 ± 0.03*	4.84 ± 0.10	4.68 ± 1.22	1.35 ± 0.37*	14.87 ± 15.78	1.15 ± 0.22*

Significant increase or decrease compared to the control are indicated by * ($p < 0.05$). T1= treatment with *Bifidobacterium longum* BB46; T2= treatment with BB46 and pectin; T3= treatment with pectin. [A], unclassified genera of *Lachnospiraceae* family; [B], different unclassified genera of *Lachnospiraceae* family.

Final Considerations

Several studies have proved the influence of gut microbiota composition and its metabolites on human health, resulting in improvement of an individual‘s health or in the development of certain diseases. Studies aiming to investigate the effects of certain substances, such as, for example, prebiotics and probiotics, on the composition and metabolism of the gut microbiota have become more common and have been helpful in the management of several diseases. To better understanding the complex gut microbial community and its interaction with diet and host, the most recent studies have explored more complex molecular analysis and microbiome models.

This study allowed us to make the observation that despite being useful in the enrichment of food products, both the acerola by-product and the citric pectin, usually discarded by the food industries, are helpful in improving the survival of certain probiotic strains under gastrointestinal adverse conditions. Moreover, the acerola by-product and the citric pectin combined with the probiotic *B. longum* BB-46 were shown to have a positive impact on the composition and metabolism of gut microbiota using the SHIME® model. However, further studies using 16S rRNA sequencing are welcome to better elucidate the effects of the acerola by-product in different microbiotas.

The use of robust tools (16S rRNA sequencing) in the analysis of the microbiome allowed us to have a clearer view of the probiotic, pectin and host interaction. We showed that both the pectin and the *B. longum* BB-46, in combination or not, are able to differently modulate the obese and weight-healthy microbiota. This study also allowed us to observe that the stimulation

or inhibition of certain bacterial families or genera is also dependent on the initial composition of the microbiota.

Finally, despite this study indicates that the specific citric pectin, combined or not with *B. longum* BB-46, may have a positive role in relieving symptoms related to obesity (due to the large increase in bacteria with potential anti-inflammatory effects (*Succinivibrionaceae* members), increase in SCFA production, and decrease in the *Lachnospiraceae* family and *Bacteroides* genus), clinical studies including blood parameters and weight control are required to prove such effects.

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Appendix I – Request permissions to include the published articles in the PhD thesis

From “Applied Microbiology and Biotechnology”

From: Alexander Steinbüchel <steinbu@uni-muenster.de>
Date: Dom 06/01/2019, 21:20
To: Fernanda Bianchi <febianchi@hotmail.com>
Subject: Request permission

Dear Fernanda Bianchi,

This will be absolutely not problem. Please include the two publications in your PhD thesis and refer to the bibliographic data.

All the best
Alexander Steinbüchel

From: Fernanda Bianchi <febianchi@hotmail.com>
Date: Dom 06/01/2019, 15:10
To: Alexander Steinbüchel <steinbu@uni-muenster.de>
Subject: Request permission

Dear Professor Alexander Steinbüchel

Editor-in-Chief of Applied Microbiology and Biotechnology

I gently would like to request the permission to include in my PhD thesis the two full articles I published in Applied Microbiology and Biotechnology in 2018:

“Gut microbiome approaches to treat obesity in humans” (DOI: https://doi.org/10.1007/s00253-018-9570-8) and

“Modulation of gut microbiota from obese individuals by in-vitro fermentation of citrus pectin in combination with Bifidobacterium longum BB-46” (DOI: 10.1007/s00253-018-9234-8)”

Kind regards,
Fernanda Bianchi

From "Food Research International"

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Title: In vitro modulation of human gut microbiota composition and metabolites by Bifidobacterium longum BB-46 and a citric pectin

Author: Fernanda Bianchi,Nadja Larsen,Thatiana de Mello Tieghi,Maria Angela T. Adorno,Susana M.I. Saad,Lene Jespersen,Katia Sivieri

Publication: Food Research International

Publisher: Elsevier

Date: Available online 9 November 2018

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From "International Journal of Food Sciences and Nutrition"

Our Ref: AF/IIJF/P19/0076

11 January 2019

Dear Fernanda Bianchi,

Material requested: 'Impact of combining acerola by-product with a probiotic strain on a gut microbiome model' by Fernanda Bianchi, Natalia Pontin Lopes, Maria Angela Tallarico Adorno, Isabel Kimiko Sakamoto, Maria Inés Genovese, Susana Marta Isay Saad & Katia Sivieri *International Journal of Food Sciences and Nutrition* pp. 1-14 Published Online: 30 August 2018.

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