CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

PORTO

STUDY OF THE IMPACT OF EDIBLE MUSHROOM BIOMASS OBTAINED FROM BY-PRODUCTS UPCYCLING ON THE HUMAN INTESTINAL MICROBIOTA

by

Arlindo André e André Cima

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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

Arlindo André e André Cima

Supervision: Professor Maria Manuela Estevez Pintado

September, 2023

To my mother´s grandfather, Olímpio Pires André

“Nada morre

Tudo se transforma,

No céu nasce mais

Uma estrela.”

Resumo

Nos últimos anos, a associação entre prebióticos e saúde humana tem sido cada vez mais forte, tornando-se um grande foco devido à capacidade de modular positivamente a composição e metabolismo da microbiota intestinal e impactar favoravelmente na saúde do consumidor. O consumo de cogumelos tem aumentado nos últimos anos e com ele também aumentou a produção dos subprodutos disponíveis, que podem representar uma nova fonte potencial para fibra alimentar que está diretamente correlacionada com o potencial prebiótico.

Este estudo teve como principal objetivo avaliar o efeito potencial prebiótico da biomassa de cogumelos obtida a partir de subprodutos usando um modelo de fermentação de fezes humanas. Foi caraterizada a composição de 4 farinhas obtidas do resíduo de extração aquosa (2 métodos de extração – M1 e M2) de subprodutos de 2 cogumelos - *Pleurotus ostreatus* e *Pleurotus eryngii*. Posteriormente, as duas farinhas de *Pleurotus ostreatus* (escolhida pelo melhor perfil de carboidratos) foram submetidas (juntamente com controlo positivo e negativo) a um processo digestivo simulado, e testadas num modelo de fermentação fecal humana *in vitro*, sendo avaliada para 5 tempos de fermentação a evolução de 5 grupos de bactérias: *Firmicutes*, *Bacteroidetes*, *Lactobacillus*, *Bifidobacterium* e *Clostridium*, bem como a formação de ácidos gordos de cadeia curta (SCFAs) e outros ácidos relevantes e representativos do metabolismo da microbiota.

Em relação à caracterização o foco principal foi avaliar principalmente a composição nutricional, em particular a fibra e carboidratos estruturais sabendo que os glucanos estão relacionados diretamente com o potencial prébiótico (utilizados como substrato para gerar metabólitos). Esta caracterização permitiu observar que *P. ostreatus* apresentou ligeira vantagem sobre *P. eryngii* no caso da fibra (*P. ostreatus*: 36.39 – 41.01 g/100 g DW, *P. eryngii*: 35.19 – 39.69 g/100 g DW). No caso dos β-glucanos, *P. ostreatus* é mais concentrado (50.92 – 52.29 g/100 g DW) comparativamente com *P. eryngii* (41.96 – 43.64 g/100 g DW). Comparando as farinhas, observa-se que o método de extração M2 gerou uma farinha com concentrações superiores de fibras e carboidratos estruturais.

Os resultados mostraram que a razão *Firmicutes : Bacteroidetes* ao longo do tempo se manteve estável e próxima de um. A produção de SCFAs (acetato, butirato e propionato), ácido lático e succínico revelou uma modulação positiva do metabolismo da microbiota, com evidencia de aumento de todos os metabolitos (menos ácido lático) em relação aos controles, o que é um indicador positivo do potencial prebiótico. Apesar dos resultados das comunidades bacterianas não estarem correlacionados diretamente com os metabolitos produzidos, é possível sugerir que a gama de propriedades bioativas associadas à biomassa de *P. ostreatus* pode estar relacionada com a potencial atividade prebiótica exibida na microbiota intestinal humana. Ainda, assegurou-se a valorização de subprodutos de cogumelos para obtenção de ingredientes funcionais.

Palavras-chave: *P. ostreatus*; Fibra alimentar; Simulação da digestão gastrointestinal; Microbiota intestinal; fermentação fecal humana *in vitro*; Subprodutos de cogumelos; Prebióticos; Ácidos gordos de cadeia curta (SCFAs).

Abstract

In recent years, the association between prebiotics and human health has been increasingly strong, becoming a major focus due to their ability to positively modulate the composition and metabolism of the intestinal microbiota and favorably impact consumer health. Mushroom consumption has increased in recent years and so the production of available by-products, which may represent a potential new source of dietary fibre that is directly correlated with prebiotic potential.

This study aimed to evaluate the potential prebiotic effect of mushroom biomass obtained from by-products using a human feces fermentation model. The composition of 4 flours derived from aqueous extraction (2 extraction methods – M1 and M2) of by-products from 2 mushrooms: *Pleurotus ostreatus* and *Pleurotus eryngii*, was characterized. Subsequently, two *Pleurotus ostreatus* flours (chosen for the best carbohydrate profile) were subjected (together with positive and negative controls) to a simulated digestive process, and tested in an *in vitro* human fecal fermentation model, being evaluated at 5 fermentation times for 5 groups of bacteria: *Firmicutes*, *Bacteroidetes*, *Lactobacillus*, *Bifidobacterium* and *Clostridium*, as well as the formation of short-chain fatty acids (SCFAs) and other relevant fatty acids representative of microbiota metabolism.

Regarding the characterization, the main focus was to evaluate mainly the nutritional composition and in particular the fibre, the structural carbohydrates including the glucans that are directly related to the prebiotic potential knowing in advance that the bacterial groups use them as a substrate to grow and generate metabolites. This characterization showed that *P. ostreatus* had a slight advantage over *P. eryngii* in the case of fiber (*P. ostreatus*: 36.39 – 41.01 g/100 g DW, *P. eryngii*: 35.19 – 39.69 g/100 g DW). In the case of β-glucans, *P. ostreatus* flours were higher (50.92 – 52.29 g/100 g DW) than for *P. eryngii* (41.96 – 43.64 g/100 g DW). Comparing the flours, it was observed that the M2 extraction method generated a flour with higher concentrations of fibre and structural carbohydrates.

The results showed that the *Firmicutes*: *Bacteroidetes* ratio over time remained stable and close to one, a positive indicator for M1 and M2 flours. The production of SCFAs (acetate, butyrate and propionate), lactic and succinic acid revealed a positive modulation of the microbiota metabolism, with evidence of an increase in all metabolites (except lactic acid) in relation to controls, which is a very positive indicator of the prebiotic potential of the flours. Although the results of bacterial communities are not directly correlated with the metabolites produced, it is possible to suggest that the range of bioactive properties associated with *P. ostreatus* biomass may be associated with the potential prebiotic activity displayed in the human intestinal microbiota. Also, the valorization of mushroom by-products allowed to obtain functional ingredients for the development of new health-promoting foods.

Keywords: *P. ostreatus*; Dietary fibre; Gastrointestinal digestion simulation; Gut microbiota; *in vitro* human fecal fermentation; Mushroom by-products; Prebiotics; Short-chain fatty acids (SCFAs).

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List of abbreviations

AAPH - (2,2'-azobis(2-methylpropionamidine) dihydrochloride

ABTS - 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

AcMZPS - acidic extracted mycelia zinc polysaccharide

AIMZPS - alkali extracted mycelia zinc polysaccharide

AOAC - Association of Official Analytical Chemists

APTR - alkali-extraction of polysaccharides from sclerotium of *P. tuber-regium*.

ASD - Autism spectrum disorder

BGP - β-glucan-enriched polysaccharide

CMP - *P. citrinopileatus* mycelia polysaccharide

CoA - coenzyme A

DNA - deoxyribonucleic acid

DP - degree of polymerization

DPPH - 2,2-diphenyl-1-picrylhydrazyl

DW - dry weight

EDTA - ethylenediaminetetraacetic acid

EPA - homogeneous polysaccharide with the molecular weight of 9.97×10^{4}Da

FAD - acid fibre detergent

FND - neutral fibre detergent

FOS - fructooligosaccharides

GAE - gallic acid equivalents

gDNA - genomic deoxyribonucleic acid

GI - gastrointestinal

GOS - galactooligosaccharides

HMO - human milk oligosaccharide

HPLC - High-Performance Liquid Chromatography

IgA - immunoglobulin A

IMO - isomalooligosaccharides

INFOGEST - consensus static digestion method

IPS - intracellular polysaccharides

ISAPP - International Scientific Association for Probiotics and Prebiotics

KOMAP - water-soluble polysaccharide from an alkaline extract of *P. ostreatus*

MAC - microbiota-accessible carbohydrates

NPEP - novel polysaccharide of 274 KDa molecular weight composed of glucose and β-type glycosidic linkages

NREL - National Renewable Energy Laboratory

ORAC - oxygen Radical Absorbance Capacity

PBS - phosphate-buffered solution

PCP - *P. citrinopileatus* polysaccharide

PCR - Polymerase Chain Reaction

PEIsR - mannogalactan derived from *P. sajor-caju*

PEP - heteropolysaccharides

PEPS - polysaccharides extracted from *P. eryngii*

PNA - alkali extractable polysaccharide of *P. citrinopileatus*

PN-S - *P. nebrodensis* polysaccharide

POP - *P. ostreatus* polysaccharides

RNA - ribonucleic acid

RPS - purified from crude polysaccharide

rRNA - ribosomal ribonucleic acid

SCFA - short-chain fatty acid

Se-POP - Selenium-enriched *P. ostreatus* polysaccharides

SGD - Simulated gastrointestinal digestion

SMS - spent mushroom substrate

S-PEPS - sulphonate- polysaccharides extracted from *P. eryngii*

STD - standard deviation

TE - tris and EDTA

TE* - trolox equivalent

TPC - total phenolic content

Tris - tris(hydroxymethyl)aminomethane

WPEP - novel polysaccharide of 167 KDa molecular weight composed of glucose and β-type glycosidic linkages

WPOPA - acidic polysaccharide extracted from *P. ostreatus*

W-PTR - water extraction of polysaccharide from sclerotium of *P. tuber-regium*

XOS - xylooligosaccharides

1. Introduction

1.1 Mushroom evolution

Mushroom research began long before the famous mind of Charles Darwin, and it took part in the evolution of biology in history. By faith in the creation account found in the Bible, the first mycologists produced a meticulous and ordered description of mushroom fruit bodies that highlighted their similarities and differences. In that era, these pioneers made remarkable strides in mushroom documentation. This was the unwitting beginning of fungal taxonomy.

Throughout a number of years, the only scientific interests focused on ancient culture and origins, connections, and similarities. As a result of the evidence to observe that similar mushrooms tend to generate similar types of toxins and other bioactive molecules, it was subsequently determined that this area possesses significant practical value.

Supported by multiple studies, it is possible to assert that all mushroom species and stripes are accurate genetic transmitters. Initially, basidiomycetes were the yeast-growing organisms. With this basic, single-celled form mechanism, fungi growth is very simple and undemanding, so it is possible for fungi to proliferate and grow in soils, freshwater ecosystems, the ocean, tree bark, and flower petals.

Despite the vast quantity and variety, there is no straightforward explanation. The primary explanation is due to the fruit body's obvious flexibility; consequently, organisms have numerous ways to adapt and accommodate to various spore release mechanisms (Money, 2022).

There are approximately 5,1 million fungal species in the world, of which 12,000 to 14,000 are considered mushrooms and only 2,000 are considered palatable. Five genera account for 85% of mushroom growth worldwide: *Agaricus*, *Pleurotus*, *Lentinula*, *Auricularia* and *Flammulina* (Royse, 2014).

Currently, it is feasible to observe an annual increase in consumption. In fact, the current per capita consumption varies considerably. In China approximately is 10 kilograms, compared to Europe 3,5 kilograms (Cheute *et al*., 2022). The outlier is Netherlands than surpass even China. Their associated health benefits and increasing consumer awareness have contributed to this trend (Aida *et al*., 2009; Royse, 2014), where mushroom is a valuable source of protein and energy that contrasts with low calorie and fat content. In addition, they contain an abundance of dietary fibre, proteins, and minerals (Fazenda *et al*., 2008; Manzi and Pizzoferrato, 2000). Considering all of these characteristics, the incorporation of this food into our diet provides us with numerous nutritional and health benefits and a distinct flavour that contributes to the enhancement of human health and longevity.

Mushrooms are available in fresh, extract, and biomass forms. The mushroom extract is a concentrated extract of the reproductive bodies of the mushroom. Mycelium and primordia require a sterile substrate to prevent contamination during the production of biomass. Since the biomass granules and cells

represent a structural barrier (where bioactive constituents are protected from digestion) and it contains not only the typical polysaccharides, but also active enzymes, it is possible to demonstrate that there exists a substance that is more resistant to gastric fluids than the extracts. On the other hand, the extraction procedure denatures proteins, resulting in the loss of enzymatic activity (Barros *et al*., 2016; Cruz *et al*., 2016; Ferrão *et al*., 2017).

As summarized by Reis *et al*. (2017), numerous mushroom-based supplements are currently being developed, including: naturally grown, dried mushroom fruiting bodies in the form of capsules or tablets; biomass or extracts from mycelium harvested from liquid culture grown in bioreactors or fermentation tanks; dried and pulverized preparations of the combined substrate, mycelium and primordial mushroom; hot water or alcohol extracts of fruiting bodies; spores and their extracts.

1.2 *Pleurotus spp.* Properties

The *Pleurotus* genus is the third most cultivated mushroom in the world and is considered edible despite possessing various medicinal properties (Fernandes *et al*., 2015; Khan and Tania, 2012). These species have a multi-coloured, shell or oyster-shaped basidiocarp and are therefore known as oyster mushrooms (Singh and Singh, 2011). Therefore, *Pleurotus spp*. are valued for their nutritional and sensory qualities. Their composition comprises terpenes, lactones, amino acids, and carbohydrates, which gives their fruiting body and mycelial biomass a wide spectrum of aromas and distinct flavour characteristics (Corrêa *et al*., 2016).

They are used as medicinal mushrooms because they contain numerous compounds with pharmacological and nutritional benefits. Valverde *et al*. (2015) identified lectins with immunomodulatory, antiproliferative, and antitumor activities, phenolic compounds with antioxidant activities, and polysaccharides with immunoenhancing and antitumor activities. Various bioactivities have been associated with *Pleurotus spp*. Some have immunomodulatory (Yan *et al*., 2019), anti-inflammatory (Taofiq *et al*., 2015), antioxidant (Zhang *et al*., 2016), and immunostimulatory (Liu et al., 2019) properties. In Table 1.1, it is possible to see that there are numerous prospective benefits.

Table 1.1 Pleurotus spp. properties and respective bioactive compounds.

Properties	Bioactive Compounds	References
Antitumor	PEP-2	Wang *et al*., (2021)
	KOMAP	Al-Saffar *et al*., (2020)
	Se-POP-3	Zhang *et al*., (2020)
	PNA-2	Wisbeck *et al*., (2017)
	PCP	Cui *et al*., (2016)
	β-glucan	Ren *et al*., (2016)
	Mycelium polysaccharide fraction (F II)	Cao *et al*., (2015)
		Liu *et al*., (2015)

Anti-inflammatory	S-PEPS PEIsR WPEP and NPEP α- and β- glucans	Ma *et al*., (2020) Castro-Alves & do Nascimento, (2018) Li & Shah, (2016) Silveira *et al*., (2015)
Immunomodulatory	PN-S APTR, BGP EPA-1	Castro-Alves & do Nascimento, (2018) Xu *et al*., (2016) Hai-Yan *et al*., (2015) Nworu *et al*., (2015)
Antioxidant	W-PTR and A-PTR PEPS30, PEPS60, PEPS80 RPS S-PEPS Se-POP IPS, IPS-1 and IPS-2 WPOPA AcMZPS1, AcMZPS2, AIMZPS1, AIMZPS2	Barbosa *et al*., (2020) Liu, Li, *et al*., (2019) Yan, Zhu, *et al*., (2019) Wu *et al*., (2013, 2014) Ma *et al*., (2018) Xu *et al*., (2017) He *et al*., (2016) Zhang, Li, *et al*., (2016) Zhang, Liu, *et al*., (2016) Ren *et al*., (2015) Li & Shah, (2014) Zhang *et al*., (2014)
Anti-diabetic	POP CMP PEP	Hao *et al*., (2020) Chen *et al*., (2016) Choi *et al*., (2016) Zhang, Hu, *et al*., (2016) Zhang *et al*., (2015)

AcMZPS- acidic extracted mycelia zinc polysaccharide; AIMZPS- alkali extracted mycelia zinc polysaccharide; APTR- alkali-extraction of polysaccharides from sclerotium of *P. tuber-regium*.; BGP- β-glucan-enriched polysaccharide; CMP- *P. citrinopileatus* mycelia polysaccharide; EPA- homogeneous polysaccharide with the molecular weight of 9.97×10^4Da; GOS- galactooligosaccharides; HMO- human milk oligosaccharide; IPS- intracellular polysaccharides; KOMAP- water-soluble polysaccharide from an alkaline extract of *P. ostreatus*; MAC- microbiota-accessible carbohydrates; NPEP- novel polysaccharide of 274 KDa molecular weight composed of glucose and β-type glycosidic linkages; PCP- *P. citrinopileatus* polysaccharide; PEIsR- mannogalactan derived from *P. sajor-caju*; PEP- heteropolysaccharides; PEPS- polysaccharides extracted from *P. eryngii*; PNA- alkali extractable polysaccharide of *P. citrinopileatus*; PN-S- *P. nebrodensis* polysaccharide; POP- *P. ostreatus* polysaccharides; RPS- purified from crude polysaccharide; Se-POP- Selenium-enriched *P. ostreatus* polysaccharides; S-PEPS- sulphonate-polysaccharides extracted from *P. eryngii*; WPEP- novel polysaccharide of 167 KDa molecular weight composed of glucose and β-type glycosidic linkages; WPOPA- acidic polysaccharide extracted from *P. ostreatus*; W-PTR- water extraction of polysaccharide from sclerotium of *P. tuber-regium*.

1.3 Mushroom by-products

Today, the food industry is extremely concerned with environmental issues and is expected to develop strategies to reduce waste and losses caused by food system activities.

An approach is to increase the value of agro-industrial residues, which could contribute to economic growth and environmental protection while fostering a circular economy. Mushroom by-products pose a disposal problem, but they are also prospective sources of valuable compounds that can be utilized due to their functional and nutritional properties. Diverse disciplines of study have been conducted in an effort to obtain value-added solutions for mushroom production and processing byproducts (Antunes *et al*., 2020).

1.3.1 Food uses

There is a dearth of scientific research in this field, despite the immense potential for food applications of mushroom byproducts. Mycelium derived from solid substrate fermentation mushroom production has the potential to be incorporated into culinary products to add functionality, nutritional characteristics, and ultimately enhance sensory properties. Polysaccharides from edible mushrooms are extraordinary molecular targets for potential and existing applications in pharmaceuticals, nutraceuticals, and functional foods. Their numerous therapeutic properties have been intensively researched, and it appears that there is a great deal of potential for exploiting these biopolymers in food formulations to improve human health (Giavasis, 2014).

Recently, Bilbao-Sainz *et al*. (2018) recovered chitin from mushroom stipe offcuts and converted into chitosan to generate edible coatings.

Atlast food, an American food technology company (https://www.atlastfood.co/), employs solid state fermentation to produce mycelium for meatless alternatives. The mycelium grows out of and up through the organic detritus. The mixture is deposited in growth chambers that replicate the conditions of soil. Mycelium obtained resembles the fibers of muscle tissue present in whole cuts of meat. This is a novel and economically viable method that satisfies both environmental concerns and consumer demand for meat-free products (Antunes *et al*., 2020).

1.3.2 Animal Feed

Multiple resistances to a number of conventional antimicrobials in animal feed formulations have raised concerns. Promoting and conducting research on natural alternatives.

To formulate animal feed containing mushroom by-products, research has been conducted.

As ruminant feed, the nutritive value of SMS (spent mushroom substrate) from *A. bisporus* mushroom production was determined (Fazaeli and Masoodi, 2006). The effects of fermented king oyster mushroom (P. eryngii) by-products on pork meat qualitative characteristics during refrigerated storage (Chu *et al*., 2012), effects of a diet containing fermented king oyster mushroom by-products on the growth performance (Moon and Lo, 2014). According to the findings, fermented king oyster mushroom byproducts may be a valuable ingredient that can reduce the cost of feeding and directly the animal

production. Had a positive effect on sensory attributes, namely meat flavor and acceptability (Chang *et al*., 2016).

1.3.3 Bioremediation and Biological Treatments

Some studies describe the use of SMS for bioremediation by degrading environmental pollutants with extracellular enzymes. Spent mushroom substrate were *P. eryngii* had the maximum removal rate for acetaminophen and sulfonamides (Chang *et al*., 2018).

Lau *et al*. (2003) obtained complete removal, by degradation, of individual naphthalene, phenanthrene, benzo(a)pyrene and benzo perylene. The outcomes demonstrated the viability of using SMS in ex situ bioremediation.

Dye removal through the enzymatic activity of SMS from solid substrate fermentation and fermentation broth submerged liquid fermentation, respectively, is another area of bioremediation that has been investigated. Physical, chemical, and biological methods have been primarily used to remediate dye wastewater up until now. The biological treatment method had been extensively concerned and studied because of its low processing cost and environment friendliness. SMS, as a result of its abundant surface hydroxyl, carbonyl, carboxyl, amide, and phosphate groups, could be considered a cost-effective adsorbent for the remediation of wastewater (Wu *et al*., 2018).

Due to their effectiveness and low cost, the use of agricultural residues as biosorbents is gaining importance in bioremediation of heavy metal-polluted water and soils. The study of Frutos *et al*. (2016) assessed the Cd, Pb and Cu adsorption capacity of the primary materials employed in the production of substrates for mushroom production (*A. bisporus* and *P. ostreatus*) and the SMS, based on the functional groups of their organic carbon. The results demonstrated the high potential of SMS to absorb heavy metals from polluted environments and were useful for developing novel biosorbents based on agricultural wastes.

1.3.4 Cosmetics and Cosmeceuticals

Cosmeceuticals are the newest trend in the skin care industry. They are primarily creams and moisturizers containing biologically active compounds that influence the biological function of the skin by supplying the essential nutrients for healthy skin. Several bioactive metabolites, ranging from large molecular weight polysaccharides and glycoproteins to phenolic compounds, terpenoids, steroids, and lipid components, have been linked to skin health (Taofiq *et al*., 2019a).

Several extracts derived from mushroom by-products, as well as their secondary metabolites, have been reported to exhibit essential biological functions, including antioxidant, anti-inflammatory, antitumor, antimicrobial, anti-aging, and photoprotective effects (Taofiq *et al*., 2017; Wu *et al*., 2016; Hyde *et al*., 2010). As a consequence of the negative effects associated with synthetic ingredients, the cosmetics industry is constantly in search of natural active biomolecules, such as those derived from mushroom byproducts, that are capable of treating these conditions. Residues produced during mushroom production remain an essential source of bioactive metabolites that can be used as cosmeceutical ingredients from a sustainable standpoint. Several of these findings indicate that mushroom by-products are valuable sources of bioactive extracts or individual metabolites that can be incorporated into topical

formulations for the maintenance of healthy skin (Fangkrathok *et al*., 2013; Huang *et al*., 2014; Chien *et al*., 2008; Taofiq *et al*., 2019b).

1.3.5 Bio-Based Materials

In order to convert low-cost organic wastes into economically viable and environmentally favorable materials, mycelium composites use biological growth rather than costly energy processes. Since an increasing number of developed nations are employing the use of sustainable materials as a strategy to reduce environmental pollution, mycelium-based materials support this strategy tremendously. The developed mycelium-materials require minimal energy for production (auto-growth), and their characteristics are modifiable by modifying their nutrient substrates. Several potential applications, including acoustic absorbers, super absorbents, paper, textiles, and structural and electronic components, have been proposed (Jones *et al*., 2017; Haneef *et al*., 2017).

Mycelium mechanical properties are affected by genetic modification and environmental growth conditions influencing mycelium density (Appels *et al*., 2018). Mycelium of the wild type resembled leather, whereas the genetically modified mycelium resembled thermoplastics (e.g. polyethylene, polypropylene, polyvinyl chloride). This was primarily due to the materials increased density and not its increased strength. This study demonstrated that mycelium can be chemically or physically modified to attain the desired characteristics in fungal materials. The results demonstrated a significant application potential as an alternative insulation material for building and infrastructure construction, particularly in frigid regions as a lightweight backfill material for geoengineering applications (Yang *et al*., 2017).

Islam *et al*. (2017) described the morphological and mechanical properties of an innovative biomaterial derived from fungal mycelium. The results of the experiment disclosed the most notable properties of mycelium under tension and compression. Under tension, the material response is linear elastic at low strain, and then strain hardening occurs prior to rupture.

In the last few years, research has been conducted on fungi-based mushroom packaging material as a cheaper alternative to conventional plastic and standard foams. Incorporating mycelium-based packaging materials could aid in reducing polystyrene foam consumption and pave the way for eco-friendly packaging, thereby augmenting sustainability without compromising cost or performance (Abhijith *et al*., 2018).

In terms of both sustainability and circular economy, these biomaterials offer a promising alternative for the design and production of products.

1.4 Prebiotics

The popularity of functional nutrients has increased dramatically as our age in the past years. Due to their ability to inhibit exogenous pathogens and manipulate the composition of colonic microbiota in the human gut to enhance host health, prebiotics have become a major focus (Roberfroid, 2002; Roberfroid, 2000; Rycroft *et al*., 2001).

Since the introduction of the term prebiotic (Gibson and Roberfroid, 1995), they have been defined as "a non-digestible food ingredient that has a beneficial effect on the host by selectively stimulating the growth and/or activity of a limited number of bacteria in the colon." With this definition, they observed that there are overlaps with the definition of dietary fibre, with the exception of its selectivity for particular species. Cummings *et al*. (2001), categorized prebiotics as carbohydrates with comparatively short chain lengths. The definition was revised again by Gibson *et al*. (2004) to include "selectively fermented ingredients that enable specific changes in the composition and/or activity of the gastrointestinal microbiota that confers health benefits on the host". The International Scientific Association for Probiotics and Prebiotics (ISAPP) defines a prebiotic as a substrate that is selectively utilized by host microorganisms and confers a health benefit. This definition of "substrate" precludes compounds that function as antimicrobial substances or other microorganisms because it refers to a substance from which an organism obtains nourishment.

As it was remarked earlier, prebiotics were formerly equated with dietary fibres. However, only a subset of dietary fibres qualifies as prebiotics, and prebiotics can also be derived from non-fibre substances such as polyphenols. According to the scientific consensus definition, a prebiotic compound must impart a beneficial physiological effect on the host, with at least a portion of this effect deriving from the microbes' utilization of the compound. Although early prebiotics targeted resident *Bifidobacterium* and *Lactobacillus*, research on the microbiome has cast light on other groups of health-related microbes that may be prebiotic targets. To qualify under this definition, however, the prebiotic can be any substance that must affect a limited group of microorganisms in the host, as opposed to the entire microbial ecosystem, thus fulfilling the criterion of being "selectively" utilized (Gibson *et al*., 2017).

Prebiotics under the class of polysaccharides are classified according to their origin and/or composition. Monosaccharide composition, the degree of polymerization (DP), chain length, and the type of linkage between monomers are the parameters that determine where and how it will interact with the microbiota, which is directly related to the species that will be stimulated and the region of the gut in which fermentation will take place.

They have been studied for a variety of health effects, including the reduction of incidence and duration of intestinal infections, impact on lipid metabolism, enhancement of immune function, improvement of colonic integrity, down-regulation of allergic response, and improvement of digestion and elimination of feces, reduction of colorectal cancer rates, and enhancement of mineral bioavailability (Charalampopoulos and Rastall, 2012; Douglas and Sanders, 2008; Everard *et al*., 2011; Fotschki *et al*., 2022; Patel and Goyal, 2012; Pineiro *et al*., 2008; Sharma and Puri, 2016; Slavin, 2013).

Inulin, galactooligosaccharides (GOS), and fructooligosaccharides (FOS) are the recognized and most frequently studied prebiotics. Other oligosaccharides, such as xylooligosaccharides (XOS) and isomalooligosaccharides (IMO), are considered emerging prebiotics (Jain *et al*., 2015; Gibson *et al*., 2017). In Table 1.2, it can be found some of these examples, their obtention and characteristics.

Table 1.2 Most important and recognize prebiotics. Adapted from (Aida et al., 2009).

Prebiotics	Production Method	DP (degree of polymerization)	References
Inulin	Hot water extraction from chicory root	11-65	Roberfroid, 2005 Coppa *et al*., 2002 Franck, 2002 Roberfroid, 2002
Fructo-oligosaccharides (FOS)	Tranfructosylation from sucrose, or hydrolysis of chicory inulin	2-10	L'Homme *et al*., 2003 Losada and Olleros, 2002
Galacto-oligosaccharides (GOS)	Produced from lactose by b-galactosidase	3-5	Ziegler *et al*, 2007 Alander *et al*., 2001
Soya-oligosaccharides (SOS)	Extracted from soya bean whey	3-5	Jaskari, 1998 Crittendan and Playne, 1996 Hayakawa *et al*., 1990
Xylo-oligosaccharides (XOS)	Enzymic hydrolysis of xylan	2-4	Crittendan and Playne, 2002 Yamada, 1993
Isomalto-oligosaccharides (IMO)	Transgalactosylation of maltose	2-8	Kaneko *et al*., 1995 Kohmoto *et al*.,1991

Inulin has a DP greater than 10, whereas the other oligosaccharides are composed of 3 to 10 monomers in short chains. Prebiotic compounds may affect the organoleptic properties of foods, which must be considered. Due to its ability to form gels, inulin has been extensively used in the food industry as a fat substitute and/or texture modifier, but it is not associated with a sweet taste. FOS has a high solubility and is primarily used as a sugar substitute. GOS are utilized primarily in infant formulations, and their resistance to high temperatures and acidic conditions facilitates their incorporation into food products. Whole grains and some dietary fibres, resistant starches, arabinoxylan, and non-carbohydrates with gut microbiota modulating effect are potential prebiotic candidates in the future (Aachary and Prapulla, 2011; Bindels *et al*., 2015; de Souza Oliveira *et al*., 2011; Patel and Goyal, 2012; Quigley, 2010).

Prebiotics will influence the microbiota composition and, consequently, metabolic activity through incorporation into foods or by existing in foods themselves. To achieve this, they must pass through the human gastrointestinal (GI) tract without being broken down by human enzymes and arrive at the colon almost unchanged, where they are fermented by host living bacteria. Most of them are fermented by *Bifidobacterium*, which is strongly associated with a healthy gut environment in which an increase in *Bifidobacterium* levels inhibits the growth of Gram-negative pathogenic bacteria, related to the organic acids production (Béghin *et al*., 2021; Christensen *et al*., 2013; Dahiya and Nigam, 2022; Vandenplas *et al*., 2015; Yoo and Kim, 2016; Wang *et al*., 2021).

1.5 The gut microbiota

The (GI) tract is one of the greatest interfaces (250–400 m^2) between the human body and the external environment. In the course of an average human life, approximately 60 tonnes of food and an abundance of microorganisms from the environment pose a significant hazard to gut integrity (Bengmark, 1998). In 2016, a study estimated and suggested that the proportion of human cells to bacterial cells is closer to 1:1 (Sender *et al*., 2016).

Huge health advantages can be provided by microbiota to the host. There is a vast array of physiological functions, such as enhancing gut integrity or shaping the intestinal epithelium (Natividad and Verdu, 2013), modulating host immunity (Gensollen, 2016), and defending against pathogens (Baumler and Sperandio, 2016). However, these mechanisms may be disrupted by dysbiosis, a condition characterized by an altered microbial composition.

1.5.1 Composition and structure

In the past few decades, culture methods have been the primary source of information regarding the microbiota of the adult human intestine (Moore and Holdeman, 1974). Our ability to survey the diversity of gut microbiota has vastly improved in recent years. Since this gene is present in all bacteria and archaea and contains nine highly variable regions (V1–V9), allowing species to be easily distinguished, it is currently the most well-known and widely used method. Former methods focused on sequencing the complete 16S rRNA gene. The emphasis of 16S rRNA sequencing has altered over the years, such that today the analysis is shorter and more in-depth in the gene's subregions; however, the use of shorter read lengths can introduce several errors. Over time, it has become evident that whole-genome shotgun metagenomics is more reliable and accurate for estimating and extrapolating microbiota composition due to the higher resolution and sensitivity of these techniques (Poretsky *et al*., 2014; Mizrahi-Man *et al*., 2013; Suau *et al*., 1999).

According to studies and reports, it was possible to identify 2172 species isolated from humans, which were classified into 12 distinct phyla, with 93.5% belonging to *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. Three of the twelve identified phyla contained only a single species isolated from humans, including *Akkermansia muciniphila*, the sole known representative of the *Verrucomicrobia phylum*, which is an intestinal species. A total of 386 of the identified species in humans are exclusively anaerobic and are typically found in mucosal regions such as the oral cavity and gastrointestinal tract (Hugon *et al*., 2015; Li *et al*., 2014).

The diversity of gut microbiota is not as rich and expansive as other´s microbial communities from other regions of the body, and a high degree of functional redundancy is evident (Schluter and Foster, 2012; Costello *et al*., 2009; Pérez-Cobas *et al*., 2012).

In 2014, it was possible to catalogue the functional capacity of the human intestinal microbiome, as 9 879 896 genes were identified using a combination of 249 newly sequenced samples and 1018 previously published samples. According to their findings, it is possible to identify patterns such as country-specific microbial signatures, indicating that the composition of the gut microbiota is influenced

by environmental factors, such as diet, and potentially also by host genetics. On the other hand, it is essential to note that functional microbiotas that differ in composition can produce similar protein or metabolite profiles (Li *et al*., 2014; Moya and Ferrer, 2016).

1.5.2 Biogeography

The composition of microbiota in the gastrointestinal tract reflects the physiological properties of a specific region and is stratified along both transverse and longitudinal axes (Macpherson and McCoy, 2013). Due to differences in chemical, nutritional, and immunological gradients, the microbiota density and composition vary along the length of the intestine. In the small intestine, there are typically large concentrations of acids, oxygen, and antimicrobials, and transit time is significantly shorter. According to Donaldson *et al*. (2015), only fast-growing, facultative anaerobes with the ability to adhere to epithelia/mucus are believed to be able to survive.

In terms of microbiota composition, it is possible to assert that the composition may vary between various GI organs, despite the fact that the composition and diversity of the microbiota of different colorectal mucosal regions within the same individual are spatially conserved. In contrast, faecal, luminal, and mucosal compositions differ significantly (Eckburg, 2005; Lavelle *et al*., 2015).

Bacteroidetes appear to be more abundant in faecal/luminal samples than in the mucosa, whereas Firmicutes, particularly the *Clostridium* cluster, are enriched in the mucus layer relative to the lumen (Eckburg, 2005; Van den Abbeele *et al*., 2012).

It is hypothesized that inter-individual differences in species and subspecies arrangement outweigh intra-individual differences in community structure. Several hypotheses have been advanced regarding the existence of a 'core microbiota,' which is hypothesized to consist of the same plentiful organisms found in all individuals. However, microbial genes were found to be more similar between individuals than the taxonomic profile, indicating that the core microbiota may be better defined at a functional level as opposed to an organism level (Eckburg, 2005; Turnbaugh *et al*., 2009; Jakobsson *et al*., 2010).

Individual microbiota arrangements can now be categorized into 'community varieties' that are predictive of one another and associated with background. However, Jeffery et al. (2012) noted that the existence and identification of these enterotypes are surrounded by considerable controversy and debate. In the adult microbiome, however, three major enterotypes have been identified: Bacteroides (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3) (Arumugam *et al*., 2011; Quigley, 2013; Wu *et al*., 2011).

Eukaryotic organisms and viruses (particularly siphophages and bacteriophages) are additional hosts in the human intestine. As bacteriophages infect and replicate within bacteria, they may affect the population of bacteria. In addition, each individual has a unique virome, the composition of which can be affected by diet (Breitbart et al., 2003, Marchesi, 2010, Minot *et al*., 2011). In addition to prokaryotes, such as fungi and protozoa, eukaryotes colonize the intestine. While Ascomycota and Basidiomycota comprise the preponderance of fungi, the diversity of fungi in the intestine differs from that of feces (Marchesi, 2010).

In recent years, evidence has increased suggesting that some protozoa may be commensal residents of the human intestine, promoting a discussion about the need to re-evaluate the role of protozoa as parasites, as some may have a beneficial effect. Blastocystis, for instance, is frequently found in healthy individuals (Luke *et al*., 2015; Chabe *et al*., 2017; Chudnovskiy *et al*., 2016; Wei *et al*., 2020; Ianiro *et al*., 2022; Dubik *et al*., 2022).

1.5.3 Shaping factors

Microbiota composition varies depending on host and environmental selective pressures. To prevent injury and maintain homeostasis, the GI tract employs a multifactorial and dynamic intestinal barrier to limit the exposure of the host immune system to microbiota. Physical (the epithelial and mucus layers), biochemical (enzymes and antimicrobial proteins), and immunological (IgA and epithelia-associated immune cells) factors are incorporated into the barrier (Hooper and Macherder, 2010). Due to the limited number of biochemical niches available in the intestine in comparison to other microbial-rich environments, gut microbiota must be adapted to a particular lifestyle. In the colon, energy is typically derived from fermentation and sulphate reduction of dietary and host carbohydrates (Ley *et al*., 2006; Travisano and Velicer, 2004; Cockburn and Koropatkin, 2016; Bajinka *et al*., 2020; Dordević *et al*., 2021). Several studies have shown a direct correlation between diet and microbiota, and that ileal microbiota is determined by the ability of microbial members to metabolize simple sugars, which reflects the adaptation to the nutrient availability in the small intestine (Deyaert *et al*., 2022; Zhang *et al*., 2022; Donaldson *et al*., 2015; Zoetendal *et al*., 2012).

Dietary fibre contains microbiota-accessible carbohydrates (MAC), which are the most important factor modulating the colonic microbiota. Extreme 'animal-based' or 'plant-based' diets result in extensive changes to the intestinal microbiota in humans (David *et al*., 2013). Diets high in resistant starch or non-starch polysaccharide fibre (wheat bran) resulted in a strong and reproducible enrichment of diverse bacterial species in the human gut (Walker *et al*., 2011) demonstrated the influence of fibre. The method of infant feeding can also influence the abundance of certain bacterial groups in the infant gut microbiota. Fucosylated oligosaccharides present in human milk can be utilized by *Bifidobacterium longum* and several species of *Bacteroides*, allowing them to outcompete other bacteria such as *E. coli* and *Clostridium perfringens* (Roager *et al*., 2023; Singh *et al*., 2022; Yu *et al*., 2012; Marcobal *et al*., 2011).

Intestinal mucus is also a source of carbohydrates for the microbiota of the intestine (Sauvaitre *et al*., 2021). Mucus layers are constructed around a large, highly glycosylated, gel-forming mucin. Mucins have diverse and complex glycan structures that are composed of four primary mucin-type O-glycans containing N-acetylgalactosamine, galactose, and N-acetylglucosamine. These core structures are further elongated and frequently terminated by sugar residues containing fucose and sialic acid. Mucus is present throughout the GI tract and is heaviest in the colon, where it is essential for mediating the relationship between the host and microbiota. Normalization of the intestinal mucus layers of the host necessitates protracted microbial colonization. Colonic mucus is composed of two layers: an inner layer that is dense and impermeable and an outer layer that is permeable by microorganisms. Whilst the inner layer is virtually sterile, in the outer layer, the mucin proteins, which are decorated by a rich and wide range of O-glycans, provide an energy source and preferential binding sites for commensal bacteria

(Tailford *et al*., 2015a; Arike and Hansson, 2016; Ouwerkerk *et al*., 2013; Johansson *et al*., 2010; Gustafsson *et al*., 2011; Johansson *et al*., 2015; Juge, 2012; Tailford *et al*., 2015b).

Diversification and modification of the microbial population can occur, for instance, through mutation or gene transfer (Bjedov, 2003; Xu *et al*., 2007; Rodríguez-Beltrán *et al*., 2021; Arnold *et al*., 2022), distribution of bile acids in the small and large intestine is reported to affect the bacterial community dynamics (Ridlon *et al*., 2014; Staley *et al*., 2017; Van Best *et al*., 2020), the host immune system (Ley *et al*., 2006; Hooper *et al*., 2012; Teixeira *et al*., 2019; Kesika *et a*l., 2021), several environmental factors (Gacesa *et al*., 2022; Rothschild *et al*., 2018; Rodríguez *et al*., 2015; Biedermann *et al*., 2013; Jiang *et al*., 2015; Tyakht *et al*., 2013) and geography (Yatsunenko *et al*., 2021).

1.5.4 Role in health

Health and gastrointestinal microbiota go hand in hand because the microbiota possess a variety of advantageous properties for the host wellbeing. Important functions of these organisms include maintaining the integrity of the mucosal barrier, providing nutrients, and protecting against pathogens. In addition, effective immune function depends on the interaction between commensal microbiota and the mucosal immune system. Colonic bacteria express carbohydrate-active enzymes, enabling them to ferment complex carbohydrates and produce metabolites such as SCFAs (Wardman *et al*., 2022; Musso *et al*., 2010). Propionate, butyrate, and acetate are typically found in a ratio of 1:1:3 in the gastrointestinal tract (Talwar *et al*., 2022; Louis *et al*., 2014). These SCFAs are swiftly absorbed by epithelial cells in the gastrointestinal tract, where they regulate cellular processes including gene expression, chemotaxis, differentiation, proliferation, and apoptosis (Corrêa-Oliveira *et al*., 2016). Acetate is produced by most anaerobes in the intestine, while propionate and butyrate are produced by distinct subpopulations of gut bacteria via distinct molecular pathways. There are two pathways for the formation of propionate, the succinate or propanediol (Kircher *et al*., 2022; Louis and Flint, 2017). Propionate is primarily produced by *Bacteroidetes* in the human intestine, whereas butyrate is primarily produced by *Firmicutes*. It has been documented that acetate is a cofactor for the growth of certain microbial species, and it is primarily produced by enteric bacteria through the hydrolysis of acetyl-CoA as a by-product of carbohydrate fermentation. It can also be produced by acetogenic bacteria using hydrogen, carbon dioxide, or formic acid via the Wood-Ljungdahl pathway in a much lesser quantity (Ros-Covián *et al*., 2016).

Acetate is released and largely absorbed going into peripheral tissues, whereas propionate is predominantly absorbed by the liver (Anachad et al., 2023; da Silva et al., 2020; Louis and Flint, 2017; Morrison and Preston, 2016). Butyrate is widely recognized for its anti-inflammatory and anti-cancer properties. SCFAs appear to regulate hepatic lipid and glucose homeostasis, the immune system and inflammatory response, appetite regulation, and energy intake (May and den Hartigh, 2023; Morrison and Preston, 2016; Lin and Zhang, 2017; Chambers *et al*., 2014; Flint *et al*., 2014; Rowland *et al*., 2017).

The GI microbiota is also essential for the synthesis of essential micronutrients that the host cannot produce. Lactic acid bacteria are essential for the synthesis of vitamin B12, which cannot be produced by animals, plants, or fungi. *Bifidobacterium* are the primary producers of folate, a vitamin required for vital metabolic processes in the host, such as DNA synthesis and repair. Vitamin K, riboflavin, biotin,

nicotinic acid, pantothenic acid, pyridoxine, and thiamine are several others (Pham *et al*., 2022; LeBlanc *et al*., 2013; Pompei *et al*., 2007).

1.6 Work objective

Worldwide, year by year wellbeing and healthcare is displaying an increasingly important role in human diet and lifestyle. Consequently, consumption and development of prebiotic-like ingredients from alternative sources have gained research relevance because of their ability to modulate the gut microbiota, bringing some health-related benefits treating problems like diarrhoea, inflammatory bowel disease, respiratory tract infection, cardiovascular disease and obesity (Jenkins *et al*., 2022).

Based on these social and research trends, the main objective of this research work was to chemically characterize, evaluate the potential antioxidant and prebiotic effects of biomasses obtained from *Pleurotus ostreatus* processed byproducts after *in vitro* gastrointestinal digestion on human gut microbiota using a human faecal fermentation model. It is expected to establish the impact of the main non-digested molecules on the main gut microbial groups including metabolism markers such as short chain fatty acids (SCFA).

2. Materials and Methods

2.1 Chemicals

DPPH (2,2-diphenyl-1-picrylhydrazyl), AAPH (2,2′-azobis(2-methylpropionamidine) dihydrochloride), fluorescein, methanol, α-amylase, bile salts, pancreatin, calcium chloride dihydrate ($CaCl_2$•$2H_2O$), magnesium chloride hexahydrate, ($MgCl_2$•$6H_2O$), pepsin and sodium hydrogen carbonate ($NaHCO_3$). Standards of glucose, fructose, arabinose, mannose, galactose, xylose, acetic acid, propionic acid, lactic acid, tartaric acid, succinic acid, isobutyric acid, trolox, gallic acid, vanillin, quercetin, p-coumaric, protocatechuic and caffeic acid and were obtained from Sigma-Aldrich (Sintra, Portugal).

ABTS diammonium salt (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), anhydrous sodium carbonate (Na_2CO_3), Folin-Ciocalteu's reagent, hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchase from Merck (Algés, Portugal).

Kjeldahl catalyst (Cu) (0.3% in $CuSO_4.5H_2O$) from PanReac AppliChem (Darmstadt, Germany).

2.2 Mushroom samples

The samples used were flours obtained from processed by-products of the project "MICOBIOEXTRACT - valorization of by-products resulting from mushroom production to improve the quality, safety and sustainability of the product (POCI-01-0247-FEDER-033939)". The samples were obtained from mushroom byproducts (losses resulting from non-conform mushrooms) from two different species *Pleurotus eryngii* and *ostreatus*. The residual biomasses transformed in flours were obtained as residual flours from the direct extraction M1 (Method 1: hot extraction 90 °C, 1 h, 500 rpm) and indirect extraction M2 (Method 2: extraction at room temperature and the resulting residue was further extracted with hot water 90 °C, 1 h, 500 rpm) were obtained. The two resulting solid residues from two different species *Pleurotus eryngii* and *ostreatus* were then freeze-dried (method based on the direct transition of a substance from the solid state to the gaseous state, called sublimation. Initially, the product is frozen and then dried by sublimation under reduced pressure) under controlled parameters (condenser temperature: -78.5 ºC, vacuum pressure 0.085 mBar).

2.3 Biochemical composition

2.3.1 Proximate composition

The moisture content (AOAC, 1997) was calculated by drying 2 g of each sample in the oven at 105 ºC for 24 hours until constant weight. Ash content was determined by carbon removal using an initial 2 g of each sample to be incinerated in a muffle furnace at approximately 550 °C for 24 hours (AOAC, 1980). The protein content was determined with a conversion factor of 4,38 (Ritota and Manzi, 2019), with the

determination of total nitrogen using the Kjeldahl method (AOAC, 1997). The analysis was done in triplicate and the results was g/100 g dry weight (DW).

2.3.2 Detergent Fibre quantification

In this analysis, the protocol followed was that of Goering and Van Soest (1970). This protocol is divided in two major steps: determination of the content of neutral fibre detergent (FND) and acid fibre detergent (FAD).

FAD measures the content of cellulose and lignin and FND measures all insoluble cell wall compounds which includes hemicelluloses, cellulose, and lignin. The analysis was done in duplicate and the results was g/100 g dry weight (DW).

2.3.3 Glucans quantification

The glucans were determined using Megazyme β-Glucan Assay Kit (Yeast & Mushroom). This kit allows to determine 1,3:1,6-β-glucan and α-glucan in yeast and mushroom preparations. The analysis was done in duplicate and the results was g/100 g dry weight (DW).

2.3.4 Minerals determination

The "microwave digestion system MARS 6" from the "CEM" brand was the digester that was utilized for the analysis. This digester comes with a compendium of pre-defined procedures that are employed for various types of materials. After that the digested samples were analysed using an optical emission spectrometer Model Optima 7000 DV™ ICP-OES (Dual View, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). The analysis was done in duplicate and the results was g/100 g dry weight (DW).

2.3.5 Antioxidant activity and total phenolics assays

Initially, the samples were prepared as a methanolic extract. 25 mL of solution (methanol/water) (80% v/v) and 2.5 g of each sample were homogenized with the Ultra-Turrax apparatus (T18, Wilmington, USA) at 24,000 rpm for 1 minute. The mixture was stirred continuously at 300 rpm for 30 minutes at room temperature. Each resulting sample was centrifuged at 4,480 g for 15 minutes at 4 ºC, and the supernatants were collected and filtered through a 0.45 m (Orange Scientific, Braine-l'Alleud, Belgium) membrane. Only 15 mL of the extracts were evaporated to dryness in the RCV 2-18 speed-vacuum evaporator (Chris. Osterode am Harz. Germany) and stored at -20 ºC until analysis.

The total phenolic content was determined in microplates using the method based on the Folin-Ciocalteu reagent described in Oliveira *et al*. (2016). For the antioxidant activity determination, three methods were used: ABTS, DPPH and ORAC.

The ABTS analysis was conducted according to Cano *et al*. (2000), DPPH (Alexandre *et al*., 2019) and ORAC (Oliveira *et al*., 2016). For total phenolic content (TPC), the results were expressed in mg gallic acid equivalents (GAE)/ g DW. For antioxidant activity the results expressed in μM Trolox-equivalents (TE*)/ g DW.

The results were expressed in mg/g dry weight (DW).

2.3.6 Structural Carbohydrates (cellulose and hemicellulose) and lignin

Through the protocol used in the NREL laboratory (Sluiter *et al*., 2008) it was possible to analyse cellulose (such as glucose), hemicellulose (such as arabinose, mannose, galactose and xylose) and lignin (soluble and insoluble) with HPLC coupling (high performance liquid chromatography) with a column carbohydrate analysis (Aminex HPX-87P, 300–7.8 mm, particle size 9 µm; pH: 5-9; Bio-Rad, flow rate: 0.6 mL/min; detector: refractive index). The insoluble lignin content was calculated gravimetrically after hydrolysis residue filtration, and soluble lignin was estimated by UV spectrophotometry at 340 nm. The analysis was done in octuplicate and the results were given as mg/g dry weight (DW).

2.4 Gastrointestinal digestion and dialysis

In digestion, the INFOGEST 2.0 (consensus static digestion method) protocol was used (Brodkorb *et al*., 2019; Salsinha *et al*., 2023; Vilas-Boas *et al*., 2022) with some changes (lipase from rabbit gastric extract was not used because of the very low concentration of lipids in *P. ostrestus*) where the three main phases were mimicked: oral phase; gastric phase and intestinal phase. In these phases, complex fluids are used to try to get close as possible to the fluids we segregate.

In brief, mouth digestion involves the dilution of food 1:1 (w/w) with synthetic salivary fluid and the addition of human salivary-amylase. Two minutes are spent simulating the mastication of food using an Orbital Shaker MaxQ 6000 at 200 rpm and 37 °C. In accordance with the INFOGEST protocol, the oral bolus is diluted 1:1 (v/v) with synthetic gastric fluid and gastric enzyme (pepsin) and agitated in the specified orbital shaker at 130 rpm, 37 °C, and pH 3.0 for 2 hours. The resulting gastric chyme is diluted 1:1 (v/v) with simulated intestinal fluid, bile salts, and pancreatic enzyme (pancreatin from the porcine pancreas) and incubated at pH 7.0, 45 rpm, and 37 °C for 2 hours in an orbital shaker. For each sample (5 g/sample), two replicates and a negative control consisting of distilled water were conducted.

Then the dialysis takes place to simulate the absorption of our organism. A segment of dialysis tube with a molecular weight of 3.5 kDa was filled with the digested samples, placed inside a recipient filled with 800 mL of distilled water. Then incubated overnight at 37 ºC and 60 rpm in an orbital shaker, simulating peristaltic movements. The purpose of the dialysis procedure was to simulate the passage of digested samples through the duodenum and jejunum. At the conclusion of the procedure, the solution left outside the dialysis membrane represented the absorbable sample (bloodstream) and the solution left inside the membrane represented the non-absorbable sample (colon-available) (Gómez-García *et al*., 2022).

The sample present in this last part was then lyophilized, being the sample year to start the process of *in vitro* human faecal fermentation.

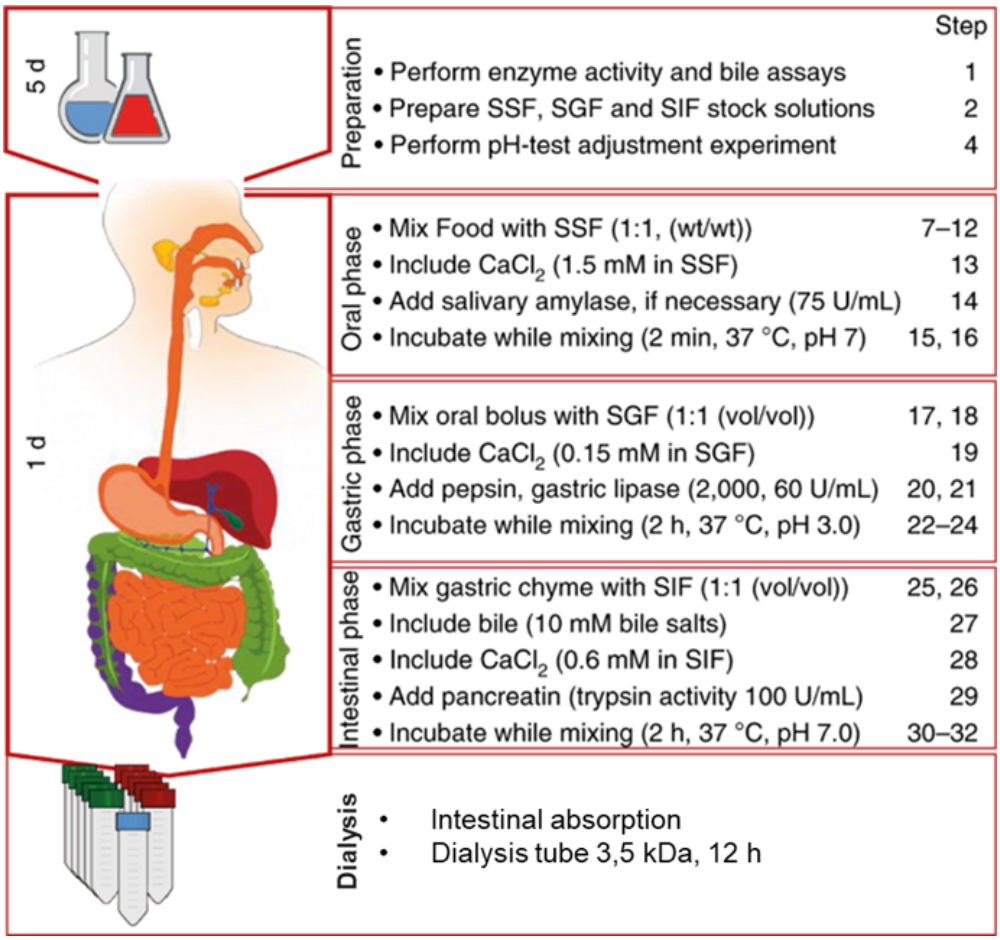


Figure 2.1 Explanatory diagram of the three main phases: oral phase; gastric phase and intestinal phase with dialysis process (Adapted from Brodkorb et al., 2019).

2.5 *In vitro* human faecal fermentation

This protocol was developed and adapted according Gullón *et al*. (2015) and Gullón *et al*. (2014) following the next steps:

2.5.1 Collection and preparation of faecal inocula

Fresh faecal samples were collected from five healthy donors (A-E, ages between 23 to 35 years), who had not ingested any probiotic/prebiotic supplements or antibiotics in the last 6 months (Annex 1 and 2 -Informed Consent Form and Sample Collection). The faecal samples were maintained under on the morning of the assay. Every donor was according to the specific needs for the trial (normal omnivorous diets, not ingested any antibiotics or other medicines known to affect the microbiota for at least 6 months before the study and cannot consume in a dairy basis probiotics or prebiotics).

The faecal inocula was then prepared, by diluting the faecal matter in Reduced Physiological Salt Solution that was already prepared (constituted by 0.5 g/L Cystein-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). For

homogenization was used a stomacher (Serward, Worthing, UK) for 2 min at 460 paddle beats per minute.

2.5.2 Nutrient Base Medium preparation

The fermentation medium used for the fermentations consisted of 5.0 g/L of trypticase soy broth without dextrose, 5.0 g/L of bactopeptone, 5.0 g/L of yeast nitrogen base, 0.5 g/L cysteine hydrochloride, supplemented with a 1% (v/v) salt solution A (100 g/L of Ammonium chloride (NH_4Cl), 10 g/L of magnesium chloride hexahydrate ($MgCl_2$ $6H_2O$) and 10 g/L of $CaCl_2$ $2H_2O$), 0.2% (v/v) of a salt solution B (200 g/L of Potassium phosphate dibasic trihydrate (K_2HPO_4 $3H_2O$), 0.2% (v/v) of 0.5 g/L resazurin solution and a 10 mL/L of a trace minerals solution. The final pH was adjusted to 6.8 and before sterilization the medium was placed under a nitrogen stream.

2.5.3 Faecal fermentations

The flasks that were prepared in previous step were then inoculated at 2% (v/v) with faecal inocula and incubated for 48 hours at 37 ºC under anaerobic atmosphere (10% CO_2, 5% H_2 and 85% N_2). Samples were collected after 0, 6, 12, 24 and 48 hours of incubation (0 hours only in the negative control) 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). At the sample collection, 2 aliquots of 2 ml each were taken from all times. The positive and negative controls were respectively designated as C+ (FOS) and C- (only faecal inocula), while the residual flour of *P. ostreatus* was identified as PO. Afterwards, the samples were stored at -30 ºC until analysis. All the procedures were performed in an anaerobic workstation (Don Whitley Scientific, West Yorkshire, UK).

2.5.4 Sample's preparation for DNA extraction

Aliquots (4 mL) were centrifuged for 10 min. The pellets were used to extract the total genomic DNA and carry through Real Time Polymerase Chain Reaction (PCR). The supernatants were used to evaluate organic acid production with High performance liquid chromatography (HPLC).

2.6 Short-chain fatty acids

Short-chain and branched-chain fatty analysis were determined in supernatants resulted from the faecal fermentation (Gullon *et al*., 2015). The analyses were performed using a Beckman Coulter System Gold HPLC (Knauer, Berlin, Germany) coupled to IR and UV detector using Aminex 37-H column (Bio-rad, Berkeley, USA) at 55 ºC and 5 mmol L H_2SO_4 as mobile phase (flow rate: 0.6 mL/min). The calibration curve for citric, lactic, acetic, propionic and isobutyric acid was 0.3-20 mg/mL, for tartaric and succinic acid was 0.3-2,5 mg/mL.

2.7 DNA extraction

Briefly, for the extraction of DNA 4 mL of the fecal fermentation sample was centrifuged at 4000 rpm for 10 mins at 4 ºC. The resultant pellet was dissolved in 1 mL of TE buffer (1X). The solution was centrifuged at 4000 rpm for 10 mins at 4ºC and 180 µL of a lysozyme solution (10 mg/mL in TE) was added to the pellet. Then was incubated at 37ºC for 2 hours. The solution was further centrifuge at 4000 rpm for 10 mins at 4ºC and the resulting pellet was used to DNA extraction using the NZYTissue gDNA isolation kit according to the manufacturers' instructions (NZYTech, Lisbon, Portugal). 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).

2.8 Real Time 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.

PCR stage	Temperature	Time		
Initial denaturation/ enzyme activation	95 ºC	10 min		
Denaturation	95 ºC	10 s	45 cycles	
Annealing	Specific temperature for each primer	1 min		
Extension *	72 ºC	15 s		
Melting curve*	60-97 ºC, with an increment of 0.5 ºC for 0.05 min	0.05 s		

*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) and 1 µL of forward and reverse primers (100 nM) targeting the 16S rRNA gene. The primers used were obtained from STABvida (Lisbon, Portugal) are listed in Table 2.2. Standard curves were constructed using tenfold dilutions (overall from 1/2 log to 6/7 log of number of copies of 16S rRNA gene/µL) of bacterial genomic DNA standards. All assays were performed in quadruplicate. All the procedure was under conditions adapted and optimized from previous studies (Bonifácio-Lopes *et al*., 2022; Ribeiro *et al*., 2021). To have an idea in Annex 4 there is a protocol which is very similar.

Table 2.2 Primer sequences targeting bacterial community, annealing temperature and genomic DNA standards.

Target group	Primer´s sequence (5'-3')	Annealing temperature (ºC)	Genomic DNA standard	
Firmicutes	**F**: ATG TGG TTT AAT TCG AAG CA **R**: AGC TGA CGA CAA CCA TGC AC	55	*Lactobacillus Gasseri* (ATCC 33323)	DSM20243
Bacteroidetes	**F**: CAT GTG GTT TAA TTC GAT GAT **R**: AGC TGA CGA CAA CCA TGC AG	55	*Bacteroides Vulgatus* (ATCC 8482)	DSM 1447
Lactobacillus	**F**: CAC CGC TAC ACA TGG AG **R**: AGC AGT AGG GAA TCT TCC A	59	*Lactobacillus Gasseri* (ATCC 33323)	DSMZ20243
Bifidobacterium	**F**: CGC GTC YGG TGT GAA AG **R**: CCC CAC ATC CAG CAT CCA	60	*Bifidobacterium Longum* subsp. infantis	DSM 20088
Clostridium leptum	**F**: GCA CAA GCA GTG GAG T **R**: CTT CCT CCG TTT TGT CAA	58	*Clostridium Leptum*	DSM 753

F- forward primer; **R**- reverse primer

3. Results and Discussion

The 4 samples obtained from mushroom byproducts (non-conform mushrooms) from two different species namely *P. eryngii* and *P. ostreatus* and the compositional analysis of the most important components was determined.

3.1 Proximate composition

The proximate composition including the content on protein, ash, moisture and detergent fibre is presented in Table 3.1.

Table 3.1 Proximate composition of P.eryngii and P.ostreatus (mean ± standard deviation).

Samples	***P. eryngii***		***P. ostreatus***	
(g/100 g DW)	*M1*	*M2*	*M1*	*M2*
Moisture	6,95 ± 0,10	6,64 ± 0,12	6,82 ± 0,16	7,22 ± 0,14
Ash	0,22 ± 0,01	0,18 ± 0,01	0,19 ± 0,01	0,11 ± 0,00
Protein	13,76±0,11	14,88±0,02	10,8±0,004	11,58±0,05
Neutral fibre detergent (FND)	35,19±1,15	39,69±0,58	36,39±0,41	41,01±1,23
Acid fibre detergent (FAD)	15,03±0,24	17,28±0,20	9,72±0,45	12,11±0,27

In the flours (dry weight mushrooms residual biomasses), the moisture content it is ranging between 6 and 7% per dry matter, what is in accordance with Kalač, (2013). Concerning the ash contents for both varieties of mushrooms, the value that was obtained was low compared to other results in same species mushroom (Ritota and Manzi, 2019). to the since ranged between 0.11 and 0.22% by dry matter. These results are justified since we were not using entire mushrooms but flours obtained as a residual biomass after an aqueous extract, and minerals as soluble components were extracted and only traces were present the residual biomasses tested in this study. Concerning extraction method, the M2 flours showed lower content since this extraction combined two sequence extractions, one at room temperature and other at 95 ºC, which allowed to extract more minerals towards the liquid fraction.

In terms of the moisture, it is not possible to compare with fresh samples because the state is already dry flour, although these results (low content) is a good indicator as self-life stability (Nasir *et al*., 2003).

Kjeldahl technique was used to measure total nitrogen, and a conversion factor of 4,38 was applied in order to calculate the amount of protein present in the sample species (Ritota and Manzi, 2019). Results showed a distinct difference between the two species, specifically that *P. eryngii* possesses a higher protein concentration (13,76-14,88 g/ 100 g DW) compared with *P. ostreatus* (10,8-11,58 g/ 100 g DW). Although we have a residual biomass obtained after extraction, results goes according Ritota and Manzi, (2019). However, there are also reports were *P. eryngii* have lower protein content (Sharma and Sharma, 2018) since these depends on different external cultivation conditions. Despite that, the content of protein is 20-30% lower compared, since as these samples result after aqueous extraction, soluble protein was extracted towards the liquid extract and this protein fraction was reduced in the residual flours. When we compare the extraction method M1 and M2 it is evident that M2 extracted slightly less protein and allowing to retain higher contents in the residual flour. Although there were 2 sequential extractions in M2, the second extraction from the residue using high temperature may have created denatured and protein aggregates that allowed slightly higher content in the residual flour.

In terms of fibre determination, with the scope of this investigation, the methodology developed by Goering and Van Soest (1970) was utilized. The results are presented in Table 3.1, where it is possible to observe that the species *P. ostreatus* (36.39 – 41.01 g/ 100 g DW) is composed by a higher percentage of neutral detergent fibre in comparison to *P. eryngii* (35.19 – 39.69 g/ 100 g DW).

In acid detergent fibre, however, the proportion of *P. ostreatus* to *P. eryngii* is reversed, so that the former can be found in greater abundance than the latter. According to this methodology, the nutritional content of dietary fibre can be accurately predicted using neutral detergent fibre as a good indicator, and as evident also in these results, mushrooms are an excellent source of fibre.

When comparing the methods M1 and M2, the M2 method showed a higher content than the M1, and this tendency holds true regardless of the species. These is explained by the two sequential extractions performed in M2 that probably extracted more soluble polysaccharides and soluble sugars from the mushroom, allowing a proportional increase of fiber in the residual flour.

3.2 Glucans

The results obtained for glucan quantification of both mushrooms and for both extraction methods are depicted in Table 3.2. It is highly reported that the mushrooms contain a significant quantity of glucans, comprising the most abundant polysaccharides in the cell walls of fungi, and their structures are highly variable. Accordingly, their glucose moieties may be joined through either or both alpha (α) or beta (β) linkages, they are either lineal or branched, and amorphous or microfibrillar (Kumar *et al*., 2021).

As is shown, β-glucans are clearly the most abundant component in each sample, regardless of the species or extraction technique used. It is clear that *P. ostreatus* has a higher content when compared to *P. eryngii* and goes along with the findings of Synytsya *et al*. (2009). The factor that varies the most is the value of the beta-glucans, which has a direct impact on the total glucans. Comparing the extraction method appositively to what happened in the total fiber, the M2 method allowed to retain less glucans

in the residual flours, which means that the two sequential extractions allowed to extract more glucans towards the liquid extract and reduced in the residual fraction. It is evident that the alfa glucans were extracted more for the liquid fraction and proportionally they were more reduced in the solid residual flours (ca. 50% less in M2 compared to M1).

Table 3.2 Total glucans, α-glucans and β-glucans profile of P.eryngii and P.ostreatus (mean ± standard deviation).

Samples			**(g/100g DW)**
P. eryngii	M1	Total glucans	50,70±0,09
		α-glucans	7,06±0,05
		β-glucans	43,64±0,04
	M2	Total glucans	45,74±0,13
		α-glucans	3,78±0,01
		β-glucans	41,96±0,14
P. ostreatus	M1	Total glucans	56,00±0,14
		α-glucans	5,08±0,01
		β-glucans	50,92±0,14
	M2	Total glucans	54,97±0,02
		α-glucans	2,67±0,00
		β-glucans	52,29±0,03

3.3 Minerals

In light of the information shown in Table 3.3 related to mineral profile, when comparing M1 and M2 values, the values of M1 are consistently higher than those of M2 (as already discussed for the ash composition) with the exception of Fe in *P. eryngii*. M1 in both species are the only samples that contains sodium.

The species *P. eryngii* has a higher concentration of the element's potassium, magnesium, and phosphorus. *P. ostreatus* has a higher concentration of the elements zinc, manganese, iron, calcium, copper, and sodium. Ca, Cu, and Al are the three minerals that are found in *P. ostreatus* but not in *P. eryngii*.

Depending on the species, potassium and phosphorus or magnesium are the most abundant minerals in mushrooms (Guillamón *et al*., 2010). In smaller amounts, they also contain calcium, iron, copper, zinc, manganese, selenium, and sodium (Guillamón *et al*., 2010). Mushrooms are an intriguing food product

for the prevention and dietary treatment of hypertension (Kalač, 2013) due to their high and low potassium and sodium content, respectively.

Due to their capacity to accumulate heavy metals in their tissues, the only risk associated with mushroom consumption is the eventual prevalence of heavy metals in some substrates where mushrooms could grow (Guillamón *et al*., 2010).

Table 3.3 Mineral profile of P.eryngii and P.ostreatus (mean ± standard deviation).

Elements	*P. eryngii*		*P. ostreatus*	
	M1	M2	M1	M2
Mo	0	0	0	0
Zn	1,851 ± 0,002	1,685 ± 0,016	1,894 ± 0,067	2,264 ± 0,308
Cd	0	0	0	0
P	146,340±0,51	129,538±0,196	104,712±0,449	71,756±1,702
Pb	0	0	0	0
Ni	0	0	0	0
Co	0	0	0	0
Mn	0,193±0,003	0,190±0,002	0,290±0,004	0,285±0,034
Fe	0,317±0,01	0,544±0,031	1,986±0,004	1,970±0,154
Mg	26,029±0,04	25,040±0,036	25,130±0,342	21,363±2,294
Ca	0	0	4,067±0,101	2,808±0,451
Cu	0	0	0,313±0,055	0,155±0,030
Al	0	0	0,242±0,018	0,145±0,013
Na	2,500±0,35	0	5,673±0,268	0
K	499,305±4,09	379,003±0,123	387,213±1,283	191,832±3,464

3.4 Structural carbohydrates (cellulose and hemicellulose) and lignin

According to the findings, glucose possesses a significant advantage over the other components in the composition of both species. *P. ostreatus* presented higher concentration than *P. eryngii* overall. Xylose is the most important component in terms of hemicellulose. M1 in the case of *P. ostreatus* showed a lower concentration in comparison to M2. The opposite happens in *P. eryngii* (Table 3.4).

Only the *P. eryngii* species has been found to have arabinose, despite the fact that it is found in lower concentrations than in the other species. Comparing the extraction methods, M2 shows ca. 5 times more than M1.

In terms of lignin characterization, both fractions are richer in Acid Soluble Lignin (ASL), derived from cell wall, approximately 5 to 6 times higher than the Acid Insoluble Lignin (AIL), middle lamella, as depicted in Table 3.5.

Table 3.4 Proximal structural carbohydrates composition of P.eryngii and P.ostreatus (mean ± standard deviation, anhydrous correction).

Samples	*P. eryngii*				*P. ostreatus*			
	M1		**M2**		**M1**		**M2**	
	mg/g DW	anhydrous correction	mg/g DW	anhydrous correction	mg/g DW	anhydrous correction	mg/g DW	anhydrous correction
Glucose	942,34±0,36	848,11	819,63±0,27	737,66	1095,53±0,33	985,98	1129,35±0,22	1016,41
Xilose	236,07±0,10	207,74	198,65±0,06	174,81	235,84±0,06	207,54	247,16±0,09	217,50
Arabinose	2,03±0,00	1,78	10,61±0,01	9,33				

Table 3.5 Lignin composition of P.eryngii and P.ostreatus (mean ± standard deviation).

Samples	***P. eryngii***		***P. ostreatus***	
(g/ 100 g DW)	*M1*	*M2*	*M1*	*M2*
ASL	5,14 ± 0,08	6,15 ± 0,10	5,25 ± 0,03	4,94 ± 0,04
AIL	0,83 ± 0,08	0,96 ± 0,01	1,11 ± 0,07	1,31 ± 0,04

3.5 Antioxidant activity and total phenolics

The total phenolic content was evaluated using the method based on the Folin-Ciocalteu and results are presented in Table 3.6. It is noticed that the free phenolic compounds are present in higher concentration than bond phenolics (3 to 6 times more). However, the total phenolic content was found to be similar between the two species. At the level of the species, the amount of free phenolics is higher in *P. eryngii* and in both cases.

Comparing the extraction methods, the flour M1 (1,50-2,05 mg GAE/g DW) showed higher content than M2 (0,92-1,53 mg GAE/g DW).. These results are not in agreement with another species, *Auricularia*

auricula and *Tremella fuciformis* (Tu *et al*., 2021), however we are working with residual flours obtained after aqueous extraction that impact the initial composition.

An antioxidant molecule can exert the antioxidant through different mechanisms it is advisable to use methods with different oxidation systems to guarantee the measurement of different antioxidants, so we have used three different methods: ABTS, DPPH, and ORAC.

In the ABTS analysis that was carried out in accordance with Cano *et al*. (2000), it is possible to detect that antioxidant activity related with free and bond polyphenols is always higher in M1 flours and in the *P. eryngii* species Both the DPPH (Alexandre et al., 2019) and the ORAC methods (Oliveira *et al*., 2016) provide evidence to support this pattern.

In light of this, it would indicate that the species *P. eryngii* and M1 have the most potential for phenolic and antioxidant activity (Table 3.6).

However, mushroom dietary fibre might negatively affect the bio accessibility of the phenolic compounds and the digestion of other nutrients when they are incorporated in the products. They can create associations with polyphenols before and during gastrointestinal digestion. Some of the phenolic compounds are available in the stomach and upper intestine to reduce the free radicals present. The remaining phenolic compounds pass into the colon and are available to be bio-transformed to metabolites via fermentation by colonic microbiota (Jakobek and Matić, 2019; Barros *et al*., 2020; Blanco Canalis *et al*., 2020).

Table 3.6 Total phenolics and antioxidant activity (mean ± standard deviation).

Samples			Total phenolics (mg GAE/g DW)	ABTS (mg TE*/g DW)	DPPH (mg TE*/g DW)	ORAC (mmol TE*/g DW)
P. eryngii	Free	M1	2,05 ± 0,01	0,41 ± 0,02	2802,39 ± 98,99	31,27 ± 0,80
	Free	M2	1,53±0,02	0,35±0,01	1763,46±128,36	28,27±1,38
P. ostreatus	Free	M1	1,50±0,04	0,30±0,01	1354,58±91,01	24,52±1,62
	Free	M2	0,92±0,06	0,18±0,01	1022,56±19,15	23,54±1,54
P. eryngii	Bond	M1	0,36±0,01	0,35±0,01	2391,56±207,36	7,17±0,20
	Bond	M2	0,23±0,01	0,20±0,01	941,82±40,06	5,00±0,09
	Bond	M1	0,38±0,03	0,22±0,02	1344,87±65,98	7,23±0,12

P. ostreatus	Bond	M2	0,33±0,02	0,13±0,01	782,08±75,06	6,47±0,29

3.6 Human faecal microbial communities

An *in vitro* fermentation model using the feces of five different donors was used in order to execute a simulation of the fermentation by gut microbiota and to predict the prebiotic potential of the samples under study. In order to reduce samples, we have selected one of the species, and as *P. ostreatus was richer in glucans, it was selected to study the impact of mushrooms byproducts flours obtained as two different extraction methods.* The participants to provide the feces samples were chosen based on a set of criteria that included the diet they followed, their age, their current health state, any food intolerances or allergies they had, whether or not they had consumed prebiotics, probiotics, or antibiotics in the preceding six months, and their household and family relationships. It is generally agreed that these factors do have a major impact over the composition of the microbiota found in the gut. They have been shown to exert a protective role where they compete with pathogens to produce direct antimicrobial effects and indirectly enhance intestinal barrier function. Bacteria primarily belonging to the genera *Bifidobacterium* and *Lactobacillus* are capable of producing bacteriocins, which are antibacterial molecules that are highly effective against foodborne pathogens such as *Listeria monocytogenes*. The probiotic effects of the genus *Lactobacillus*, in particular, have been the subject of a significant amount of research during the past ten years. Clinical studies and animal experiments have shown that there are significant differences in the abundance of *Lactobacillus* between diseased and disease-free hosts, and that the administration of specific *Lactobacillus spp.* improves clinical symptoms and/or prevents relapse of several intestinal disorders (Yang *et al*., 2022; Jeong ***et al***., 2022; Darbandi *et al*., 2022). These findings have been demonstrated both in human subjects and in animals. Recent research has shown that certain strains of *Lactobacillius* can be effective in treating colorectal cancer, either on their own or in combination with chemotherapy (Ghorbani ***et al***., 2022). In addition, *Lactobacillus spp*. may be able to ameliorate disorders such as gastrointestinal diseases, allergies, and liver diseases through a variety of methods. These mechanisms encompasses the generation of metabolites that can directly inhibit pathogens, demonstrating immunomodulatory effects, and altering the composition of the microbiota in the intestinal tract (Jeong ***et al***., 2022). Concerning the genus *Bifidobacterium*, numerous probiotic *Bifidobacterium* have been found to have good effects on humans or animals. These effects include fighting infections and depression, modulating the immune system of the host, and making it easier for the host to absorb nutrition (Chen *et al*., 2021).

Table 3.7 Overall variation, for the 5 different donors, of the distinct bacterial communities. Values presented as log10 16S rRNA gene copies/ ng of DNA (mean ± standard deviation).

	Bacteroidetes					***Bifidobacterium***					***Clostridium leptum***				
Time	0 h	6 h	12 h	24 h	48 h	0 h	6 h	12 h	24 h	48 h	0 h	6 h	12 h	24 h	48 h
C-	0,257 ±0,012	0,278 ±0,013	0,299 ±0,017	0,305 ±0,021	0,316 ±0,025	0,277 ±0,018	0,343 ±0,032	0,341 ±0,030	0,333 ±0,013	0,312 ±0,028	0,261 ±0,100	0,287 ±0,113	0,293 ±0,111	0,282 ±0,112	0,285 ±0,110
C+		0,284 ±0,031	0,305 ±0,031	0,303 ±0,019	0,301 ±0,026		0,350 ±0,030	0,357 ±0,023	0,366 ±0,030	0,347 ±0,022		0,266 ±0,101	0,299 ±0,112	0,220 ±0,097	0,170 ±0,110
M1		0,270 ±0,031	0,290 ±0,028	0,292 ±0,037	0,310 ±0,032		0,217 ±0,021	0,206 ±0,025	0,195 ±0,024	0,200 ±0,015		0,192 ±0,073	0,217 ±0,081	0,179 ±0,069	0,172 ±0,066
M2		0,259 ±0,014	0,310 ±0,034	0,297 ±0,027	0,252 ±0,20		0,209 ±0,024	0,192 ±0,023	0,205 ±0,024	0,186 ±0,060		0,204 ±0,078	0,211 ±0,80	0,191 ±0,076	0,169 ±0,064
	Firmicutes					***Lactobacillus***					***Lactobacillus donor E***				
Time	0 h	6 h	12 h	24 h	48 h	0 h	6 h	12 h	24 h	48 h	0 h	6 h	12 h	24 h	48 h
C-	0,317 ±0,110	0,332 ±0,120	0,369 ±0,138	0,349 ±0,132	0,352 ±0,131	0,062 ±0,015	0,040 ±0,012	0,046 ±0,015	0,041 ±0,006	0,054 ±0,017	0,142	0,137	0,137	0,120	0,131
C+		0,299 ±0,112	0,298 ±0,112	0,307 ±0,114	0,298 ±0,111		0,102 ±0,013	0,114 ±0,009	0,108 ±0,006	0,121 ±0,005		0,170	0,190	0,233	0,277
M1		0,299 ±0,113	0,291 ±0,110	0,275 ±0,102	0,315 ±0,119		0,061 ±0,007	0,051 ±0,013	0,037 ±0,004	0,050 ±0,018		0,137	0,116	0,101	0,100
M2		0,285 ±0,108	0,294 ±0,111	0,289 ±0,108	0,311 ±0,117		0,035 ±0,007	0,047 ±0,003	0,040 ±0,009	0,051 ±0,008		0,143	0,132	0,105	0,111

As shown in Table 3.7, *Bacteroidetes*, *Bifidobacterium*, *Clostridium leptum*, *Firmicutes* and *Lactobacillus* (* Except donor E) exhibited significant differences. The only exceptions are *Bacteroidetes* negative control 0 hours, 6 hours and *Bifidobacterium* negative control 24 hours. In these results, donor E was separated in *Lactobacillus* due to the much higher concentration so as not to interfere with the results as an outlier.

Positive control (enriched with FOS, fructooligosaccharides, are well-known prebiotics, which is often used to be positive controls in fermentation experiments *in vitro* because of prebiotic effect on the gut microbiota) had consistently higher 16s rRNA copies than negative control, M1 and M2 at 6,12, 24, and 48 hours for *Bacteroidetes* and *Bifidobacterium* (except 24 hours and 48 hours in *Bacteroidetes*). In the other cases negative control showed the higher value.

Throughout the different communities for M1 and M2 flours showed that the results are very homogenous which precludes any kind of conclusion regarding evolution over time. The research conducted by Liu *et al.* (2017) on the effect of various prebiotics on the feces of 36 participants reported that the prebiotics had a bifidogenic effect on the feces of all but three of the participants. In contrast, the responses of other bacterial groups were found to exhibit a significant amount of inter-individual variability when exposed to the prebiotics. This is in line with the results of the current study which found that the heterogeneity across donors did not allow any inferences from being taken regarding the

influence of *P. ostreatus* on the bacterial groups that were investigated, or even a comparison to FOS. Also, Saxami *et al.* (2023) have investigated the effects of *P. eryngii* and *P. ostreatus* mushrooms as potential novel prebiotics with possible beneficial effects on ASD (Autism spectrum disorder) children where, according to the results, edible mushrooms may be able to control *in vitro* the population of gut bacteria as well as metabolic activity, particularly in the ASD group. Fermentation of both types of mushrooms resulted in a considerable increase in *Bifidobacterium* levels, however *P. eryngii* mushrooms had no such effect.

Researchers from Rodrigues *et al.* (2016) used *in vitro* fecal fermentations for 24 hours to study the effect that an extract of the fungus *Pholiota nameko* had on the gut microbiota of three different donors. They discovered that the mushroom extract had an influence on the gut microbiota. In particular, there was a rise in the population of *Bifidobacterium* when compared to the control that was negative. These results are contradictory because it´s possible to observe that the concentration of samples (M1 and M2) has a tendency to go down. On the other hand, it was also reported that *Lactobacillus* population remained unchanged, which is consistent with the results above.

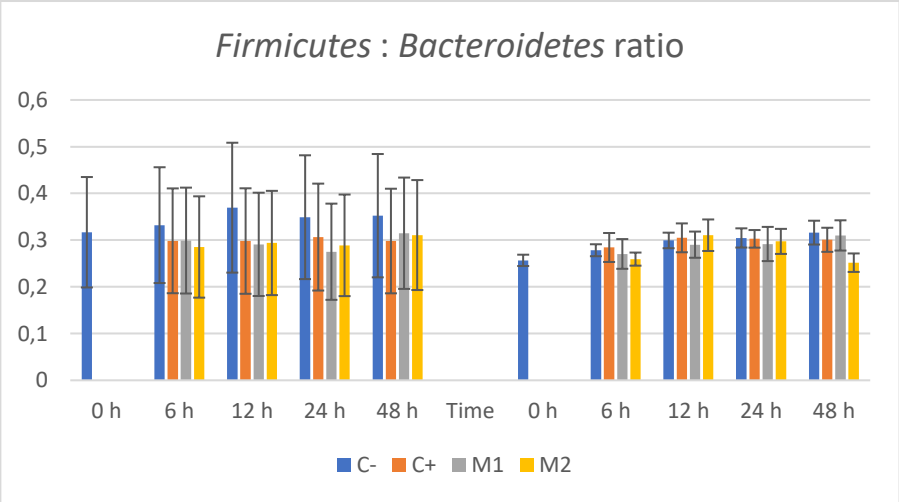


Figure 3.1 Firmicutes: Bacteroidetes ratio values evaluation during fermentation with human faeces. Samples fermented with pineapple peel flour, pineapple stem flour and frutooligosaccharides (FOS).

According to Simpson and Campbell (2015), the maintenance of the relative abundance of *Firmicutes* and *Bacteroidetes*, has greater significance than the stimulation of the amount of *Firmicutes* and *Bacteroidetes* produced. An increase or reduction in the ratio of *Firmicutes* to *Bacteroidetes* has been connected with obesity and weight loss, respectively. In general, healthy persons have a ratio of roughly 1:1 of *Firmicutes* to *Bacteroidetes* (Simpson and Campbell, 2015; Koliada *et al.* 2017; Cockburn and Koropatkin, 2016). By utilizing M1, M2 and FOS, the ratio was kept constant and near to one, thereby preventing significant variations (Figure 3.1).

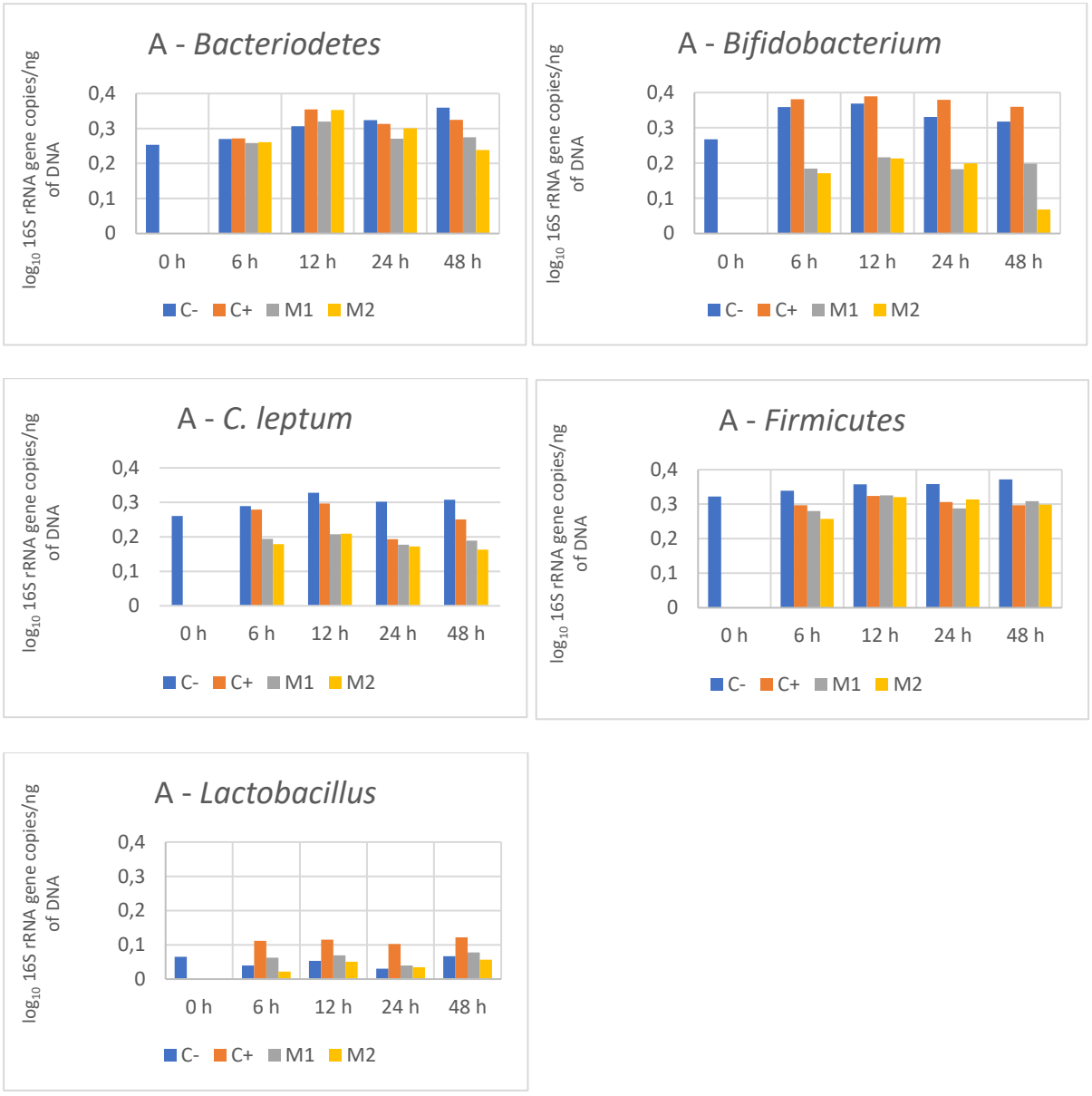


Figure 3.2 Variation of microbiota in donor A. Values presented as log10 16S rRNA gene copies/ ng of DNA through time.

In donor A is possible to see (Figure 3.2) that M1 and M2 exhibited the lowest values of 16s rRNA copies, with the overall values specially in case of *Bifidobacterium* and *C. leptum* being compared to the negative control. In the case *Bacteroidetes* is possible to observed that both samples M1 and M2 have a high concentration near the controls. The maximum peak is at 12 hours, where the samples present higher concentration than controls and since that they start to decrease. M2 in 12 hours and 24 hours demonstrate higher concentration than M1. Overall, there isn't a huge decrease from 12 to 24 hours and 48 hours.

For *Firmicutes* the same analysis is applied. The only difference is that in no fermentation point the concentration of samples was higher than the negative control. Between 24 and 48 hours the decrease

apparently is not detected. The results seem to be homogenous between positive control, M1 and M2 except 6 hours.

C. *leptum* and *Bifidobacterium* displays a similar profile that differs from *Bacteriodetes* and *Firmicutes*, the concentration in samples (M1 and M2) are much lower than the comparative controls. Also, the higher peak of concentration occurred at 12 hours that was a common trend in all bacterial communities. *Bifidobacterium* was the community that presented lower concentration in samples in correlation with controls.

Lactobacillus were the less expressed community in the donor A gut microbiota with very low values, where is possible to see at 12 hours and 48 hours the higher concentration.

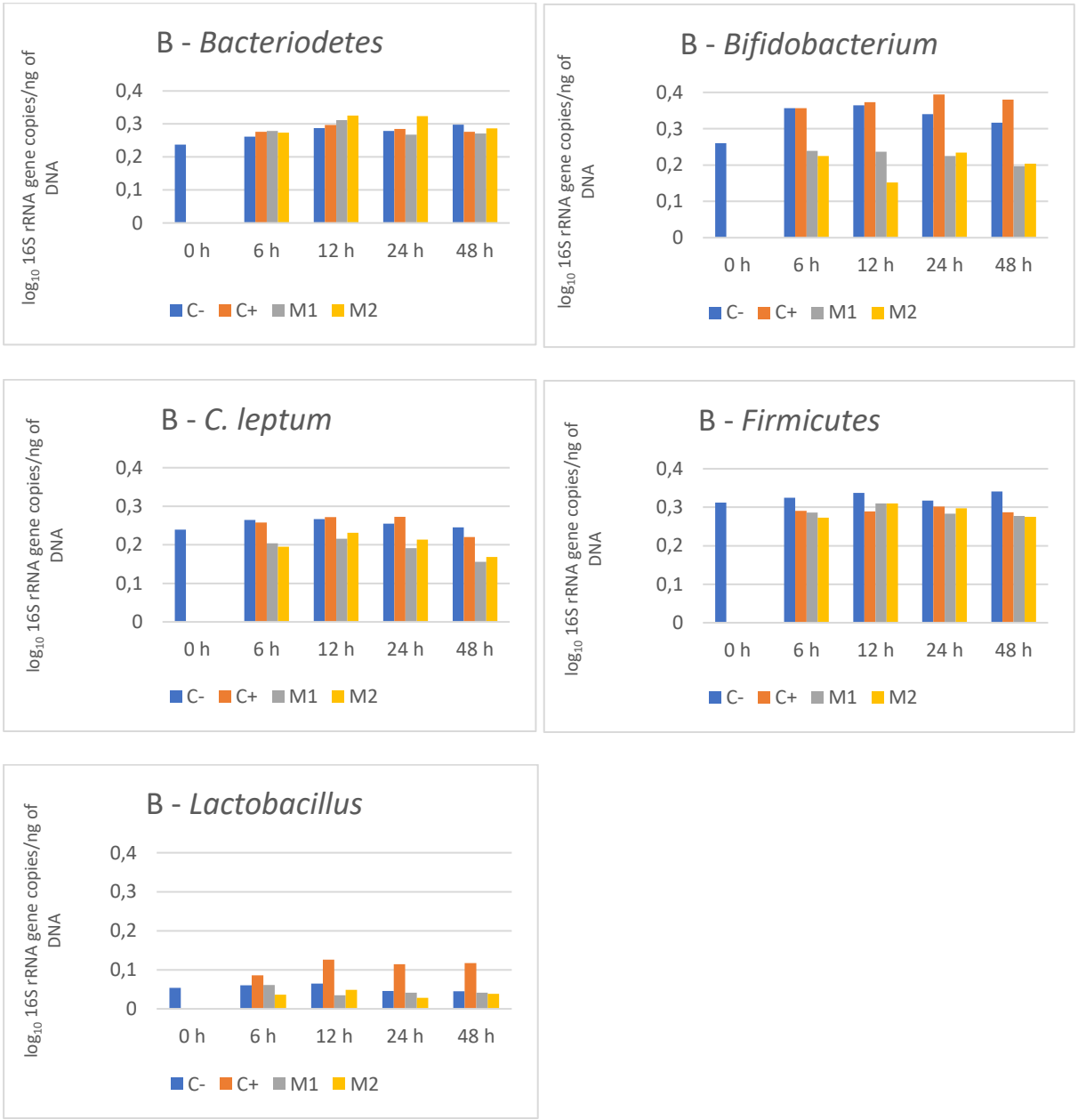


Figure 3.3 Variation of microbiota in donor B. Values presented as log10 16S rRNA gene copies/ ng of DNA through time.

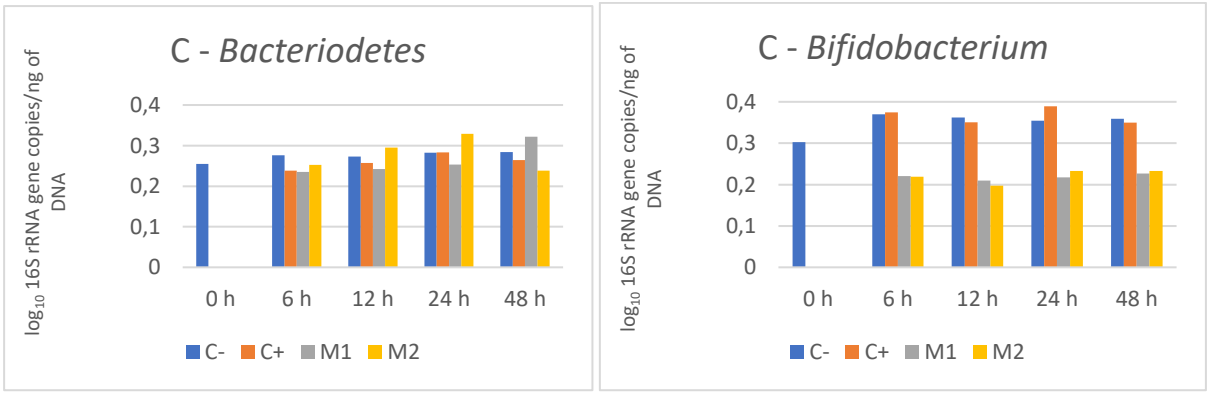
The graphics, in Figure 3.3, indicate similar trend throughout the bacterial communities of the donor B to donor A.

The samples M1 and M2 demonstrated the lowest values of 16s rRNA copies, with the overall values particularly in the case of *Bifidobacterium* and *C. leptum* being compared to the negative control, with the exception of *Bacteroidetes*. It is evident that both sample M1 and sample M2 have a high concentration at 6, 12, and 24 hours, respectively, in the genus *Bacteroidetes*. The largest peak occurred at 12 hours, where the samples present higher concentration than the controls, and since then, they have started to gradually decrease. The maximum peak occurred at 12 hours. M2 has a significantly higher concentration after 12, 24, and 48 hours compared to M1. The findings, in general, remained consistent over the course of the study, although there was a slight decline in performance beginning at 12 hours and continuing throughout 24 and 48 hours.

The same kind of analysis was used for *Firmicutes.* The first difference is that the concentration of the samples is never greater than that of the negative control, and the second difference is that the samples only overlap the positive control at 12 hours. In 12, 24, and 48 hours, the decrease begins to become apparent. The peak of concentration for both samples was at 12 hours.

The concentration of *C. leptum* and *Bifidobacterium* in the samples (M1 and M2) is significantly lower compared to the controls, in particular for *Bifidobacterium*. In terms of *Bacteriodetes* and *Firmicutes* the discrepancy to controls does not occur. In the case of *C. leptum* and *Bifidobacterium* M2, the highest peak of concentration occurred at 12 hours *Bifidobacterium* was the group that showed a significant lower concentration in samples when compared to controls.

In *Lactobacillus* it is possible to see that at 6 hours is where M1 presented higher concentration and M2 at 12 hours. This community is the least expressed in the gut microbiota of the donor B. They have low values in comparison with other communities.



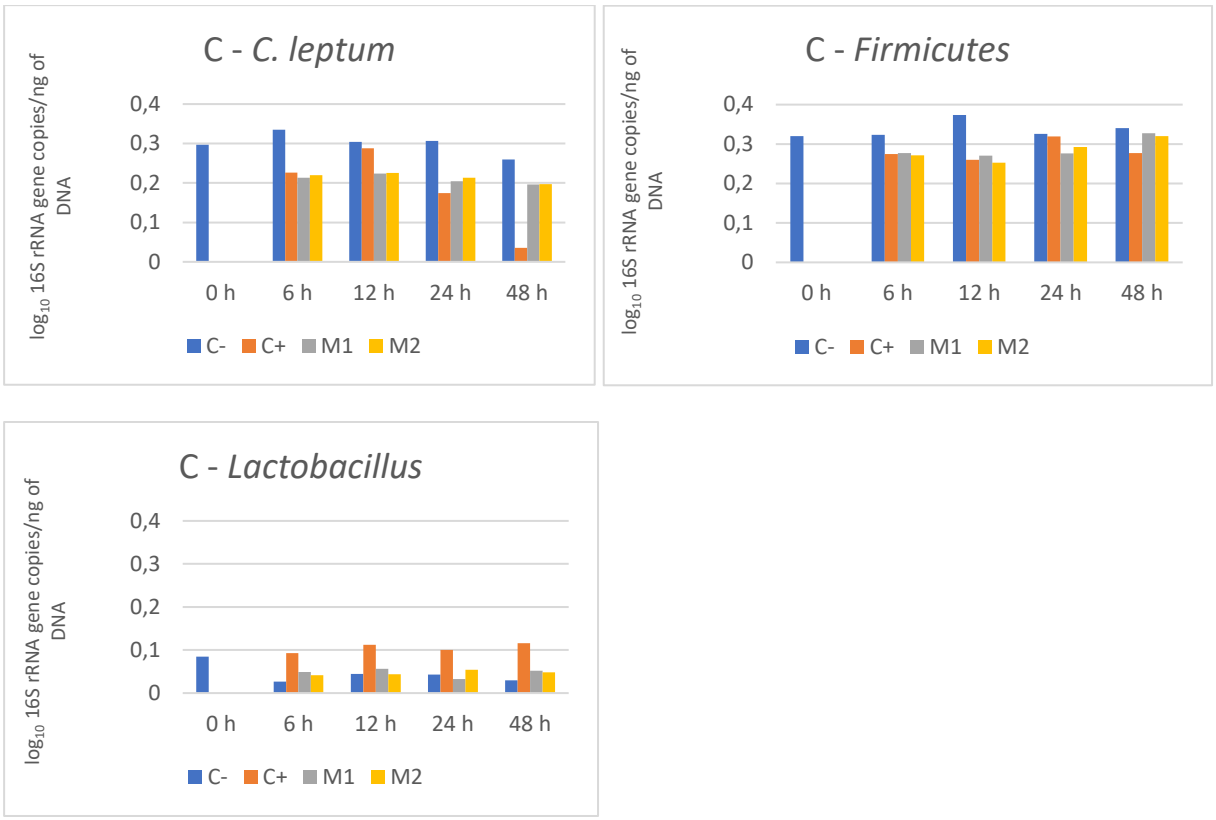


Figure 3.4 Variation of microbiota in donor C. Values presented as log10 16S rRNA gene copies/ ng of DNA through time.

According to the Figure 3.4, the outcomes for donors A and B were very similar.

M1 and M2 samples had the lowest values of 16s rRNA copies when compared to the negative control, especially in the case of *Bifidobacterium* and C. *leptum* (except for *Bacteroidetes*). In *Bacteroidetes*, M1 showed a high concentration at 48 hours, while M2 at 24 hours. In both instances, the concentration is markedly greater than that of the controls. The concentration of the M1 sample tends to increase steadily over time, whereas for the M2 sample the concentration start decreasing at 24 hours and continues until 48 hours.

For *Firmicutes*, an identical profile was observed. The primary difference is that samples have attained a higher concentration at 48 hours. There is a rise from 12 hours to 24 hours and 48 hours for donor A but it is not verified at the donor B.

Bifidobacterium differs from *Bacteriodetes* and *Firmicutes* in sample concentrations. M1 and M2 are significantly lower than the control. Also, the highest concentration peak for *Bifidobacterium* M1 occurred at 48 hours. M2 reached his optimum at 24 hours and kept up to 48 hours. *Bifidobacterium* was the group with the lowest sample concentration in comparison to controls. Despite this, the results between M1 and M2 were consistent throughout time.

In previous donors, *C. leptum* was comparable to *Bifidobacterium*, but in this instance, a different pattern was observed. In the beginning (6 hours and 12 hours), the concentrations of the positive and control

samples (M1 and M2) were higher than those of the samples (M1 and M2). At 24 hours, the negative control showed the highest number, while the positive control began to decline at 48 hours. The samples peaked at 12 hours and then began to decline.

Lactobacillus maintain the tendency to be the least expressed community in the gastrointestinal microbiota of donor C, with low values compared to the others, where is possible to see that M1 displayed higher concentration at 12 hours and M2 at 24 hours.

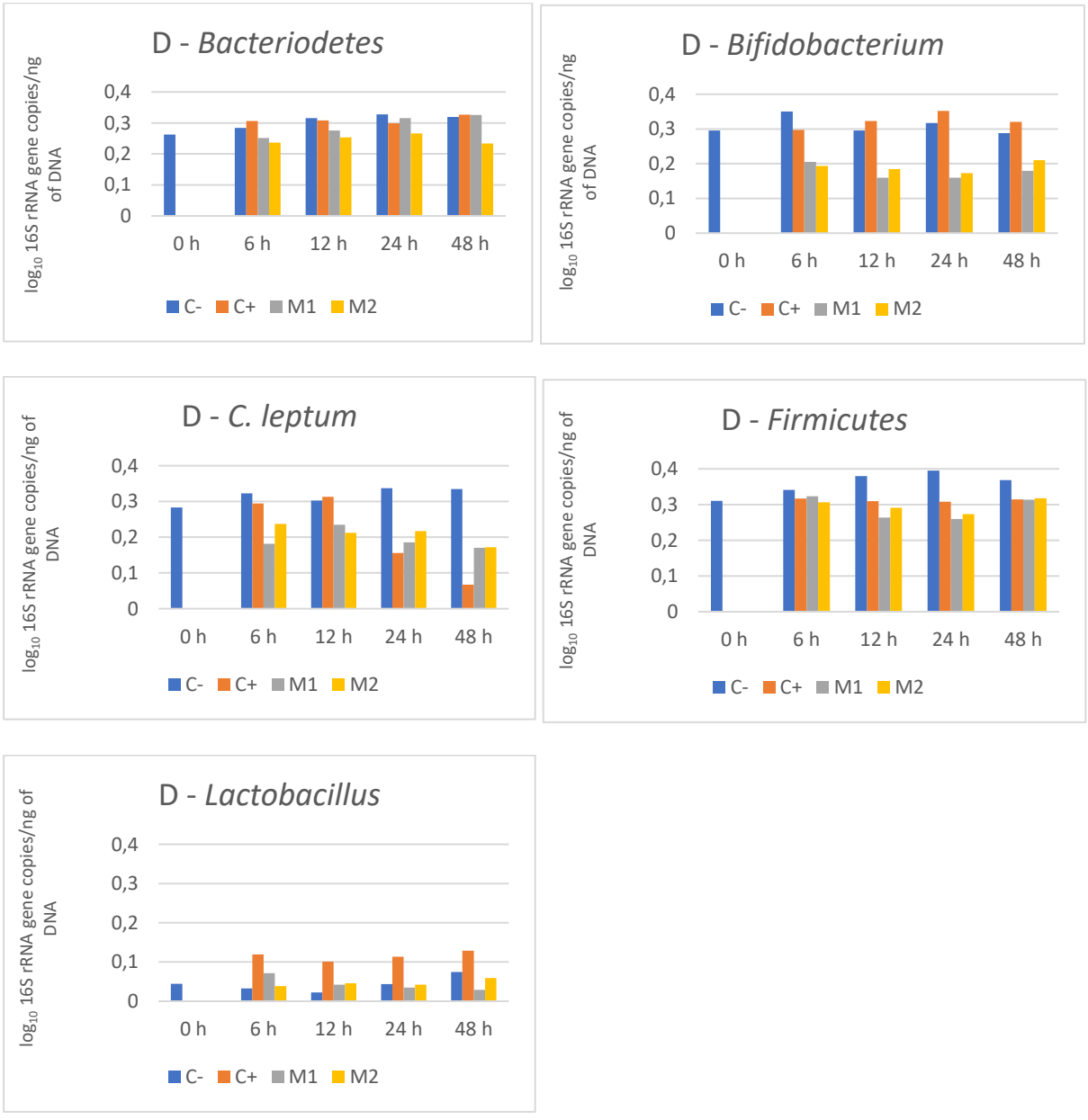


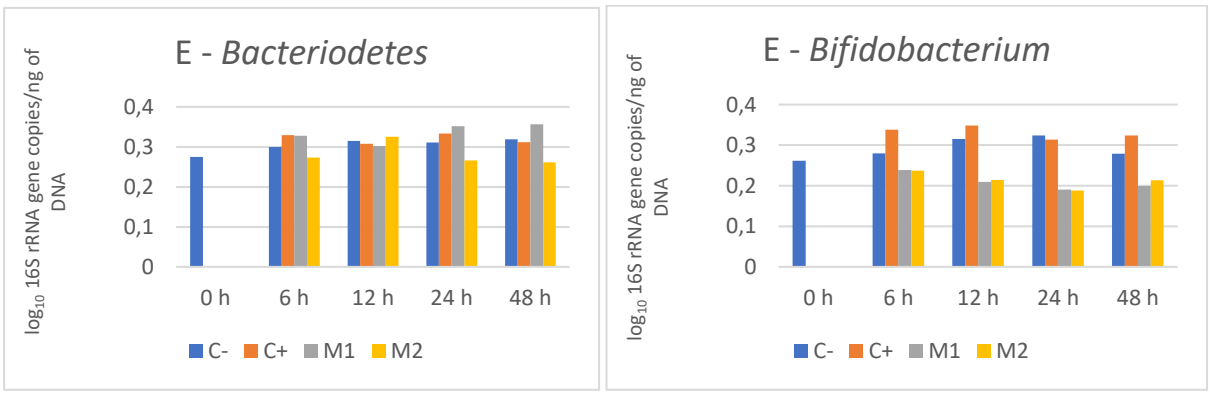
Figure 3.5 Variation of microbiota in donor D. Values presented as log10 16S rRNA gene copies/ ng of DNA through time.

According to the graphics in Figure 3.5, the results were very similar to donor A, B and C.

When the overall results are compared to the negative control, it is obvious to note that M1 and M2 displayed the lowest values of 16s rRNA copies. This is especially true in the case of *Bifidobacterium* and *C. leptum*. It is possible to demonstrate that M1 have a high concentration in *Bacteroidetes* at 48 hours, whereas M2 have a high concentration in *Bacteroidetes* at 24 hours. In the case of M1, the the level was even lower than in the negative control and was similar to what was found in the positive control. The concentration of M2, on the other hand, is significantly lower than that of the controls. The M1 sample exhibited a trend that steadily becomes greater as time passes, displaying this pattern. At the 24 hours M2 started reducing up to 48 hours.

Concerning *Firmicutes* follows the same pattern as the previous described. The most significant difference is that M1 reached its maximum concentration after only six hours, but M2 required forty-eight hours to reach its maximum concentration. The concentration of the samples was initially higher at 6 hours, and then it steadily declined until it reached 24 hours, following which it increased until it reaches 48 hours. Unlikely donors A and B exists an increase from 24 hours to 48 hours.

When compared to *Bacteriodetes* and *Firmicutes*, the level of the bacteria in the samples (M1 and M2) is significantly lower than the controls. This is one of the distinguishing characteristics that *Bifidobacterium* possesses, which sets it apart from the other two groups. In addition, the time at which the concentration of *Bifidobacterium* M1 reached its maximum peak was at 6 hours. M2 attain the higher concentration at 48 hours. After 12 hours and possibly even up to 48 hours it is possible to observe a higher level in M2 samples. Bifidobacterium was the community that had a reduced level in samples in conjunction with controls. In the preceding donors A and B, *C. leptum* was similar to *Bifidobacterium*, however, in this instance, the tendency is similar to donor C. At the beginning of the experiment, we were able to see that the level of the positive group as well as the control group is higher than that of the samples (M1 and M2). When 24 hours had passed, the negative control prevailed with the highest level. On the other hand, the positive control had started to decrease, which was further demonstrated at 48 hours. The highest did not occur at the same time in each of these samples, but it did not occur in the donor C sample until 24 hours later. M1 showed this at 12 hours, while M2 at 6 hours.



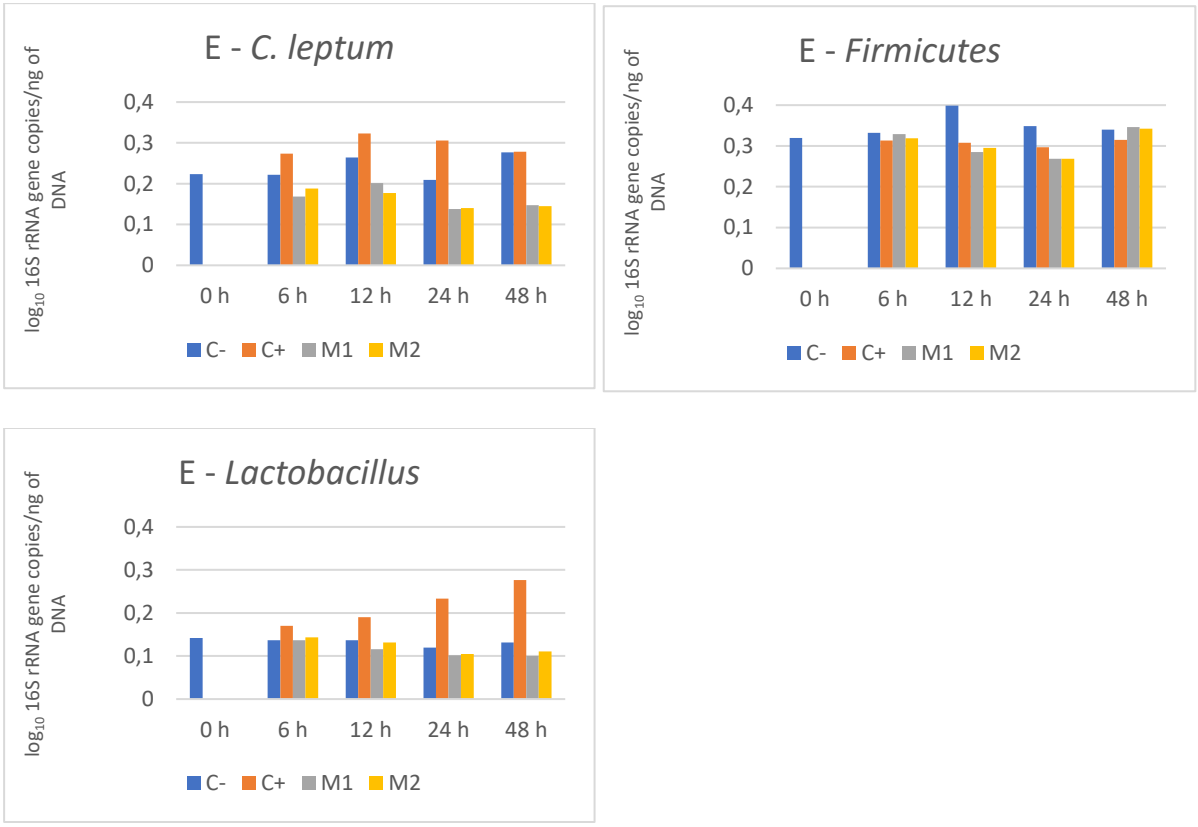


Figure 3.6 Variation of microbiota in donor E. Values presented as log10 16S rRNA gene copies/ ng of DNA through time.

Figure 3.6 shows the graphics of donor E and they are very comparable to donor A, B, C, and D's.

When the total values of *Bifidobacterium* and C. *leptum* are compared to the negative control, it is clear that M1 and M2 showed the lowest levels of 16s rRNA copies. In *Bacteroidetes* is possible to see that M1 had a high level at 48 hours but at 24 hours was very similar and M2 at 12 hours. In the case of M1 the level was higher than negative control at 6, 24 and 48 hours. Similar to positive control at 6 hours and above at 24 and 48 hours. On the other hand, M2 displays concentration inferior to controls, except at 12 hours.

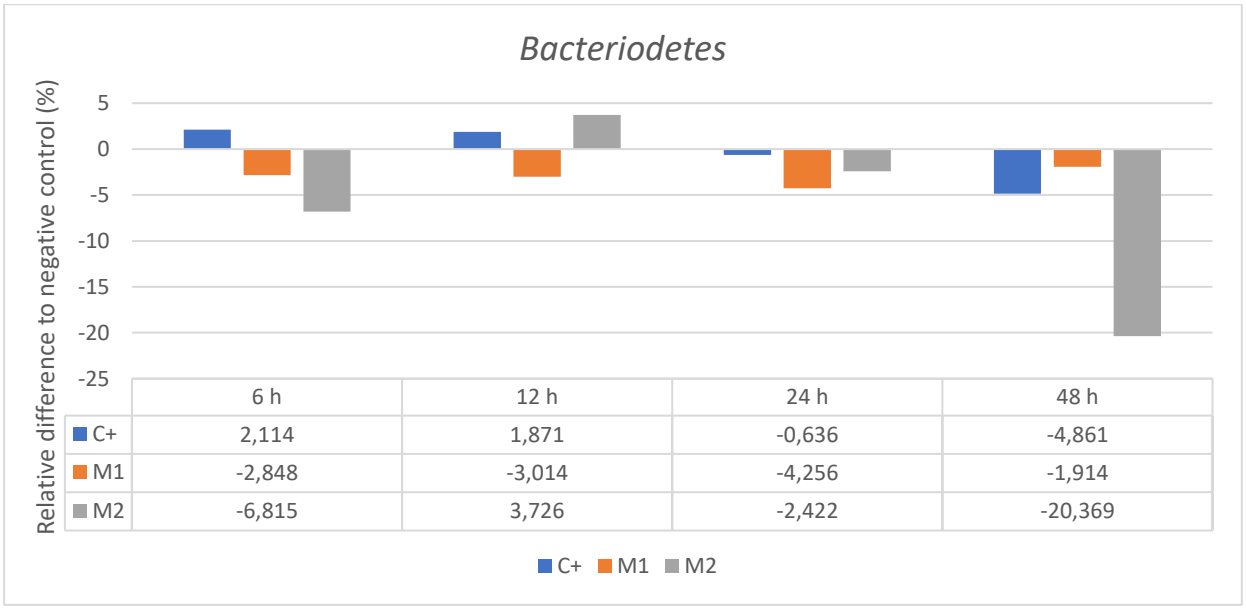
A similar pattern was observed for *Firmicutes*. The significant difference is that for both samples, the higher concentration was reached at 48 hours. The negative control and samples had a higher correlation.

It is easy to see how the samples' greater concentration starts at 6 hours and fades to 24 hours, after which there was an uptick until 48 hours. There is a rise in the number of unlikely donors A and B from 24 to 48 hours. The outcomes for M1 and M2 are rather uniform.

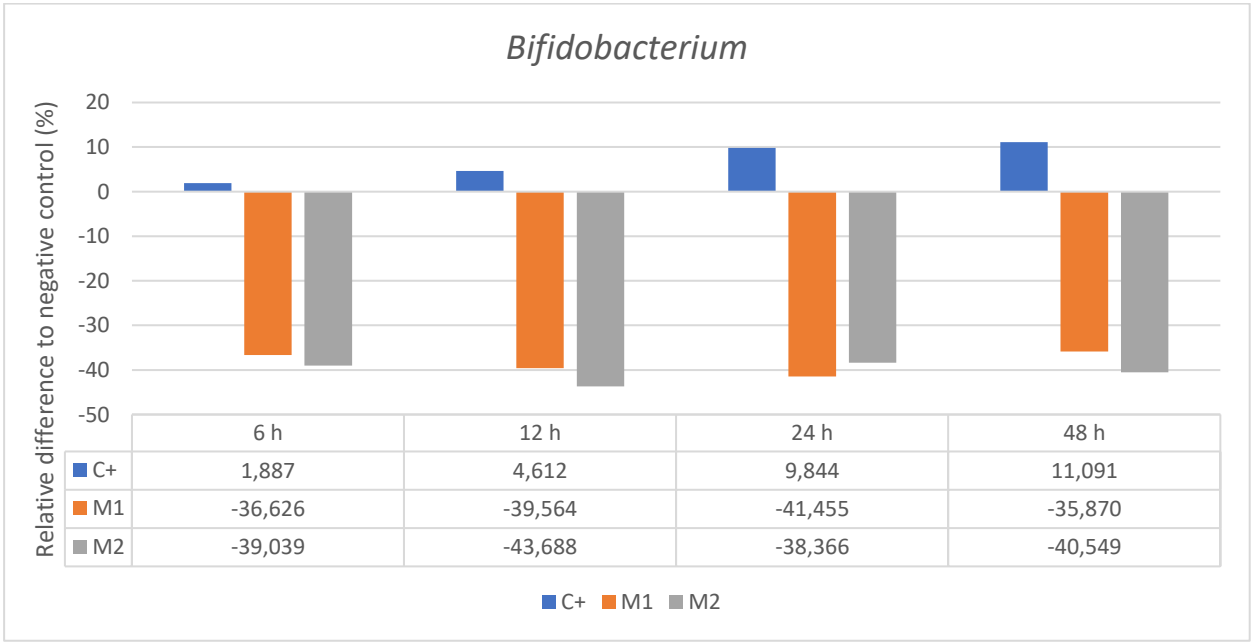
The level of *C. leptum* and *Bifidobacterium* in the samples (M1 and M2) is significantly lower than that of the controls when compared, which is different from *Bacteriodetes* and *Firmicutes*. Additionally, the higher level was reached for *C. leptum* M2 and *Bifidobacterium* at 6 hours, and for M1 was at 12 hours

in the case of *C. leptum*. The group with the lower abundance in samples compared to controls was *Bifidobacterium*.

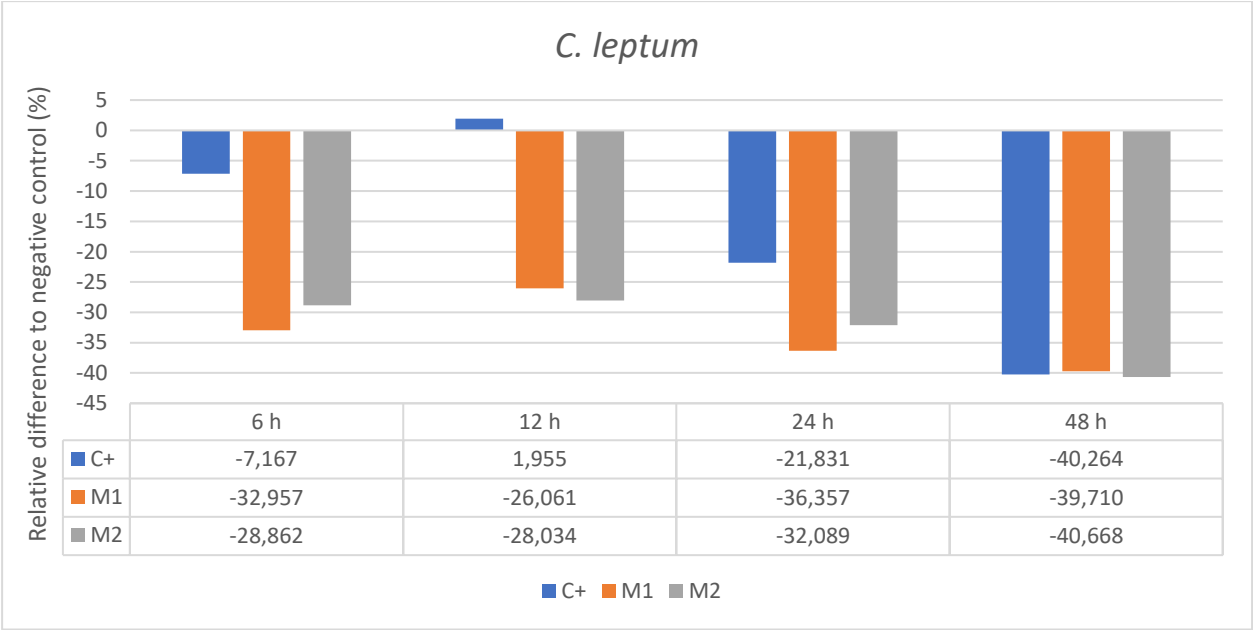
In the donor E gut microbiota, *Lactobacillus* continued to be the least abundant community, but in this particular donor, the values were almost three times greater, and it is clear that at 6 hours, a larger concentration was noted.



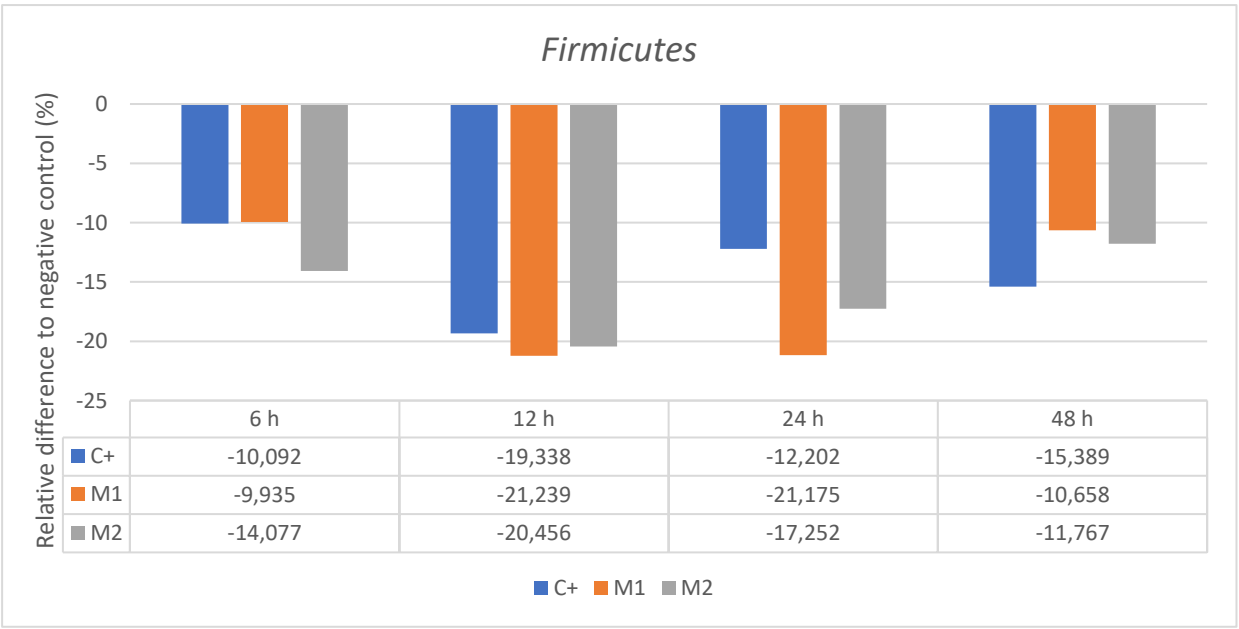
	6 h	12 h	24 h	48 h
C+	2,114	1,871	-0,636	-4,861
M1	-2,848	-3,014	-4,256	-1,914
M2	-6,815	3,726	-2,422	-20,369



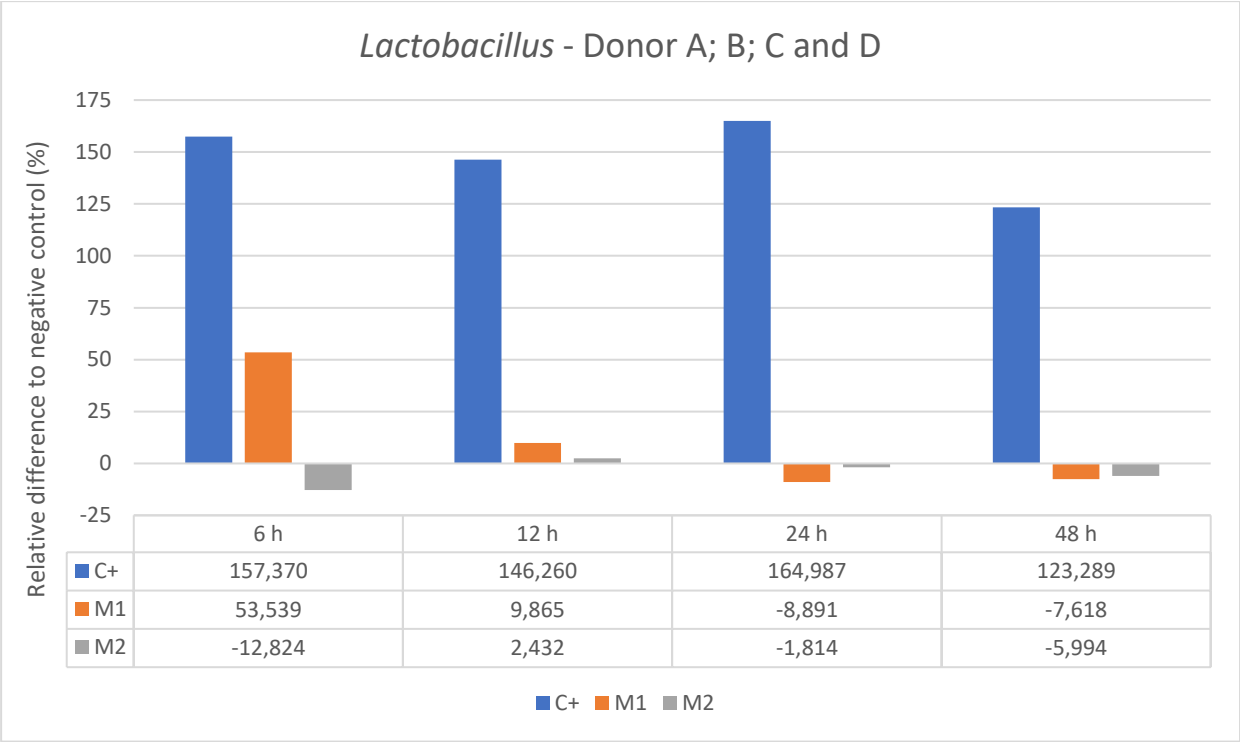
	6 h	12 h	24 h	48 h
C+	1,887	4,612	9,844	11,091
M1	-36,626	-39,564	-41,455	-35,870
M2	-39,039	-43,688	-38,366	-40,549



	6 h	12 h	24 h	48 h
C+	-7,167	1,955	-21,831	-40,264
M1	-32,957	-26,061	-36,357	-39,710
M2	-28,862	-28,034	-32,089	-40,668



	6 h	12 h	24 h	48 h
C+	-10,092	-19,338	-12,202	-15,389
M1	-9,935	-21,239	-21,175	-10,658
M2	-14,077	-20,456	-17,252	-11,767



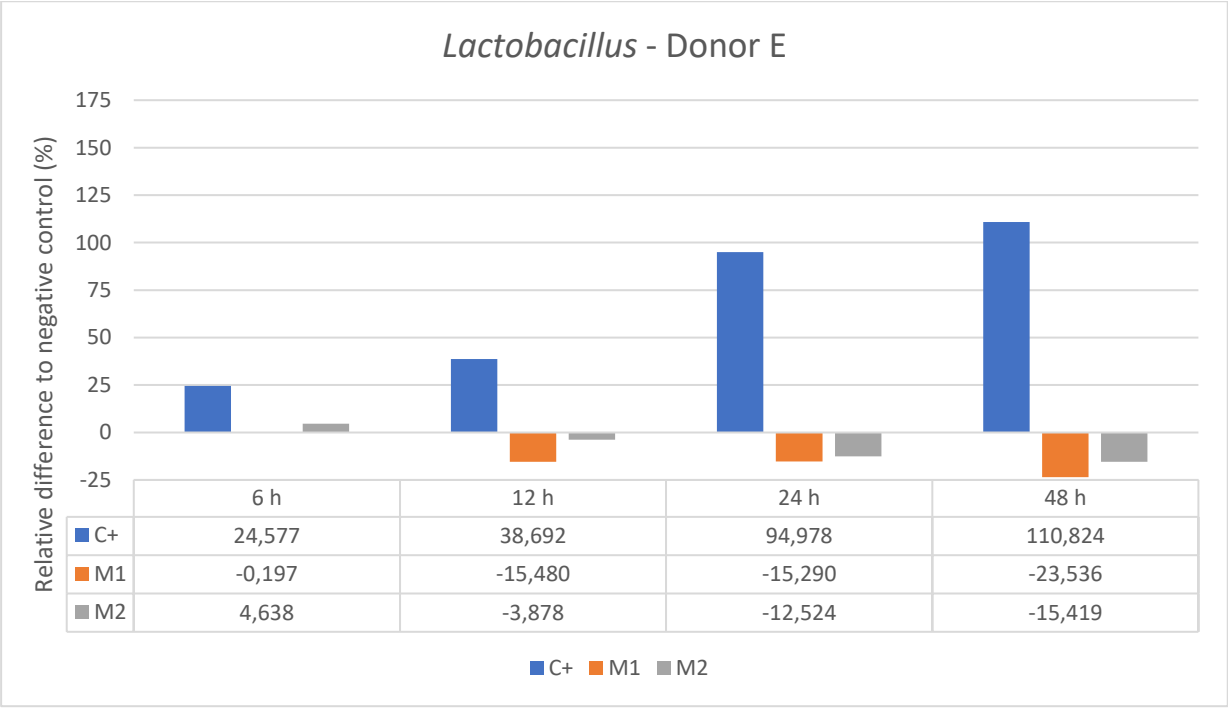


Figure 3.7 Relative differences to negative control of human faeces fermentation. Data presented in percentage during fermentation times of 6, 12, 24 and 48 hours. The results are the means of five different donors, except Lacatobacillus.

According to reported studies, the gut microbiota is essentially a two-phyla system, with *Firmicutes* constituting half to 80% of the gut microbial composition with over 250 genera known, followed by *Bacteroidetes* (Clemente *et al*., 2012, Rajilić-Stojanović and de Vos, 2014). The abundance of *Firmicutes* and *Bacteroidetes* was in this study in accordance with the findings of previous researches.

In this case *Firmicutes* levels of positive control and samples (M1 and M2) were much lower than negative control (Figure 3.7). Everard *et al.* (2011) and Parnell and Reimer (2011) reported the same effect with an *in vivo* rehearsal. On contrary, in another study involving obese women, Firmicutes levels increased after supplementation with inulin-type fructans, an effect they attributed to an increase in Clostridium clusters IV and XIV (Dewulf *et al.*, 2012). The results of this study aren't aligned with Dewulf *et al.* (2012) as mentioned above and also *C. leptum* had also lower level. In perspective, the huge range of genera makes very difficult to predict their behaviour and response.

Bacteroidetes community had no tendency throughout time. The only positive correlation with negative control was positive control at 6 hours and 12 hours, then it started to decay till 48 hours. In samples, M2 at 12 hours was the only point that has a positive percentage. Observing the two samples (M1 and M2), is noticeable that M1 is more linear with the results than M2 was.

In addition, according to Li *et al*. (2015), *Bacteroidetes* are capable of metabolizing a wide variety of substrates. Consequently, it would be anticipated that samples supplemented with FOS or *P. ostreatus* would consistently have more gene copies than the negative control.

Based on the results presented in Figure 3.7 it is not possible to draw the same conclusion, being in fact the opposite, negative control has the higher concentration.

A factor that may partially explain the results obtained is *Bacteroides'* sensitivity to acidic pH levels. Colonic pH values are known to range between 5.5 and 7.5, but *in vitro* fermentation models are associated with a more acidic environment (Holscher, 2017). This factor had eventually affected M2 at the 48 hours leading to a huge decline.

The levels of *Bifidobacterium* were significantly lower than *Bacteroidetes* and *Firmicutes*. According to Rodriguez *et al*. (2015) and Rajilić-Stojanović and De Vos (2014) *Actinobacteria*, which is primarily made up of *Bifidobacterium*, is reported as the third most prevalent phylum, accounting for up to 10% of all microorganisms (Rodriguez et al., 2015). *Bifidobacterium* is one of the conventional prebiotic targets, but other bacterial groups have also been shown to benefit from the presence of prebiotics (Cammarota *et al*., 2014). In terms of effect that can be observed in Figure 3.7 it is possible to see that there wasn´t a consistent bifidogenic effect. The positive control is the only that present a light bifidogenic effect. This is inconsistent with the research experiments conducted by Cruz *et al.* (2016), who reported a beneficial effect. Rodrigues *et al*. (2016) fermented a different mushroom species (*P. nameko*) and reported a stimulatory effect when compared to the negative control.

Kerezoudi *et al.* (2021) data suggested that high counts of *Bifidobacterium spp.*, after fermentation and demonstrated an advantageous prebiotic effect especially in the osteopenic group. Furthermore, fermentation significantly enhanced the growth of *F. prausnitzii* and was accompanied by a substantial increase in butyrate production. As stated by Roberfroid *et al*. (1998), a daily dose of a prebiotic does not necessarily result in a prebiotic effect. In fact, the extension of a bifidogenic effect is highly dependent on the overall microbiota composition prior to prebiotic ingestion, specifically the presence of *Bifidobacterium* in the original microbiota composition, which may explain why bifidogenic effect doesn't exist in M1 and M2. Parallel to this, the different response to FOS and *P. ostreatus* may be explained

by their nature because is reported that the length of the polysaccharides chain, its degree of polymerization, and the branching of the fibre affect the bacteria's ability to metabolize (Holscher, 2017; Scott *et al*., 2013). *Bifidobacterium* is one of the most important bacterial groups and one of the earliest colonizers of the human GI tract.

These results are partially in accordance with the literature because in this study *C. leptum* is really close where should be the fourth community present in the gut microbiota. *C. leptum* that were found to be lower than those of Firmicutes are in accordance with the findings of Lay *et al*. (2005) and Kabeerdoss *et al.* (2013), who stated that *C. leptum* (which also belongs to the phylum of *Firmicutes*) contributes 16-25% of the gut microbiota in humans. This particular cluster is classified and also known as *Clostridium* cluster IV. It is a member of the genus *Clostridium*, which contains a total of 19 distinct clusters. Therefore, the relatively high abundance of this subgroup could be anticipated, especially given that it is a significant and highly abundant population present in the gastrointestinal tract. Also have been strongly associated with the production of butyrate and may therefore play an important role in the host's health (Kabeerdoss *et al.*, 2013). Taking into account the outcomes observed in the presence of FOS and *P. ostreatus* samples (M1 and M2), it is plausible to conclude that substrate was not optimal for these bacteria, which have been reported to prefer resistant starch for their butyrogenic metabolism (Pryde *et al.*, 2002; Flint, 2012). In addition, it was reported that despite the bifidogenic effect of FOS, it had a negative impact on the glucose metabolism of butyrogenic bacteria, some of which belonged to the *C. leptum* subgroup. It has been hypothesized that only certain bacterial species within this subgroup are capable of metabolizing FOS (Liu *et al.*, 2017). On other hand, there are also studies such as Fu *et al.* (2019), where the synergy that exists between bacterial communities is direct correlated with the production of SCFAs.

In general, the levels of *Lactobacillus* varied significantly between donors. Despite that it is possible to see that the donors A, B, C and D presented an initial effect at 6 hours and 12 hours in both samples. In donor E the only time that have a tenuous effect is 6 hours for M2. Cruz *et al*. 2016, stated that although *L. paracasei* L26 had the ability to grow in vitro. With FOS supplementation, a recognized prebiotic present in positive control, it is possible to see the huge stimulation in every donor. These suggests that prebiotics, as selectively fermented constituents, may be utilized by some species but not others, suggesting that variations in species' composition, even within the same genus, may result in varying susceptibilities to prebiotic action.

Overall, when comparing the obtained results to the literature, exists some point that have to be made.

First one is that there are no specific studies according to this, that makes it difficult to compare results. There are some studies in the area with another species of mushroom. Yu *et al*. (2013a), where tested extracted *C. Versicolor* polysaccharide on the gut microbiota using a faecal model. Biomass consisted of mycelium and primordia (young fruit body), whereas extracts were concentrated forms of the fruit body's soluble constituents. This indicates that the biomass because of the nature and composition, is more resistant to the action of gastric fluid due to enzymatic protection.

Second, mushrooms biomass also possesses non-digestible carbohydrates (essentially dietary fibres). This resistant fraction, upon reaching the colon, is subjected to the action of the local microbiota, which may use them as part of their fermentative process, resulting in the production of metabolites that have been linked to a variety of potential health effects, both positive and negative (Ferro *et al.*, 2017).

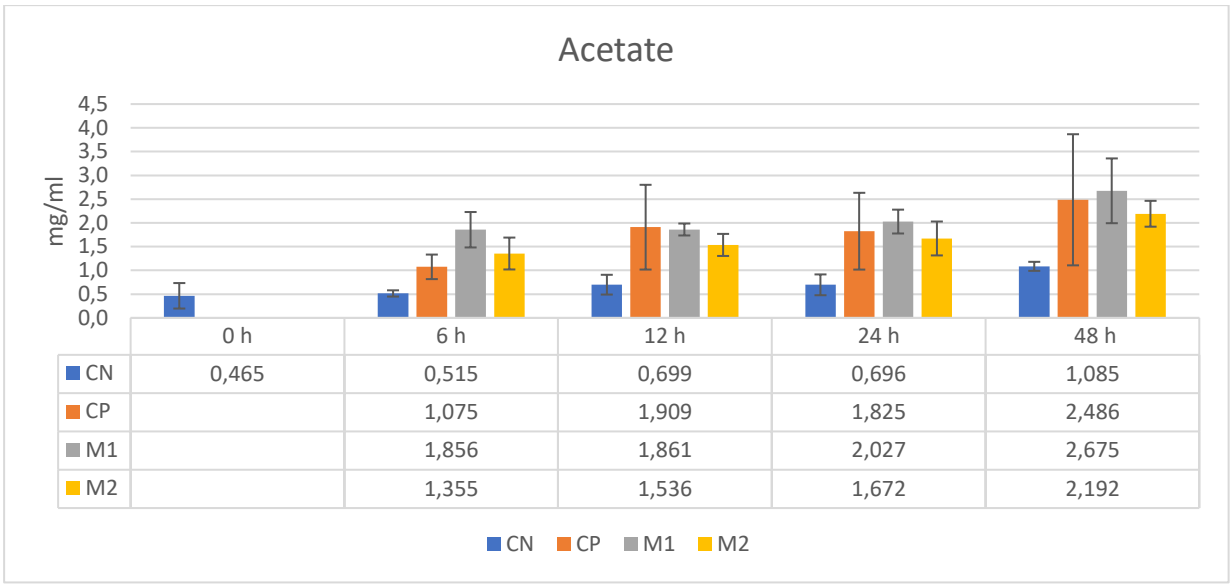
Third, long-term diet has a substantial effect on the composition of the gut microbiota (Riaz Rajoka *et al.*, 2017), and comparing with results of this study, a long-term administration/supplementation may be necessary to observe substantial changes and prebiotic effect.

Fourth, when considering the generality of the observed results, it is crucial to keep in mind the limitations of the methodology, from sample size to faeces collection and fermentation, and several factors such as age, diet, contamination, hormonal status, and lifestyle, which can result in more or less differences in the baseline composition of the intestinal microbiota. Dissecting the factors mentioned before, the sample size is decisive when extrapolating the results to the entire population, so only 5 donors can be short to mirror reality.

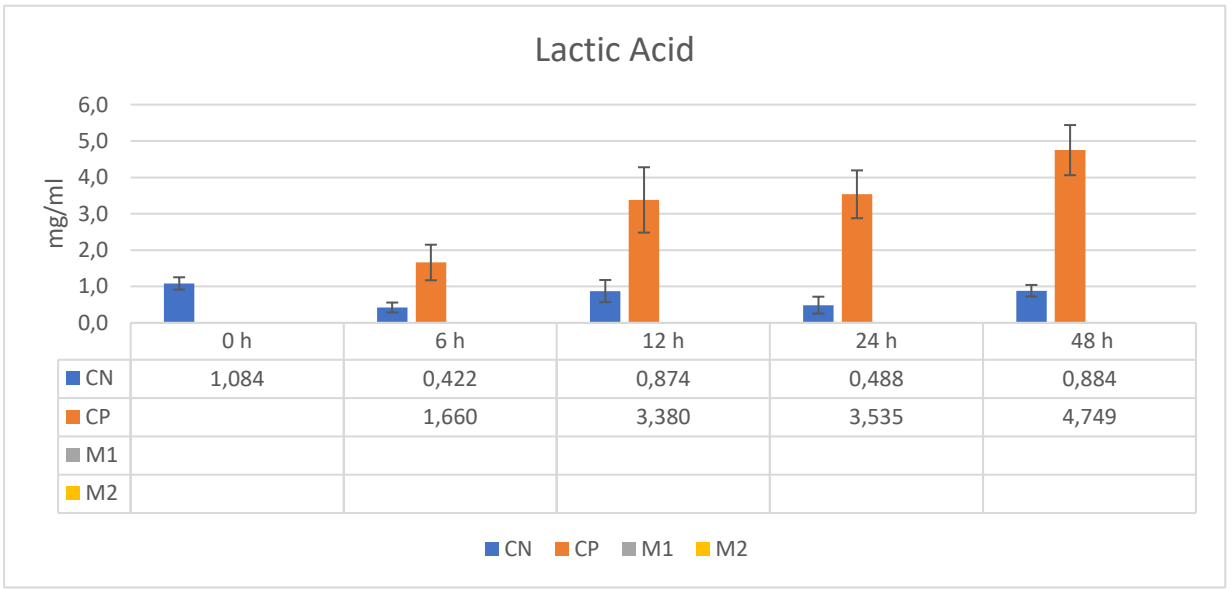
Fifth, the limitation of substrate available and the effect of pH reduction have throughout the course of fermentation (Payne *et al.*, 2012; McDonald, 2017; de Andrade *et al*., 2020).

3.7 SCFAs production analysis

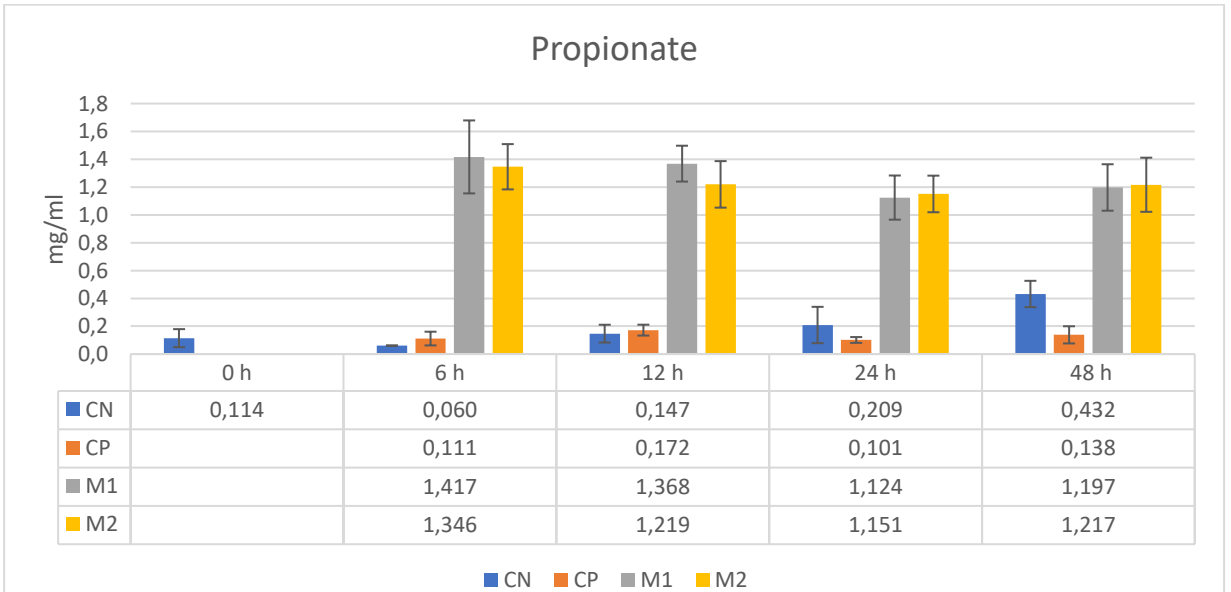
Succinic and lactic acid are not technically a short-chain fatty acid, but it's produced by gut bacteria and makes valuable contributions to the health of the colon. SCFAs, such as acetate, propionate, butyrate and succinic lactic acid are volatile fatty acids produced by the gut microbiota in the colon as fermentation products from dietary components that are unabsorbed/undigested in the small intestine (Ríos-Covián *et al.*, 2016). Seong *et al*. (2019) reported that these SCFAs can be produced during growth phases, be present in medium composition (presence and absence of amino acids) and be produced by metabolic cross-feeding. Butyrate and propionate originate solely from bacterial metabolism, whereas acetate may have an endogenous source (Tabernero and de Cedrón, 2017).



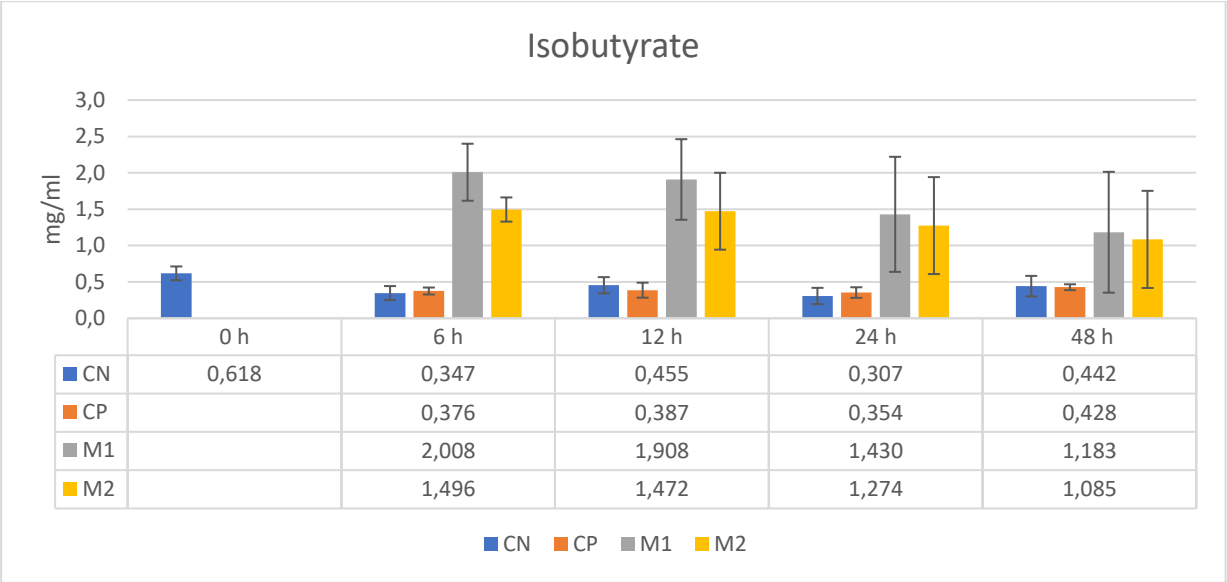
	0 h	6 h	12 h	24 h	48 h
CN	0,465	0,515	0,699	0,696	1,085
CP		1,075	1,909	1,825	2,486
M1		1,856	1,861	2,027	2,675
M2		1,355	1,536	1,672	2,192



	0 h	6 h	12 h	24 h	48 h
CN	1,084	0,422	0,874	0,488	0,884
CP		1,660	3,380	3,535	4,749
M1					
M2					



	0 h	6 h	12 h	24 h	48 h
CN	0,114	0,060	0,147	0,209	0,432
CP		0,111	0,172	0,101	0,138
M1		1,417	1,368	1,124	1,197
M2		1,346	1,219	1,151	1,217



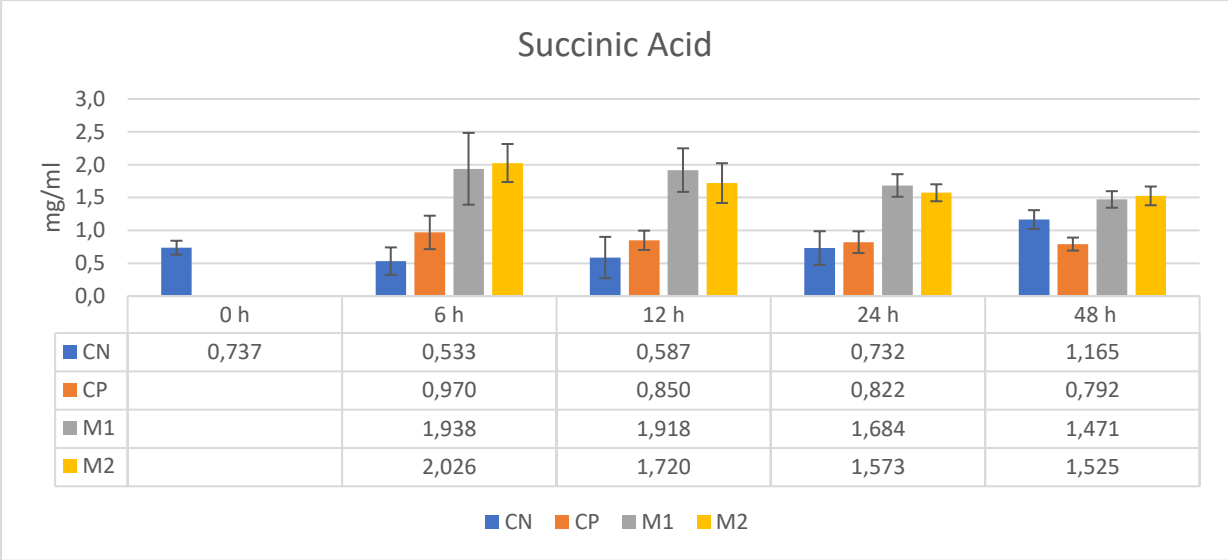


Figure 3.8 Concentrations of short-chain fatty acids (SCFAs) and succinic and lactic acid produced after 0 ,6 ,12 , 24 and 48 hours in vitro faecal fermentation of negative control, positive control and samples (M1 and M2). The results are the means of five different donors.

Figure 3.8 depicts the evolution SCFAs and succinic and lactic acid throughout time produced by feces microbiota at different stages of fermentation and for the controls and samples.

Lactic acid is a microbial metabolite. To put it another way, the digestion of carbohydrates results in the production of lactate as well as SCFAs by some of the bacteria that live in your gut. It is beneficial to the overall health of your digestive tract, and the bacteria that are responsible for its production offer some degree of resistance against the disease. Similar to acetate, certain bacterial species can utilize lactate to produce butyrate. Therefore, maintaining a high *Lactobacillus* population can increase lactate production. By feeding your butyrate-producing bacteria, you will indirectly aid in maintaining the integrity of your intestinal lining and even reduce inflammation (Bourriaud *et al.*, 2005).

During the entire fermentation, lactic acid was observed and noticed only in the controls (negative and positive – FOS), which is consistent with the increase of lactate-producing bacteria.

This increase in lactic acid production leads to a pH decrease, specially at 48 hours (Figure 3.9). The accumulation of lactic acid in positive control suggests that it was not utilized as a substrate during fermentation. Previous studies using analogous *in vitro* fermentation models support these findings (Bonifácio-Lopes *et al*., 2022; Gómez-Garcia *et al*., 2022; Ribeiro *et al*., 2021). The significant decrease in pH in the positive control after 24 hours can prevent the growth of lactate-utilizing bacteria or lactobacillus in this case. Despite that, itis possible to see (Figure 3.7) that *Lactobacillus* community exhibited a continuous increase throughout time. In donor E it was gradual in every point-time. In the rest donors at 24 hours reach the peak and start to regress towards 48 hours where the most important decrease in pH was observed (Figure 3.9). To corroborate this possible hypothesis, the inhibition of proliferation of these bacteria has been previously reported at pH levels below 5.2 (Belenguer et al., 2007). Also, in negative control is possible to see the existence of lactic acid in low concentration compared to the positive control. For the samples (M1 and M2) it was not possible to quantify lactic acid that can be justified by the fact that lactate can be used by certain bacterial species to produce butyrate (Seong *et al*., 2019), which in fact was increasing for these two samples.

Bifidobacterium and *Lactobacillus* are the predominant producers of acetate, but *Akkermansia muciniphila*, *Prevotella spp*., and *Ruminococcus* also produce it. *Clostridium* are also known for acetate production (Akhtar *et al*., 2022). When you consume fibre, it travels throughout your GI tract and reaches the gut, where these bacteria convert it into acetate. Members of the *Firmicutes* family can then use this SCFA to produce butyrate, which is an essential source of energy for gastrointestinal cells (Bourassa *et al*., 2016). Acetate is an essential pH regulator in the intestine. It helps maintain environmental stability. According to the results presented in Figure 3.8 it is possible to observe that for M1 concentration of acetate is higher than for M2. The result at 6 and 12 hours were very similar and when reached the 24 hours the values started to grow steadily. However, when results from Figure 3.7 are compared with Figure 3.8 according to what was described above, the opposite happened. There was production of acetate, with higher concentrations in the samples (M1 and M2), which should also be reflected in the increase of *Bifidobacterium*, *Lactobacillus* and *C. leptum* communities what did not happen. This may indicate that other minor species made acetate production. If we analyse the results in relation to the negative control, there was a reduced increase of these bacteria, but other groups could be involved or other metabolic conversion in the media.

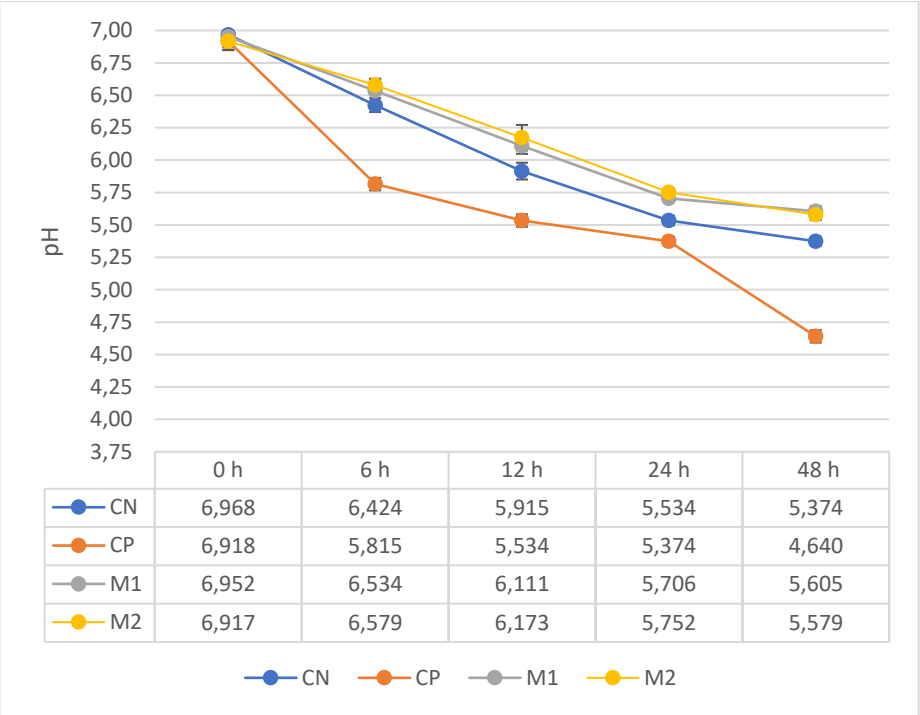
Propionate, like acetate mentioned before, is a by-product of the bacterial degradation of dietary fibre. It is produced when bacteria, including those from the *Bacteroidetes*, *Firmicutes*, and *Lachnospiraceae*, degrade carbohydrates. It offers numerous health benefits like cholesterol-lowering, fat-reduction, anti-cancer, and anti-inflammatory properties. It results from bacterial fermentation in the large intestine. As more people around the world are diagnosed with obesity, propionate's potential function as an appetite suppressant is gaining increasing attention. Propionate, stimulates the secretion of the hormone’s peptide YY and GLP-1, which indicate a satiety promoter (Byrne *et al*., 2015; Psichas *et al*., 2015). This SCFA was the one that showed more difference between the results of the controls and the samples.

Samples have approximately 22X to 23X more at 6 hours until it gradually declines to 3X at 48 hours. Despite the results of the concentrations if we analysed the results presented in Figure 3.7 and 3.8 it is possible to see that *Bacteroidetes* and *Firmicutes* communities did not grow compared to the negative control to obtain this kind of result in propionate production. These results for the samples means a very positive impact of these flours modulating gut metabolic activity.

Butyrate is the SCFA less produced, normally. Studies indicate that it an essential SCFA to promote mainly gut health. It is excellent for combating inflammation, which is a developing problem because it damages the cells and raises the risk of chronic diseases. In fact, increasing the intake of prebiotic dietary fibres is a simple way to increase butyrate production in your gut and may help prevent gut dysbiosis (imbalances in your microbiome), which has been linked to a variety of diseases, digestive issues, and even mental health. Other important feature is the improvement of the health of the intestinal membrane by regenerating it even under aggression (Hussey *et al*., 2017).

Family *Firmicutes* is well-known for producing this SCFA. Anaerobic bacteria such as *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and *Roseburia spp*. are the primary producers of butyrate. Comparing the results of Figure 3.7 and Figure 3.8 the relation between *Firmicutes* communities and butyrate is not in agreement because it is possible to see that the concentration of isobutyrate in samples (M1 and M2) is higher than the controls. When compared with the relative concentration with the negative control is possible to see that the *Firmicutes* community did not step up as it should.

For last, succinic acid has garnered substantial attention as a proinflammatory mediator and profibrotic marker in intestinal inflammation. Recent research suggests that lactic acid, an additional SCFA precursor, may be a signalling molecule that affects the immune regulation of the intestinal mucosa (Kaczmarczyk *et al*., 2021). The trend of the results is like butyrate, with the biggest difference being that the controls have slightly higher concentrations. The maximum concentration for positive control, and samples (M1 and M2) was observed at 6 hours, for negative control the peak was at 48 hours (Figure 3.7).



	0 h	6 h	12 h	24 h	48 h
CN	6,968	6,424	5,915	5,534	5,374
CP	6,918	5,815	5,534	5,374	4,640
M1	6,952	6,534	6,111	5,706	5,605
M2	6,917	6,579	6,173	5,752	5,579

Figure 3.9 pH values at different fermentation times (0, 12, 24 and 48 hours) for M1, M2, negative control (CN) and positive control (CP). The results are the means of five different donors.

4. Conclusion

The main aim of this research was to have a better understanding of the effect of *P. ostreatus* biomass (obtained as a residual biomass of *P. ostreatus* byproducts after aqueous extractions) on the human gastrointestinal microbiota. For this was essential to understand the composition of the residual flours, and after digestion, observe throughout time the specific and complex interactions with different bacterial communities to try to establish some prebiotic potential.

First, because samples M1 and M2 (obtained as residual biomasses according two different aqueous extraction approaches) were obtained from 2 different species of mushroom, a biochemical analysis of the composition was performed to evaluate primarily the nutritional composition and in particular β-glucans related to the prebiotic potential knowing in advance that the bacterial groups use it as a substrate to grow and generate metabolites such as the SCFA that were also analysed. This characterization allowed to observed that *P. ostreatus* presented a slight advantage over *P. eryngii* in case of neutral detergent fibre, which can be accurately predicting the nutritional content of dietary fibre. Among the dietary fibre sources, β-glucans have been shown to be one of the most effective fibre types to prevent type 2 diabetes and cardiovascular diseases. This is likely due to their ability to effectively lower the postprandial glucose response, as well as to improve long-term blood cholesterol levels, a known risk factor for cardiovascular diseases.

The same occurred with structural carbohydrates, where the xylose value is similar between the 2 species but in the main component (glucose) there is still a considerable difference. Comparing M1 and M2 samples it was also possible to obtain a trend, where M2 always obtained higher concentrations (fibre and structural carbohydrates), since it was subject to two aqueous extractions compared with one extraction in M1, and this allowed to extract more soluble components towards the aqueous extract, allowing to increase the insoluble carbohydrates in M2 residual biomasses.

In fecal fermentation, it was decided to proceed only with residual biomasses (M1 and M2) from *P. ostreatus* since it was the richer in potential prebiotic carbohydrates. The *in vitro* fecal fermentation model was performed using the feces of 5 donors, and evaluating microbial growth and metabolism of key groups in DNA extraction has been made to analyze quantify by real time PCR 5 groups of bacteria: *Firmicutes*, *Bacteroidetes*, *Lactobacillus*, *Bifidobacterium* and *Clostridium*. The results did not exhibit a prebiotic potential regarding the growth of the bacterial communities. The unique compositions and substrate preferences of each bacterial community may account for additional differences between FOS supplementation and our sample. It is important to note that FOS is an ingredient with a high concentration of FOS, whereas *P. ostreatus* biomass consists of a broad variety of compounds in which prebiotic substrates may be present in low concentrations.

Additionally, the production of SCFAs (acetate, butyrate and propionate) and lactic and succinic acids reveals a positive prebiotic potential. In all donors for both M1 and M2 (less lactic acid) the metabolite presented higher concentration than controls which is a very positive indicator. The lactic acid results

can be explained as a precursor and being utilized as substrate for the production of butyrate. As mentioned in 3.7 these metabolites bring many benefits in term of health and well-being.

Acetic and propionic acids, as products of the bacterial degradation of dietary fibre, displays a very positive impact in modulating gut metabolic activity, where propionate at 6 hours had 22X to 23X more concentration than negative control which rapidly decreases until 48 hours (3X). In acetate the correlation was 3X more than negative control at 6 hours. However, the concentrations were more constant over time.

Succinate results were also positive were at 6 hours the concentration was the highest (4X more than negative control). Throughout time occur the same effect that happens in acetate, the concentration decreases in a gradually and constant form.

Overall, the pH acidification has high interference with the lactic acid production. The accumulation of lactic acid in the positive control indicates that the substrate was not utilized during fermentation. According literature to the inhibition of proliferation of these bacteria has been previously reported at pH levels below 5.2. Despite pH at beginning was over 5.2 and over time the acidification became more accentuated which may have affected the production of lactic acid.

In conclusion, despite the results of bacterial communities are not directly correlated with the metabolites produced, is possible to suggest that the range of bioactive properties associated with *P. ostreatus* biomasses may be expanded due to the potential prebiotic activity exhibited on the human gut microbiota. Besides, it important to highlight that the valorisation of mushroom byproducts in a circular economy approach (zero waste) allowed to produce aqueous extracts, and the residual biomasses could be valorised as functional prebiotic ingredient.

5. Future Work

The present study investigated the potential prebiotic effect of *P. ostreatus* biomasses obtained by valorisation of its byproducts using an *in vitro* faecal fermentation model, where 5 different donors were used.

In this procedure would be important to establish a pH-control system as faeces are alkaline and the observed acidification of the media may introduce a significant positive impact in the microbial community development throughout time.

It would also be interesting to expand the bacterial communities because in the results it is not possible to directly interconnect the SCFAs production with the productive bacteria. Therefore, expanding the spectrum would be interesting to observe more specifically which sample has greater production and consequently impact.

Expand the donors pool, estipulate a consensus hour to collect so that the faeces are all in the same condition or *in vivo* models with mice should be an interesting prospective work.

Although it would be difficult and complex to find donors, but to select volunteers with diet based on mushrooms and make a comparison with common donors would be interesting to demonstrate the prebiotic impact. As mentioned in bibliography, exposure to a certain food for a continuous period of time leads to shaping the composition of microbiota community levels and consequently the level of metabolites.

Finally, it would be interesting to carry out interim analyses at each stage of digestion, especially protein and total phenolics and antioxidants to see the degree of absorption and the availability, and better correlate the final digested composition with prebiotic effect.

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7. Annexes

Annex 1. Informed Consent Form

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

Título do projeto de investigação: Estudo do impacto da biomassa de cogumelos edíveis e seus subprodutos na microbiota intestinal humana	
Supervisor: Prof. Dra. Manuela Pintado	
Nome do investigador: Arlindo André e André Cima	
(A completar pelo participante) 1. Leu a ficha informativa deste estudo?	SIM / NÃO
2. Se colocou questões, recebeu respostas adequadas?	SIM / NÃO / Não aplicável
3. Compreende que é livre de abandonar este estudo, sem necessidade de dar uma justificação?	SIM / NÃO
4. Aceita participar neste estudo?	SIM / NÃO
Assinatura (participante):	Data
Nome do participante (letras maiúsculas):	
Assinatura do investigador:	Data

Annex 2. 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 prébiótico de um extracto 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 extracto digerido. Por fim, será avaliado o crescimento de determinados grupos bacterianos, bem como alterações na composição da microbiota.

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 Bífidos) ou antibióticos nos últimos 6 meses.

Instruções para colheita

1. 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:
2. Calçar as luvas;
3. Levantar a 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.
4. Baixar o aro da sanita, e colocar o saco de plástico cortado em cima da película aderente.

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

Annex 3. Material list and protocol for in vitro faecal fermentation.

This document is written in Portuguese.

Material, check-list e protoclo de fermentações

KIT

- Consentimentos e instruções
- Película aderente, sacos de stomacher, tesoura, caixa, luvas, saqueta de anaerobiose, elástico.

Material

- Máscaras
- Caixa de luvas nitrilo
- Sacos para descartar
- Balança com proteção
- Espátulas para fezes (6 espátulas; 1a por dador+ 1 em excesso)
- Micro Espátula para pesar amostra
- Sacos de stomacher
- Rolo papel
- Álcool
- Fita de carpinteiro
- Seringas e agulhas
- Eppendorfs 4mL autoclavados
- RPS (100 mL)
- Tesoura
- Filtros estéreis
- Tubos de 50 mL estéreis (para colocar os inóculos fecais)
- Tubos de 15 mL estéreis (para retirar o ponto t_{0h} de 10 mL)
- Caneta de acetato

Procedimento Check-list

➢ **Preparar RPS (Cystein-HCl 0,5g/L; NaCl 8,5 g/L**; pH 6.8) e autoclavar (N.B. Não ajustar mesmo para 6.8 porque após autoclavar, acidifica consideravelmente)

➢ **Preparar o meio de fermentação**

Para 1L em água desionizada:

- 5.0 g/L **trypticase soy broth (TSB) without dextrose**
- 5.0 g/L **bactopeptone**
- 5.0 g/L **yeast nitrogen base** (pesado directamente para o shcott)
- 0.5 g/L **cysteine hydrochloride** (pesado directamente para o shcott)
- 1.0% (v/v) of **salt solution A:**
  - 100.0 g/L NH4Cl
  - 10.0 g/L MgCl2.6H2O
  - 10.0 g/L CaCl2.2H2O
- 0.2% (v/v) of **salt solution B:**
  - 200.0 g/L K2HPO4 ·3H2O

Soluções salinas A e B e resazurina já preparadas previamente

- 0.2% (v/v) of 0.5 g/L resazurin
- **Trace minerals solution** (10ml/L) **(encontra-se pronto a usar no frigorifico)**
    - Trisodium nitrilotriacetic acid, 1.5 g/L; ou EDTA (disodium salt) 500 mg/L (armário X)
    - Iron sulphate heptahydrate $FeSO_4.7H_2O$, 200 mg/L (armário C ou B94)
    - Cobalt(II) chloride hexahydrate $CoCl_2.6H_2O$, 0.1 g/L; (B24)
    - Manganese(II) Chloride Tetrahydrate $MnCl_2$-$4H_2O$, 3 mg/L (B32)
    - Sodium molybdate dihydrate $Na_2MoO_4.2H_2O$, 0.1 g/L; (B65)
    - Zinc Sulfate Heptahydrate $ZnSO_4.7H_2O$, 0.1 g/L; (B111)
    - Nickel(II) chloride $NiCl_2.6H_2O$, 0.1 g/L;
    - boric acid H_3BO_3, 0.01 g/L; (C)
    - Copper(II) chloride dihydrate 1 mg/L (B26)

The final pH of the medium was adjusted at 6.8 (if lower use NaOH 0.1M if higher use HCL 0.1 M)

- **Borbulhar** o meio com N_2

A experiencia inclui três condições: **amostra** a testar, **controlo positivo** (C+; FOS) e **controlo negativo** (C-; apenas inoculo fecal). São retirados pontos às **0h, 12h, 24h e 48h**. O ponto 0h é retirado do frasco do C-. Como tal é preparado um maior volume de reacção para o C- (60 mL), por oposição ao C+ e amostras (50 mL)

- Colocar **49 mL de meio** nos frascos de fermentação da **amostra e C+**; Colocar **58,8 mL** de meio nos **C-**

C+ e amostras: 49 mL de meio de fermentação
C- : 58, 8 mL de meio de fermentação

- **Preparar as amostras e controlo positivo** (FOS)- a **2% (w/v)**

Pesar 1 g de amostra/ FOS para o interior do frasco

- Cravar
- Autoclavar

Na sala suja

- Ligar os canais da que libertam a mistura gasosa a filtros estereis; Fazer dois furos com agulhas estéreis no septo: um ligado ao filtro, por onde entra a mistura gasosa; outro

Na câmara de anaerobiose

- Preparar o **Inóculo fecal (100g/L, em RPS, com pH 6.8) : pesar 1 g de fezes para 10 mL de RPS ;** Colocar num saco de stomacher e homogeneizar
- **Inocular os frascos a 2% (v/v):** com agulha e seringa, retirar 1 mL e injectar nos frascos de fermentação

➢

C+ e Amostras= 1ml Inóculo fecal + 1g de amostra/FOS + 49 ml meio

C- = 1,2 ml de inóculo fecal + 58,8 mL de meio

Retirar o ponto 0h do **C- (retirar 10 mL para um tubo de 15 mL);**

NOTAS: Os frascos devem ter duas seringas ligadas: uma para retirar os pontos, outra para contagem dos volumes de gases libertados. Não retirar os pontos através de uma agulha/seringa já utilizada. Deixar os frascos na parte de baixo da câmara.

Esquema dos frascos para 1 dador

-58,8 mL meio;

-1,2 mL de Inóculo fecal

V_i=60 mL

t_{0h}= 10 mL

t_{12h}= 4 mL

t_{24h}= 4 mL

t_{48h}= 4 mL (fim) vf= 38 mL

-49 mL meio;

-1 g de FOS

-1 mL de inóculo fecal;

V_i=50 mL

t_{12h}= 4 mL

t_{24h}= 4mL

t_{48h}= 4 mL(fim);vf= 38 mL

-49 mL meio;

-1 g de FOS

-1 mL de inóculo fecal;

V_i=50 mL

t_{12h}= 4 mL

t_{24h}= 4 mL

t_{48h}= 4 mL(fim);vf= 38 mL

Legenda: C- Controlo negativo; **C+** Controlo positivo (FOS); **A** Amostra a analisar

Annex 4. DNA extraction protocol (NZY tissue gDNA isolation kit).

nzytech
genes & enzymes

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.

Annex 4. PCR quantification protocol.

This protocol is presented in Portuguese.

PCR

Material

- Placas
- Películas selantes
- Master Mix (fotosensível)
- Água ultrapura (autoclavada 2x; aliquotada e congelada)
- Primers (previamente preparados para uma concentração de 100 nM no poço)
- Pontas (autoclavadas 2x; principalmente de 10 µL)
- Pipetas próprias para biologia molecular
- Eppendorfs para PCR (autoclavados 2x)
- gDNA (quantidade equilibrada)

Procedimento

O volume por poço é de 10 µL. **As proporções por poço** são:

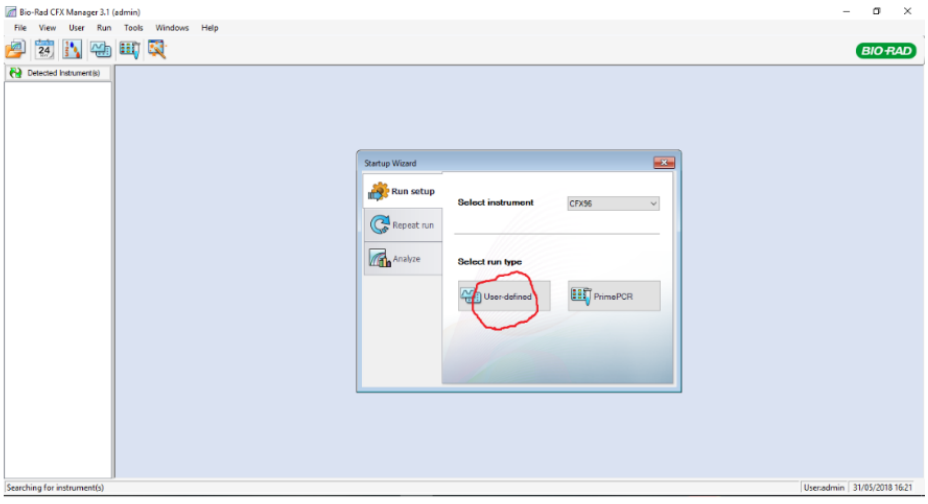
5 µL Master mix
1 µL cada primer
1 µL gDNA
2 µL Água ultra pura

Preparar a quantidade equivalente a mais uma réplica do que o pretendido (ex. para fazer 4 réplicas, preparar mistura para 5 réplicas)

Carregar a placa, e colocar a película selante.

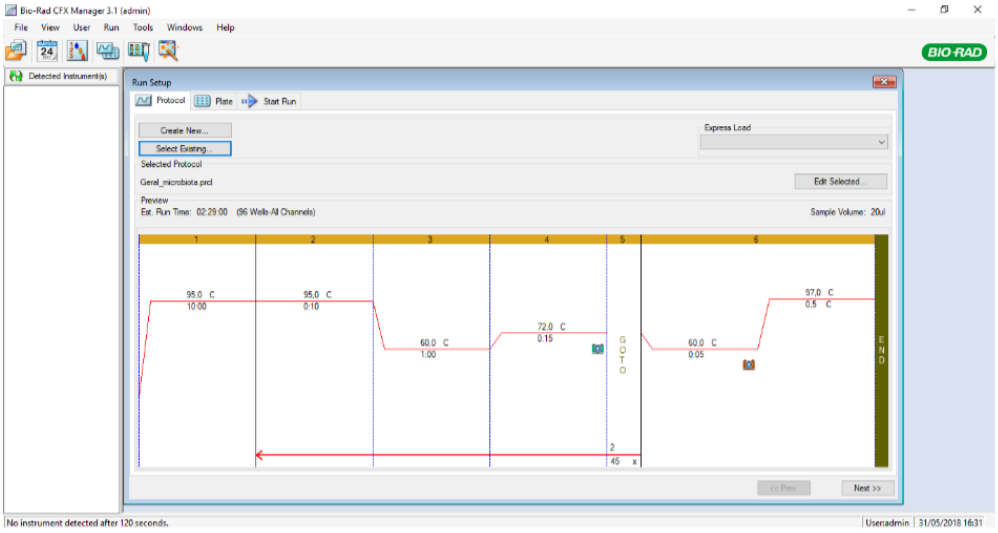
PROGRAMA:

- Selecionar **"user-defined"**



1ºEtapa: Protocol

- Selecionar um protocolo pre-existente gravado (**"Select existing"; no caso da microbiota pasta MP, "Geral microbiota"**) ou criar um novo (**Create new**)



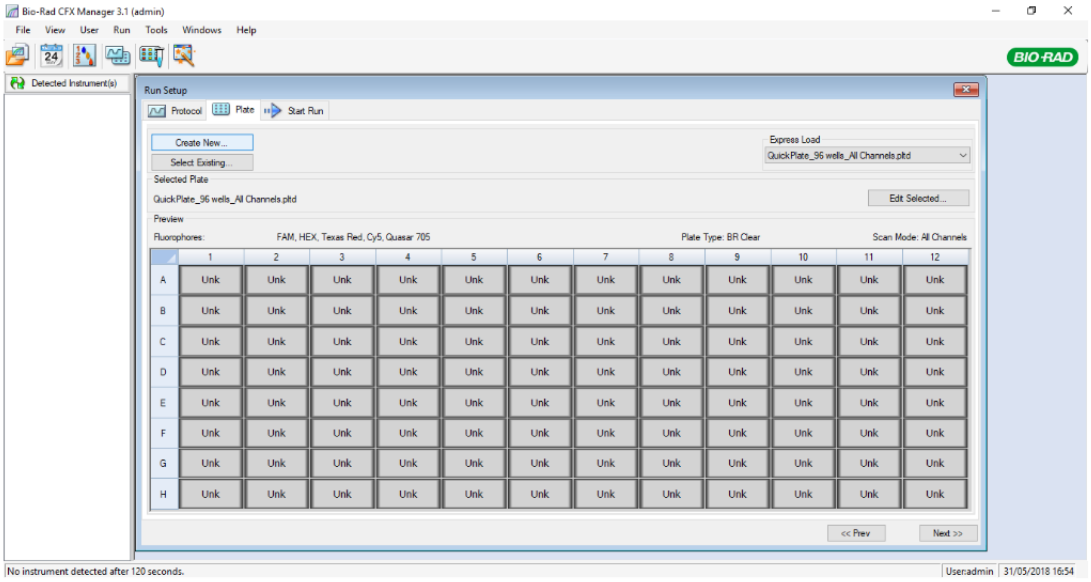
Se selecionar um protocolo pré exisente, depois selecionar a opção "**Edit selected**" para editar o necessário.

Condições utilizadas (Nota: definir o volume de reação/poço)

PCR stage	**Temperature/time**	
Initial denaturation/enzyme activation	95ºC for 10 min	
Denaturation	95ºC for 0.10 min	45 cycles
Annealing	XXºC, for 1 min	
Extension *	72ºC, for 0.15 min	
Melt curve*	60-97ºC, with na increment 0.5 ºC for 0.05 min	

2ª Etapa: Layout

- Selecionar um layout pre-existente gravado (“**Select existing**”) ou criar um novo (“**Create new**”)



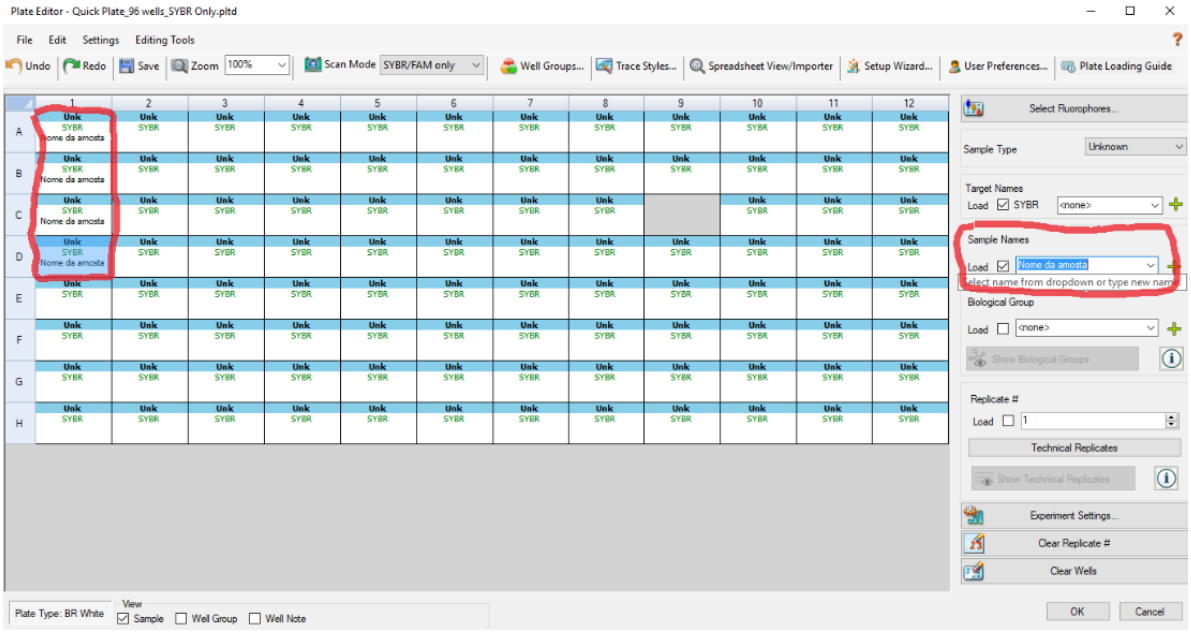
- Indicar o tipo de placa a utilizar: opção **“Settings”**, **“Plate type”** (ex. **“White”** marcar a placa como branca).

- Indicar o fluoroforo a utilizar. Opção **“Select Fluorophores”**, marcar a opção **“SYBR”**. O nome do Gene de referência também pode ser indicado, por exemplo, caso se testem vários genes. Selecionar a opção no menu lateral “**Target name**”, ou na opção **“experiment settings”** (ver mais à frente).

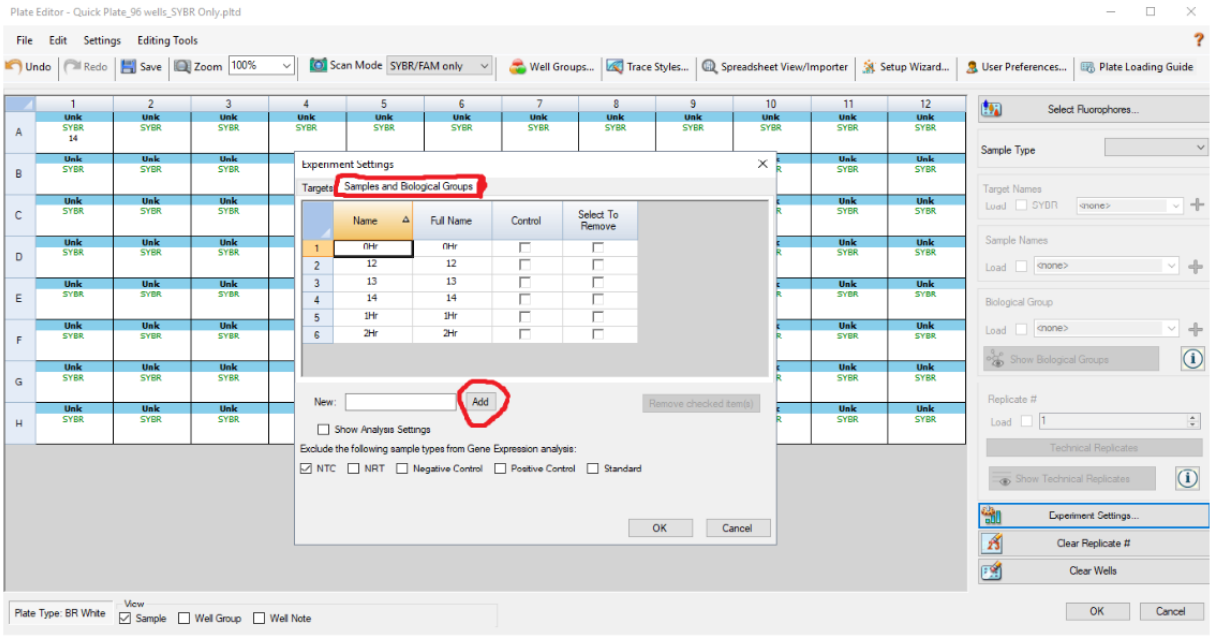
- Indicar o tipo de amostra em “Sample type”, consoante seja desconhecida (**“Unknown”**) ou um padrão (**“Standard”**)

- Nomear as amostras. Pode ser feito de dois modos:

(1) Selecionando os poços e escrevendo directamente na barra lateral, na opção **“Sample names”**

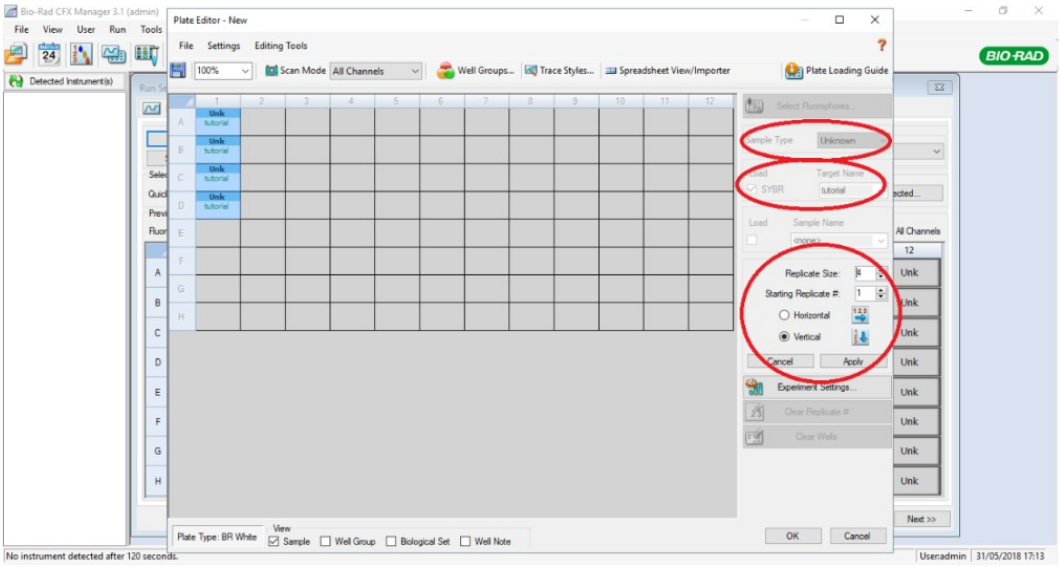


(2) ou abrindo a janela **“Experiment settings”. “Samples and biological groups”** para nomear amostras, ou **“Targets”** para indicar os genes de referência.



Escrever o nome de cada amostra e selecionar “**Add**”

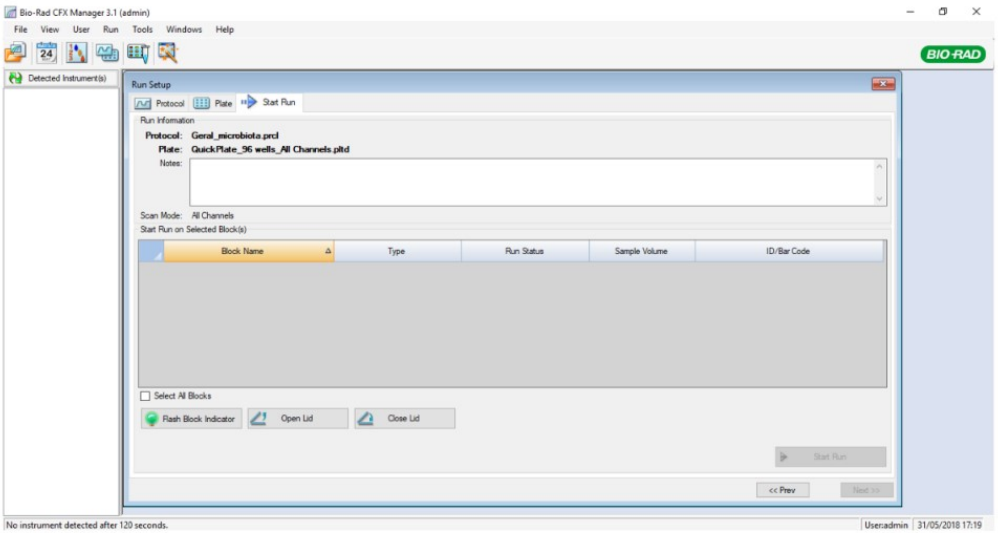
- Marcar em **“Technical replicates”**, o número de réplicas **(“replicate size”)** e a orientação.



- Depois, a cada novo conjunto de poços selecionados, repetir a indicação do nome da amostra (**Sample names**, na barra lateral) e aumentar o número à opção **“Replicate #”.**

No fim, selecionar **OK**, e gravar o layout no computador.

3ª Etapa: Start run



- Após gravar o layout, selecionar **“open lid”**, colocar a placa no termociclador e carregar em **“close lid”**.
- Selecionar a opção **“Start run”** e guardar a corrida no computador, indicando um nome. Aguardar até aparecer a palavra **“Running”**, para garantir que o sistema não dá erros.

- ***Preparação dos primers***

1. Fazer um spin down dos tubos antes de abrir, para garantir que não há pó nas paredes nem na tampa do tubo.

2. Preparar solução stock a 100 µM. Multiplicar o número de nanomoles de oligos, presente no rótulo de cada primer e multiplicar por 10. Esse produto será o volume a adicionar de agua ultrapura.

3. Consoante a frequencia de utilização, armazenar em aliquotas a -20ºC.

- ***Diluição dos primers para o qPCR***

As intruções de utilização da mix recomendam 100-500 nM de cada primer.

A proporção a utilizar é:

Retirar 10 uL da solução stock e perfazer com 990 uL de TE. Utilizar esta solução na preparação da PCR mixture.

- ***Tabela de tempeaturas de annealing***

Calculadas com base no simulador de temperaturas de annealing do software da biorad

NOTA ISTO FOI PARA A MINHA SEQUENCIA DE PRIMERS, LOGO É ESPECÍFICO DE CADA TRABALHO.

Target group	**Annealing temperature/ ºC**
Universal	48
Firmicutes	55
Bacteroidetes	55
Clostridium Leptum	49
Bacteroides	49
Enterococcus	52
Prevotella	56
Roseburia	56
Bifidobacterium	56
Lactobacillus	58
Akkermansia	62

Annex 5. Bacterial calibration curves for qPCR

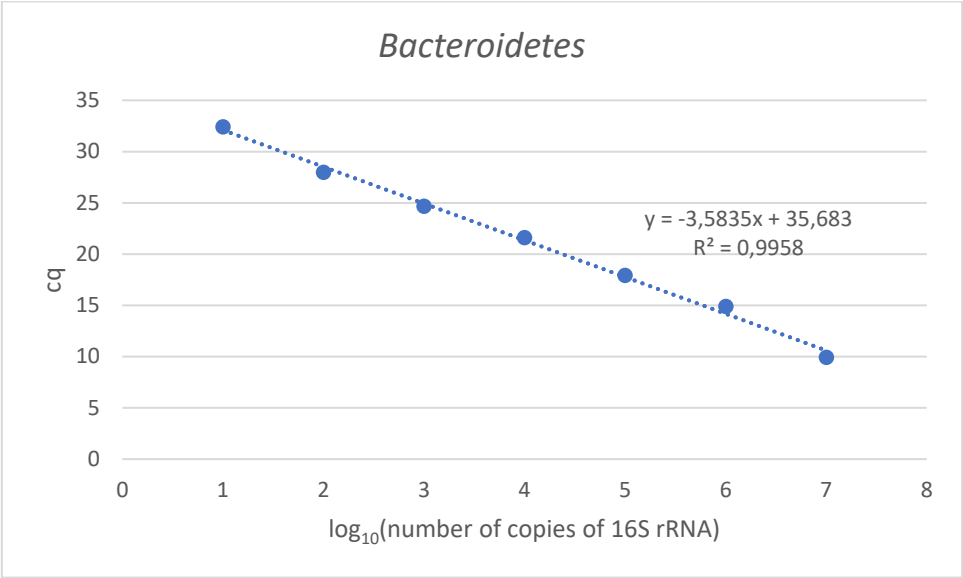


Figure 7.1 Calibration curve for Bacteroides using gDNA solutions from Bacteroides Vulgatus.

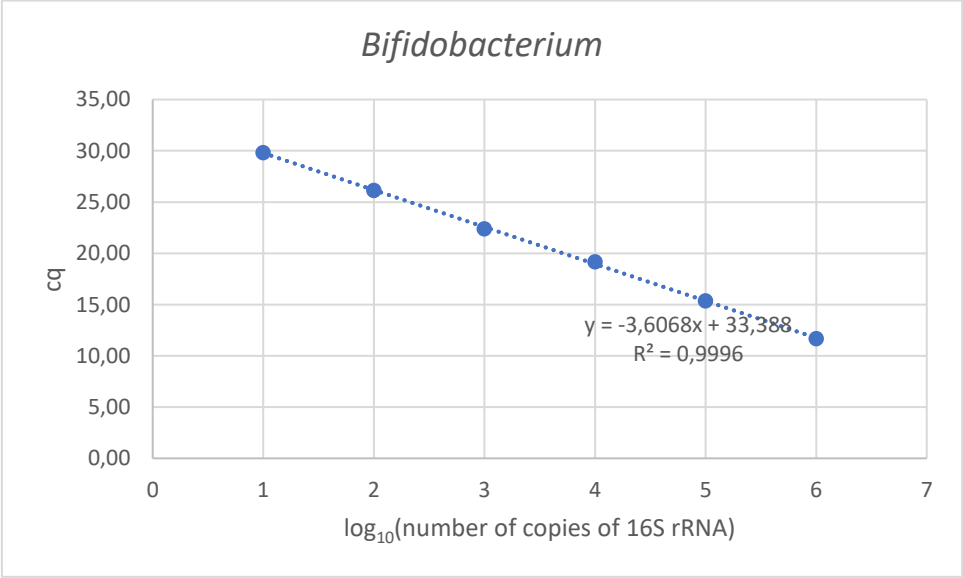


Figure 7.2 Calibration curve for Bifidobacterium using gDNA solutions from Bifidobacterium Longum subsp. Infantis.

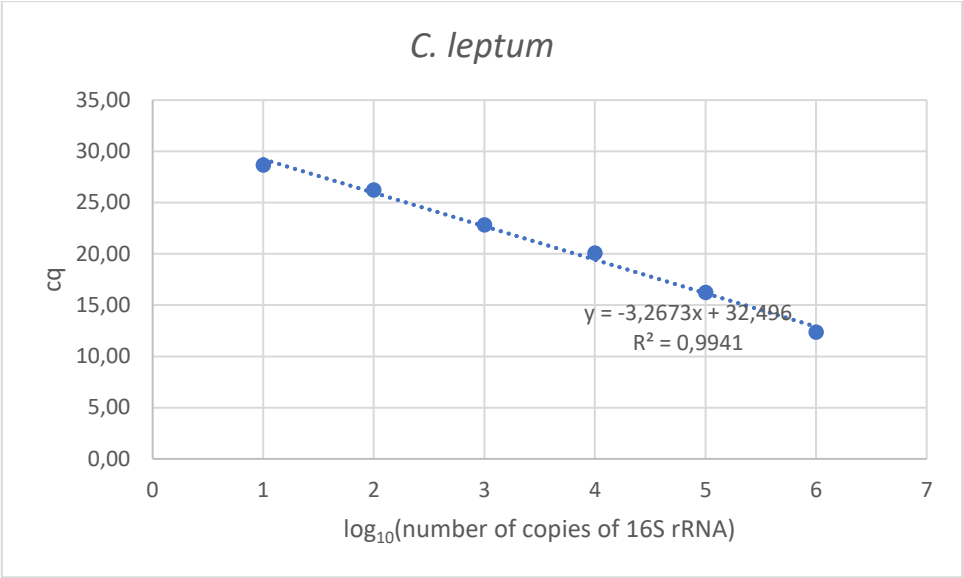


Figure 7.3 Calibration curve for C. leptum using gDNA solutions from C. leptum.

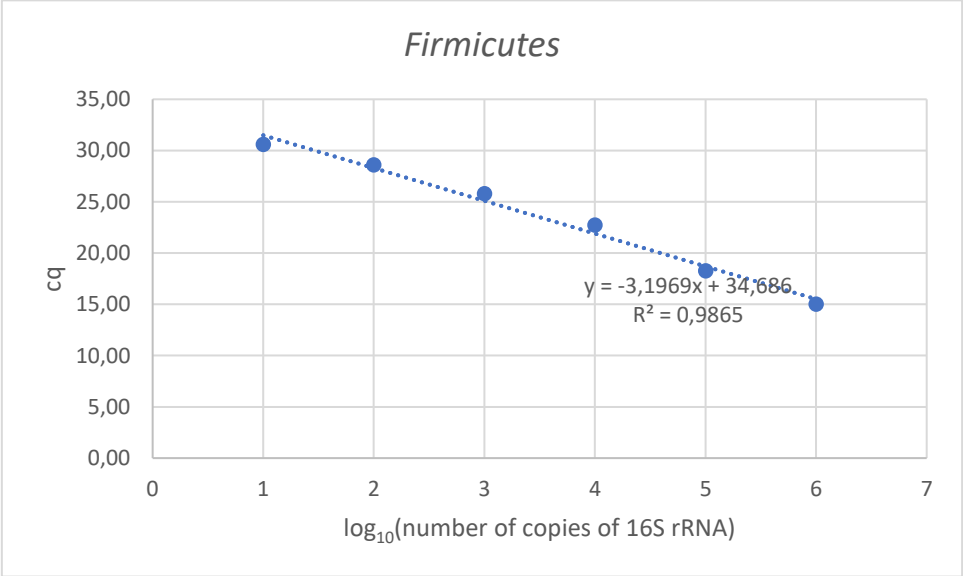


Figure 7.4 Calibration curve for Firmicutes population using gDNA solutions from Lactobacillus Gasseri.

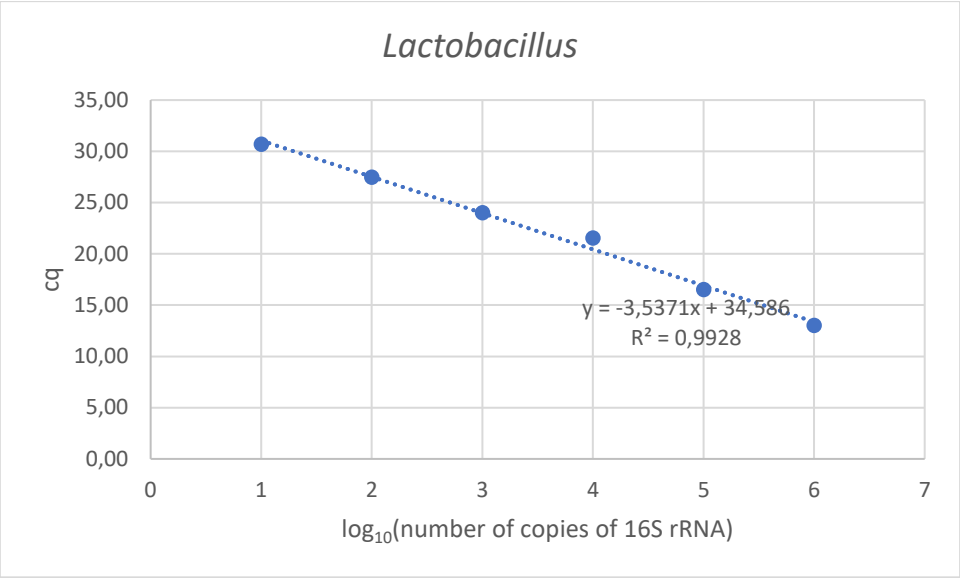


Figure 7.5 Calibration curve for Lactobacillus using gDNA solutions from Lactobacillus Gasseri.

Annex 6. SCFAs produced during the fermentation. The values are presented in mg/mL (average of the five donors, including two injections of each of sample in HPLC).

Table 7.1 SCFAs produced during the fermentation. The values are presented in mg/mL (average of the five donors, including two injections of each of sample in HPLC).

Hours	**Acetate mg/mL**				**Lactic Acid mg/mL**				**Propionate mg/mL**			
Mean	CN	CP	M1	M2	CN	CP	M1	M2	CN	CP	M1	M2
0 h	0,465	n.d.	n.d.	n.d.	1,084	n.d.	n.d.	n.d.	0,114	n.d.	n.d.	n.d.
6 h	0,515	1,075	1,856	1,355	0,422	1,660	n.d.	n.d.	0,060	0,111	1,417	1,346
12 h	0,699	1,909	1,861	1,536	0,874	3,380	n.d.	n.d.	0,147	0,172	1,368	1,219
24 h	0,696	1,825	2,027	1,672	0,488	3,535	n.d.	n.d.	0,209	0,101	1,124	1,151
48 h	1,085	2,486	2,675	2,192	0,884	4,749	n.d.	n.d.	0,432	0,138	1,197	1,217
STD												
0 h	0,268	n.d.	n.d.	n.d.	0,170	n.d.	n.d.	n.d.	0,065	n.d.	n.d.	n.d.
6 h	0,066	0,258	0,373	0,336	0,137	0,489	n.d.	n.d.	0,000	0,049	0,262	0,163
12 h	0,208	0,892	0,126	0,232	0,304	0,898	n.d.	n.d.	0,064	0,039	0,129	0,167
24 h	0,219	0,808	0,251	0,357	0,232	0,658	n.d.	n.d.	0,130	0,021	0,159	0,132
48 h	0,095	1,380	0,682	0,272	0,159	0,689	n.d.	n.d.	0,095	0,062	0,167	0,194

Hours	**Isobutyrate mg/mL**				**Succinic Acid mg/mL**			
Mean	CN	CP	M1	M2	CN	CP	M1	M2
0 h	0,618	n.d.	n.d.	n.d.	0,737	n.d.	n.d.	n.d.
6 h	0,347	0,376	2,008	1,496	0,533	0,970	1,938	2,026
12 h	0,455	0,387	1,908	1,472	0,587	0,850	1,918	1,720
24 h	0,307	0,354	1,430	1,274	0,732	0,822	1,684	1,573
48 h	0,442	0,428	1,183	1,085	1,165	0,792	1,471	1,525
STD								
0 h	0,095	n.d.	n.d.	n.d.	0,106	n.d.	n.d.	n.d.
6 h	0,096	0,048	0,392	0,166	0,209	0,254	0,546	0,290
12 h	0,111	0,103	0,554	0,528	0,315	0,147	0,331	0,302
24 h	0,112	0,073	0,791	0,666	0,256	0,165	0,171	0,129
48 h	0,141	0,040	0,831	0,668	0,143	0,099	0,126	0,143