CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

PORTO

EVALUATION OF THE PREBIOTIC POTENTIAL OF *CORIOLUS VERSICOLOR* – EFFECT UPON THE HUMAN GUT MICROBIOTA

by

Célia Maria da Silva Freitas Costa

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master in Science degree in Applied Microbiology

by

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In loving memory of my grandparents,

Domingos José da Costa

Maria Augusta Ferreira de Lemos

Maria Armanda Vieira da Silva

Rui António Morais de Freitas

You may write me down in history, with your bitter, twisted lies; You may trod me in the very dirt. But still, like dust, I'll rise.

Maya Angelou

Resumo

A microbiota intestinal humana tem-se tornado progressivamente mais relevante no estudo da saúde humana e de certas doenças, devido à sua ligação com o sistema imunitário e com o metabolismo humano. Um modo de modelar a microbiota intestinal é através da ingestão de prebióticos, que podem ser fermentados por bactérias benéficas presentes no cólon, resultando na produção de certos metabolitos considerados benignos, tais como os ácidos gordos de cadeia curta. O *Coriolus versicolor* é um cogumelo que, embora seja conhecido pelas suas propriedades imunomoduladoras e anticarcinogénicas, também foi anteriormente associado a uma potencial atividade prebiótica. No entanto, os estudos publicados focam se nas potencialidades dos extratos de C. versicolor, enquanto as propriedades da biomassa permanecem pouco estudadas. Assim, o principal objetivo do presente trabalho foi a avaliação do potencial prebiótico de um suplemento comercial de biomassa de *C. versicolor*, utilizando um modelo fecal *in vitro*. A fim de atingir o objetivo proposto, a biomassa foi primeiramente submetida a uma simulação do processo digestivo, após a qual esta foi utilizada para realizar fermentações usando fezes de cinco dadores distintos. Durante a fermentação foram recolhidas amostras às 0, 12, 24, e 48 h para realizar medições dos valores de pH e de as caracterizar relativamente à produção de ácidos orgânicos, particularmente, os ácidos gordos de cadeia curta (através de HPLC) e ao seu perfil microbiano (através de qPCR).

Apesar da variabilidade intrínseca associada à microbiota intestinal humana entre indivíduos, a presença da biomassa de *C. versicolor* resultou num aumento do número de cópias do gene 16S rRNA de *Bifidobacterium* para todos os dadores, em comparação com o controlo negativo, ainda que o aumento global tenha sido inferior ao do controlo positivo (FOS). Este comportamento foi observado após 24 h (controlo negativo- 0,203 ± 0,017; controlo positivo- 0,282 ± 0,011; *C. versicolor*- 0,238 ± 0,008, expresso como log do número de cópias de 16S rRNA por ng de ADN) e após 48 h (controlo negativo- 0,212 ± 0,016; controlo positivo – 0,301 ± 0,027; *C. versicolor*- 0,268 ± 0,011, expresso como log do número de cópias de 16S rRNA por ng de ADN), mas não foram encontradas diferenças significativas entre as condições testadas após 12 h de incubação. Relativamente a outros grupos bacterianos (Firmicutes, Bacteroidetes, Bacteroides, *Clostridium leptum* e *Lactobacillus*), não foi possível estabelecer uma tendência geral, mas apenas comportamentos específicos de cada dador. De um modo geral, quer o *C. versicolor* quer o FOS, resultaram numa diminuição dos valores de pH, por oposição ao controlo negativo, o que confirmou a produção de ácidos, como consequência do metabolismo bacteriano. No que se refere à quantificação dos ácidos gordos de cadeia curta e do ácido láctico, no fim da fermentação, a biomassa de *C. versicolor* levou à produção de ácido acético (0,212 ± 0,088 mg mL^{-1}), seguida de ácido láctico (0,032 ± 0,002 mg mL^{-1}), e do ácido propiónico (0,018 ± 0,007 mg mL^{-1}). Em suma, é possível concluir que a biomassa de *C. versciolor* teve um efeito bifidogénico, relevante na demonstração do seu potencial prebiótico.

Palavras-chave: Microbiota intestinal; Prebióticos; Biomassa de cogumelos; *Coriolus versicolor*; SCFA

Abstract

The human gut microbiota has become increasingly relevant when considering human health and disease due to its connection with the immune system and human metabolism. One way of actively modulating the gut microbiota is through the ingestion of prebiotics, that can be fermented by beneficial members of the colonic population, resulting in the production of certain metabolites regarded as beneficial, such as Short Chain Fatty Acids (SCFA). *Coriolus versicolor* is a mushroom that, while known for its immunomodulatory and anticarcinogenic properties has also been previously described as possessing some prebiotic potential. However, most studies focus on the potentialities of *C. versicolor* extracts, leaving the properties of its biomass unstudied. Hence, the main goal of the present work was to evaluate the prebiotic potential in a commercial supplement of *C. versicolor* biomass using an *in vitro* faecal model. In order to achieve the proposed goal, the biomass was first submitted to a digestion simulation after which, it was used to perform human faecal fermentations using faeces from five distinct donors. Samples were collected at 0, 12, 24 and 48 h, for pH measurements and to be characterized regarding organic acid production, with emphasis on SCFA (through HPLC), as well as characterize the microbial profile (through qPCR).

Despite the intrinsic variability of the human gut microbiota between individuals, the presence of *C. versicolor* biomass consistently resulted in an increase in the number of *Bifidobacterium*'s 16S rRNA gene copies, for all donors when comparing to the negative control, although the overall increase was lower than the one registered for the positive control (*i.e.* FOS). This behaviour was observed after 24 h (negative control- 0.203 ± 0.017; positive control- 0.282 ± 0.011; *C. versicolor*- 0.238 ± 0.008, expressed in log of copy numbers of 16S rRNA per ng of DNA) and 48 h (negative control- 0.212 ± 0.016; positive control- 0.301 ± 0.027; *C. versicolor*- 0.268 ± 0.011, expressed in log of copy numbers of 16S rRNA per ng of DNA), but no significant differences were found between the assayed conditions after 12 h incubation. For the remaining bacterial groups characterized (Firmicutes, Bacteroidetes, Bacteroides, *Clostridium leptum* and *Lactobacillus*) it was not possible to observe a general trend but only donor specific behaviours. Overall, both *C. versicolor* and FOS resulted in a decrease of the pH values, in opposition to the negative control, which confirmed the production of acids as a result of bacterial metabolism. As for SCFA and lactic acid quantification, at the end of the fermentation, *C. versicolor* biomass led to the production of acetic acid (0.212 ± 0.088 mg mL^{-1}), lactic acid (0.032 ± 0.002 mg mL^{-1}) and propionic acid (0.0184 ± 0.007 mg mL^{-1}). In sum, it is possible to conclude that *C. versicolor* biomass had a bifidogenic effect and, therefore, could be interesting to its establishment as a prebiotic.

Keywords: Gut microbiota, Prebiotics, Mushroom biomass, *Coriolus versicolor*, SCFA.

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List of Abbreviations

16S rRNA .... Small Subunit Ribosomal Ribonucleic Acid

ALD .... Alcoholic Liver Disease

ANOVA .... Analysis of Variance

AU .... Absorbance Units

CFU .... Colony Forming Units

CNS .... Central Nervous System

CoA .... Coenzyme A

Cq .... Quantification Cycle

CRC .... Colorectal Cancer

DB .... Degree of branching

DGGE .... Denaturating Gradient Gel Electrophoresis

DMSO .... Dimethyl Sulfoxide

DNA .... Deoxyribonucleic Acid

DP .... Degree of Polymerization

EDTA .... Ethylenediamine Tetra Acetic Acid

EFSA .... European Food Safety Authority

F/B .... Firmicutes to Bacteroidetes Ratio

FAO .... Food and Agriculture Organization for the United Nations

FDA .... Food and Drug Administration

FI .... Faecal Inocula

FISH .... Fluorescent *in situ* Hybridization

FOS .... Fructooligosaccharides

FPLC .... Fast Protein Liquid Chromatography

GI .... Gastrointestinal

GOS .... Galactooligosaccharides

HPLC .... High Performance Liquid Chromatography

HSV .... Herpes Simplex Virus

IBD .... Inflammatory Bowel Disease

IgA .... Immunoglobulin A

IM .... Isomaloologosaccharide

ISAPP .... International Scientific Association for Probiotics and Prebiotics

OPA .... Phthaldialdehyde

MW .... Molecular Weight

NAFLD .... Non-alcoholic Liver Disease

pH .... Cologarithm of the hydrogen cation concentration

PSK .... Polysaccharopeptide Krestin

PSP .... Polysaccharopeptide

qPCR .... Real-time Polymerase Chain Reaction

RFU .... Relative Fluorescence Units

RNA .... Ribonucleic Acid

RPS .... Reduced Physiological Salt

RT-PCR .... Reverse Transcriptase Polymerase Chain Reaction

SCFA .... Short Chain Fatty Acids

TE .... Tris Ethylenediamine Tetra Acetic Acid buffer

TGGE .... Temperature Gradient Gel Electrophoresis

T-RFLP .... Terminal Restriction Fragment Length Polymorphism

v/v % .... volume/ volume percentage

XOS .... Xyloologosaccharide

1. Introduction

1.1. The gut microbiota

Over the last decade, the importance of the human gut microbiota in metabolic homeostasis and health has been widely recognized and due to its importance has been recently named as the “forgotten organ”. A deeper understanding of the microbiota’s functions, composition and interactions with the host has made some authors compare it to the immune system, since it is comprised of a group of different cells that, together, can either promote health or trigger disease (Marchesi *et al.*, 2016, Sun *et al.*, 2004). Moreover, the composition of the gastrointestinal (GI) microbiota varies along the GI ecosystem with the stomach and proximal small intestine containing a small number of bacteria (due to digestive secretions), while in the ileum and jejunum viable cell numbers may reach *ca.* 10^9 CFU mL^{-1} (mostly aerobic microorganisms) and in the colon *ca.* 10^{12} CFU mL^{-1} viable cell counts may be found, with the local microbiota being comprised of mainly anaerobic microorganisms (Million *et al.*, 2013, Quigley, 2013).

From a composition standpoint, the gut microbiota is mainly comprised by bacteria with several papers reporting on this composition, but virus and eukaryotic organisms can also be found in the gut microbiome (Arumugam *et al.*, 2011, Eckburg *et al.*, 2005, Qin *et al.*, 2010). The human GI microbiota has been described as encompassing 300 to 500 different bacterial species that, despite being frequently regarded as a two phyla ecosystem (Firmicutes and Bacteroidetes), is comprised of at least ten different phyla such as Actinobacteria, Proteobacteria, Fusobacteria or Verrucomicrobia, among others. However, the actual composition of the microbiota is still somewhat unknown as, in spite of the possible combination of molecular techniques and culture based identification methods, there are still many microorganisms that lack taxonomic classification, notwithstanding the intrinsic variations between individuals (Rajilić-Stojanović and de Vos, 2014) (**Figure 1.1.**). In addition to the increasingly consensual phylum level composition of the human gut and to the idea of the microbiota profile as unique as a fingerprint, the concept of clustering microbiota in enterotypes has gained ground. An example of this, is the work of Arumugam *et al.* (2011) who hypothesized if the inter-individual variability was only a result of slight changes in the microbial community or if the microbiota could be clustered around certain stable microbial communities. Nevertheless, factors determining the enterotype clustering are still somewhat controversial (sex, body mass index, age, *etc*) although, three main clusters have already been described in the adult microbiome: *Prevotella*, *Ruminococcus* or *Bacteroides* (Quigley, 2013, Wu *et al.*, 2011).

In addition to bacteria, the human gut is also colonized by viruses (in particular siphophages and prophages, both bacteriophages) and eukaryotic organisms. As bacteriophages infect and replicate within bacteria, they may influence the bacterial population. Furthermore, it has been reported that each individual has a distinct virome whose composition can be affected by diet (Breitbart *et al.*, 2003, Marchesi, 2010, Minot *et al.*, 2011). In parallel, the gut is also colonized by eukaryotes such as fungal species and even some protozoa. While most fungi are Ascomycota and Basidiomycota, fungal diversity varies throughout the gut and is distinct from faeces (Marchesi, 2010). In later years there has been an

increase in evidences that demonstrate that some protozoa may be commensal residents of the human gut which has led to a discussion on the need to re-evaluate the role of protozoa as parasites, since some may have a beneficial effect. For example, reports have shown that *Blastocystis* can be frequently found in healthy individuals (Lukeš *et al.*, 2015, Chabe *et al.*, 2017, Chudnovskiy *et al.*, 2016).

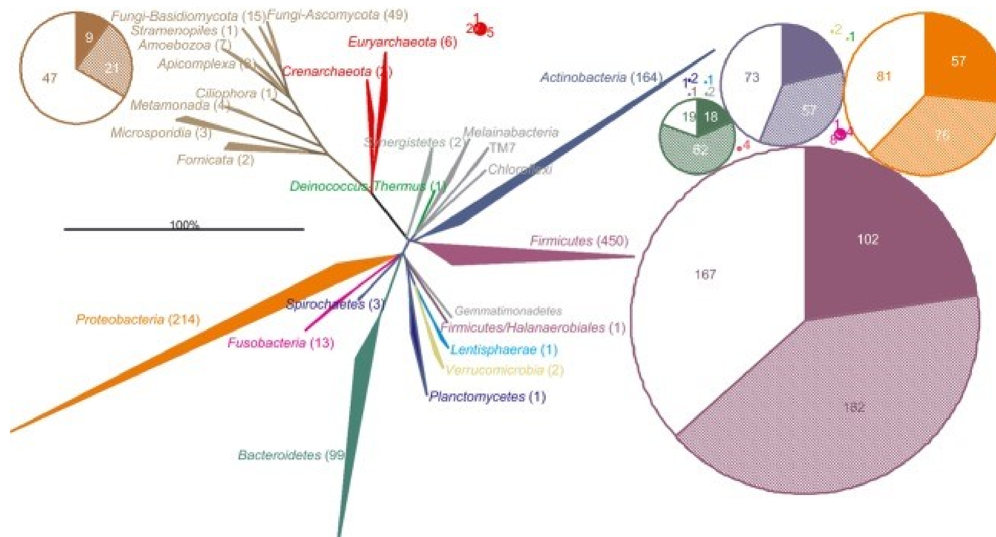

Figure 1.1. *Phylogenetic tree of the human gut microbiota. The numbers in parentheses indicate the number of cultured species per phylum. Reprinted from Rajilić-Stojanović and de Vos (2014).*

In order to understand the impact of the gut microbiota upon host homeostasis, and therefore its role in health and disease, it is necessary to comprehend the range of functions it plays (**Table 1.1.**). As an example of the proposed range of influence of the gut microbiota, several authors have suggested the existence of a gut-brain axis, *i.e.* it has been hypothesized that the microbiota may play a relevant role in the functioning and development of the Central Nervous System (CNS), influencing emotions, human behaviour and even contributing to the amelioration of some neurodegenerative conditions (Szablewski, 2018, Hu *et al.*, 2016, Klingelhoefer and Reichmann, 2015, Mayer *et al.*, 2015, Carabotti *et al.*, 2015, Cryan and Dinan, 2012). Furthermore, it has been suggested that some bacteria produce neurotransmitters and neuromodulators that can affect gut functions like motility (Bienenstock *et al.*, 2015, Carabotti *et al.*, 2015, Sampson and Mazmanian, 2015) or impact inflammation and acid production in the gut (Strandwitz, 2018, Mittal *et al.*, 2017). From a different perspective, since the nervous system communicates with the immune system, the impact of microbiota-brain interactions may also result in immunomodulatory effects, that go beyond those previously associated with microbiota (Wang and Kasper, 2014, El Aidy *et al.*, 2014, Cryan and Dinan, 2012).

Table 3.1. *Main functions associated with the gut microbiota. Adapted from Quigley (2013).*

Function	
1. Metabolite production	• Produces SCFA • Produces arginine and glutamine • Synthesis of vitamins K and B • Participates in drug metabolism • Rescues calories
2. Deconjugation of bile acids	
3. Immunologic effects	• Stimulates Immunoglobulin A (IgA) production • Promotes anti-inflammatory cytokines and down regulates the proinflammatory ones • Induces regulatory T cells
4. Prevention of pathogens colonization	

1.1.1. Influencing factors and modulators of gut microbiota

Many endogenous or exogenous factors can influence the microbiota composition (*e.g.* age, ethnicity, diet, genetic markers or geographic location) and consequently influence the host's wellbeing. For example, the microbiota of a newborn has a low diversity, gaining complexity as the child ages until it reaches a completely developed and distinct microbial profile, similar to that of an adult, after *ca.* 2.5 years. Furthermore, the gut microbiota can be actively modulated by diet, which in turn it is influenced by the geographic location, in particular concerning fibres, proteins, sugar and fat intake. As such, De Filippo *et al.* (2010) noticed differences on the microbiota of African and Italian children with the latter registering higher protein and starch intakes and the former possessing a higher vegetable fibre intake. Similarly, in a study conducted with obese adults, the change from a high-fat/low fibre diet to a low fat/high fibre one, induced changes in the gut microbiota profile, therefore confirming the influence of diet upon gut bacterial composition (Clemente *et al.*, 2012, Conlon and Bird, 2015, Wu *et al.*, 2013). On a different perspective, the consumption of prebiotics, probiotics or antibiotics can induce changes in microbiota composition (Million *et al.*, 2013). Prebiotics are food ingredients/components that can be selectively fermented by gut microbiota and alter it, promoting an increase in beneficial bacteria (Bindels *et al.*, 2015). Probiotics are live bacteria, whose consumption is perceived as beneficial to the host with effects that are, typically, strain specific (Quigley, 2010). Both prebiotics and probiotics have shown to improve *Lactobacillus* and *Bifidobacterium* levels in the gut microbiota, but the composition changes observed are relatively small and temporary (Angelakis *et al.*, 2013, Conlon and Bird, 2015). Antibiotics, whether prescribed or ingested through the food chain, can negatively impact the gut microbiota and, while some bacterial species have fast recovery rates, others suffer harder effects (Conlon and Bird, 2015). In fact, prolonged antibiotic consumption and/or high therapeutic dosages may even cause long-term dysbiosis, a condition characterized by a reduction in microbial diversity with loss of important taxa and consequent metabolic and homeostatic alterations (Lange *et al.*, 2016, Quigley, 2013).

1.1.2. Microbiota metabolites and host interaction

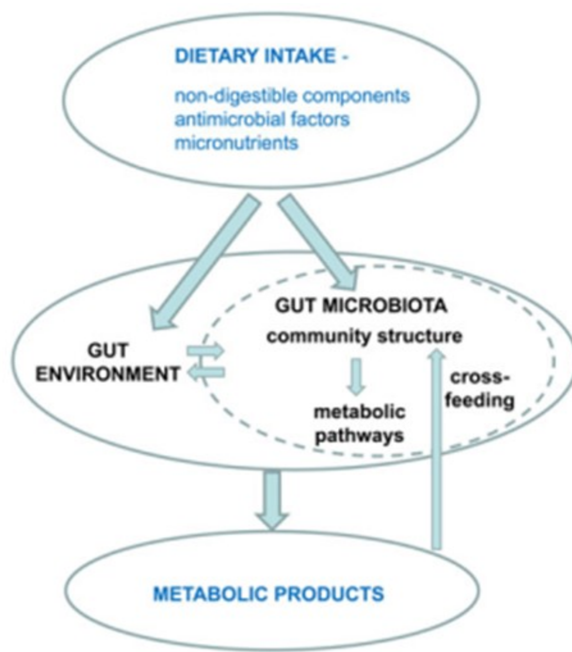

Figure 1.2. *Relation between diet, gut and metabolism. Reprinted from Flint et al. (2015).*

Gut microbiota has a major role in host's metabolism and physiology, particularly regarding energy use and the synthesis of absorbable components. Overall, gut microbiota contains *ca.* 3 million genes, several of which encode information for enzymes that, while important, are not present in the human genetic information and therefore represent a significant contribution for the human metabolism (Rowland *et al.*, 2017, Flint *et al.*, 2015). Ingested dietary components may have a wide range of influence in the gut (schematically represented in **Figure 1.2.**). The gut microbiota metabolizes undigested food components (*e.g.* dietary fibres and indigestible proteins), with preference for the fermentation of carbohydrates. This fermentative process may result in the production of beneficial metabolites, typically Short Chain Fatty Acids (SCFA) like propionate and butyrate. In contrast, the fermentation of undigested proteins and fat results also in the production of SCFA, but with greater variety of metabolites such as ammonia, amines, thiols, indoles, phenols, H_2S, H_2 and CO_2, many of which have potentially toxic effects (Rowland *et al.*, 2017, Ríos-Covián *et al.*, 2016).

1.1.2.1. Short chain fatty acids

Short Chain Fatty Acids are molecules with less than 6 carbons chains (linear or branched) that can be produced by different bacteria through an array of pathways (**Figure 1.3.**). The ingestion of fibres leads to the production of SCFA which in turn lowers the pH values in the colon. This pH oscillation induces alterations in the overall bacterial composition and inhibits the growth of pathogenic bacteria like Enterobacteriaceae or Clostridia (den Besten *et al.*, 2013, Flint *et al.*, 2012, Sun and O'Riordan, 2013). So, as the SCFA concentration decreases from proximal colon to distal colon, the pH value increases. From a different perspective, the production of SCFA has also been associated with the preservation of the gut barrier, a multi-layer complex system which constitutes a physical and functional barrier, as butyrate, for example, has been related to an increased mucin production and to lead to stronger tight-junctions (Ríos-Covián *et al.*, 2016, den Besten *et al.*, 2013, Viggiano *et al.*, 2015). Different SCFA, particularly acetate, butyrate and propionate, have been reported to exert specific effects in the host.

As above mentioned, butyrate has been associated with an increased mucin production and with the improvement of the integrity of tight-junctions. Once formed, this SCFA may be absorbed by the colonocytes and used as the preferred energy source or enter the circulatory system to then be metabolized in the liver. Additionally, butyrate has been associated with anti-inflammatory and anti-

carcinogenic activities as well as with the activation of intestinal gluconeogenesis (Flint *et al.*, 2015, Rowland *et al.*, 2017). The minority that is not absorbed by the colonocytes is transported to the liver where it participates in lipid biosynthesis. Butyrate production is commonly associated with Firmicutes, including members of Clostridium IV and XIVa clusters, and it can be synthetized through two distinct pathways: Butyrate kinase and Coenzyme A transferase.

Acetate, one of the most abundant SCFA is regarded as essential to the growth of gut bacteria and has been reported to be a co-factor in the growth of certain microbial species. Moreover, it represents a minor energy source for colonocytes and so, when it reaches the liver, it is introduced into the lipid's biosynthesis pathway. Acetate is mostly produced by enteric bacteria, namely Bacteroidetes and *Bifidobacterium*, as a result of carbohydrate's fermentation, through the hydrolysis of acetyl-CoA. A smaller portion is synthetized by acetogenic bacteria that utilize hydrogen, carbon dioxide or formic acid, through the Wood-Ljungdahl pathway (Ríos-Covián *et al.*, 2016).

Propionate, which can be produced through three different pathways (acrylate, succinate or propanediol pathway), represents a minor energy source for colonocytes, but has been known for its interaction with the immune system, exerting an anti-inflammatory effect. When taken up by the liver, it interferes with liver gluconeogenesis (Flint *et al.*, 2015, Rowland *et al.*, 2017). Propionate's production is mainly associated to Bacteroidetes, Verrucomicrobia and to bacteria belonging to *Negativutes*.

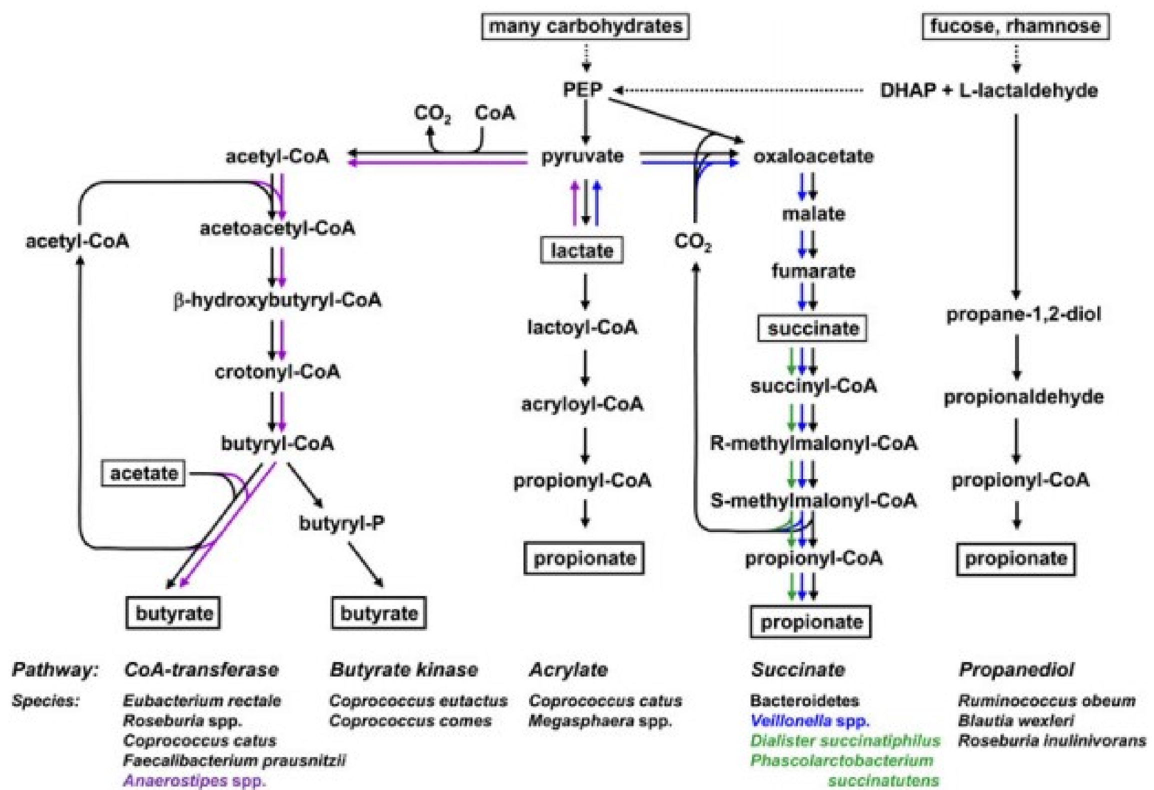

Figure 1.3. *Example of the metabolic pathways of acetate, butyrate and propionate production. Reprinted from Flint et al. (2015).*

1.1.3. Gut microbiota and health

The microbiota has been described as essential for the development of the mucosa and the immune system while also constituting a physical barrier and directly competing with pathogens for colonizing space and nutrients. Therefore, the microbiota is considered to play an important role in well-being, with dysbiosis playing a role in several pathologies such as obesity, liver diseases, Inflammatory Bowel Disease (IBD) or even colorectal cancer (CRC) (Marchesi *et al.*, 2016).

As stated previously, the gut microbiota is often regarded as a two phyla system (Firmicutes and Bacteroidetes) with an imbalance between these two groups often being associated with some health conditions. In fact, an abundance of Firmicutes and low Bacteroidetes levels has been reported to be linked with several obese phenotypes, with authors hypothesizing that a modulation of the first could contribute to the prevention/amelioration of obesity, an increasingly frequent pathology (Bastien *et al.*, 2014, Hruby and Hu, 2015, Morgen and Sørensen, 2014). Nevertheless, the Firmicutes/Bacteroidetes ratio (F/B) and its association with obesity is controversial since contradictory evidences can be found in literature, *i.e.* obese volunteers' microbiota did not exhibit high levels of Firmicutes and low levels of Bacteroidetes (Schwiertz *et al.*, 2010, Duncan *et al.*, 2008, Finucane *et al.*, 2014). Overall, obesity is still considered a multifactorial pathology that results from a continuous imbalance between energy intake and expense, and therefore is highly dependent on the individual's lifestyle, which makes it difficult to consider the gut microbiota as a direct and single cause or solution for this problem (Wang *et al.*, 2017).

A dysbiotic state can be triggered by genetic or environmental factors such as diet or alcohol consumption, with the liver being one of the most affected organs by this state (most of the liver's blood supply comes from the intestines through the portal vein) (Guinane and Cotter, 2013). In alcohol induced dysbiosis the liquor consumption has been reported to cause an overgrowth of certain gut bacteria, in detriment of other beneficial ones. While some authors mention the general overgrowth of Gram negative bacteria, due to the increase of endotoxin levels (Hartmann *et al.*, 2013, Purohit *et al.*, 2008), Engen *et al.* (2015) described an increase in Proteobacteria, Fusobacteria and Firmicutes, particularly in Bacilli. The stimulated bacteria metabolize alcohol into other substances that affect mucin production and tight junction proteins' expression. This, in turn, results in an increase of the gut barrier's permeability to bacteria and their endotoxins, which will affect the liver's homeostasis and stimulate the production of pro-inflammatory cytokines, leading to a pathology known as Alcoholic Liver Disease (ADL) (Cassard *et al.*, 2017, Hartmann *et al.*, 2015). Genetic factors, such as the mutation of patatin-like phospholipase (highly related to liver fat accumulation) and/or environmental factors which are related to dysbiosis (such as obesity, diabetes or hypertriglyceridemia) can also contribute to the development and progression of Non-Alcoholic Fatty Liver Disease (NAFLD). In fact, the role of the intestinal microbiota has been reported to be crucial to the pathogenesis of NAFLD, through the association with obese phenotypes, the promotion of liver inflammation and the production of metabolites, like ethanol, which are toxic to hepatocytes (Gkolfakis *et al.*, 2015, Boursier *et al.*, 2016). Prebiotics and probiotics, acknowledged gut microbiota modulators, may pose an interesting therapeutic resource in the amelioration/control of both ADL and NAFLD, but it still requires a better insight into the mechanisms involving gut bacteria and liver damages (Dubinkina *et al.*, 2017, Boursier *et al.*, 2016).

Dysbiosis has also been associated with other pathologies such as Inflammatory Bowel Disease (IBD), which is characterized by the chronic inflammation of the GI tract in response to environmental factors, in genetically susceptible hosts. Inflammatory Bowel Disease is a term for two conditions: Crohn's disease and Ulcerative Colitis. The most commonly observed pattern for this condition is an overall decrease in bacterial diversity (particularly Firmicutes) coupled with an increase in Proteobacteria. Although some studies point at a causative role of gut bacteria in IBD, it is unclear if dysbiosis is a cause or a consequence of the intestinal inflammation (Matsuoka and Kanai, 2015, Hold *et al.*, 2014). Regardless, the modulation of the gut microbiota, through prebiotic and probiotic consumption or even faecal transplantation, are perceived as good potential therapeutic strategies for these pathologies (Marchesi *et al.*, 2016, Cassard *et al.*, 2017, Matsuoka and Kanai, 2015, Kamada *et al.*, 2013b).

There is accumulating evidence suggesting the involvement of dysbiosis in colorectal carcinogenesis development. Once again, this dysbiotic state can be triggered by environmental factors, such as a high fat/high sugar diet, giving CRC a geographic incidence on western countries. In addition, the host may bear a genetic predisposition to carcinogenesis (Gagnière *et al.*, 2016). It is becoming increasingly accepted that certain pathogens may pose a pro-carcinogenic stimuli through their capacity to alter colonocytes' metabolism via their adherent and invasive properties and their metabolic output (which is perceived to promote continuous inflammatory responses). Some particular bacterial species have been identified as potentially linked to CRC such as, *Streptococcus bovis*, *Fusobacterium* spp., *Helicobacter pylori*, *Bacteroides fragillis* or *Escherichia coli* (Gagnière *et al.*, 2016, Louis *et al.*, 2014). Diet can then play an important role in CRC prevention, since high fibre intakes can lead to an increase in SCFA production, which are essential to the gut homeostasis, some have anti-carcinogenic activity and promote cellular mechanisms that contribute to the tissue integrity (Wang *et al.*, 2017, Conlon and Bird, 2015, den Besten *et al.*, 2013).

1.1.4. Methods for gut microbiota monitorization

For many years, culture-dependent methods were the gold standard for identifying bacteria. However, despite being a cheap and quantitative approach, these methods are not only labour intensive but they disregard non-culturable bacteria and therefore introduce a significant bias in the results (Fraher *et al.*, 2012). Therefore, the development of culture-independent methods, particularly sequencing techniques, allowed for a better insight into the microbial environment. Most of the commonly used techniques are either based on the comparison of highly conserved sequences, such as the small subunit ribosomal Ribonucleic Acid (16S rRNA) or in a shotgun approach (Clarridge, 2004, Lozupone *et al.*, 2012). For instance, Queipo-Ortuno *et al.* (2013) used Real Time Polymerase Chain Reaction (qPCR) to evaluate differences in 16S rRNA and compare the composition of the gut microbiota of rat models with distinct diets and physical activity levels. On the other hand, Yatsunenko *et al.* (2012) reported a shotgun approach to characterize bacterial population of human faecal samples, and evaluate the gene content. De Filippo *et al.* (2010) compared the faecal microbiota of European and African children using a high-throughput 16S rRNA sequencing approach combined with biochemical analysis.

The 16S rRNA, one of the most commonly used genes, contains both highly conserved and variable regions and allows for the identification of microorganisms down to the species level. The sequencing

or amplification of this gene allows for an assessment of the microbial diversity (using either a qualitative or quantitative approach) and therefore the monitorization of microbial population shifts and their subsequent association with altered metabolic states. Some examples of techniques using conserved sequences are qPCR, Reverse Transcription Polymerase Chain Reaction (RT-PCR), Terminal Restriction Fragment Length Polymorphism (T-RFLP) and techniques that also rely on fluorescence emission like Fluorescent *in situ* Hybridization (FISH) and Flow Cytometry. On the other hand, the random shotgun approach implies the sequencing of the entire microbiota community's Deoxyribonucleic Acid (DNA), with it being broken into small constant-sized fragments that can then be compared with existing databases. This high throughput approach allows for the quantification of the relative microorganism's proportions as well as for the establishment of a phylogenetic profile. However, on the down side, and although the costs are decreasing, these techniques are still expensive, and the large amounts of data typically generated frequently require the application of bioinformatic tools. Some of these techniques are summarized in the **Table 1.2.** along with their advantages and disadvantages (Fraher *et al.*, 2012, Ngom-Bru and Barretto, 2012, Thomas *et al.*, 2015).

Table 1.2. *Example of techniques used to describe gut microbiota. Adapted from (Fraher et al., 2012).*

Technique	Advantages	Disadvantages
qPCR	Phylogenetic identification Quantitative approach Fast analysis	PCR bias Unable to identify unknown species
DGGE/TGGE	Semi-quantitative approach Bands can be retrieved for further analysis Fast analysis	No phylogenetic identification PCR bias
T-RFLP	Semi-quantitative approach Low cost Fast analysis	No phylogenetic identification PCR bias
FISH	Phylogenetic identification Semi-quantitative data No PCR bias	Dependent on probe sequences Unable to identify unknown species
DNA microarrays	Phylogenetic identification Semi-quantitative Fast procedure	Cross hybridization, PCR bias, can be difficult to detect in low amounts
Cloned 16S rRNA gene sequencing	Phylogenetic identification Quantitative data	PCR bias, difficult, expensive, cloning bias
Direct sequencing of 16S rRNA amplicons	Phylogenetic identification Quantitative data Fast procedure Identification of unknown bacteria	PCR bias, expensive, difficult
Microbiome shotgun sequencing	Phylogenetic identification Quantitative data	Expensive; analysis of data is difficult

qPCR – Real Time Polymerase Chain Reaction; DGGE – Denaturing Gradient Gel Electrophoresis; TGGE – Temperature Gradient Gel Electrophoresis; T-RFLP – Terminal Restriction Fragment Length Polymorphism; FISH – Fluorescent *in situ* Hybridization;

1.2. Prebiotics

As a result of a growing concern with dietary habits and their impact upon the health and wellbeing, functional foods and additives that may have a beneficial health effect, namely through intestinal microbiota modulation, have gathered the interest of the scientific community, the industry and the consumers. As previously mentioned in section 1.1.1. prebiotics are a specific example of this phenomena. In December of 2016, the International Scientific Association for Probiotics and Prebiotics (ISAPP) reviewed the latest definition of prebiotic defining it as a substrate that is selectively utilized by host microorganisms conferring a health benefit. The word 'substrate' refers to a substance from which an organism obtains nourishment and therefore excludes compounds that function as antimicrobial substances or other microorganisms; *i.e.* the 'substrate' should be a part of the normal metabolism of the bacterial cells and not function as an inhibitor. The concept of selectivity goes beyond the specific stimulation of bifidobacteria or lactobacilli. It can actually encompass more than one bacterial group as long as the microorganisms affected, and metabolites produced, are related to a health benefit and do not lead to gas production. Finally, the notion of health benefit implied in the prebiotic concept is difficult to prove since the effects of prebiotics vary between individuals. Regardless, there is a conceptual need to establish a causality relation between a specific action on microbial population and a positive health outcome, with all of this having in mind that the potential health benefits may occur beyond the GI tract, such as in the cardiovascular (*e.g.* blood lipid levels modulation) and nervous systems (*e.g.* through metabolites that influence brain function) (Gibson *et al.*, 2017).

On a different note, some prebiotic related concepts need to be clarified, such as the similarity/differences between prebiotics, dietary fibres, and bioactive polysaccharides, particularly as they are frequently claimed to have the same beneficial effects and/or have the same nature (carbohydrates). Bioactive polysaccharides are carbohydrates, from a natural source, that show specific biological activity (Bindels *et al.*, 2015, Quigley, 2010). The term dietary fibre refers to carbohydrate polymers, with ten or more monomeric units, which are hydrolysed by colonic bacteria exogenous enzymes (Bindels *et al.*, 2015, Quigley, 2010). The Food and Agriculture Organization (FAO) stated that a prebiotic can be a dietary fibre, but a fibre does not have to be a prebiotic. Regulatory agencies around the world, such as the U.S. Food and Drug Administration (FDA) or Europe's European Food Safety Authority (EFSA), have distinct criteria when defining prebiotics, a fact that significantly impacts the labelling of foodstuffs. Therefore the food industry is interested in developing a stable and universal definition of prebiotic, particularly as this concept gains the consumer's acceptance (Hutkins *et al.*, 2016).

The categories of prebiotics vary according to their origin and/or structure. Their monosaccharide composition, the degree of polymerization (DP), chain length and the type of linkage between monomers determines where and how it will interact with the microbiota, since they affect which species will be stimulated and in which portion of the gut the fermentation will occur. Prebiotics are frequently classified according to their number of monomeric units and the nature of the first residue (fructose or glucose). The most commonly recognized prebiotics in Europe are inulin, galactooligosaccharides (GOS),

fructooligosaccharides (FOS), polydextrose and lactulose while other oligosaccharides, such as xylooligosaccharides (XOS) and isomalooligosaccharides (IMO) are considered emerging prebiotics (Jain *et al.*, 2015, Gibson *et al.*, 2017). Inulin has a DP above 10 whereas the other oligosaccharides are short chains of 3 to 10 monomers. It is important to consider that prebiotic compounds may affect the organoleptic properties of foods. For example, inulin has been widely used by the food industry as a fat replacer and/or a texture modifier due to its ability to form gels, but it is not associated with a sweet flavour. FOS has a good solubility and is mainly used as a sugar replacement and for its prebiotic properties. GOS is known for being very resistant to acidic conditions and high temperatures, which facilitates its incorporation into warm or acid foodstuffs although their main application is in infant formulas. Whole grains and some dietary fibres, resistant starches, arabinoxylan and non-carbohydrates, with a gut microbiota modulating effect, are potential candidates to be recognized as prebiotics (Patel and Goyal, 2012, Bindels *et al.*, 2015, Quigley, 2010, De Souza Oliveira *et al.*, 2011, Aachary and Prapulla, 2011).

As previously mentioned, prebiotics, either naturally occurring or incorporated into foods, will affect the microbiota composition and its metabolic activity. To accomplish this, they must pass through most of the human GI tract without being digested by human enzymes and therefore reach the colon almost unaltered, where they can then be fermented by certain bacterial species. The majority of prebiotics target bifidobacteria which are strongly associated with healthy gut environment (Christensen *et al.*, 2013, Vandenplas *et al.*, 2015). Thus, most prebiotics increase bifidobacteria levels, in turn inhibiting the growth of pathogenic bacteria (Yoo and Kim, 2016). Prebiotic's consumption has also been associated with a positive impact upon lipid metabolism (and a subsequent cholesterol reduction) and with a beneficial effect upon the immune system as they have been reported to alleviate the inflammatory responses in conditions such as IBD. Moreover, prebiotic's ingestion may also affect cellular apoptosis, consequently reducing the rates of CRC, as well as affect the bioavailability of minerals like calcium (Pineiro *et al.*, 2008, Patel and Goyal, 2012, Slavin, 2013, Charalampopoulos and Rastall, 2012).

1.3. Mushrooms as functional ingredients

Mushrooms' consumption has begun many centuries ago in Asia, but nowadays it has spread worldwide, becoming more relevant in our nutrition. In fact, the current worldwide consumption *per capita* reaches 4 kg per person. Their associated health benefits and a deeper consumer awareness have contributed to this tendency (Royse, 2014, Aida *et al.*, 2009). Overall, there are around 5.1 million fungal species in world with 12 000 to 14 000 of these being considered mushrooms of which only 2 000 species are considered edible. Five genera constitute 85% of the world's mushroom growth namely *Agaricus*, *Pleurotus*, *Lentinula*, *Auricularia* and *Flammulina* (Royse, 2014, Cruz *et al.*, 2016).

Mushrooms can be found as fresh, biomass or extract. The biomass is comprised of the mycelium and the primordia and is produced using a sterilized substrate to prevent contamination with other fungi and

ensure the absence of heavy metals and pesticides. Mushroom biomass is also more resistant to gastric fluids than extracts, since the biomass granules and cells represent a structural barrier (protecting bioactive ingredients from digestion) and it possesses not only the typical polysaccharides, but also active enzymes, which prevent oxidative stress, enhance immunity, *etc*. This product is usually considered a food or dietary supplement. On the other hand, mushroom extract is a concentrated extract of the mushroom's fruiting bodies. This product has been more extensively studied and has a higher market value. Nevertheless, they lack enzymatic activity because the extraction process results in the denaturation of proteins. The extracts are often associated with the concept of 'nutraceuticals' or 'pharmaceutical compounds'. However, it is important to understand the lack of consistency regarding the application of terminology such as 'functional food', 'nutraceutical' and 'dietary supplement', since the criteria varies from country to country (Ferrão L, 2017, Cruz *et al.*, 2016, Barros *et al.*, 2016b).

Currently, there are several mushroom based supplements as summarized by Reis *et al.* (2017): powder, hot water or alcoholic extracts from artificially cultivated fruiting bodies; combined preparations of substrate, mycelium and primordial mushroom, dried and pulverized; biomass or extracts from mycelium harvested from liquid culture grown in bioreactors; naturally grown and dried mushroom fruiting bodies, as tablets or capsules or spores and their extracts.

Mushrooms are made up of enzymes (*e.g.* peroxidase, *β*-glucanase), polysaccharides (*e.g. α*- and *β*-glucans, hemicellulose, chitin), glycoproteins (*e.g*. Polysaccharide Krestin (PSK), Polysaccharopeptide (PSP)), lipids (*e.g.* cholesterol), minerals (*e.g.* germanium), vitamins (*e.g.* B1, B2, D2, C), terpenes (*e.g.* triterpenes) and phenolic compounds (*e.g.* flavonoids). They are often considered a low-calorie food, due to their high-water, proteins and fibre content and low amounts of fat. These components, in particular polysaccharides, have many potential benefits associated, which are listed in **Table 1.3.** (Cruz *et al.*, 2016, Aida *et al.*, 2009).

Table 1.4. *Mushroom properties and respective bioactive compounds.*

Properties	Bioactive Compounds	Reference
Antitumor	Lentinan Glucan Mushroom biomass Glucan *Hericium erinaceus* extracts	Okamoto *et al.* (2004), Kim *et al.* (2004), Zhang *et al.* (2009), Li *et al.* (2014)
Antiviral	anti-HSV protein polysaccharide Fruiting bodies	Gu *et al.* (2007), Faccin *et al.* (2007), Hobbs (2000)
Immunomodulation	*β*-D-Glucan Cordycepin Mycelium polysaccharide Crude polysaccharide	Kim *et al.* (2005), El Enshasy and Hatti-Kaul (2013), Yang *et al.* (2004), Chen *et al.* (2012), Bimczok *et al.* (2009)
Antioxidant	Phenolic compounds *G. lucidum* peptide Ethanolic extract Polysaccharides	Jayakumar *et al.* (2009), Sun *et al.* (2004), Liu *et al.* (2013), Ker *et al.* (2011)
Prebiotic	*β*-Glucans PSP	Synytsya *et al.* (2009), Chen *et al.* (2013), (Pallav *et al.*, 2014)

PSP - Polysaccharopeptide; HSV - Herpes Simplex Virus

1.3.2. *Coriolus versicolor*

Coriolus versicolor, also known as *Trametes versicolor* or *Polyporus versicolor,* belongs to the genus *Coriolus*, family Polyporaceae, order Polyporales and division Basidiomycotina. It is an obligate aerobe fungus and has a fan-shaped wavy margin and coloured concentric zones (**Figure 1.4.**). It can be found the entire year, on tree trunks, branches and stumps in temperate forests of the northern hemisphere (Asia, Europe and North America). The fruiting body of this mushroom, known as Yun-zhi, has been used for centuries in traditional Chinese medicine. Chu *et al.* (2002) reports that it is documented that *C. versicolor* exerts effects on the host's immune function increasing energy, removing toxins, lifting the spirit, among others. In fact, *C. versicolor* derived preparations have been included in the modern clinical practice of Asian countries such as Japan or Korea. In a different perspective, this species has also been studied for its potential to bioconvert

Figure 1.4. Coriolus versicolor. *Reprinted from Midwest Mycological Information.*

lignocellulosic wastes (Cruz *et al.*, 2016, Chen *et al.*, 2013, Cui and Chisti, 2003, Dashtban *et al.*, 2009, López *et al.*, 2002).

Like for other mushrooms, *C. versicolor* can be commercially found either as an extract or as biomass formulations which have distinct compositions and are produced using different parts of the mushroom. Extracts are the most widely used and studied with the most common ones being PSP or PSK), which are among the most commercially successful supplements in the world (Cui and Chisti, 2003, Chang and Buswell, 2008). On the other hand, as previously mentioned, biomass contains other biologically relevant compounds such as enzymes, which in turn, given their presence within the mushroom tissues, are more protected from the digestion's effect.

Coriolus versicolor polysaccharides (both intra and extracellular) have been associated with an array of health benefits namely antitumoral (Luo *et al.*, 2014, Lee *et al.*, 2006, Sekhon *et al.*, 2013), antioxidant (Pang *et al.*, 2000, Kozarski *et al.*, 2012), prebiotic (Chen *et al.*, 2013, Pallav *et al.*, 2014), antiviral (Collins and Ng, 1997), antidiabetic (Hsu *et al.*, 2013) and immunomodulatory activities (Yang *et al.*, 2015). Polysaccharide Krestin and PSP obtained from CM-101 and Cov-1 strains respectively, are known as biological response modifiers that can complement conventional therapies. The oral administration of these extracts seems to have an effect in controlling the proliferation of certain carcinomas as well as potentiating the immune response by inducing the production of macrophages, T-lymphocytes, interferons and immunoglobulins. Moreover, it has been reported as reducing tumours inducing immunosuppression and these extracts also seem to be beneficial regarding intestinal disorders (Fisher and Yang, 2002, Cui and Chisti, 2003). As for the prebiotic potential, the fact that *C. versicolor's β*-glucans, PSP and PSK, are resistant to the digestive process, means that they are likely to reach the microbiota and may, therefore, exert some type of influence over it. Chen *et al.* (2013) suggested that PSP promoted the growth of beneficial bacteria (lactobacilli and bifidobacteria) while Pallav *et al.* (2014) stated that PSP could modulate the gut microbiota. PSK were also reported to have an antioxidant effect (Cruz *et al.*, 2016). Additionally, when it comes to *β*-glucans, it is important to stand out that more studies are necessary to consider them as established prebiotics.

1.4. Work objectives

The gut microbiota has been reported as playing a vital role in human health and wellbeing, with prebiotics gaining the attention of the scientific community given their ability to modulate it. In parallel, mushrooms have been associated to a wide range of beneficial effects on the human health. However, most studies focus on extract's properties, leaving the biomass unstudied. As such, the objective of this research work was to evaluate the potential prebiotic effect of *Coriolus versicolor* biomass in the human gut microbiota using a human faecal fermentation model. To accomplish this, the mushroom biomass was subjected to a simulated digestive process and then used to evaluate the evolution of specific bacterial groups, while monitoring the formation of SCFA, which are essential to the proper functioning of the large intestine.

2. Materials and Methods

2.1. Sample information

The *C. versicolor* biomass was kindly provided by Mycology Research Laboratories (UK). This biomass was produced using CV-OH1 strain and had an appearance of a medium beige free flowing powder. The product was packed in a white 200 mL opaque container and 1 g silicagel desiccant bag. The sample batch number was 17B13H and the batch size was 450. The ingredients listed were: *C. versicolor* biomass, Microcrystalline Cellulose, Silica and Vegetable Magnesium Sterate. The nutritional information available *per* 100 g of product was: 396 kcal, 6.34 g of proteins, 3.63 g of fat and 84.55 g of carbohydrates.

2.2. *In vitro* digestion simulation

The digestion's simulation was performed according to Madureira *et al.* (2005) with some modifications. Initially, 2 g of *Coriolus versicolor* powder were suspended in 250 mL of tap water, in agreement with the manufacture's indication for daily consumption. In order to simulate mouth digestion (as the samples' pH values were between 5.6 and 6.9 no adjustments were necessary) an *α*-amylase (Sigma-Aldrich Chemistry, St. Louis, USA) solution (117.5 U mg^{-1}) was added to the suspension at a rate of 0.6 mL min^{-1} of digestion, to mimic the action of saliva. The sample was incubated for 1 min, at 37 ºC with agitation (200 rpm). Afterwards, to mimic stomach conditions, the pH value was adjusted to 2.0 using HCl (Merck, Darmstadt, Germany) 1 M and a solution that simulates the gastric juice was added (25 mg mL^{-1} pepsin, Sigma-Aldrich Chemistry, St. Louis, USA) at a ratio of 0.05 mL mL^{-1} of sample. The resulting mixture was then incubated (37 ºC, 130 rpm) for 60 min. Following this, the pH value was adjusted to 6.0 using $NaHCO_3$ (Sigma-Aldrich Chemistry, St. Louis, USA) 1 M and a solution of pancreatin (2 g L^{-1}; Sigma-Aldrich Chemistry, St. Louis, USA) and bile salts (12 g L^{-1}; Fulka Analytical, Morris Plains, USA) was added at a ratio of 0.25 mL mL^{-1} of sample. The solutions were then incubated for 120 min at 37 ºC at 45 rpm. Finally, intestinal absorption was simulated using a semi permeable dialysis membrane (Biotech Cellulose Ester Dialysis Membranes 100-500 Da; Spectrum Laboratories, Inc, Waltham, USA) that was submerged in distilled water (with constant stirring) for 48 h, with water being replaced after the initial 24 h period. Samples were collected at each point throughout the assay for further characterization and the final content of the dialysis membranes was freeze dried using a Christ freeze dryer (Alpha 1-4, Osterode Am Harz, Germany). The resulting powder was used later for the gut microbiota fermentations. All assays were performed in duplicate. All pH measurements were undertaken using a pH meter equipped with a Hach 52-07 pH electrode (Loveland, USA).

2.3. Protein quantification and characterization

The collected samples were thawed at room temperature and centrifuged at 6026 g, for 15 min. Before analysis, the supernatant was filtered through 0.22 µm filters (Milipore, Darmstadt, Germany). Protein quantification and characterization was performed using a Fast Protein Liquid Chromatography (FPLC) system (AKTA pure GE Healthcare Life Sciences, Chicago, USA), connected to a Superdex™ 200 Increase 10/300 GL column (GE Healthcare Life Sciences, Chicago, USA). The system was operated with a phosphate buffer (pH 7) containing NaCl and NaN_3 at a flow rate of 0.5 mL min^{-1}. The absorbance measurements (280 nm) were carried out in duplicate and the results expressed in Absorbance Units (AU) per volume (mL).

2.4. Free amino acids analysis

2.4.3. Mobile phase and reagent preparation

The chromatographic analysis was carried out using two distinct eluents, A and B. Eluent A consisted of 10 g L^{-1} sodium phosphate dibasic dihydrate (Sigma-Aldrich Chemistry, St. Louis, USA), 7.4 g L^{-1} propionic acid (Sigma-Aldrich Chemistry, St. Louis, USA), 20 mL L^{-1} dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemistry, St. Louis, USA), 65 mL L^{-1} acetonitrile (Fisher Chemical, Waltham, USA) and ultrapure water, with the final pH value being adjusted to 6.65 using NaOH 4 M. Eluent B was composed of 330 mL L^{-1} methanol (Fisher Chemical, Waltham, USA), 70 mL L^{-1} DMSO, 400 mL L^{-1} acetonitrile and ultrapure water. Both eluents were filtered under vacuum and degassed. The reagent A (25 mL) consisted of 3 mL of a previously prepared internal standard solution (20 mg mL^{-1} of homoserine and norvaline (Sigma-Aldrich Chemistry, St. Louis, USA) in 0.1 M HCl), 120 µL mercaptoetanol (Fluka Analytical, St. Louis, Missouri, EUA), 500 mg of sodium tetraphenylborate (Merck, Darmstadt, Germany) and 25 mL of borate buffer. Reagent B (100 mL) was comprised of 3.5 g of iodoacetic acid (Sigma-Aldrich Chemistry, St. Louis, USA), 50 mL of borate buffer, adjusted to a pH value of 9.5 with NaOH 4 M and the volume was completed with borate buffer. Finally, 50 mL of reagent C were prepared by mixing 225 mg of OPA (Phthaldialdehyde) (Sigma-Aldrich Chemistry, St. Louis, USA), 5 mL of methanol and completed to 50 mL with borate buffer. Then, 0.5 mL of mercaptoethanol were added and the solution was bubbled with N_2.

2.4.2 Chromatographic analysis

The characterization and quantification of the free amino acids was performed using a liquid chromatography apparatus (HPLC Gold 128 Solvent module, Beckman Coulter, Brea, USA) with a High Resolution Fluorescence Detector ($\lambda_{excitation}$ 356 nm; $\lambda_{emission}$ 445 nm; Waters 474, Milford, USA) and an autosampler (model 410 Varian prostar, Agilent technologies, Santa Clara, USA). The system was connected to a Chromolith® Performance RP18 (4.6 × 100 mm) (Merck, Darmstadt, Germany) column, operating at a flow rate of 0.8 mL min^{-1}. From the filtered samples (prepared as described in section 2.2.), 100 µL were mixed with 250 µL of reagent A, and 250 µL of reagent B. After 3 min, 250 µL of

reagent C were added and 10 µL of the mixture was injected into the HPLC system. All samples were injected in duplicate.

2.5. *In vitro* faecal fermentations

2.5.1. Collection and preparation of faecal inocula

Fresh faecal samples were collected from five healthy donors (A-E, three men and two women, ages between 23 to 63 years old), who had fulfill the criteria stablished (**Appendix I** and **II**- Informed consent form and Instructions for stool specimen collection). The faecal samples were maintained under anaerobic conditions, for a maximum of 2 h before being used. The faecal inocula (FI) was then prepared, by diluting the faecal matter in Reduced Physiological Salt solution (RPS) (constituted by 0.5 g L^{-1} cysteine-HCl (Merck, Darmstadt, Germany) and 8.5 g L^{-1} NaCl (LabChem, Zelienople, USA)) with a final pH value of 6.8, at 100 g L^{-1} in an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK) (10% CO_2, 5% H_2 and 85% N_2).

2.5.2. Nutrient Base Medium preparation

Nutrient Base Medium was used to carry out the faecal fermentations. The medium was comprised of 5.0 g L^{-1} trypticase soy broth without dextrose (Fluka Analytical, St. Louis, Missouri, EUA), 5.0 g L^{-1} bactopeptone (Becton Dickinson Biosciences, New Jersey, USA), 0.5 g L^{-1} cysteine-HCl (Merck, Darmstadt, Germany), 1.0% (v/v) of salt solution A [100.0 g L^{-1} NH_4Cl (Merck, Darmstadt, Germany), 10.0 g L^{-1} $MgCl_2 \cdot 6H_2O$ (Merck, Darmstadt, Germany), 10.0 g L^{-1} $CaCl_2 \cdot 2H_2O$ (Carlo Erba, Chaussée du Vexin, France)], 1.0% (v/v) of trace mineral solution (ATCC, Virginia, USA), 0.2% (v/v) of salt solution B [200.0 g L^{-1} $K_2HPO_4 \cdot 3H_2O$ (Merck, Darmstadt, Germany)] and 0.2% (v/v) of a 0.5 g L^{-1} resazurin solution (Sigma-Aldrich Chemistry, St. Louis, USA). The medium final pH value was adjusted to 6.8 and was then bubbled with N_2 until it presented a translucent/yellowish colour. Following this, 50 mL were then distributed into several containers. Fructooligosaccharides (FOS) (Nutripar, Matosinhos, Portugal) and the freeze-dried digested *C. versicolor* biomass were added to the respective vessels at a final concentration of 20 g.L^{-1}. The bottles were capped and autoclaved. Following sterilization, and before adding the faecal inocula, the atmosphere of each flask was refluxed with a gas mixture (10% CO_2, 5% H_2 and 85% N_2) sterilized using a 0.22 µm filter (Millipore, Burlington, USA).

2.5.3 Faecal fermentations

The flasks prepared in 2.4.2. were inoculated at 2% (v/v) with faecal inocula (section 2.4.1) and incubated for 48 h at 37 ºC under anaerobic atmosphere (10% CO_2, 5% H_2 and 85% N_2). Samples were collected after 0, 12, 24 and 48 h of incubation and the pH values were measured using a MicropH 2002 pH meter (Crison, Barcelona, Spain), equipped with a 52-07 pH electrode (Crison, Barcelona, Spain). The positive and negative controls were respectively designated as C+ (FOS) and C- (only faecal inocula), while the *C. versicolor* digested biomass was dubbed Cv. Afterwards, the samples were stored at -30 ºC until analysis. All the steps considered in this section were carried out inside an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK).

2.5.4. Faecal fermentation sample's processing

Aliquots (4 mL) of each sample collected in section 2.4.3. were centrifuged for 6 min. The resulting supernatants were used to evaluate organic acid production and the pellet used to extract the genomic DNA.

2.6. Bacterial population analysis

2.6.1. DNA extraction

DNA was extracted using a NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal) according to the manufacturers' instructions, with some adaptations. Briefly, pellets were washed with TE (pH 8.0; Tris EDTA buffer), vortexed and centrifuged at 4000 g for 10 min, a process that was repeated until the supernatant was colourless. Then, 180 µL of a freshly prepared lysozyme solution (10 mg mL^{-1} lysozyme in a NaCl-EDTA solution; 30 mM NaCl and 10 mM EDTA) were added and incubated for a period of 1 h, at 37 ºC, with periodic shaking. Afterwards, 350 µL of NT1- buffer were added to samples which were then vortexed and incubated at 95 ºC. After 10 min, samples were centrifuged (11000 g, 10 min, 4 ºC), supernatants (200 µL) were mixed with 25 µL of proteinase K, and incubate at 70 ºC during 10 min. The remaining steps were performed accordingly to the manufacturer's instructions (**Annex I -** NZY tissue gDNA isolation kit). After extraction, DNA's purity and concentration were assessed using a Thermo Scientific™ µDrop™ Plate coupled with a Thermo Scientific™ Multiskan™ FC Microplate Photometer (Thermo Fisher Scientifc, Waltham, USA).

2.6.2. Real Time Quantitative Polymerase Chain Reaction

Real-time PCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA), under the conditions described in Table 2.1.

Table 2.1. *Real-time PCR conditions*

<table>
<tr><th>PCR stage</th><th>Temperature</th><th>Time</th><th>Number of Cycles</th></tr>
<tr><td>Initial denaturation/ enzyme activation</td><td>95 ºC</td><td>10 min</td><td rowspan="4">45</td></tr>
<tr><td>Denaturation</td><td>95 ºC</td><td>10 s</td></tr>
<tr><td>Annealing</td><td>Specific temperature for each primer</td><td>1 min</td></tr>
<tr><td>Extension *</td><td>72 ºC</td><td>15 s</td></tr>
<tr><td>Melting curve*</td><td>60-97 ºC, with an increment of 0.5 ºC for 0.05 min</td><td>0.05 s</td><td></td></tr>
</table>

*Stages in which fluorescence is measured

The PCR reaction mixture comprised of 5 µL of 2x iQTM SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Hercules, USA), 2 µL of ultrapure water, 1 µL of sample DNA (equilibrated to 20 ng μL^{-1}) and 1 µL of forward and reverse primers (100 nM) targeting the 16S rRNA gene. The primers used were obtained from STABvida (Lisbon, Portugal) and are listed in **Table 2.2.**. Standard curves were constructed using tenfold dilutions (from 2 log to 6 log of number of copies of 16S rRNA gene μL^{-1}) of bacterial genomic DNA standards (DSMZ, Braunschweig, Germany) (**Table 2.2.**) and were drawn using the number of copies of the 16S rRNA gene of each bacterial strain in the relation to the quantification cycles (Cq) (**Appendix III-** Bacterial calibration curves for qPCR). Melting curve analysis was performed for each PCR in order to evaluate the specificity of the amplification, considering a temperature interval from 60 to 97 ºC. All assays were performed in quadruplicate.

Table 2.2. *Primer sequences targeting bacterial groups, genomic DNA standards and PCR product size. Adapted from Marques et al. (2016).*

Target group	Primer sequence (5'-3')	Genomic DNA standard	PCR Product size	Annealing temperatures (ºC)	References
Firmicutes	F ATG TGG TTT AAT TCG AAG CA R AGC TGA CGA CAA CCA TGC AC	*Lactobacillus gasseri* ATCC 33323	126	45	Queipo-Ortuno *et al.* (2013)
Bacteroidetes	F CAT GTG GTT TAA TTC GAT GAT R AGC TGA CGA CAA CCA TGC AG	*Bacteroides vulgatus* ATCC 8482	126	45	Queipo-Ortuno *et al.* (2013)
Bacteroides	F ATA GCC TTT CGA AAG RAA GAT R CCA GTA TCA ACT GCA ATT TTA	*Bacteroides vulgatus* ATCC 8482	495	45	Matsuki *et al.* (2004)
Clostridium leptum subgroup	F GCA CAA GCA GTG GAG T R CTT CCT CCG TTT TGT CAA	*Clostridium leptum*	239	45	Matsuki *et al.* (2004)
Lactobacillus	F GAG GCA GCA GTA GGG AAT CTT C R GGC CAG TTA CTA CCT CTA TCC TTC TTC	*Lactobacillus gasseri* ATCC 33323	126	55	Delroisse *et al.* (2008)
Bifidobacterium	F CGC GTC YGG TGT GAA AG R CCC CAC ATC CAG CAT CCA	*Bifidobacterium longum subsp. Infantis* ATCC 15697	244	50	Delroisse *et al.* (2008)

F- forward primer; R- reverse primer

2.7. Sugars and SCFA analysis

Sugar consumption and organic acid production during faecal fermentation were analysed using an HPLC system comprised of a Knauer K-1001 pump (Berlin, Germany), an ion exchange Aminex HPX-87H (300 x 7.8 mm) (Bio-Rad, Hercules, USA) column and two detectors assembled in series, namely a UV-vis detector (220 nm) and a refractive index detector, both from Knauer (Berlin, Germany) at a temperature of 65 ºC. An isocratic gradient was used (13 mM sulfuric acid; Merck, Darmstadt, Germany), at a flow rate of 0.6 mL min^{-1}. The injection volume was of 40 µL and the running time was 30 min. Fermentation supernatants were filtered through a 0.22 µm syringe filter and each sample was injected in duplicate.

2.8. Statistical analysis

Statistical analysis of the data was performed using IBM SPSS Statistics v21.0 (IBM, Chicago, USA). The normality of the data's distribution was evaluated through Shapiro-Wilk's test. As the data proved to follow a normal distribution, One-way ANOVA, coupled with Tukey's post hoc test was used to determine the significance of *C. versicolor* biomass' effect on bacterial populations, at each time point. Repeated Measures ANOVA was used to evaluate the effect of *C. versicolor* biomass on the bacterial population trough time. Differences were considered significant for p-values ≤ 0.05.

3. Results and Discussion

3.1. Simulation of the gastrointestinal tract conditions

A simulation of the digestive process was performed several times in order to achieve the mass needed for the faecal fermentations. In order to get a better insight on the impact of the digestive process on the sample, a FPLC analysis was performed to characterize the protein content. In **Figure 3.1.** it is possible to observe the FPLC profiles of the samples collected after each digestion step, in which 5 different sets of peaks were identified.

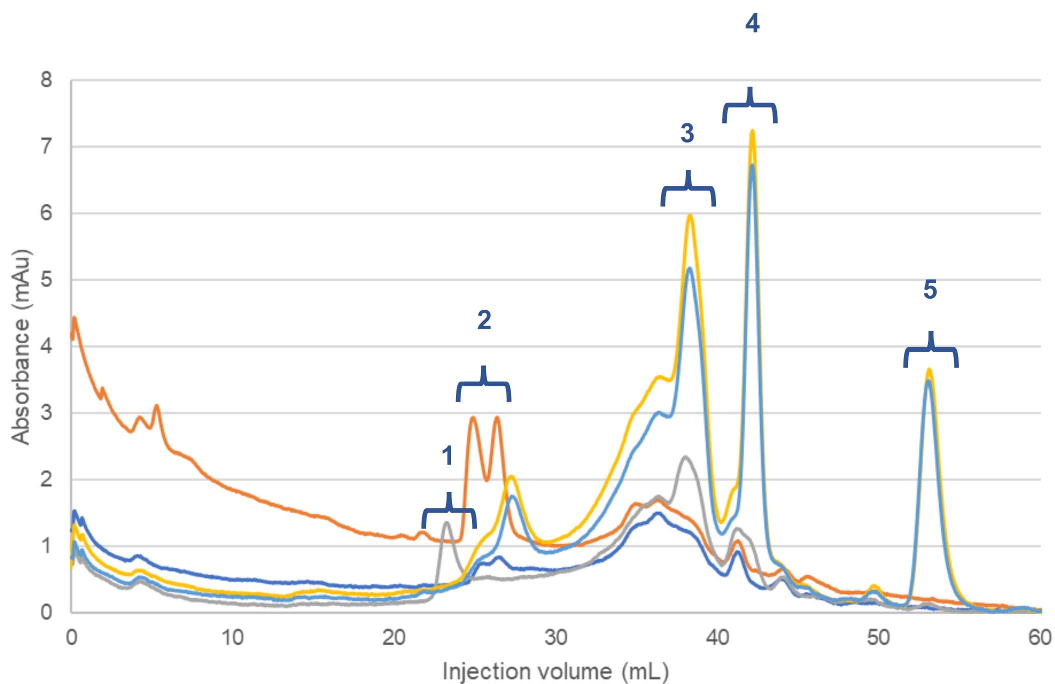

Figure 3.1. *FPLC profile of* C. versicolor *biomass throughout the digestion simulation.— Initial sample;— Mouth digestion;— Stomach digestion;— Intestinal digestion;— After dialysis.*

Overall, it was possible to observe that there was an increase in the prevalence of low molecular weight fragments throughout the digestive process, which indicates a proteolytic effect of the enzymatic solutions added upon the sample in the different GI tract compartments. The initial sample and the one collected after mouth digestion exhibited similar profiles regarding the lower molecular weight fractions, which could be expected since only *α*-amylase was added. Moreover, the profiles after intestine simulation and after dialysis were also very similar, which could also be expected, since no proteolytic enzymatic solutions were added between these steps. However, in the initial sample two distinct peaks of 44 and 29 kDa (**Figure 3.1.** 2) were observed. After stomach digestion, a peak of *ca.* 72 kDa (**Figure 3.1.** 1), not present in the other samples, was observed along with several other peaks which correspond to fragments with a molecular weight lower than 1.2 kDa that may be the result of the proteolytic activity of pepsin. After intestinal digestion and after dialysis only fragments smaller than 1.2 kDa were detected (**Figure 3.1.** 3 to 5). The product information sheets provided by the supplier reports that *α*-amylase has a molecular mass between 58 and 62 kDa, pepsin mass is approximately 35 kDa, and tripsin has a molecular mass of 23.3 kDa, so, none of the enzymatic solutions added corresponds to the 72 kDa peak observed. The salivary *α*-amylase present in mouth digestion is known for having an optimal pH of 6.8 and it is thought to be denaturated when it reaches the stomach due to the acidic environment (Minekus *et al.*, 2014, Alpers, 2003). Additionally, Yadav and Prakash (2011) reported an optimal *α*-amylase activity within the pH range of 4.7 to 7 and reported a decrease in the enzymatic activity bellow 4.7. The authors also reported a gradual protein unfolding, as a consequence of the acidic stomach environment, along with a process of protein aggregation. So, it can be hypothesized that the 72 kDa peak observed may be a result of *α*-amylase aggregation. Cruz (2015) also performed a FPLC analysis of *C. versicolor* biomass and the sample exhibited the same profile throughout digestion and had a peak between 12 and 22 kDa. These results are not in line with the present results, since a distinct profile was observed through digestion and the initial sample had 44 and 29 kDa peaks and a 72 kDa peak after stomach simulation. It can be hypothesized that the differences between these protein profiles of the same mushroom biomass can be due to differences in the mushroom composition.

Mushrooms have been reported to have a low caloric, sodium and fat contents while being rich in protein, carbohydrates, fibres, vitamins, minerals (*e.g.* Mg, K, Zn, Cu), and unsaturated fatty acids (Cruz *et al.*, 2016, Aida *et al.*, 2009, Stachowiak and Reguła, 2012). Moreover, edible mushrooms possess several proteins, among which are several enzymes , with an interesting bioactive potential like lectins, laccases, ribosome inactivating proteins, peroxidases, ribonucleases, *etc* (Xu *et al.*, 2011). Rau *et al.* (2009) reported that, fruiting bodies' extracts of *C. versicolor*, possess three different protein fractions, 1200, 150 and 15 kDa, with the last being the richest in terms of overall protein content. These protein fractions were not observed in the FPLC analysis of *C. versicolor* biomass as the range of the calibration curve was between 66 and 1 kDa, so it was not possible to verify the existence of 1200 and 150 kDa fragments. Additionally, the protein fractions above mentioned were determined using mushrooms extracts and not biomass. Moreover, it is difficult to establish any comparisons between what has been reported in literature and those observed in the present work as the fractions reported were characterized using purified protein extracts while the present work focused on the soluble fractions. Regardless, Singh *et al.* (2014) stated that lectins from edible mushrooms ranged from 12 to 68 kDa and Johansson and

Nyman (1993) isolated a manganese (III) peroxidase with 44 kDa, which correspond to molecular weights that are among those observed in **Figure 3.1.**.

To further characterize the impact of the digestive process upon the protein fraction of the sample, the free amino acids' profile was characterized in order to understand if the proteolysis, consequential of the digestion simulation, resulted in a progressive release of amino acids. As can be seen in **Table 3.1.**, there was an increase in the number of identified amino acids throughout the digestion, particularly between stomach and intestinal simulation. When comparing the initial sample with those after mouth and stomach simulation no consistent trend was observed regarding the amino acids' concentration, however when comparing the sample after intestinal digestion and dialysis there was an overall decrease in amino acid concentration which demonstrated their diffusion throughout the membrane. Some authors reported that *C. versicolor* has 18 different amino acids (most of them being acidic or neutral) such as aspartate, glutamate, glycine, serine, threonine, alanine, valine and leucine (Cruz *et al.*, 2016, Cui and Chisti, 2003). This stands in line with the results observed for the initial sample as the most abundant amino acids were those previously mentioned except threonine which was detected but not at quantifiable levels. Overall, as the digestion simulation progressed (up until after intestine simulation), the concentrations of other amino acids, such as arginine, tyrosine, phenylalanine and isoleucine, increased which is a likely consequence of the action of the proteolytic enzymes upon the *C. versicolor* biomass.

Table 3.1. *Amino acid identification and quantification of the samples collected initially and throughout digestion. Each sample was injected in duplicate.*

Amino acids	mg g^{-1} Sample				
	Initial	Mouth digestion	Stomach digestion	Intestinal digestion	After Dialysis
Asp	0.297 ± 0.186	0.185 ± 0.048	0.359 ± 0.092	3.050 ± 0.331	2.631 ± 0.465
Glu	0.214 ± 0.008	0.225 ± 0.056	0.479 ± 0.064	6.461 ± 0.390	5.499 ± 0.264
Cys	nd	nd	nq	0.803 ± 0.543	0.199 ± 0.190
Asn	nq	nq	nq	3.453 ± 0.892	2.454 ± 0.936
Ser	0.357 ± 0.340	nq	0.125 ± 0.018	3.882 ± 0.410	3.369 ± 0.524
His	nd	nd	nd	1.847 ± 0.590	1.920 ± 0.484
Gln	0.412 ± 0.068	0.420 ± 0.092	0.254 ± 0.012	6.489 ± 2.493	4.259 ± 0.242
Thr	nq	nq	nq	3.726 ± 0.348	3.051 ± 0.363
Arg	0.097 ± 0.067	nd	0.298 ± 0.039	11.107 ± 2.231	8.049 ± 1.720
Ala	0.117 ± 0.011	0.146 ± 0.023	0.097 ± 0.085	4.225 ± 0.242	3.730 ± 0.892
Tyr	0.198 ± 0.083	nq	0.159 ± 0.045	6.564 ± 1.441	4.767 ± 0.418
Val	0.122 ± 0.007	0.149 ± 0.022	nq	5.530 ± 1.109	4.067 ± 3.770
Met	nd	nd	nq	0.660 ± 0.190	0.676 ± 0.151
Trp	nd	nd	nd	1.267 ± 0.046	1.084 ± 0.282
Phe	0.115 ± 0.007	0.090 ± 0.010	0.254 ± 0.045	4.923 ± 0.438	3.495 ± 0.813
Ile	0.065 ± 0.011	0.089 ± 0.002	nq	4.145 ± 1.093	3.334 ± 0.321
Leu	0.125 ± 0.015	0.142 ± 0.026	0.177 ± 0.017	6.233 ± 0.125	5.491 ± 1.010

Asp- Aspartate; Glu- Glutamate; Cys- Cysteine; Asn- Asparagine; Ser- Serine; His- Histidine; Gln- Glutamine; Thr- Threonine; Arg- Arginine; Ala- Alanine; Tyr- Tyrosine, Val- Valine; Met- Methionine; Trp- Tryptophan; Phe- Phenylalanine; Ile- Isoleucine; Leu- Leucine;

nd- not detected; nq- detected but bellow the quantification limit;

3.2. Impact of *Coriolus versicolor* biomass on gut microbiota

3.2.1. Microbial population modulation

In order to assess the potential prebiotic effect of *C. versicolor* biomass, a simulation of gut microbiota fermentation was performed through an *in vitro* fermentation model, using faeces from five donors. The participants were selected according to a set of criteria regarding the diet followed, age, health status, food intolerances or allergies, absence of prebiotic's, probiotic's or antibiotic's consumption in the last 6 months and household/family relations. These factors are widely acknowledged as having a significant impact upon the gut microbiota composition. For instance, Zimmer *et al.* (2012) compared the diet of vegans and vegetarians with individuals that followed a typical western diet and concluded that the microbiota was distinct in both groups, and the faecal pH values were more acidic in the vegetarians and vegans than the group that followed an omnivore diet. Additionally, as previously mentioned, there are several studies comparing the microbiota of African individuals with European or US American groups. It was noted an increase in *Prevotella*, and in some cases, in Bacteroidetes' population in the individuals who followed a fibre based diet (De Filippo *et al.*, 2010, Ou *et al.*, 2013, Schnorr *et al.*, 2014). As such, it was important to select donors with a similar diet, or at least that did not follow a restricted food regime, like vegetarianism. The age factor was also taken into consideration in the selection process, in which it was established that the participants should not be older than 65 years old. The microbiota composition changes with age, and as such the bacterial population of the elderly is distinct from the one of an adult. For example, Claesson *et al.* (2011) reported a shift in the gut microbiota among individuals over 65 years old. In another study regarding the microbiota of older individuals, an increase in the abundance of Bacteroidetes and in *Clostridium* cluster IV was also reported (Thursby and Juge, 2017). Moreover, Odamaki *et al.* (2016) observed some patterns and transition points in the bacterial populations with age, in particular, a high abundance of Bacteroidetes in the elderly cluster. Other studies also report an overall reduction in bacterial diversity and similarities between the elderly and infants regarding some phyla. Additionally, another criterion of the participants was to be healthy. It has been suggested that several diseases may have a link with an altered structure of the gut bacterial community, although is not fully understood if it is a relation of consequence or causality (Buttó and Haller, 2016). Wang *et al.* (2017) reviewed some of the major human diseases that are thought to be linked to gut microbiota, which range from infectious, liver, metabolic and autoimmune diseases, to GI cancers, among others. Previously, Hur and Lee (2015) also affirmed the role of the gut microbiota as modulator of lipidemia, insulin signaling, inflammation and food intake. Considering this, it was important for the faecal donors to not present any health condition since some diseases could influence the structure of the gut microbial community. It was also determined as a specific criterion for the participants to not have any known food intolerances or allergies, since it has been hypothesized that one of the underlying causes for this problem may be related to the microbial community in the gut. The most common intolerances are related to impairments in the digestion and intestinal absorption of carbohydrates, often due to a lack of luminal or mucosal enzymes but also because of the bacterial overgrowth or even bacterial translocation to the small intestine (Zopf *et al.*, 2009, Gigante *et al.*, 2011). Another important requirement stablished was the guarantee that the participants' microbiota had not been

influenced by the consumption of prebiotics, probiotics or antibiotics in the previous 6 months. As previously said, prebiotics and probiotics have the ability to beneficially modulate the gut microbiota, in opposition to antibiotics which have been suggested to induce dysbiosis and to have prolonged effect (Million *et al.*, 2013, Conlon and Bird, 2015). Finally, it was also determined that the participants should not belong to the same household. Although the microbiota of an individual displays an unique profile, it is also accepted that is more similar within the same family than with unrelated individuals (Schloss *et al.*, 2014). For instance, Song *et al.* (2013) reported that household members, in particular couples, had a more similar microbiota when in comparison to individuals from outside the household. As so, it was important for the donors to be from distinct families to better mimic the differences in a broader population.

As can be seen in **Table 3.2.**, Firmicutes, Bacteroidetes, Bacteroides and *Clostridium leptum* presented no significant differences ($p > 0.05$) between the controls and the Cv regardless of the time point while for *Lactobacillus* and *Bifidobacterium* groups some significant differences ($p < 0.05$) were observed. Concerning the levels of *Lactobacillus* there was an increase in C+ after 48 h. For *Bifidobacterium*, at 12, 24 and 48 h, C+ consistently presented the higher ($p < 0.05$) 16S rRNA copies than C-, and Cv exhibited significantly higher values ($p < 0.05$) than C-. The significantly higher levels of *Bifidobacterium* (at 24 and 48 h) observed in the presence of Cv, hint at a consistent bifidogenic effect, as this trend was significant when considering the results for all faecal donors. This tendency followed the behaviour observed for C+ (FOS) which is a recognized prebiotic known for the ability to stimulate *Bifidobacterium* and *Lactobacillus* proliferation (Mendlik *et al.*, 2012, Rossi *et al.*, 2005). A visual representation of the overall variation and fluctuations of the bacterial groups in each condition through time can be seen in **Figure 3.2.** The total values were considered taking into account the bacterial groups tested in the current work.

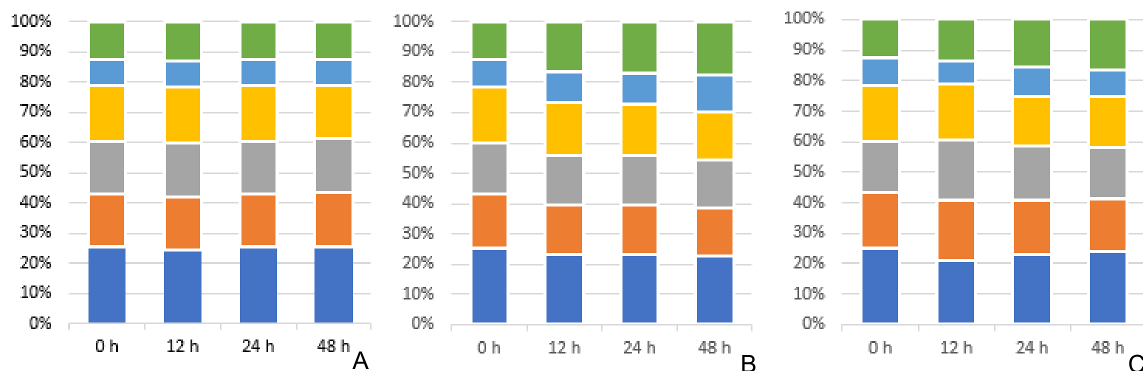

Figure 3.2. *Overall variation of the distinct bacterial groups. (A) Negative Control; (B) Positive Control; (C) C. versicolor digested biomass.* ■ *Firmicutes;* ■ *Bacteroidetes;* ■ *Bacteroides;* ■ C. leptum*;* ■ Lactobacillus*;* ■ Bifidobacterium

Table 3.2. *Overall variation, considering the 5 different donors, of the distinct bacterial groups (mean ± standard deviation).*

Time	Condition	Firmicutes	Bacteroidetes	Bacteroides	*C. leptum*	*Lactobacillus*	*Bifidobacterium*
		log 16S rRNA gene copies/ng of DNA					
0 h	C-	0.443 ± 0.021 [a]	0.312 ± 0.025 [a]	0.303 ± 0.021 [a]	0.326 ± 0.021 [a]	0.154 ± 0.030 [a]	0.221 ± 0.019 [a]
	C+	0.443 ± 0.021 [a]	0.312 ± 0.025 [a]	0.295 ± 0.021 [a]	0.326 ± 0.021 [a]	0.154 ± 0.030 [a]	0.221 ± 0.019 [a]
	Cv	0.443 ± 0.021 [a]	0.312 ± 0.025 [a]	0.295 ± 0.021 [a]	0.326 ± 0.021 [a]	0.154 ± 0.030 [a]	0.221 ± 0.019 [a]
12 h	C-	0.408 ± 0.023 [a]	0.295 ± 0.014 [a]	0.301 ± 0.008 [a]	0.315 ± 0.017 [a]	0.143 ± 0.034 [a]	0.219 ± 0.013 [a]
	C+	0.386 ± 0.031 [a]	0.273 ± 0.020 [a]	0.273 ± 0.016 [a]	0.289 ± 0.024 [a]	0.171 ± 0.037 [a]	0.271 ± 0.024 [b]
	Cv	0.376 ± 0.019 [a]	0.353 ± 0.106 [a]	0.345 ± 0.083 [a]	0.328 ± 0.119 [a]	0.135 ± 0.033 [a]	0.241 ± 0.018 [a,b]
24 h	C-	0.405 ± 0.020 [a]	0.279 ± 0.028 [a]	0.283 ± 0.019 [a]	0.294 ± 0.011 [a]	0.134 ± 0.026 [a]	0.203 ± 0.017 [a]
	C+	0.385 ± 0.031 [a]	0.269 ± 0.023 [a]	0.267 ± 0.021 [a]	0.277 ± 0.022 [b]	0.168 ± 0.040 [a]	0.282 ± 0.011 [b]
	Cv	0.359 ± 0.026 [a]	0.279 ± 0.045 [a]	0.274 ± 0.044 [a]	0.253 ± 0.029 [a,b]	0.154 ± 0.012 [a]	0.238 ± 0.008 [c]
48 h	C-	0.434 ± 0.017 [a]	0.305 ± 0.008 [a]	0.302 ± 0.017 [a]	0.302 ± 0.008 [a]	0.145 ± 0.015 [a]	0.212 ± 0.016 [a]
	C+	0.392 ± 0.013 [a]	0.275 ± 0.017 [a]	0.269 ± 0.030 [a]	0.274 ± 0.024 [a]	0.212 ± 0.056 [b]	0.301 ± 0.027 [b]
	Cv	0.399 ± 0.026 [a]	0.283 ± 0.059 [a]	0.267 ± 0.057 [a]	0.278 ± 0.028 [a]	0.146 ± 0.017 [a]	0.268 ± 0.011 [c]

Letters indicate significant differences ($p < 0.05$) between the controls and the *C. versicolor* digested biomass, at each sampling time. C-, negative control; C+, positive control (FOS); Cv, *Coriolus versicolor* digested biomass.

While to the best of our knowledge no research work has described the impact of *C. versicolor* biomass upon these particular groups, several authors studied the effect of FOS or other mushroom species extracts on the gut microbiota, focusing on certain bacterial groups. For instance, Liu *et al.* (2017) tested the effect of different prebiotics using the faeces of 36 donors, and concluded that the prebiotics had a bifidogenic effect in all participants, except for three. Nevertheless, it was also observed that, regarding other bacterial groups, there was a high interpersonal variability in the response to prebiotics. This stands in line with the results of the present work since the inter donor variability did not allow to draw any conclusions regarding the effect of *C. versicolor* biomass in the bacterial groups studied, or even compare it to FOS, except for *Bifidobacterium*. Xu and Zhang (2015) analyzed the effect of the ingestion of a *Lentinula edodes* derived polysaccharide on mice gut microbiota and reported an intense response from bacteria belonging to Bacteroidetes. Regardless of the differences between this study and the present work, this was not in accordance with the results presented above, as no differences in the Bacteroidetes population were observed between sample and controls. Rodrigues *et al.* (2016) tested the influence of an extract of mushroom *Pholiota nameko* on the gut microbiota of three donors, through a 24 h *in vitro* faecal fermentations. It was reported that the extract had an effect on the gut microbiota, namely, there was an increase in *Bifidobacterium* in comparison to the negative control, but no significant changes were observed in *Lactobacillus* population. Additionally, *Bacteroides* population was increased, comparing to negative control and FOS. These results are in part in accordance to the present results, since, at 24 h, *C. versicolor* also stimulated *Bifidobacterium* population and no changes were observed in terms of *Lactobacillus* population, although Bacteroidetes population exhibited no changes in response to Cv sample or in the controls. Nevertheless, Yu *et al.* (2013) evaluated the effect of PSP, a polysaccharide extracted from *C. versicolor,* performing *in vitro* fermentations using faeces from 8 donors. Once again, there was a consistent bifidogenic effect in addition to an increase in *Lactobacillus* community, in response to PSP. Yet, no differences between controls or PSP regarding the Bacteroides community were observed. This is partially in accordance with the results presented, because Cv also exhibited a bifidogenic effect, and no differences were observed regarding Bacteroides population, although Cv did not promote the growth of *Lactobacillus*, as it would be expected.

It is widely accepted that the microbiota profile is specific for each individual and it is the result of an array of different factors such as gender (Mueller *et al.*, 2006), age (Claesson *et al.*, 2012, Claesson *et al.*, 2011, Mariat *et al.*, 2009), diet or geographic location (De Filippo *et al.*, 2010, Flint *et al.*, 2015, Thursby and Juge, 2017), as previously mentioned. As the donor's set used in the present work included both men and women of different ages, the establishment of an overall effect of *C. versicolor* may be a complex subject if taking into account the unique microbial profile of each participant, and the fact that the results obtained displayed considerably different behaviors between donors. As such, in an attempt to better understand the underlying variations, a donor-by-donor analysis of the results was also carried out.

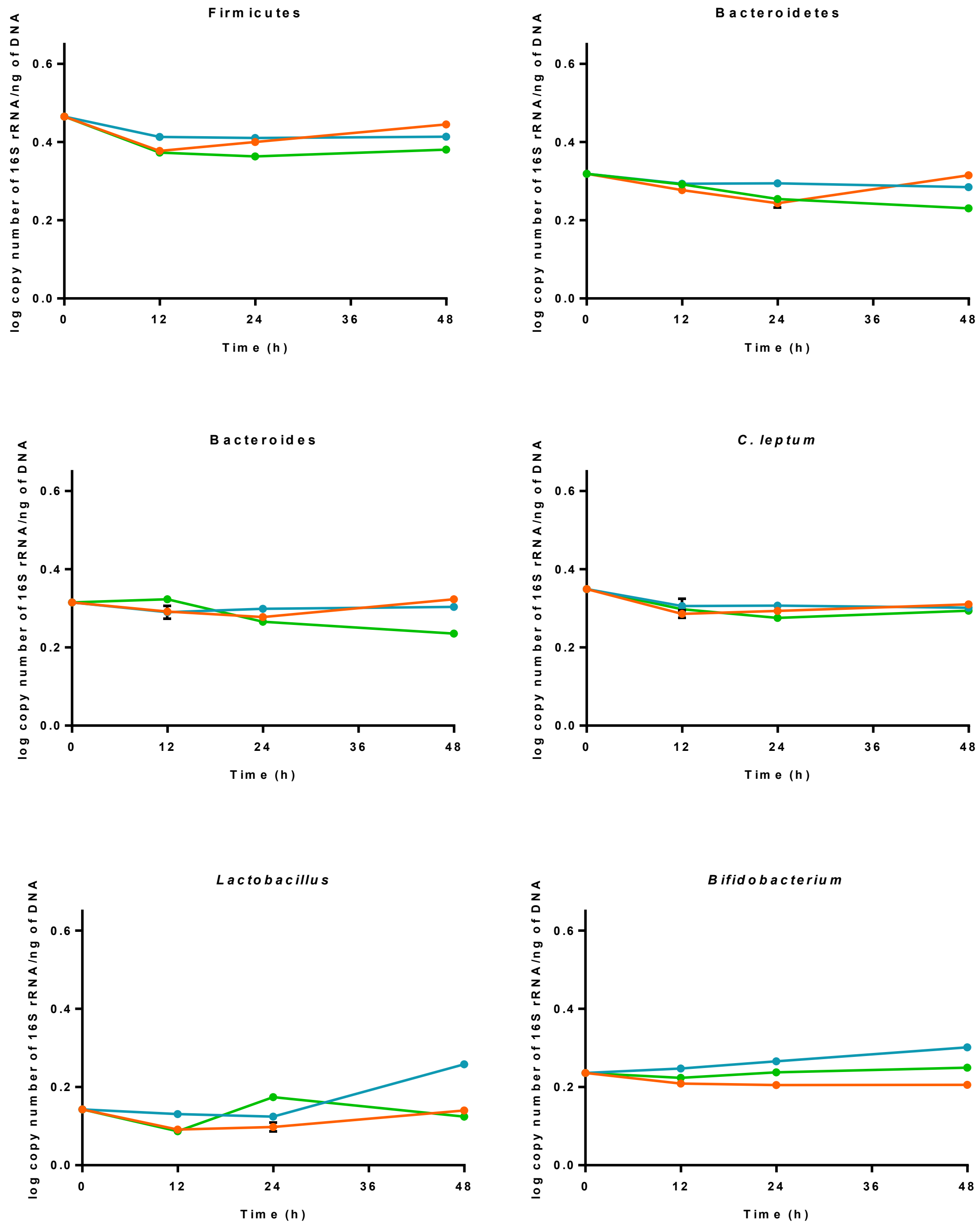

Figure 3.3. *Variation of microbiota composition in donor A. Values presented as log 16S rRNA gene copies/ng of DNA through time, with standard deviation error bars.* ●*C-, negative control;* ●*C+, positive control;* ●*Cv,* C. versicolor.

For donor A (**Figure 3.3.**) it can be observed that for Firmicutes, Bacteroidetes, Bacteroides and *C. leptum* the samples with *C. versicolor* digested biomass exhibited lowest values of 16S rRNA copies, with the overall values being similar to the negative control or even lower (**Appendix IV-** Real time PCR results and statistical analysis). In Cv, Bacteroides exhibited a slight peak at 12 h, followed by a decrease until the end of the fermentation, while the number of gene copies for Bacteroidetes decreased until 24 h, and then increased at 48 h. For *Lactobacillus,* at 12 h, both Cv and C- displayed similar levels ($p > 0.05$). However, after 24 h, the Cv sample, exhibited *Lactobacillus* levels that were higher than C+. Finally, after 48 h of fermentation, there was a decrease in Cv, exhibiting values lower than C- and C+ ($p < 0.05$). Concerning the levels of *Bifidobacterium*, the overall values were higher than those observed for *Lactobacillus*, with a clear trend being observed after the 12 h mark in which Cv exhibited higher values than C- ($p < 0.05$) but lower than C+ ($p < 0.05$).

In donor A, the abundance of Firmicutes and Bacteroidetes was in accordance with reports which describe the gut microbiota essentially as a two phyla system, with Firmicutes constituting 50% to 80% of gut microbial composition, followed by Bacteroidetes (Clemente *et al.*, 2012, Rajilić-Stojanović and de Vos, 2014). *Bifidobacterium* levels were less abundant than Firmicutes and slightly lower than Bacteroidetes. This is in accordance with literature since Actinobacteria (which is mainly comprised of *Bifidobacterium*) is reported as the third most abundant phylum (up to 10%) (Rodríguez *et al.*, 2015, Rajilić-Stojanović and de Vos, 2014). Concerning the *Lactobacillus'* content, it can be seen that it is less abundant than Firmicutes, which could be expected as lactobacilli are only one of the members of this phylum that may be found in the gut. The *C. leptum* levels' observed were lower than Firmicutes, which is also in agreement with the work of Lay *et al.* (2005) and Kabeerdoss *et al.* (2013), who reported that *C. leptum* (which also belongs to Firmicutes' phylum) constitutes 16-25% of gut microbiota. This targeted group belongs to the genus *Clostridium* which is grouped in 19 clusters, and is generally named as *Clostridium leptum*, or *Clostridium* cluster IV. Thus, the relative abundance of this subgroup could be expected, particularly as it is a relevant and highly abundant population present in the GI tract, encompassing important species like *Faecalibacterium prauznitzii* and *Eubacterium* and *Ruminococcus*'s species whose importance has been increasingly acknowledged (Mao *et al.*, 2015, Matsuki *et al.*, 2004).

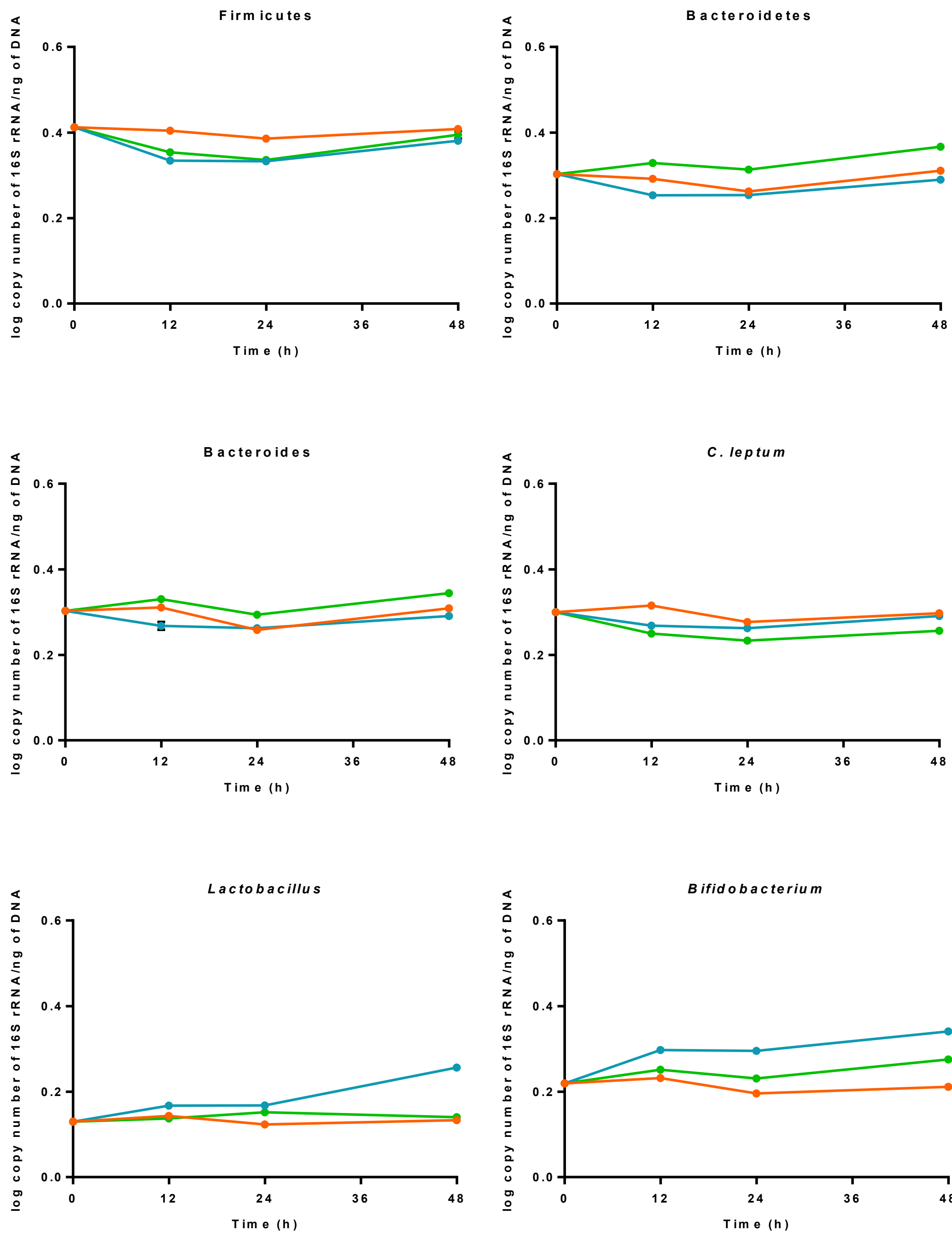

Figure 3.4. *Variation of microbiota composition in donor B. Values presented as log 16S rRNA gene copies/ng of DNA through time, with standard deviation error bars.* ●*C-, negative control;* ●*C+, positive control;* ●*Cv,* C. versicolor.

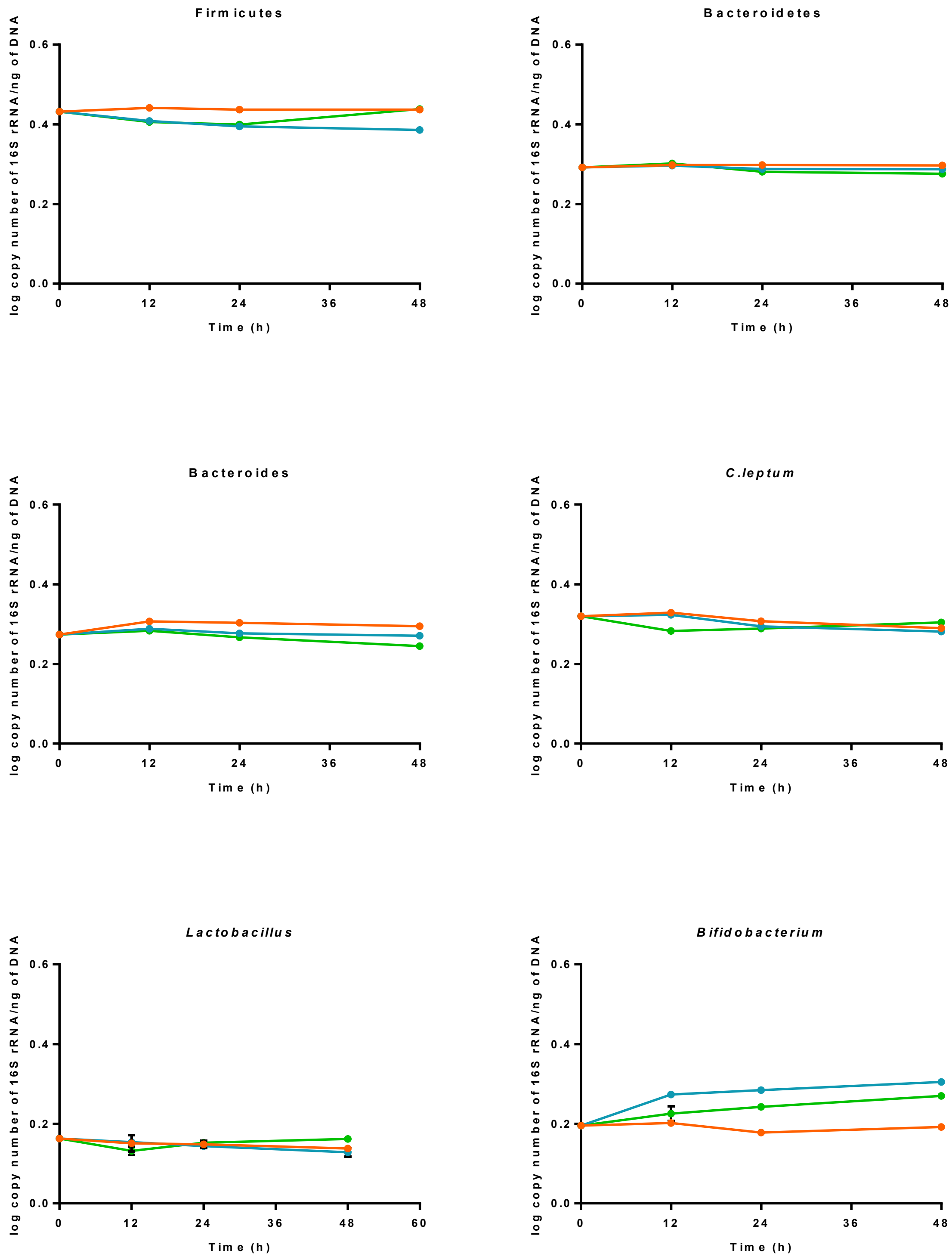

Figure 3.5. *Variation of microbiota composition in donor C. Values presented as log 16S rRNA gene copies/ng of DNA through time, with standard deviation error bars. ●C-, negative control; ●C+, positive control; ●Cv,* C. versicolor.

In what concerns donor B (**Figure 3.4.**) Cv exhibited different effects upon the bacterial groups of the microbiota, sometimes resembling C- and in others C+ (**Appendix IV-** Real time PCR results and statistical analysis). For Firmicutes, Cv followed the trend of C+, even assuming equal values at 24 h ($p > 0.05$). In Bacteroidetes and Bacteroides, Cv presented the higher levels of gene copies, with C+ registering the lowest values ($p < 0.05$). In opposition, Cv exhibited the lowest values of *C. leptum*, followed by the C+ and C- ($p < 0.05$). Regarding the levels of *Lactobacillus* there were no significant variations in the presence of Cv throughout the assay. In fact, after 24 h of fermentation, Cv exhibited similar values to C- ($p > 0.05$). On the other hand, for *Bifidobacterium*, Cv exhibited higher levels of 16S rRNA than C- ($p < 0.05$), although they remained lower than those observed for C+. Considering the relative abundance of each bacterial group, similarly to what was observed for donor A, it is in accordance with the general proportions found in literature.

In **Figure 3.5.**, regarding donor C, there were significant differences between controls and the studied sample, in each time point (**Appendix IV-** Real time PCR results and statistical analysis). In terms of Firmicutes levels', Cv exhibited a similar behaviour to C+, from 0 h to 24 h ($p > 0.05$), although assuming both the lowest values. After 48 h, there was an inversion of this trend, and Cv assumed a similar value to C- ($p > 0.05$). As for Bacteroidetes, there were little to no differences found between Cv and the controls throughout the assay, while for Bacteroides, Cv resulted in the lowest levels of 16S rRNA ($p < 0.05$), while C- resulted in the highest. *Clostridium leptum*'s bacterial community decreased from 0 to 12 h and then increased until 48 h, reaching higher levels in the presence of Cv, than both controls ($p < 0.05$). For *Lactobacillus* no differences were observed from 0 to 24 h ($p > 0.05$). However, after 48 h Cv exhibited significant higher levels of gene copies than C+ ($p < 0.05$). Regarding *Bifidobacterium* levels, once again, Cv exhibited significantly higher ($p < 0.05$) values of gene copies than C-, although significantly lower than C+ ($p < 0.05$). The general proportions of the studied bacterial groups were in accordance with other donors, as well as with the literature, as previously mentioned.

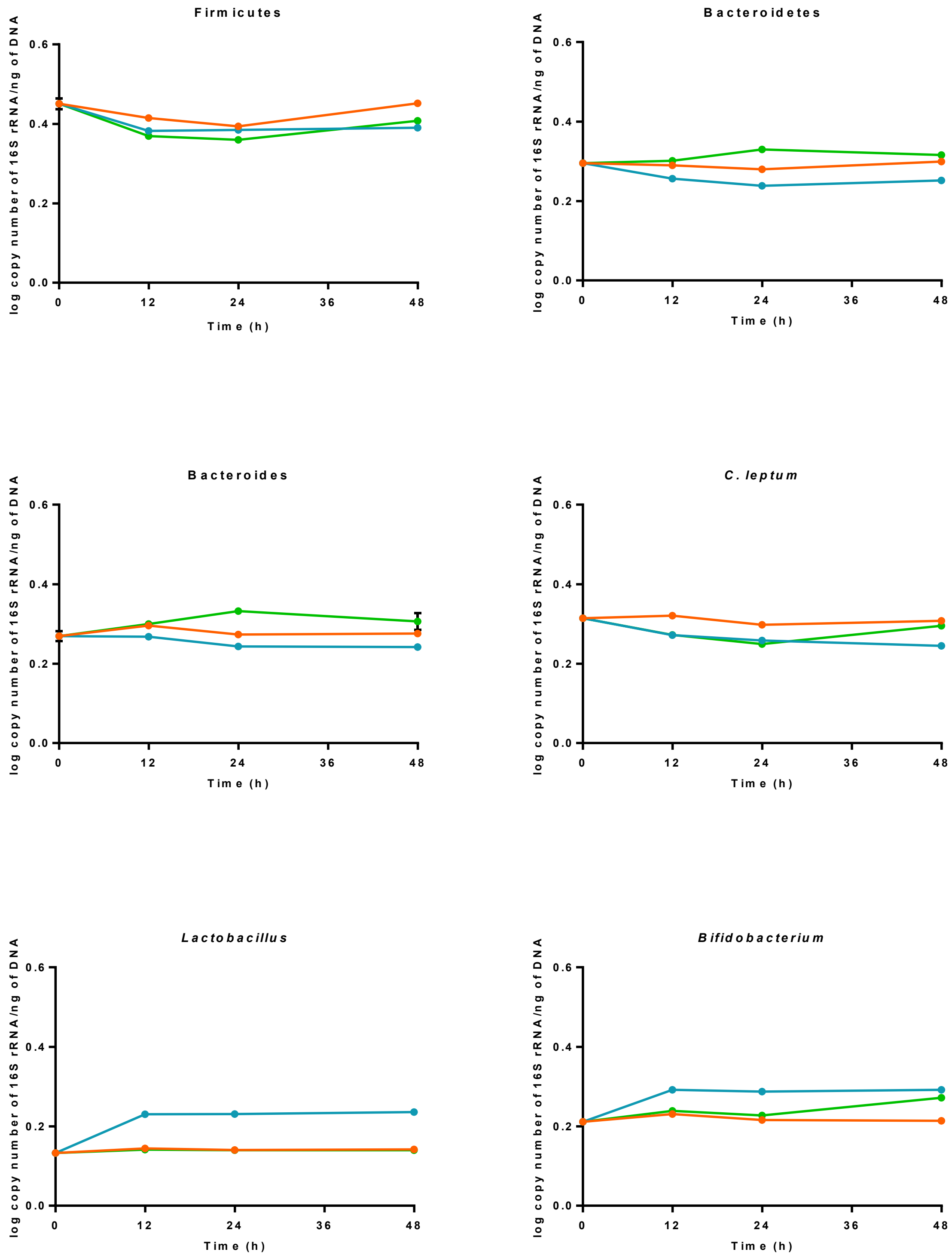

Figure 3.6. *Variation of microbiota composition in donor D. Values presented as log 16S rRNA gene copies/ ng of DNA through time, with standard deviation error bars;* ●*C-, negative control;* ●*C+, positive control;* ●*Cv,* C. versicolor.

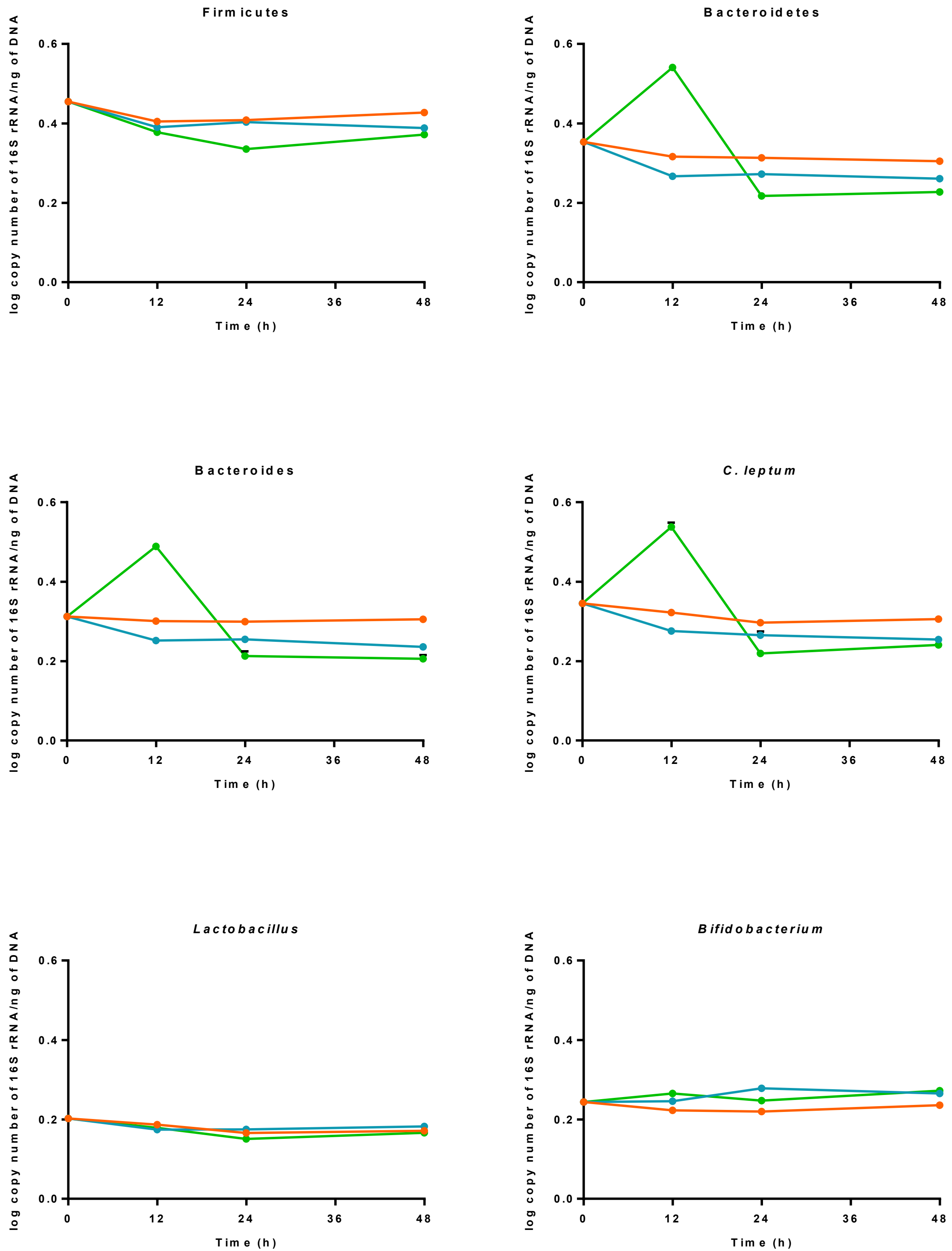

Figure 3.7. *Variation of microbiota composition in donor E. Values presented as log 16S rRNA gene copies/ng of DNA through time, with standard deviation error bars.* ●*C-, negative control;* ●*C+, positive control;* ●*Cv,* C. versicolor.

In donor D (**Figure 3.6.**), it was possible to observe that, concerning Firmicutes' levels, Cv assumed a similar behaviour to C+, although with significantly lower values ($p < 0.05$). When analysing Bacteroidetes and Bacteroides levels, the sample did not follow the behaviour of either C+ or C-, reaching a peak at 24 h and exhibiting the highest values ($p < 0.05$). As for *C. leptum* bacterial community, Cv exhibited a parallel trend to C+ but both with statistically significant lower levels of copy numbers ($p < 0.05$), than the C-. After that, it was observed an increase until 48 h, reaching closer values to C-. The sample showed the same behaviour as C-, in terms of *Lactobacillus* levels ($p > 0.05$) throughout the assay. Finally, regarding *Bifidobacterium*, as for the other donors, Cv displayed values between C- and C+, although the levels were closer to those of C-, and displaying an increase from 24 to 48 h, assuming values similar to C+. Once again, the overall relative abundance was in accordance with the previous donors and literature.

Finally, regarding donor E (**Figure 3.7.**) there is an apparent abnormality for values corresponding to the presence of Cv samples at the 12 h sample point. These values increased significantly, falling considerably outside the limits registered for the controls, as well as the values observed for other donors and the overall data collected. A possible explanation for this behaviour may reside in the DNA extraction procedure, particularly as a lower extraction yield was attained for this sample. Nevertheless, considering only the 0, 24 and 48 h results, some observations could still be made. Firmicutes population, did not respond to FOS (C+) since the values were similar to those observed for C- ($p > 0.05$), while the presence of *C. versicolor* resulted in the lowest values observed for this group ($p < 0.05$). Similarly, *C. versicolor*'s sample presented the lowest levels of Bacteroidetes, Bacteroides and *C. leptum* bacterial communities, followed by the C+. However, when considering their variation during the assay a significant ($p < 0.05$) increase in Bacteroidetes community was observed for Cv from 24 to 48 h, while the *C. leptum*'s community levels did not oscillate ($p > 0.05$). As for the *Lactobacillus*' bacterial population, there were little differences in behaviour between Cv and the controls. After 12 h, Cv was similar to C+ ($p > 0.05$), with the levels dropping after 24 h (reaching the lowest value) and, after 48 h, it exhibited similar values to those of C- ($p > 0.05$). Overall, *Lactobacillus* levels decreased significantly ($p < 0.05$) from 0 to 24 h in the presence of Cv and remained stable ($p > 0.05$) from 24 to 48 h. Finally, the *Bifidobacterium* levels' were in accordance with the results previously described for other donors, with the exception being at the 12 h mark where Cv values were higher than C+ ($p < 0.05$) and at 48 h, when Cv reached similar values to C+ ($p > 0.05$).

Bifidobacterium is one the traditional targets for prebiotic to act upon, although other bacterial groups have also proven to benefit from the presence of prebiotic compounds (Cammarota *et al.*, 2014). When considering the overall effect of *C. versicolor* supplementation, it can be seen that, in spite of the intrinsic donor variability, there was a consistent bifidogenic effect even if the *Bifidobacterium* levels did not match those observed in the presence of FOS. This stands in accordance with the research studies carried out by Cruz (2015) who reported a positive effect of the same *C. versicolor* biomass on *B. animalis* Bo and *B. longum* BG3 *in vitro* growth. Additionally, Yu *et al.* (2013), performed faecal fermentations supplemented with PSP extract and evaluated the microbial community through qPCR.

The PSP extract supplementation led to higher values of *Bifidobacterium* than the negative control, but lower than the FOS samples, which is in accordance with the above presented results. Nevertheless, this study used an isolated polysaccharide, which is distinct from the whole mushroom biomass. To the best of our knowledge no publications have reported a similar study performed using *C. versicolor* biomass. As referenced before, Rodrigues *et al.* (2016) fermented another mushroom specie (*P. nameko*) and also reported a stimulatory effect when compared to the negative control. When analyzing the donor's results, it can be seen that donor B, D and E responded better to the supplementation with *C. versicolor* digested biomass, exhibiting higher levels of *Bifidobacterium* than donors A and C. As Roberfroid *et al.* (1998) stated, a daily dose of a prebiotic *per si* does not necessarily translate into a *de facto* prebiotic effect. In fact, the extension of a bifidogenic effect is highly dependent of the overall microbiota composition before prebiotic ingestion, namely on the presence of *Bifidobacteria* in the original microbiota composition, which may explain why some donors exhibited a more pronounced bifidogenic effect than others. This hypothesis may explain the differences observed between the donors' responses to *C. versicolor* supplementation. In parallel, the different response to FOS and *C. versicolor* may be explained by the nature of these ingredients, as Holscher (2017) and Scott *et al.* (2013) stated that the polysaccharide chain length, its degree of polymerization and the fibre's branching influences the ability of bacteria metabolizing them. *Bifidobacterium* is among the earliest colonizers of the human GI tract and has been regarded as one of the most important bacterial groups. In fact, as the human host provides non-digestible carbohydrates to be metabolized by these bacteria, *Bifidobacterium* produce several potentially beneficial metabolites (Turroni *et al.*, 2014, Meyer and Stasse-Wolthuis, 2009, Russell *et al.*, 2011). Moreover, they have been associated with several health benefits such as the improvement of lactose digestion, anti-carcinogenic ability, cholesterol reduction, immunostimulatory potential and reduction of the risk of colonization by potentially pathogenic bacteria (Russell *et al.*, 2011, Rizzardini *et al.*, 2011, Kamada *et al.*, 2013a).

Overall, the levels of *Lactobacillus* displayed an inconsistent behaviour between donors. Donor D was the only case in which controls exhibited a similar trend to the one observed for *Bifidobacterium*, but revealing an identical behaviour between the *C. versicolor* biomass and the negative control. Cruz (2015), reported that while *L. paracasei* L26 was capable of growing, *in vitro,* when supplemented with *C. versicolor* biomass, *L. acidophilus* L10 was not. In fact, even FOS supplementation, an acknowledged prebiotic, did not result in a stimulation of the growth of *L. acidophilus* L10. This suggests that prebiotics, as selectively fermented ingredients may be used by certain species and not by others, suggesting that variations in species' composition, even within the same genera, may result in different susceptibilities for prebiotic action. On the other hand, Yu *et al.* (2013) reported higher levels of *Lactobacillus* in PSP supplemented samples, than those registered in fermentations with FOS. One possible explanation for this difference may be, as previously mentioned, due to the intra-genera variability regarding the capacity to metabolize carbon sources and the intrinsic donor variability concerning *Lactobacillus* species. Another possible explanation lies on a potential bias introduced by the DNA extraction procedure which may, in turn, affect the qPCR outcome. This is particularly concerning because *Lactobacillu*s possess a cell wall structure that protects them from damage and rupture (namely because of the high peptidoglycan concentration). Therefore hampers DNA extraction with some authors

reporting that cell lysis using lysozyme bears some difficulties although the current work aimed at circumvent this issue through a combination of thermic, mechanic and enzymatic methods (Alimolaei and Golchin, 2016, Atashpaz *et al.*, 2010).

Regarding *C. leptum*, in most cases, C+ and Cv presented lower levels than C-. Overall, the bacteria included in this group (such as *Eubacterium*, *Ruminococcus*, or *Faecalibacterium prauznitzii*) have been highly associated with the production of butyrate and, therefore, may play an important role in the host's health, as previously mentioned in section 1.1.2.1. (Kabeerdoss *et al.*, 2013, Saunier *et al.*, 2005). When considering the results observed in the presence of FOS *and C. versicolor* biomass it can be hypothesized that the substrate available was not the ideal for these bacteria, which have been reported to prefer resistant starch for their butyrogenic metabolism (Pryde *et al.*, 2002, Flint, 2012). Additionally, Liu *et al.* (2017) stated that regardless of the bifidogenic effect exerted by FOS, it also had an adverse effect on the glucose metabolism by butyrogenic bacteria, some of which belong to *Clostridium leptum* subgroup. In parallel, Mao *et al.* (2015) only reported alterations in *Clostridium* population when mice were subjected to a diet with high concentration of FOS (25%), but no changes were reported when submitted to a low concentration (5%) and hypothesized that only some bacterial species within this subgroup are able to metabolize FOS.

Overall Firmicutes' levels in Cv and C+ were lower than C- (with the exception of donor A), which stands in line with the results of Everard *et al.* (2011) and Parnell and Reimer (2011) who reported that prebiotic ingestion by rats both lean and obese as well as obese mice resulted in a decrease of Firmicutes' levels. On the other hand, Dewulf *et al.* (2012) reported that, in a study with obese women, Firmicutes levels' increased after supplementation with inulin type fructans, an effect that the authors associated with an increase of the *Clostridium* clusters IV and XIV. Regardless, this is not in accordance with the overall tendency observed for the donors in this study, as both Firmicutes and particularly *C. leptum* subgroup, exhibited the lower levels in the C+ and Cv samples than in the C-. Riaz Rajoka *et al.* (2017) reviewed publications regarding the effect of distinct diets on the gut microbiota composition, reporting that a western diet (low fiber/high fat) was often related to an increased Firmicutes population, in opposition to a plant based, polysaccharide rich diet, which led to a decrease in this phylum. These results are somehow in line with the present work, although it refers to prebiotic supplementation, which differs from a dietary regimen. Additionally, Holscher (2017) stated that fructans' consumption has been shown to increase butyrate concentrations, most of which are produced by bacteria belonging to Firmicutes phyla (in particular to Clostridium IV and XIVa), but the first increase after fructans' consumption belongs to non-butyrogenic bacteria. Hence, perhaps it can be hypothesized that *C. versicolor* and FOS were firstly metabolized by bacteria belonging to other groups, since it is also known the influence of the chain structure in the metabolization of prebiotics. Finally, Firmicutes are among the main phyla constituting the gut microbiota, and comprise more than 250 genera (Marciano and Vajro, 2017) which may difficult the establishment of a predictable behaviour in response to distinct prebiotics and polysaccharides.

When focusing on Bacteroidetes community, no overall consistent trend was observed among the five donors. In fact, while for donors B and D (**Figure 3.4.** and **3.6.**), Cv displayed a higher number of gene copies than C- and C+ (which exhibited the lowest values) for donors C and E (**Figure 3.5.** and **3.7.**;

regardless of the abnormal peak at 12 h) both C+ and Cv exhibited lower expression levels than C-. As referenced above, Yu *et al.* (2013), reported no differences between the negative control, FOS and PSP. Several authors have stated that prebiotic ingestion and a low fat/high fiber diet are often related to an increase of the Bacteroidetes population, and followed by a decrease in Firmicutes, which results in a reduction of the F/B ratio (Parnell and Reimer, 2011, Everard *et al.*, 2011, Geurts *et al.*, 2014). However, this effect is not universally described by all authors. For instance, as mentioned above Dewulf *et al.* (2012) observed an increased ratio between Firmicutes and Bacteroidetes in obese women that ingested prebiotics for several weeks. Nevertheless, it is important to take into account the differences between these studies and the present work, particularly as the referenced papers consider the systematic ingestion prebiotics by rats, mice or humans and the hereby described work considers only an *in vitro* fermentative model that lacks the complexity of the *in vivo* systems. Additionally, Li *et al.* (2015) stated that Bacteroidetes are able to metabolize a broad range of substrates. So, taking this into account it would be expected that FOS or Cv supplemented samples had consistently more gene copies than the negative control. Nevertheless, it has been also affirmed that Bacteroidetes are able to use proteins present in the yeast extract included in the fermentation medium, which could explain the increase in the gene copies observed in most donors (Scott *et al.*, 2013, Liu *et al.*, 2017). Another fact to consider is the sensibility of Bacteroides to acidic pH values. It is known that colonic pH values range between 5.5 and 7.5, but *in vitro* fermentation models are associated to a more acidic environment (Holscher, 2017). Regardless, as the fermentation progressed towards an acidification of the medium, this did not lead to a decrease in Bacteroidetes or *Bacteroides* levels.

As previously mentioned, when comparing the results obtained with the literature it is important to keep in mind the differences between *C. versicolor* biomass and extracts. To the best of our knowledge, there are no works characterizing the impact of *C. versicolor* biomass supplementation upon the gut microbiota, which makes it difficult to draw comparisons between results, although as previously referred, Yu *et al.* (2013) reported on the impact of PSP (a *C. versicolor* extracted polysaccharide) upon gut microbiota using a faecal model similar to the one employed in the present work. As previously stated, biomass is composed of both mycelium and primordia (young fruiting body) while the extracts are concentrated forms of the soluble constituents of the fruiting body. This means that the biomass is more resistant to the gastric fluid action (due to its own nature and composition, which protects the bioactive compounds from enzymatic action) while the active compounds present in *C. versicolor* extracts are unprotected against digestive enzymes (since there is no physical barrier). Karmali (2014) stated that after treatment with the digestive enzymes pepsin and trypsin (also used in the present work), *Ganoderma lucidum* biomass exhibited higher levels of *β*-glucans than its extract. Moreover, the extraction process may result in a denaturation of bioactive enzymes present in the original biomass and, therefore alter the bioactive potential of the extract. This effect may not be so relevant when considering the overall biomass (Barros *et al.*, 2016a). Furthermore, mushrooms' biomass also possesses non-digestible carbohydrates (*i.e.* carbohydrates that are resistant to digestive enzymes, such as dietary fibres) that are not solubilized when producing the mushroom extracts. This resistant fraction, when reaching the colon, is subjected to the action of the local microbiota which may use them as a part of their fermentative process, resulting in the production of metabolites which have been

correlated with an array of potential health effects, both positive and negative (Ferrão L, 2017). Thus, it can be hypothesized that perhaps the undigestible fraction, left out when considering the mushroom extracts, may also be an important factor contributing to their potential beneficial effect and, therefore essential when considering the overall potential of *C. versicolor*. On one hand, it is known that long-term diet has a significant impact upon the gut microbiota composition (Riaz Rajoka *et al.*, 2017), but the extent of the changes that result from a short-term dietary intervention have been questioned. In fact, while David *et al.* (2014) reported some alterations in the human gut microbiome as a response to short term specific diet and Wu *et al.* (2011) stated that despite the fact that a short term controlled diet can in fact induce detectable changes in the microbiome composition, it does not change the enterotype, meaning the overall grouping of the microbiota is specific, up to a certain point, to a certain individual. On the other hand, this work demonstrated that *C. versicolor* biomass had an impact on the gut microbiota, in particular a bifidogenic effect. Taking this in consideration, we may theorize that in order to achieve consistent beneficial effects perhaps a prolonged supplementation through time is required, with further benefits being achieved when accompanied by other lifestyle changes (for example, diet changes).

Overall, while the results observed are interesting, it is important to keep in mind the limitations of the methodology (from sample size to faeces collection and fermentation) when considering their generalization. As previously mentioned, there is a high inter-individual variability as several factors like age, diet, hormonal status and lifestyle may result in more or less subtle differences in the baseline composition of the gut microbiota (Quigley, 2013, Million *et al.*, 2013). In addition, different subjects may have distinct responses to the same stimuli, in terms of intensity or time (short-term or long-term responses). So, considering the variability factor, the sample size is a decisive when extrapolating the results to the population. This complexity is then increased when considering the sampling process as there is not a simple and direct approach (McDonald, 2017). Although, from the get-go, stool collection is an easy and non-invasive procedure, the auto-collection of the sample comprises the danger of external contamination as well as further modifications of the microbiota resulting from several factors such as exposure to oxygen and further fermentation of the faecal matter. Intestinal biopsies could solve some of these problems, but it is highly invasive procedure and therefore not one frequently used. Moreover, it is important to consider that the microbiota profile varies not only from the proximal to the distal colon, but differences have also been reported between the cellular populations adhered to the mucosa layer and those loose in the lumen (within a given section of the colon) (Payne *et al.*, 2012). Furthermore, it has been suggested that even in a specific portion of stool there is a stratified biostructure with some taxa being more abundant in the center of the stools and others more abundant in the more external part that is in contact with the intestinal mucosa (Thomas *et al.*, 2015). From a different perspective, and focusing on the fermentation process itself, the use of a batch system poses some significant limitations, so it is important to consider the limitation of substrate availability and the effect that the reduction of pH values have throughout the fermentation time. (McDonald, 2017, Payne *et al.*, 2012).

3.1. Production of SCFA

As previously mentioned, the fermentation of carbohydrates carried out by colonic bacteria results in the production of organic acids. As such, the sugars consumed, and acids produced throughout the fermentation were identified and quantified (for controls and Cv) (**Figure 3.8.**), coupled with the control of the environmental pH values (**Figure 3.9.**). Regarding the sugars and acids, glucose and arabinose were detected in Cv along with acetic, propionic, butyric and lactic acids in varying concentrations through time. The pH values measured, as can be seen in **Figure 3.9.**, decreased in Cv sample and C+ throughout the time of fermentation, from an initial value of 5.90 to 4.79 and 3.49 respectively.

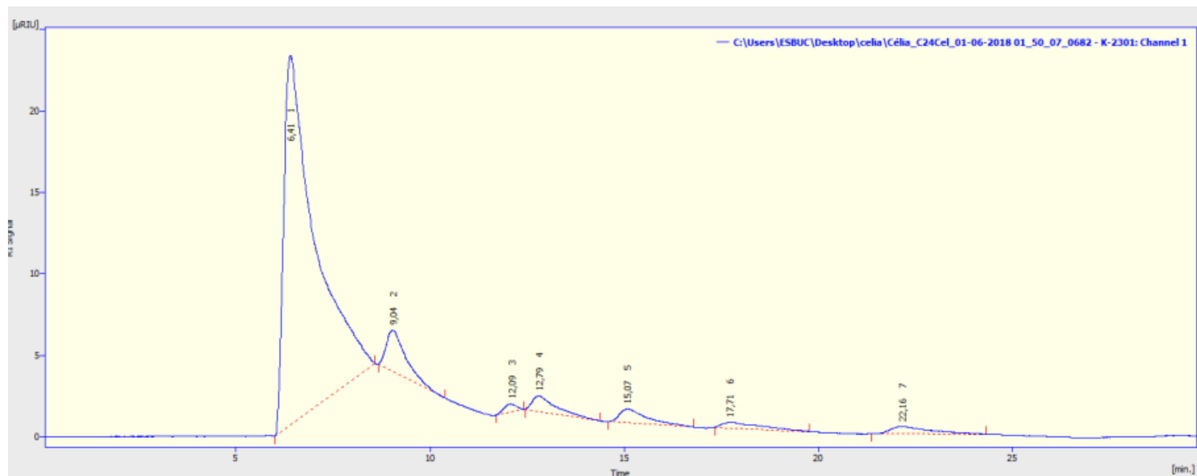

Figure 3.8. *Example of the chromatogram obtained for a sample supplemented with* C. versicolor *digested biomass, after 24 h of fermentation. Each injection was carried out in duplicate.*

As can be seen in **Table 3.3.**, in the Cv samples glucose was detected throughout the fermentation and arabinose was detected at the 12 h mark and onwards, in varying concentrations. Glucose concentration increased from 0 to 12 h and then decreased until the end of the fermentation process, while the arabinose concentration remained relatively stable through time.

Table 3.3. *Concentrations obtained for sugars consumed and organic acids produced during the fermentation of the* C. versicolor *biomass and controls. The values are presented in mg mL^{-1} and correspond to an average of the five donors, including two injections of each of each sample;*

Time	Sample	Glucose	Frutose	Sucrose	Arabinose	Propionic acid	Lactic acid	Acetic acid	Butyric acid
0 h	C-	0.199 ± 0.023	nd	nd	nd	nd	nd	nd	nd
	C+	0.199 ± 0.023	nd	nd	nd	nd	nd	nd	nd
	Cv	0.199 ± 0.023	nd	nd	nd	nd	nd	nd	nd
12 h	C-	0.238 ± 0.028	nd	nd		nd	nd	0.126 ± 0.033	nd
	C+	nq	3.448 ± 1.306	0.178 ± 0.006	nd	nd	0.018 ± 0.005	0.135 ± 0.079	nd
	Cv	0.940 ± 0.266	nd	nd	0.275 ± 0.079	0.031 ± 0.002	nd	0.097 ± 0.046	nd
24 h	C-	0.220 ± 0.027	nd	nd	nd	0.014 ± 0.004	nd	0.404 ± 0.092	0.012 ± 0.001
	C+	nq	3.240 ± 0.731	0.184 ± 0.009	nd	nd	0.084 ± 0.056	0.370 ± 0.195	0.075 ± 0.111
	Cv	0.733 ± 0.191	nd	nd	0.281 ± 0.057	0.048 ± 0.028	0.034 ± 0.026	0.171 ± 0.024	0.014 ± 0.005
48 h	C-	0.214 ± 0.016	nd	nd	nd	0.030 ± 0.007	nd	0.696 ± 0.339	0.024 ± 0.007
	C+	nq	2.636 ± 1.762	0.190 ± 0.013	nd	nd	0.097 ± 0.064	0.642 ± 0.394	0.018 ± 0.004
	Cv	0.577 ± 0.270	nd	nd	0.266 ± 0.094	0.018 ± 0.007	0.032 ± 0.022	0.212 ± 0.088	nd

nd- not detected; nq- detected, but bellow the quantification limit

Coriolus versicolor has been reported as being comprised of complex polysaccharides and polysaccharopeptides, which are mainly formed by *β*-glucans (polymers of D-glucose) in addition with other sugars like L-arabinose, L-rhamnose, D-xylose D-galactose, D-mannose and L-fucose, which may also be involved in other glyosidic linkages (Cruz *et al.*, 2016, Cui and Chisti, 2003, Yu *et al.*, 2013). While a reduction in glucose and arabinose levels could be expected throughout fermentation as, in theory, the sugars present in the medium could be metabolized by the bacteria as a part of their metabolism. However, the large *β*-glucans and other polysaccharides present in *C. versicolor* (not easily degraded by the human digestive system) reach the colon where they may either be utilized by the local flora or expelled in the stools. So, the increase in glucose from 0 to 12 h, could be explained by the action of the bacteria present in the faecal inoculum, which may have degraded glucose polymers into glucose monomers, resulting in an increase of the overall concentration. Moreover, while a similar process could explain the increase in arabinose observed during the first 12 h of fermentation, the absence of a reduction in this sugar's levels is somewhat unexpected, as many bacteria may use L-arabinose as a source of carbon and energy in their metabolism (Chang *et al.*, 2015, Agrawal *et al.*, 2017). It was hypothesized that the degradation of complex polysaccharides resulted in the release of other monosaccharides that were preferably consumed by the bacteria (namely glucose) as this type of behaviour has already been described for some bacteria (Beisel and Afroz, 2016).

Regarding the SCFA production acetic and propionic acids were detected in Cv after 12 h of fermentation, while the first increased in concentration until the end of the assay, the latter presented a production increase from 12 to 24 h and then a decrease from 24 to 48 h. As for butyric and lactic acids, production was only detected after 24 h of fermentation with lactic acid being only detected in C+ and Cv. These acids' production was followed by an acidification of the medium, represented in **Figure 3.9.**, where it can be observed a decrease in the pH values of Cv, following the same trend as C+.

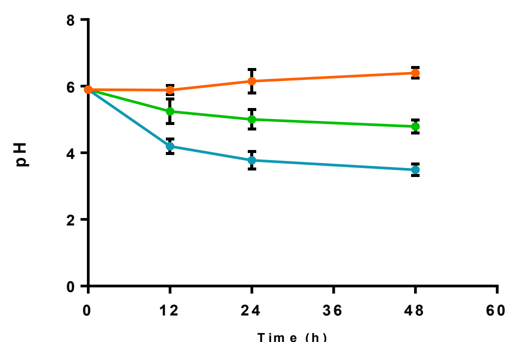

Figure 3.9. *Variation of the pH values during the fermentation process. Values of the five donors measured at 0, 12, 24 and 48 h, in duplicate.* ● *C-, negative control;* ● *C+, positive control;* ● *Cv,* C. versicolor*.*

In theory, it was expected to obtain increasing concentrations of SCFA and lactic acid throughout the fermentative process, with higher values in both C+ and Cv samples. However, some inconsistencies were observed mainly regarding propionic, butyric and acetic acids. Propionic acid was not detected in C+ throughout the fermentation and at 48 h, its concentration was higher in C- than in Cv. Regarding the acetic acid concentration, C- and C+ had very similar values, both higher than Cv. Finally, the concentrations of butyric acid at 48 h were higher in C- than C+, and it was not detected in Cv. Yu *et al.* (2013) also tested the presence of SCFA and lactic acid and reported significantly higher concentration values of these acids in *C. versicolor* polysaccharide and also in FOS supplemented samples than in the negative control. This is partially in accordance with the present results, since we also report the presence of lactic acid only in FOS and in *C. versicolor* biomass, but no trend was observed regarding propionic, acetic and butyric. Rodrigues *et al.* (2016) also evaluated the SCFA content of *P. nameko* and reported that mushroom supplementation lead to SCFA concentrations between the ones obtained for FOS and the negative control. It also reported higher values regarding acetic and propionic acid production, which may be linked to *Bifidobacterium* metabolism. This is partially in accordance to our results since acetic and propionic acids corresponded to the two highest acids concentrations among the acids detected in Cv, although there was no trend between the mushrooms and the controls. Kawakami *et al.* (2016) tested two mushrooms powders (*Agaricus bisporus,* white and brown) through the intestinal fermentation in rats, and only the white type exhibited significantly higher SCFA concentrations than C-, which was explained by the distinct sugar content of the two types. This may explain the present results, since *C. versicolor* supplementation did not result in a higher concentration of SCFA or lactic acid than C-, which could be explained by the lower sugar content.

Several authors have affirmed that the fermentation of polysaccharides often results in acetic, propionic and butyric acids in a proportion of 3:1:1, a behaviour that was not observed in the results presented here (Scott *et al.*, 2013, den Besten *et al.*, 2013). In addition to SCFA, lactic acid, is also produced by some bacteria present in the gut belonging to the *Lactobacillus* and *Bifidobacterium* genera*,* but *in vivo* it is not accumulated in the colon, since it is converted by certain bacterial species into other SCFA. However, this conversion was not observed as lactic acid concentration remained stable from 24 to 48 h. As for the SCFA, they have been shown to be formed as a result of distinct microbial metabolic pathways, with each acid being associated to more than one bacterial phylum or genus. For instance, propionate production has been related to some *Negativutes* within Firmicutes, Bacteroidetes and bacteria belonging to Verrucomicrobia, although it has been hypothesized that Bacteroidetes may be the largest propionate producer in the human gut. Additionally, butyrate production has also been linked to Firmicutes, namely *Roseburia*, *Eubacterium* and some Clostridia clusters. As for acetic acid, it has been related mainly to Bacteroidetes but also to *Bifidobacterium* (Binda *et al.*, 2018, Ríos-Covián *et al.*, 2016, Rios-Covian *et al.*, 2017). However, in this work the only relationship that was possible to establish between the abundance of the bacterial groups tested and the SCFA production was regarding Firmicutes and *C. leptum* subgroup and the butyric acid. The samples supplemented with FOS and *C. versicolor* presented a lower abundance of Firmicutes and *C. leptum* (which have a butyrogenic metabolism) than the negative control, and this behaviour corresponded to lower concentrations of butyric acid than the negative control. Furthermore, it has been proved the cross-feeding mechanisms

have significant impact on the overall SCFA balance. These mechanisms consist either in the utilization of end products of a given bacteria by other bacterial species, or in the metabolization of an energetic breakdown molecule from one bacteria by another one (substrate cross-feeding). The first cross-feeding mechanism usually consists mainly in the conversion of acetate to butyrate and from butyrate to propionate (Ríos-Covián *et al.*, 2016). Nevertheless, this behavior was not observed, as the acetic acid concentration remained stable and the butyrate was only detected at 48 h. Furthermore, it is known that the SCFA production lowers the pH value in the gut which, in turn affects the gut microbiota composition, and therefore the SCFA produced. *In vivo,* these acids produced are absorbed by the colonocytes, in exchange for bicarbonate, so the luminal pH value is a balance between this exchange and the SCFA which are not absorbed. Lower pH values are highly related to ability of inhibiting the overgrowth of pathogenic bacteria but also influence the overall bacteria growth and metabolism. For instance, butyrogenic reactions occur at pH 5.5, whereas acetate and propionate reactions usually occur at pH 6.5, although Bacteroidetes can still grow in a wide range of pH values (Ilhan *et al.*, 2017, den Besten *et al.*, 2013). It could be hypothesized that the absence of a pH controlling system, led to an acidification (**Figure 3.9.**) which was hampering to the growth of some bacteria and consequently the SCFA production. In fact, Liu *et al.* (2017) stated that the high dose prebiotics intervention promoted mainly the growth of *Bifidobacterium*, which led to the production of high amounts of lactic and acetic acid. The authors affirmed that this tendency induced a lowering of the pH values inhibiting the growth of pathogenic bacteria, but also the butyrate producing ones. This stands in line with the presented results, since it was possible to observe the stimulatory effect of both *C. versicolor* biomass and FOS, on *Bifidobacterium* population, and a lowering of the pH values, but this was not translated into high concentrations of butyrate, but instead acetate and lactate remained stable.

4. Conclusions

This work allowed to obtain a better insight into the potential effect of *C. versicolor* biomass upon the human gut microbiota and to understand the complex interactions of this mushroom with different bacterial groups, in order to further establish its prebiotic potential.

First, in result of the *in vitro* digestion of *C. versicolor* biomass, was possible to observe an increased proteolysis throughout the GI tract and a rich amino acid profile, that would be interesting to further analyse.

Secondly, and regarding the impact of *C. versicolor* digested biomass on gut microbiota, the results demonstrated a bifidogenic effect, in all donors, regardless of their intrinsic variability. When it comes to *Lactobacillus* population, it did not appear to be responsive to *C. versicolor* biomass presence, as no consistent increase of 16S rRNA gene copies was observed. Regarding other bacterial groups, no tendency was found among donors. Nevertheless, it was possible to observe that, in three out of five donors, *C. versicolor* biomass supplemented samples exhibited a similar behaviour to the FOS ones, both exhibiting a lower abundance of gene copies than the negative control, regarding Firmicutes and *Clostridium leptum* subgroup. As for Bacteroidetes and Bacteroides, in two out of five*, C. versicolor* displayed an opposite behaviour to FOS, exhibiting the highest number of 16S rRNA gene copies, while FOS displayed the lower values. Concerning the remaining donors, it was observed that *C. versicolor* digested biomass supplemented samples exhibited a lower number of 16S rRNA gene copies than negative control and FOS, in terms of Firmicutes, *C. leptum*, Bacteroidetes, Bacteroides population. The differences observed among the donors may be explained by the unique microbial profile of each individual. Further differences between FOS and sample tested may be explained by their distinct compositions, since FOS has a prebiotic *per si*, while *C. versicolor* biomass is composed by a great diversity of compounds where the prebiotic substrates (such as polysaccharides) may be in limited concentration and also by each bacterial group giving preference to different substrates.

From a metabolic point of view, glucose and arabinose were both identified and quantified but only glucose was consumed throughout time. From 12 to 48 h, acids were detected, mainly acetic, propionic and lactic acid. This may be explained by the stimulatory effect of *C. versicolor* on *Bifidobacterium*, which is related to acetic and lactic acid production. Additionally, the detection of butyric acid only at 24 h and its small concentrations may be explained by the reduced number of 16S rRNA gene copies detected for Firmicutes and *C. leptum* subgroups, which have a butyrogenic metabolism.

In summary, the results described above open the possibility to expand the range of bioactive properties associated to *C. versicolor* biomass, through the potential prebiotic activity exhibited on the human gut microbiota, in terms of the stimulation of bacterial groups and the resulting metabolites produced.

5. Future Work

The present work studied the potential prebiotic effect of *C. versicolor* biomass, focusing on its impact on the gut microbiota *in vitro*. Human faecal stools from different donors were used to carry out batch fermentations that had better mimic the complexity of the gut microbiota. However, considering the culture medium acidification observed, it would be interesting to use a pH-control system that allowed the stabilization of the pH values, as faeces are alkaline and the acidification of the media observed may introduce an important bias in the microbial groups and on their metabolites. Additionally, it would also be interesting to increase the number of bacterial groups analysed (both phyla and genera) focusing on the analysis of bacterial species known for their specific properties (for example, butyrogenic species), along with the inclusion of more donors to strengthen the consistency of the results, at a population level, instead of a donor-by-donor approach.

Additionally, an interesting follow up would also be the ingestion of *C. versicolor* biomass by human volunteers. As it is already a food supplement, it would be interesting to understand how the continuous intake could effectively modulate the human gut microbiota along with its metabolites and, given the known anti-inflammatory potential of this matrix, evaluate plasma levels of cytokines in an attempt to draw some correlations between cytokine levels, microbiota profile and its metabolites.

6. Appendixes

6.1. Appendix I- Informed consent form

Model of the informed consent form distributed to the donors in Portuguese.

FORMULÁRIO DE CONSENTIMENTO INFORMADO

<table>
<tr><td colspan="2">Título do projeto de investigação: Avaliação do potencial prebiótico do cogumelo Coriolus versicolor – efeito na microbiota intestinal humana</td></tr>
<tr><td colspan="2">Nome do investigador: Célia Freitas Costa</td></tr>
<tr><td>A completar pelo participante

1. Leu a ficha informativa deste estudo?</td><td>SIM / NÃO</td></tr>
<tr><td>2. Se colocou questões, recebeu respostas adequadas?</td><td>SIM / NÃO / Não aplicável</td></tr>
<tr><td>3. Compreende que é livre de abandonar este estudo, sem necessidade de dar uma justificação?</td><td>SIM / NÃO</td></tr>
<tr><td>4. Aceita participar neste estudo?</td><td>SIM / NÃO</td></tr>
<tr><td>Assinatura (participante):</td><td>Data</td></tr>
<tr><td>Nome do participante (letras maiúsculas):</td><td></td></tr>
<tr><td>Assinatura do investigador:</td><td>Data</td></tr>
</table>

Este projeto é supervisionado por: Prof. Dra. Manuela Pintado
Contatos: Escola Superior de Biotecnologia da Universidade Católica Portuguesa do Porto (ESB-UCP); +351 911 095 151

6.2. Appendix II- Instructions for stool specimen collection

Model of the instructions for stool specimen collection delivered to the donors, in Portuguese.

KIT Doação de fezes

O presente kit é composto por:

- Formulário de consentimento informado;
- Informação do estudo, critérios de elegibilidade e instruções;
- 1 Caixa de vácuo;
- 2 Sacos de plástico (um cortado e outro inteiro);
- 1 saqueta de anaerobiose;
- 1 par de luvas;
- 1 rolo de película aderente;
- 1 tesoura;
- 1 elástico de borracha.

Informações do estudo

O presente estudo tem como objetivo avaliar o efeito prebiótico de um extrato de cogumelo (*Coriolos versicolor*) na microbiota intestinal humana. Para isso, após simulação das condições gastrointestinais, proceder-se-á a fermentação *in vitro* de fezes humanas com o suplemento digerido. Por fim, será avaliado o crescimento de determinados grupos bacterianos e a concentração de ácidos orgânicos resultantes do metabolismo bacteriano.

Critérios de elegibilidade

Os participantes no presente estudo devem preencher os seguintes requisitos:

- Assinar o formulário de consentimento informado;
- Ser saudável;
- Ter entre 18 e 65 anos de idade;
- Não seguir nenhum regime alimentar restritivo (ex. vegetarianismo)
- Não ter intolerâncias alimentares, nem alergias alimentares severas;
- Não ter ingerido suplementos prebióticos, probióticos (incluindo iogurtes com "Bifidus") ou antibióticos nos últimos 6 meses;

Instruções para colheita

Na colheita de fezes, é importante ter em consideração que a urina, a água ou o papel higiénico podem contaminar a amostra. Assim, este procedimento deve seguir as seguintes etapas:

1. Calçar as luvas;
2. Levantar ao tampa e o aro da sanita, e cobri-la com película aderente, deixando uma folga, para a amostra assentar; Utilizar a tesoura para cortar a película, se necessário;
3. Baixar o aro da sanita, e colocar o saco de plástico cortado em cima da película aderente;

4. Defecar em cima do saco de plástico cortado;
5. Segurando nas bordas da película, levantar o aro da sanita e colocar a película com a amostra dentro do saco de plástico;
6. Colocar o saco de plástico, as luvas e a tesoura dentro da caixa de vácuo;
7. Abrir a embalagem da saqueta de anaerobiose e colocá-la dentro da caixa de vácuo;
8. Fechar a caixa de vácuo, colocando o elástico à sua volta;
9. Colocar a caixa de vácuo e o rolo de película aderente dentro do saco de plástico;

6.3. Appendix III- Bacterial calibration curves for qPCR

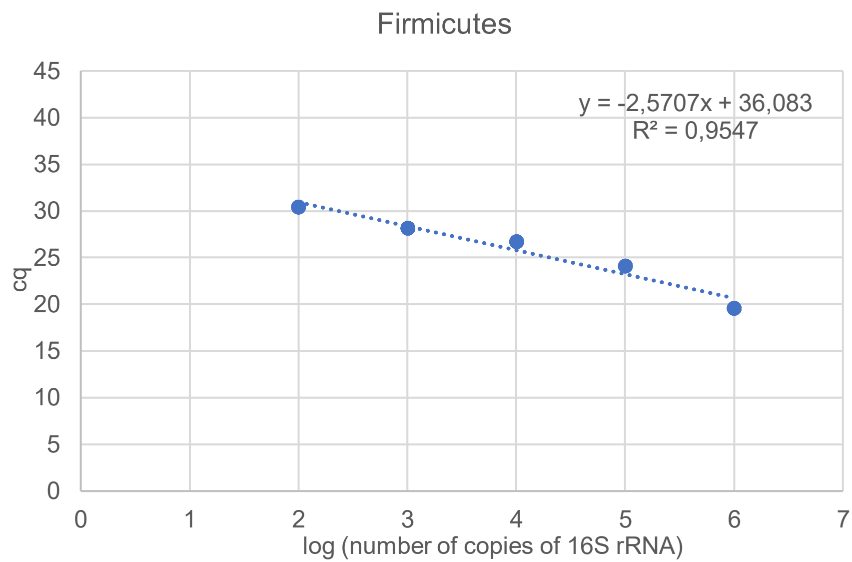

Figure 6.1. *Calibration curve for Firmicutes population prepared using gDNA solutions from* Lactobacillus gasseri.

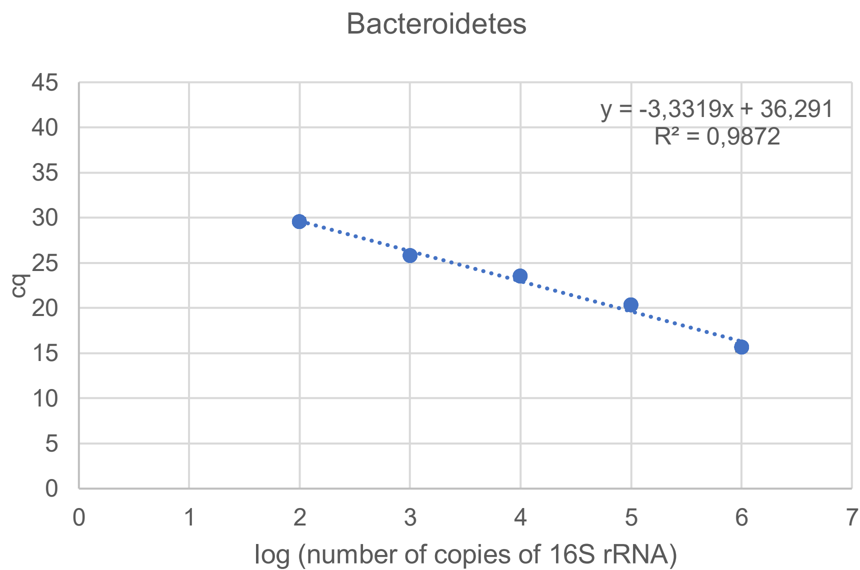

Figure 6.2. *Calibration curve for Bacteroidetes prepared using gDNA solutions from* Bacteroides vulgatus.

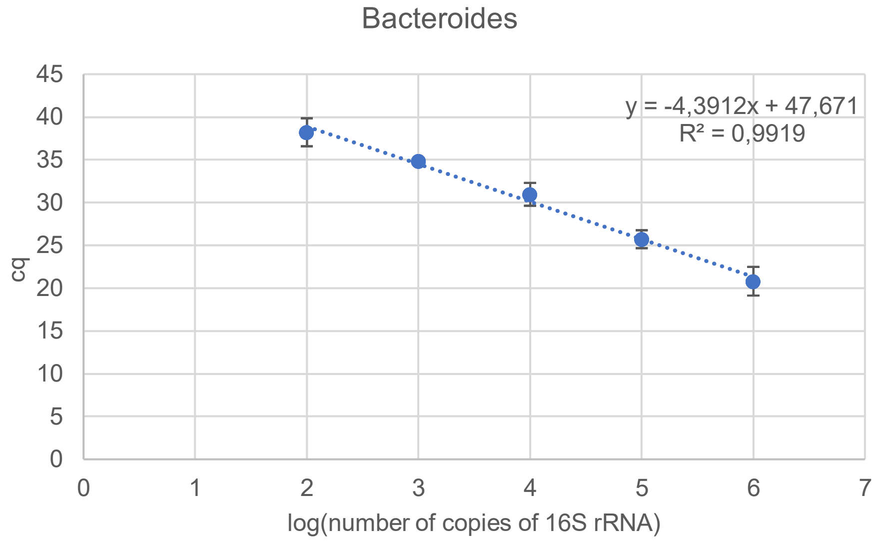

Figure 6.3. *Calibration curve for Bacteroides prepared using gDNA solutions from* Bacteroides vulgatus.

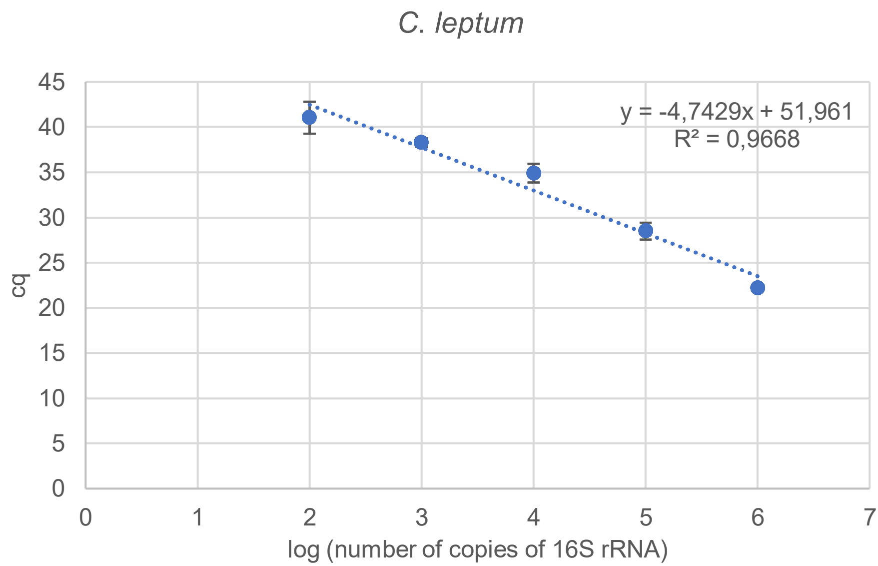

Figure 6.4. *Calibration curve for* C. leptum *subgroup prepared using gDNA solutions from* C. leptum.

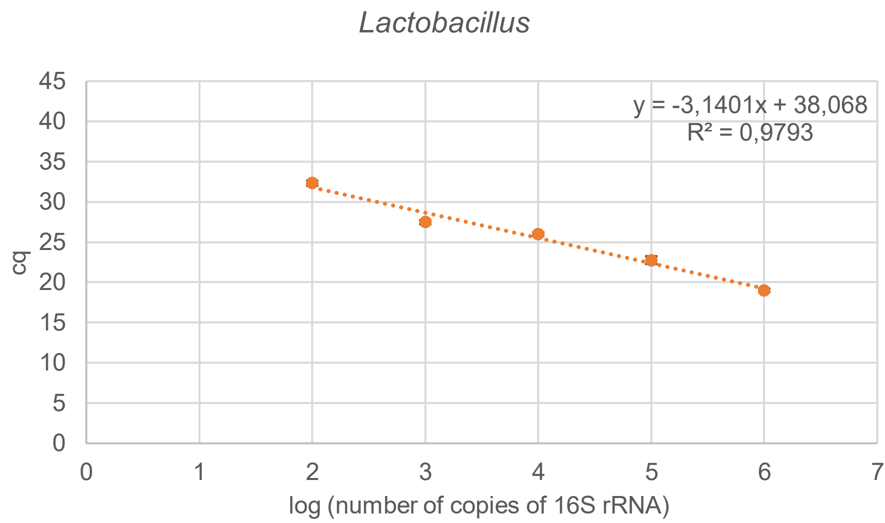

Figure 6.5. *Calibration curve for* Lactobacillus *subgroup prepared using gDNA solutions from* Lactobacillus gasseri.

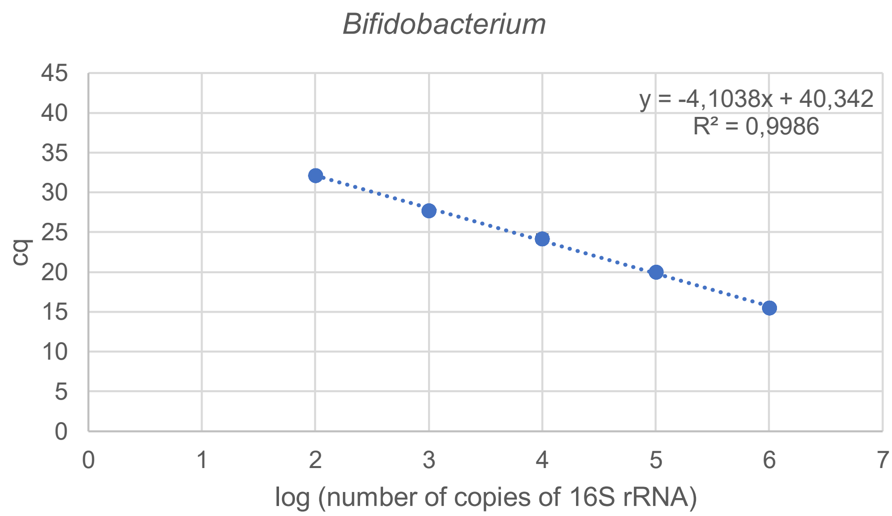

Figure 6.6. *Calibration curve for* Bifidobacterium *prepared using gDNA solutions from* Bifidobacterium longum *subs* Infantis.

6.4. Appendix IV- Real time PCR results and statistical analysis.

Table 6.1. *Variation of microbiota in donor A. Values presented as mean ± standard deviation.*

Time	Condition	log copy number of 16S rRNA/ng of DNA					
		Firmicutes	Bacteroidetes	Bacteroides	*C. leptum*	*Lactobacillus*	*Bifidobacterium*
0 h	C-	0.465 ± 0.007 [a]	0.319 ± 0.001 [a]	0.315 ± 0.009 [a]	0.349 ± 0.004 [a]	0.142 ± 0.004 [a]	0.236 ± 0.006 [a]
	C+	0.465 ± 0.007 [a]	0.319 ± 0.001 [a]	0.315 ± 0.009 [a]	0.349 ± 0.004 [a]	0.142 ± 0.004 [a]	0.236 ± 0.006 [a]
	Cv	0.465 ± 0.007 [a]	0.319 ± 0.001 [a]	0.315 ± 0.009 [a]	0.349 ± 0.004 [a]	0.142 ± 0.004 [a]	0.236 ± 0.006 [a]
12 h	C-	0.377 ± 0.001 [a]	0.277 ± 0.002 [a]	0.292 ± 0.002 [a]	0.285 ± 0.010 [a]	0.091 ± 0.009 [a]	0.209 ± 0.002 [a]
	C+	0.413 ± 0.003 [b]	0.293 ± 0.002 [b]	0.290 ± 0.016 [a]	0.306 ± 0.018 [b]	0.131 ± 0.008 [b]	0.247 ± 0.001 [b]
	Cv	0.373 ± 0.006 [a]	0.292 ± 0.008 [b]	0.323 ± 0.001 [b]	0.275 ± 0.004 [c]	0.087 ± 0.007 [a]	0.223 ± 0.005 [c]
24 h	C-	0.400 ± 0.002 [a]	0.244 ± 0.019 [a]	0.278 ± 0.002 [a]	0.293 ± 0.006 [a]	0.098 ± 0.011 [a]	0.205 ± 0.001 [a]
	C+	0.410 ± 0.005 [b]	0.294 ± 0.002 [b]	0.299 ± 0.006 [b]	0.307 ± 0.002 [b]	0.124 ± 0.005 [b]	0.266 ± 0.003 [b]
	Cv	0.363 ± 0.002 [c]	0.254 ± 0.006 [a]	0.266 ± 0.003 [c]	0.275 ± 0.003 [c]	0.174 ± 0.004 [c]	0.242 ± 0.000 [a]
48 h	C-	0.445 ± 0.003 [a]	0.315 ± 0.007 [a]	0.323 ± 0.002 [a]	0.310 ± 0.004 [a]	0.140 ± 0.005 [a]	0.206 ± 0.004 [a]
	C+	0.413 ± 0.002 [b]	0.285 ± 0.003 [b]	0.303 ± 0.001 [b]	0.302 ± 0.004 [b]	0.258 ± 0.003 [b]	0.301 ± 0.005 [b]
	Cv	0.381 ± 0.004 [c]	0.230 ± 0.002 [c]	0.235 ± 0.002 [c]	0.294 ± 0.003 [b]	0.124 ± 0.003 [c]	0.249 ± 0.003 [c]

Letters indicate significant differences ($p < 0.05$) between the controls and the *Coriolus versicolor* digested biomass, at each sampling time. Cv, *Coriolus versicolor* digested biomass; C+, positive control (FOS); C-, negative control

Table 6.2. *Variation of microbiota in donor B. Values presented as mean ± standard deviation.*

Time	Condition	Firmicutes	Bacteroidetes	Bacteroides	*C. leptum*	*Lactobacillus*	*Bifidobacterium*
		log copy number of 16S rRNA/ng of DNA					
0 h	**C-**	0.412 ± 0.002 [a]	0.302 ± 0.002 [a]	0.303 ± 0.007 [a]	0.300 ± 0.002 [a]	0.130 ± 0.005 [a]	0.219 ± 0.001 [a]
	C+	0.412 ± 0.002 [a]	0.302 ± 0.002 [a]	0.303 ± 0.007 [a]	0.300 ± 0.002 [a]	0.130 ± 0.005 [a]	0.219 ± 0.001 [a]
	Cv	0.412 ± 0.002 [a]	0.302 ± 0.002 [a]	0.303 ± 0.007 [a]	0.300 ± 0.002 [a]	0.130 ± 0.005 [a]	0.219 ± 0.001 [a]
12 h	**C-**	0.404 ± 0.000 [a]	0.292 ± 0.003 [a]	0.311 ± 0.001 [a]	0.316 ± 0.001 [a]	0.143 ± 0.001 [a]	0.232 ± 0.006 [a]
	C+	0.334 ± 0.002 [b]	0.253 ± 0.002 [b]	0.267 ± 0.009 [b]	0.268 ± 0.002 [b]	0.167 ± 0.001 [b]	0.297 ± 0.001 [b]
	Cv	0.354 ± 0.004 [c]	0.329 ± 0.003 [c]	0.330 ± 0.002 [a]	0.250 ± 0.006 [c]	0.137 ± 0.008 [a]	0.251 ± 0.003 [c]
24 h	**C-**	0.386 ± 0.003 [a]	0.262 ± 0.003 [a]	0.259 ± 0.002 [a]	0.277 ± 0.005 [a]	0.123 ± 0.008 [a]	0.196 ± 0.005 [a]
	C+	0.333 ± 0.007 [b]	0.254 ± 0.001 [b]	0.262 ± 0.006 [b]	0.262 ± 0.003 [b]	0.168 ± 0.003 [b]	0.295 ± 0.003 [b]
	Cv	0.336 ± 0.003 [c]	0.314 ± 0.003 [c]	0.294 ± 0.003 [a]	0.233 ± 0.005 [c]	0.152 ± 0.002 [a,b]	0.231 ± 0.002 [c]
48 h	**C-**	0.408 ± 0.005 [a]	0.311 ± 0.002 [a]	0.309 ± 0.003 [a]	0.298 ± 0.003 [a]	0.134 ± 0.004 [a]	0.212 ± 0.003 [a]
	C+	0.381 ± 0.001 [b]	0.290 ± 0.001 [b]	0.291 ± 0.002 [b]	0.291 ± 0.002 [b]	0.256 ± 0.006 [b]	0.341 ± 0.001 [b]
	Cv	0.395 ± 0.009 [c]	0.367 ± 0.003 [c]	0.344 ± 0.001 [c]	0.256 ± 0.000 [c]	0.140 ± 0.003 [a]	0.275 ± 0.002 [c]

Letters indicate significant differences ($p < 0.05$) between the controls and the *Coriolus versicolor* digested biomass, at each sampling time. Cv, *Coriolus versicolor* digested biomass; C+, positive control (FOS); C-, negative control.

Table 6.3. *Variation of microbiota in donor C. Values presented as mean ± standard deviation.*

		log copy number of 16S rRNA/ng of DNA					
Time	**Condition**	**Firmicutes**	**Bacteroidetes**	**Bacteroides**	***C. leptum***	***Lactobacillus***	***Bifidobacterium***
0 h	**C-**	0.432 ± 0.005 [a]	0.291 ± 0.004 [a]	0.274 ± 0.003 [a]	0.320 ± 0.003 [a]	0.163 ± 0.005 [a]	0.195 ± 0.003 [a]
	C+	0.432 ± 0.005 [a]	0.291 ± 0.004 [a]	0.274 ± 0.003 [a]	0.320 ± 0.003 [a]	0.163 ± 0.005 [a]	0.195 ± 0.003 [a]
	Cv	0.432 ± 0.005 [a]	0.291 ± 0.004 [a]	0.274 ± 0.003 [a]	0.320 ± 0.003 [a]	0.163 ± 0.005 [a]	0.195 ± 0.003 [a]
12 h	**C-**	0.441 ± 0.004 [a]	0.298 ± 0.001 [a]	0.307 ± 0.003 [a]	0.329 ± 0.002 [a]	0.151 ± 0.021 [a]	0.202 ± 0.005 [a]
	C+	0.408 ± 0.002 [b]	0.296 ± 0.002 [a]	0.288 ± 0.003 [b]	0.323 ± 0.001 [b]	0.154 ± 0.002 [a]	0.273 ± 0.006 [b]
	Cv	0.406 ± 0.008 [b]	0.302 ± 0.007 [a]	0.283 ± 0.002 [c]	0.283 ± 0.005 [c]	0.132 ± 0.010 [a]	0.226 ± 0.019 [c]
24 h	**C-**	0.437 ± 0.005 [a]	0.298 ± 0.004 [a]	0.303 ± 0.004 [a]	0.308 ± 0.001 [a]	0.148 ± 0.010 [a]	0.178 ± 0.002 [a]
	C+	0.395 ± 0.003 [b]	0.288 ± 0.002 [b]	0.277 ± 0.006 [b]	0.294 ± 0.003 [b]	0.144 ± 0.006 [a]	0.284 ± 0.003 [b]
	Cv	0.400 ± 0.009 [b]	0.281 ± 0.002 [c]	0.267 ± 0.001 [c]	0.289 ± 0.005 [a]	0.152 ± 0.004 [a]	0.242 ± 0.003 [c]
48 h	**C-**	0.436 ± 0.004 [a]	0.297 ± 0.001 [a]	0.295 ± 0.003 [a]	0.290 ± 0.002 [a]	0.138 ± 0.003 [a]	0.192 ± 0.002 [a]
	C+	0.386 ± 0.000 [b]	0.288 ± 0.001 [b]	0.271 ± 0.005 [b]	0.281 ± 0.001 [b]	0.128 ± 0.011 [b]	0.305 ± 0.001 [b]
	Cv	0.438 ± 0.009 [a]	0.276 ± 0.006 [c]	0.245 ± 0.002 [c]	0.304 ± 0.004 [c]	0.162 ± 0.006 [a]	0.270 ± 0.001 [c]

Letters indicate significant differences ($p < 0.05$) between the controls and the *Coriolus versicolor* digested biomass, at each sampling time. Cv, *Coriolus versicolor* digested biomass; C+, positive control (FOS); C-, negative control

Table 6.4. *Variation of microbiota in donor D. Values presented as mean ± standard deviation.*

Time	Condition	Firmicutes	Bacteroidetes	Bacteroides	*C. leptum*	*Lactobacillus*	*Bifidobacterium*
		log copy number of 16S rRNA/ng of DNA					
0 h	**C-**	0.451 ± 0.014 [a]	0.296 ± 0.005 [a]	0.269 ± 0.015 [a]	0.314 ± 0.002 [a]	0.132 ± 0.005 [a]	0.211 ± 0.001 [a]
	C+	0.451 ± 0.014 [a]	0.296 ± 0.005 [a]	0.269 ± 0.015 [a]	0.314 ± 0.002 [a]	0.132 ± 0.005 [a]	0.211 ± 0.001 [a]
	Cv	0.451 ± 0.014 [a]	0.296 ± 0.005 [a]	0.269 ± 0.015 [a]	0.314 ± 0.002 [a]	0.132 ± 0.005 [a]	0.211 ± 0.001 [a]
12 h	**C-**	0.415 ± 0.002 [a]	0.290 ± 0.001 [a]	0.296 ± 0.004 [a]	0.321 ± 0.001 [a]	0.144 ± 0.002 [a]	0.231 ± 0.005 [a]
	C+	0.382 ± 0.002 [b]	0.256 ± 0.002 [b]	0.268 ± 0.005 [b]	0.272 ± 0.004 [b]	0.230 ± 0.002 [b]	0.292 ± 0.001 [b]
	Cv	0.369 ± 0.002 [c]	0.301 ± 0.003 [c]	0.300 ± 0.003 [a]	0.272 ± 0.002 [b]	0.141 ± 0.006 [a]	0.239 ± 0.003 [c]
24 h	**C-**	0.394 ± 0.004 [a]	0.280 ± 0.005 [a]	0.274 ± 0.005 [a]	0.298 ± 0.003 [a]	0.140 ± 0.003 [a]	0.216 ± 0.002 [a]
	C+	0.385 ± 0.003 [b]	0.238 ± 0.003 [b]	0.244 ± 0.001 [b]	0.258 ± 0.003 [b]	0.231 ± 0.003 [b]	0.287 ± 0.001 [b]
	Cv	0.360 ± 0.003 [c]	0.330 ± 0.002 [c]	0.333 ± 0.003 [c]	0.250 ± 0.005 [c]	0.140 ± 0.004 [a]	0.227 ± 0.002 [c]
48 h	**C-**	0.452 ± 0.004 [a]	0.299 ± 0.002 [a]	0.276 ± 0.004 [a]	0.308 ± 0.003 [a]	0.142 ± 0.001 [a]	0.214 ± 0.005 [a]
	C+	0.390 ± 0.003 [b]	0.252 ± 0.003 [b]	0.242 ± 0.006 [b]	0.244 ± 0.004 [b]	0.236 ± 0.002 [b]	0.292 ± 0.002 [b]
	Cv	0.408 ± 0.006 [c]	0.315 ± 0.002 [c]	0.306 ± 0.021 [c]	0.296 ± 0.003 [c]	0.140 ± 0.002 [a]	0.272 ± 0.001 [c]

Letters indicate significant differences ($p < 0.05$) between the controls and the *Coriolus versicolor* digested biomass, at each sampling time. Cv, *Coriolus versicolor* digested biomass; C+, positive control (FOS); C-, negative control

Table 6.5. *Variation of microbiota in donor E. Values presented as mean ± standard deviation.*

		log copy number of 16S rRNA/ng of DNA					
Time	**Condition**	**Firmicutes**	**Bacteroidetes**	**Bacteroides**	***C. leptum***	***Lactobacillus***	***Bifidobacterium***
0 h	**C-**	0.455 ± 0.002 [a]	0.354 ± 0.001 [a]	0.312 ± 0.007 [a]	0.345 ± 0.002 [a]	0.202 ± 0.005 [a]	0.244 ± 0.004 [a]
	C+	0.455 ± 0.002 [a]	0.354 ± 0.001 [a]	0.312 ± 0.007 [a]	0.345 ± 0.002 [a]	0.202 ± 0.005 [a]	0.244 ± 0.004 [a]
	Cv	0.455 ± 0.002 [a]	0.354 ± 0.001 [a]	0.312 ± 0.007 [a]	0.345 ± 0.002 [a]	0.202 ± 0.005 [a]	0.244 ± 0.004 [a]
12 h	**C-**	0.405 ± 0.006 [a]	0.316 ± 0.001 [a]	0.301 ± 0.002 [a]	0.345 ± 0.002 [a]	0.187 ± 0.002 [a]	0.223 ± 0.001 [a]
	C+	0.390 ± 0.001 [b]	0.267 ± 0.002 [b]	0.252 ± 0.005 [b]	0.276 ± 0.002 [b]	0.174 ± 0.002 [b]	0.246 ± 0.001 [b]
	Cv	0.378 ± 0.002 [c]	0.541 ± 0.006 [c]	0.489 ± 0.004 [c]	0.538 ± 0.011 [c]	0.178 ± 0.002 [b]	0.265 ± 0.004 [c]
24 h	**C-**	0.408 ± 0.001 [a]	0.313 ± 0.006 [a]	0.300 ± 0.006 [a]	0.297 ± 0.002 [a]	0.166 ± 0.003 [a]	0.220 ± 0.002 [a]
	C+	0.403 ± 0.001 [a]	0.272 ± 0.001 [b]	0.255 ± 0.006 [b]	0.265 ± 0.009 [b]	0.175 ± 0.004 [b]	0.278 ± 0.001 [b]
	Cv	0.335 ± 0.004 [b]	0.217 ± 0.001 [c]	0.213 ± 0.011 [c]	0.219 ± 0.003 [c]	0.151 ± 0.002 [c]	0.247 ± 0.002 [c]
48 h	**C-**	0.427 ± 0.008 [a]	0.304 ± 0.001 [a]	0.305 ± 0.005 [a]	0.306 ± 0.004 [a]	0.171 ± 0.003 [a]	0.236 ± 0.001 [a]
	C+	0.388 ± 0.001 [b]	0.261 ± 0.002 [b]	0.236 ± 0.006 [b]	0.254 ± 0.003 [b]	0.182 ± 0.004 [b]	0.265 ± 0.001 [b]
	Cv	0.372 ± 0.006 [c]	0.228 ± 0.001 [c]	0.206 ± 0.010 [c]	0.241 ± 0.005 [c]	0.166 ± 0.005 [a]	0.272 ± 0.001 [b]

Letters indicate significant differences ($p < 0.05$) between the controls and the *Coriolus versicolor* digested biomass, at each sampling time. Cv, *Coriolus versicolor* digested biomass; C+, positive control (FOS); C-, negative control

7. Annexes

7.1. Annex I- NZY tissue gDNA isolation kit

NZY Tissue gDNA Isolation kit

Catalogue numbers: MB13502, 50 columns
MB13503, 200 columns

Description

NZY Tissue gDNA Isolation kits are designed for the simple and rapid small-scale preparation of highly pure genomic DNA from a variety of sample sources including animal cells and tissues, Gram-positive and Gram-negative bacteria, mouse tails, yeast, forensic samples and clinical samples. The method is spin column silica-based and requires no phenol or chloroform extraction. This kit uses optimized lysis buffers containing Proteinase K and SDS to release DNA from cells. After preparing the lysate, DNA is selectively absorbed into the NZYSpin Tissue Column and others impurities such as proteins and salts are removed during the washing steps. The eluted genomic DNA has a $A_{260/280}$ ratio between 1.7 and 1.9 what makes it ready to use in applications like sequencing, PCR, multiplex-PCR, genotyping and a wide range of other enzymatic manipulations.

The NZY Tissue gDNA Isolation kit is optimized to isolate up to 35 µg of DNA from up to 25 mg of tissue samples or 10^7 cells. We suggest not using more than the recommended starting material to prevent reduction in yield and purity of DNA isolated. For samples with very high RNA and protein contents (e.g. liver or spleen tissues), use only up to 15 mg of the sample. This kit is suitable for isolation of DNA from human or animal blood.

Storage conditions and reagents preparation

All kit components can be stored at room temperature (20-25 °C) and are stable till the expiry date. Before use, add 1.35 mL (MB13502/3) of Proteinase buffer to each vial of Proteinase K and vortex. Proteinase K solution is stable at -20 °C for up to 6 months. Add 28 mL (MB13502) or 112 mL (MB13503) of ethanol (96-100%) to each bottle of buffer NW2. Add 0.625 mL (MB13502) or 2.5 mL (MB13503) of water to RNase A vial. Store the RNase A solution at 4 °C for up to 3 months. For longer storage (up to 1 year), the RNAse A solution should be divided into small aliquots and stored at -20 °C. Buffers NL and NW1 contain guanidine hydrochloride. Wear gloves and goggles when using this kit.

System Components

Component	50 columns	200 columns
Buffer NT1	20 mL	80 mL
Buffer NL	15 mL	60 mL
Buffer NW1	30 mL	120 mL
Buffer NW2 (concentrate)	2 x 7 mL	2 x 28 mL
Buffer NE	15 mL	60 mL
Proteinase K (lyophilized)	30 mg	4 x 30 mg
Proteinase buffer	1.8 mL	7.2 mL
RNase A (lyophilized)	25 mg	100 mg
NZYSpin Tissue columns (light green ring)	50	200
Collection tubes (2 mL)	100	400

Standard protocol for isolating genomic DNA from animal tissues, cultured cells and bacteria cells

1. Sample preparation

Animal Tissues: Cut up to 25 mg tissue sample into small pieces, and place it in a microcentrifuge tube. Proceed with step 2.

Notes: *Tissue samples can be ground under liquid nitrogen for more efficient lysis.*
For rodent tails, place one (for rat) or two (for mouse) 0.6 cm-long pieces in a 1.5 mL tube.

Cultured Cells: Re-suspend up to 10^7 cells in 200 µL Buffer NT1. Add 25 µL Proteinase K solution and 200 µL Buffer NL*. Mix thoroughly by vortex, and incubate at 56 °C for 10-15 min. Vortex occasionally during incubation. Proceed with step 5.

**Mix Buffer NL thoroughly by shaking before use.*

Bacteria Cells: Pellet up to 1 mL bacteria culture for 5 min at 8,000 x*g*. Discard supernatant. Re-suspend cell pellet in 180 µL Buffer NT1 by pipetting up and down. Add 25 µL Proteinase K solution and vortex vigorously. Incubate at 56 °C for 1-3 hours. Mix occasionally during incubation. Proceed with step 3.

For other sample sources see the support procedures available in https://www.nzytech.com/products-services/kits-genomic-dna-purification/mb135/.

2. Pre-lysis of sample

Add 180 µL of Buffer NT1 and 25 µL Proteinase K solution to the sample. Mix thoroughly by vortex. Incubate at 56 °C for 1-3 hours and vortex occasionally during incubation.

Note: *Samples that are difficult to lyse can be incubated overnight as well.*

3. Removal of RNA (optional)

If RNA-free DNA is required, add 10 µL of RNase A solution (40 mg/mL) to each sample. Mix and incubate for 5 min at room temperature.

4. Lysis of sample

Vortex the sample. Add 200 µL Buffer NL* to the sample, and mix by vortex for 10 seconds.

Notes: *If insoluble particles are visible, centrifuge for 5 min at full speed and transfer the supernatant to a new microcentifuge tube.*
**Mix Buffer NL thoroughly by shaking before use.*

5. Addition of ethanol

Add 210 µL of 100% ethanol to the sample and mix immediately by vortex.

6. DNA binding

Transfer the mixture from step 5 into a NZYSpin Tissue Column placed in a 2 mL collection tube. Centrifuge for 1 min at > 11,000 x*g*. Discard flow-through and place the column in a new collection tube.

7. Wash silica membrane

Add 500 µL of Buffer NW1 to the column. Centrifuge for 1 min at > 11,000 x*g*. Discard flow-through and place the column back into the collection tube.

Add 600 µL of Buffer NW2 (make sure ethanol was previously added) to the column, and centrifuge for 1 min at > 11,000 x*g*. Discard flow-through.

Note: *For isolations of viral DNA from stool, we recommend to repeat the wash silica membrane step with Buffer NW2.* Add more 600 µL of Buffer NW2 to the column, and centrifuge for 1 min at > 11,000 x*g*. Discard flow-through.

8. Dry silica membrane

Place the NZYSpin Tissue Column back into the collection tube and centrifuge for 2 min at > 11,000 x*g*.

9. Elute DNA

Place the column into a clean microcentrifuge tube and add 100 µL of Buffer NE, TE buffer or sterile water (preheating of elution buffer to 70 °C may improve yield) directly in the membrane column. Incubate 1 min at room temperature and centrifuge at > 11,000 x*g* for 2 min to elute DNA. The genomic DNA can be stored at 4 °C or -20 °C.

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