Universidade do Minho
Escola de Engenharia

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Development of a novel food application using mixtures of commercial prebiotics
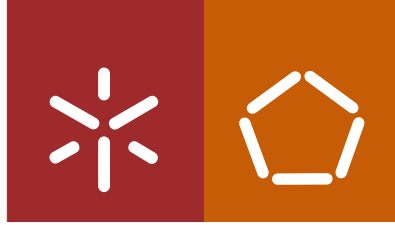
outubro de 2019

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Development of a novel food application using mixtures of commercial prebiotics

Dissertação de Mestrado
Mestrado em Biotecnologia

Trabalho efetuado sob a orientação da
Professora Doutora Lígia Raquel Marona Rodrigues
e da
Doutora Sara Isabel da Cruz Silvério

outubro de 2019

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Agradecimentos

Primeiramente agradeço à minha orientadora, Professora Doutora Lígia Rodrigues pela oportunidade de participar neste projeto, pela ajuda e disponibilidade.

Agradeço à Doutora Sara Silvério e à Doutora Cláudia Amorim por toda a disponibilidade, incentivo, integração, por todos os conselhos e pelo auxílio prontamente prestado durante a elaboração desta dissertação. Agradeço ainda à Beatriz Cardoso pelo apoio no decorrer deste trabalho.

Gostaria de agradecer a todos os amigos que de uma forma direta ou indireta, me acompanharam desde o Colégio e durante o percurso académico, pela paciência, atenção e força que prestaram em momentos menos fáceis. Também um agradecimento aos meus colegas de casa, que tornaram a minha passagem por Braga mais divertida.

Agradeço ao meu Padrinho, pela sua amizade e pela inteira disponibilidade em me acompanhar desde sempre.

Por último, mas não menos importante, aos meus pais, aos meus avós, Maria e António, pela força e pelo carinho que sempre me prestaram ao longo de toda a minha vida, a qual sem o seu apoio teria sido impossível.

A todos, enfim, reitero o meu apreço e a minha eterna gratidão

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Development of a novel food application using mixtures of commercial prebiotics

Abstract

The increased consumer awareness of the importance of a healthy lifestyle to prevent diseases and maintain the well-being led to the advance of the functional foods and beverages market, that exhibits an expected revenue of €252.079 million worldwide by 2025. Functional food is described as food consumed as part of the daily diet but has demonstrated physiological benefits beyond basic nutrition. Health and well-being improvement via gut microbiota modulation is an attractive approach to shift composition and metabolic signatures of the microbiota, namely through the ingestion of prebiotics. Prebiotics include non-digestible carbohydrates and non-carbohydrates with proven ability to modulate microbial populations, such as the gut microbiota. Fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are very well-studied prebiotic oligosaccharides with proven health benefits in humans. These compounds can be added to foods and beverages due to their nutritional value and technological properties, being used for instance to improve the mouthfeel, to replace sugar and fat content and to increase the fibre content. In this work, the prebiotic potential of commercial FOS and GOS, individually and in mixtures (GF), was studied using an *in vitro* model of static batch culture fermentations with human faecal inoculum (48h at 37◦C). The production of lactate and short-chain fatty acids (SCFAs), ammonia and gases was evaluated, as well as the pH variation. The fermentation of GOS led to the highest production of SCFAs, namely acetate and propionate, 125.10 ± 19.62 mM and 18.09 ± 5.08 mM, respectively. PH values and ammonia concentrations decreased with the supplementation of prebiotics, with the mixture GF 3:1 yielding the major ammonia reduction, namely 9-fold. Overall, the production of CO_2 increased with the addition of all prebiotics, while the CH_4 production decreased with GOS supplementation. In addition, a healthier traditional Portuguese rice cake containing GOS and GF 3:1, and a 25% sugar reduction, was herein developed. The sensory test results revealed no significant differences in appearance, aroma, taste, texture and overall impression between the original recipe and the 3 modified recipes tested. However, based on market acceptability, recipes 4 (25% sugar reduction) and 5 (25% sugar reduction and 1% GOS addition) were selected as the most accepted ones.

Keywords: FOS, functional food, GOS, prebiotics, sensory test,

Desenvolvimento de novas aplicações alimentares com misturas de prebióticos comerciais

Resumo

O aumento da consciência dos consumidores sobre a importância de um estilo de vida saudável na prevenção de doenças e na manutenção do bem-estar, levou à expansão do mercado de alimentos e bebidas funcionais, que apresenta uma receita mundial expectável de €252.079 milhões até 2025. Alimentos funcionais são alimentos consumidos numa dieta diária normal, mas que apresentam benefícios fisiológicos comprovados além da nutrição convencional. Melhoria da saúde e do bem-estar através da modulação da microbiota intestinal é uma estratégia em crescimento para alterar a composição e assinatura metabólica da mesma, que pode ser obtida pela ingestão de prebióticos. Os prebióticos incluem hidratos de carbono não digeríveis e outros compostos que não são carboidratos. Fruto-oligossacáridos (FOS) e galato-oligossacáridos (GOS), são oligossacáridos prebióticos muito bem estudados com benefícios provados para a saúde humana. Estes substratos podem ser adicionados a alimentos e bebidas funcionais pelo seu valor nutricional e pelas suas propriedades tecnológicas para, por exemplo, melhorar a textura de produtos, substituir o teor de açúcar e gordura e aumentar o teor de fibra. Neste trabalho foi estudado o potencial prebiótico de FOS e GOS, individualmente e em misturas (GF), através de um modelo *in vitro* de fermentações descontínuas inoculadas com microbiota fecal humana (48h a 37◦C). Foi avaliada a produção de lactato e ácidos gordos de cadeia curta (SCFAs), amónia e gases, assim como a variação de pH. A fermentação com GOS resultou na maior produção de SCFAs, nomeadamente de acetato e propionato com 125.10 ± 19.62 mM e 18.09 ± 5.08 mM, respetivamente. A concentração de amónia e os valores de pH diminuíram como resultado da adição de prebióticos, com a mistura GF na proporção 3:1 a resultar na maior redução de amónia, nomeadamente 9 vezes. De um modo geral, a suplementação com todos os prebióticos causou um aumento na produção de CO_2, enquanto que a produção de CH_4 diminui como resultado da suplementação com GOS. Adicionalmente, desenvolveu-se a receita de um bolo de arroz tradicional mais saudável com a incorporação de GOS e GF 3:1 e com uma redução de açúcar de 25%. Os testes sensoriais não revelaram diferenças significativas entre a receita tradicional e as 3 receitas modificadas nos parâmetros de aparência aroma, sabor, textura e impressão global. No entanto, a aceitabilidade do mercado sugere que as receitas 4 (redução de 25% de açúcar) e 5 (redução de 25% de açúcar e adição de 1% de GOS) são as mais aceites.

Palavras-chave: alimentos funcionais, FOS, GOS, prebióticos, testes sensoriais

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List of Abbreviations

GI - Gastrointestinal

ANOVA - Analysis of Variance

DNA - Deoxyribonucleic acid

DP - Degree of polymerization

FOS - Fructooligosaccharides

Fru - Fructose

Gal - Galactose

GF - GOS and FOS

Glc - Glucose

GOS - Galactooligosaccharides

HPLC - High Performance Liquid Chromatography

PBS - Phosphate Buffer Saline

rRNA - Ribosomal ribonucleic acid

SCFAs - Short Chain Fatty Acids

SD - Standard Deviation

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1. Introduction

1.1. Gut Microbiota

The gastrointestinal (GI) tract is densely populated with microorganisms that modulate metabolic, physiological, nutritional and immunological processes. Throughout its length the diversity and number of microorganisms is variable. The stomach harbours the lowest number of microorganisms, while the distal colon contains the highest with up to 10^{14} microorganisms from 1100 different species, and a distribution of at least 160 species per person. The community of microorganisms which colonize the gut is known as gut microbiota. (Power et al. 2014; Valdes et al. 2018).

The distal colon has a pH value close to neutral, the lowest concentration of oxygen in the GI tract and minimal nutrient absorption. Therefore, the maintenance of gut microbiota, among other, is ensured by the characteristics of the proximal intestine, with acidic pH values (~2-5), high oxygen concentration and constant peristalsis which provides a protective mechanism to the colonization of the gut by exogenous microorganisms (Wexler and Goodman 2017).

The gut microbiota can be thought of as an additional organ since it impacts nutrient absorption, metabolism and disease response. The composition of the gut microbiota is influenced by factors inherent to the host and by external factors. The inherent factors include genetics; age (with a stable composition in adult years); stress; disease; body temperature and composition of the intestinal fluid. On the other hand, the external factors encompass the microorganisms inherited at birth, the use of medication and diet. Diet is an external factor of utmost importance since it determines the type and number of nutrients reaching the large intestine. Changes in the gut microbiota composition have been found to occur in as little as 24 h after an alteration in the diet has been made (Hannah D. Holscher 2017; Sako, Matsumoto, and Tanaka 1999).

The identification of a high diversity of microorganisms colonizing the GI tract has been promoted by DNA-based techniques such as 16S rRNA and whole genome sequencing which also allowed the knowledge of functional characteristics. However, the identification of all species inhabiting the gut is still far due to its high complexity and interindividual diversity (Power et al. 2014). In most healthy adult individuals, the gut microbiota is composed of bacteria belonging to the phylum Firmicutes, which encompasses Gram-positive bacteria of the genera *Lactobacillus, Enterococcus, Clostridium, Eubacterium* and *Ruminococcus*, and of Gram-negative bacteria from the phylum Bacteroidetes of which the genera *Bacteroides* and *Prevotella* can be highlighted. Methanogenic Archaea, Proteobacteria and

Actinobacteria such as *Bifidobacterium* (Power et al. 2014; Wexler and Goodman 2017) are also found in the gut. It has been established that healthy individuals of the same family will have similar bacterial population in their gut, due to inherited microorganisms (from mother to child), but also due to similar diet patterns, while individuals of different cultures will, most likely have divergent taxa. For example, Europeans' gut microbiota has an abundance of Firmicutes (≥50%) followed by Bacteroidetes of which *Bacteroides* is the dominant genera. On the other hand, the Japanese population have the highest incidence of bacteria from the phylum Actinobacteria (Korpela 2018; Wexler and Goodman 2017).

Based on its pathogenic potential, gut microbiota can be organized in three categories: (1) non-pathogenic (e.g. *Lactobacillus* and *Bifidobacterium* which are complex carbohydrate-fermenting bacteria), (2) potentially pathogenic, such as some species of clostridia, and (3) commensal bacteria, for example, *Bacteroides* that can present positive or negative behaviours (Meyer and Stasse-Wolthuis 2009). Therefore, gut microbiota inherently interferes with the host's health by assisting with digestion, protecting against exogenous microorganisms and regulating metabolism and immunity. In fact, diminished microbial diversity or dysbiosis has been associated with type 1 and type 2 diabetes, obesity and coeliac diseases, while a more complex gut microbiota seems to indicate a healthier gut as it results in resistance to environmental alterations such as the transiently missing species (Valdes et al. 2018).

Bacteria in the gut harbour more than 200 enzymes able to degrade complex carbohydrate linkages making them important to ferment undigested carbohydrates which consequently makes them susceptible to modulation by diet alterations. The modulation via the consumption of non-digestible carbohydrates is a growing field of knowledge which aims to treat and prevent gut related diseases such as colon cancer (Korpela 2018; Sheflin et al. 2017).

1.2. Fermentation by gut microbiota

Gut microbiota ferment undigested dietary substrates that reach the colon to fulfil their biochemical and metabolic requirements. These substrates include resistant starch, oligosaccharides, proteins, amino acids and plant cell wall that the upper GI tract is unable to absorb being degraded by proteolytic or saccharolytic fermentation. Factors that usually influence the dietary carbohydrates' fermentation are the degree of polymerization (DP), branching and enzymes produced by gut microbiota. Saccharolytic fermentation occurs in the proximal colon where, comparing to the distal colon, there are more fermentable substrates. This fermentation results in the production of short-chain fatty acid (SCFAs). SCFAs are also end-products of proteolytic fermentation which takes place in the distal colon. However,

amines and ammonia, which are known to be toxic metabolites, are also produced. (Kolida and Gibson 2007; Power et al. 2014).

SCFAs are carbon-based anions, of which acetate (C2), propionate (C3) and butyrate (C4) make up 95% of the total SCFAs produced in the human gut. Acetate is the most abundant SCFA produced due to the wide distribution of the production pathways amongst several groups of bacteria. This metabolite is essential for the growth of different bacteria but its systemic availability of ~36%, and ability to cross the blood-brain barrier means acetate can attain peripheral tissues where it can be used for cholesterol metabolism and lipogenesis in the liver. Propionate and butyrate production pathways, as opposed to acetate, are present in a narrower group of bacteria and the production appears to be substrate dependent. Only about 9% of propionate becomes systemically available and can be used for hepatic gluconeogenesis, while the remaining is converted, in the intestine, to glucose via intestinal gluconeogenesis. Butyrate has an estimated systemic availability of about 2%, since it is the preferred energy source for colonocytes and enterocytes. This metabolite is of utmost importance in the maintenance of the hypoxia state in the colon preventing an oxygen imbalance that could lead to dysbiosis (Gill et al. 2018; Hannah D Holscher 2017; Power et al. 2014).

Another fermentation end-product commonly produced by bacteria such as *Bifidobacteria* and *Lactobacilli* is lactate. Even though lactate is not considered a SCFAs, it can be converted into SCFAs by different bacteria (e.g. *Eubacterium hallii* converts lactate into butyrate) due to cross-feeding mechanisms which prevent the excessive acidification of the colon (Flint et al. 2015; Gill et al. 2018; Hannah D. Holscher 2017; Morrison and Preston 2016; Valdes et al. 2018). Due to lactate's potential as SCFAs precursor, its analysis is relevant to better understand the gut microbiota fermentation of non-digestible food substrates (Vulevic, Rastall, and Gibson 2004). The accumulation of lactate in the distal colon causes acidification resulting in a shift in the composition of the microbiota and their metabolic fingerprint. Louis and collaborators (Louis et al. 2007) reported that decreasing the pH from 6.5 to 5.5 resulted in different SCFAs production profiles caused by shifts in the gut microbiota.

Since the gut microbiota's modulation is known to influence an individual's health, multiple *in vivo* and *in vitro* studies have been conducted under this topic. These studies include tests on the influence of dietary alterations (increased fibre intake, supplementation with oligosaccharides such as prebiotics) on gut microbiota's modulation. Lactate and SCFAs evaluation can be performed during an *in vitro* fermentation of oligosaccharides by analysing the supernatant using high performance liquid chromatography (HPLC). The determination of the overall increase in total lactate and SCFAs is helpful

to assess the prebiotic potential of the oligosaccharide under study. The *in vitro* analysis enables the understanding of the SCFAs produced in comparison to *in vivo* analysis, since it eliminates the adsorption phenomena occurring in living beings (Coulier et al. 2009).

Hydrogen (H_2) and carbon dioxide (CO_2) gases are also unavoidable fermentation end-products produced by gut bacteria such as clostridia and enterobacteria. Some individuals, who harbour methanogenic bacteria such as *Methanobrevibacter smithii*, can also produce methane (CH_4) (Ghoddusi et al. 2007). The H_2 produced may be reused by microbial species in redox reactions during the oxidation of organic material. CO_2 can also be subject to reuse via methanogenesis and acetogenesis. Unlike H_2 and CO_2, which quantity can be reduced by bacterial metabolism, CH_4 is unable to be metabolized any further and it is expelled with the other gases, causing foul smell (Probert and Gibson 2002).

Gas accumulation in the large intestine is associated with gas distention issues which constitute a clinical disincentive to non-digestible carbohydrate supplementation. These issues may result from the administration of high doses, which highlights the need to determine a recommended daily dose (Gibson et al. 2017). The gas production profiles can be determined using batch fermentation of gut microbiota in response to fermentable carbohydrates.

1.3. Prebiotics: definition and examples

In 1995, prebiotics were defined as "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon" (Gibson et al. 2017). These bacteria would include bifidobacteria and lactobacilli as preferential targets, since they were extensively studied at the time for their probiotic abilities. In 2004, the same authors updated the definition to "selectively fermented ingredients that allow specific changes, both in composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Gibson et al. 2017). According to this definition, prebiotics were required to fulfil three criteria: (a) resistance to the digestive process, (b) fermentability by gut microbiota and (c) selective stimulation of growth and activity of said microorganisms. In 2010, prebiotics were redefined as "a selectively fermented ingredient that results in specific changes in the composition and/or activity of gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al. 2017). This definition accompanied the advances in molecular techniques that allowed a better understanding of the microorganisms comprising the gut microbiota, beyond lactobacilli and bifidobacteria, associated with health benefits.

Prebiotics have been recently defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al. 2017). This definition takes into consideration that all microbiologically colonized body sites can benefit from fermentable carbohydrates to improve the composition of the microbiota present by increasing the number of beneficial bacteria. However, when the prebiotic concept was originally introduced by Gibson and Roberfroid, it only considered the effects in the GI tract.

Even though the concept of prebiotic has expanded beyond their application to the GI tract, for the purpose of this dissertation, the term prebiotic will refer only to prebiotic of food grade.

Supplementation with prebiotic is a strategy that promotes the growth of beneficial gut microbiota to restore the normal flora after dysbiosis, for example. It is viable due to the ability of some bacteria to utilize different carbohydrates to obtain energy and thrive. This ability results from the secretion by healthful bacteria of several enzymes, such as hydrolases, glycosyltransferases, polysaccharide lyases, and carbohydrate esterases (Hannah D. Holscher 2017).

Currently, the most well-established prebiotics are inulin-type fructans, such as fructooligosaccharides (FOS), and galactooligosaccharides (GOS) which are substrates that have been regarded as safe, dominating the prebiotic market. Additionally, their prebiotic properties have been recognized, for humans and other animals (e.g. cats and farmed fish), for over 20 years. Both substrates cannot be hydrolysed by digestive enzymes of the upper GI tract, reaching the colon undigested. The prebiotic market is still growing with an expected profit of € 6.743 million by 2023 representing a global growth of 10.4%, with the Asian region presenting the highest expected growth (Amorim et al. 2019; Gibson et al. 2017; Kovács et al. 2013; Mano et al. 2018).

FOS are inulin-type fructans consisting of fructose (Fru) moieties linked to each other through β-(2→1) linkages and to a terminal glucose (Glc) residue by α-linkages. These are the most studied and best implemented prebiotics at the European market and are naturally occurring, although in low quantities, in several biological materials from which they can be extracted including asparagus, sugar beet, agave, honey and Jerusalem artichoke. To fulfil the market's needs, FOS can be obtained by hydrolysis of inulin or produced via transfructosylation of sucrose (Nobre, Teixeira, and Rodrigues 2015). Inulin-type fructans can differ in the degree of polymerization with inulin varying from 2 to 60 and FOS varying from 2 to 10. Due to its composition FOS are commonly represented as Glc-Fru$_n$ (n represents the number of fructose moieties) being mainly composed of kestose (Glc-Fru$_2$), nystose (Glc-Fru$_3$) and β-

fructofuranosyl nystose (Glc-Fru$_4$) (Dominguez et al. 2014; Marín-Navarro, Talens-Perales, and Polaina 2015).

Galactooligosaccharides (GOS) are non-digestible oligosaccharides made up of galactose (Gal) moieties linked to each other and linked terminally to a glucose (Glc) residue via β-linkages according to the formula Gal$_n$-Glc, with a DP varying from 2 to 8. Similarly to FOS, GOS are considered well-established prebiotics and are widely used in the food industry. Other oligosaccharides are naturally found in plants such as sugar beet and soya beans. However, the structure of these GOS makes them easily degraded by many bacteria diminishing its specificity. Hence, commercially available GOS are produced via enzymatic transglycosylation of lactose derived from whey, for example, using β-galactosidases from microbial origin which results in GOS with a more complex structure (Kovács et al. 2013; Sangwan et al. 2011; Torres et al. 2010; Wilson and Whelan 2017).

The modulation of the colonic bacteria by prebiotic supplementation results in several health benefits to the GI tract, some of which are represented in Figure 1 (Bali et al. 2015; Kolida and Gibson 2007).

Pathogen inhibition and prevention of infection

Growth of probiotic bacteria

Production of beneficial metabolites (SCFAs) and vitamins

Support normal gut functions

Improved bioavailability of essential minerals (Mg^{2+}, Zn^{2+} and Ca^{2+})

Reduced antibiotic associated diarrhoea

Removal of potentially carcinogenic toxins
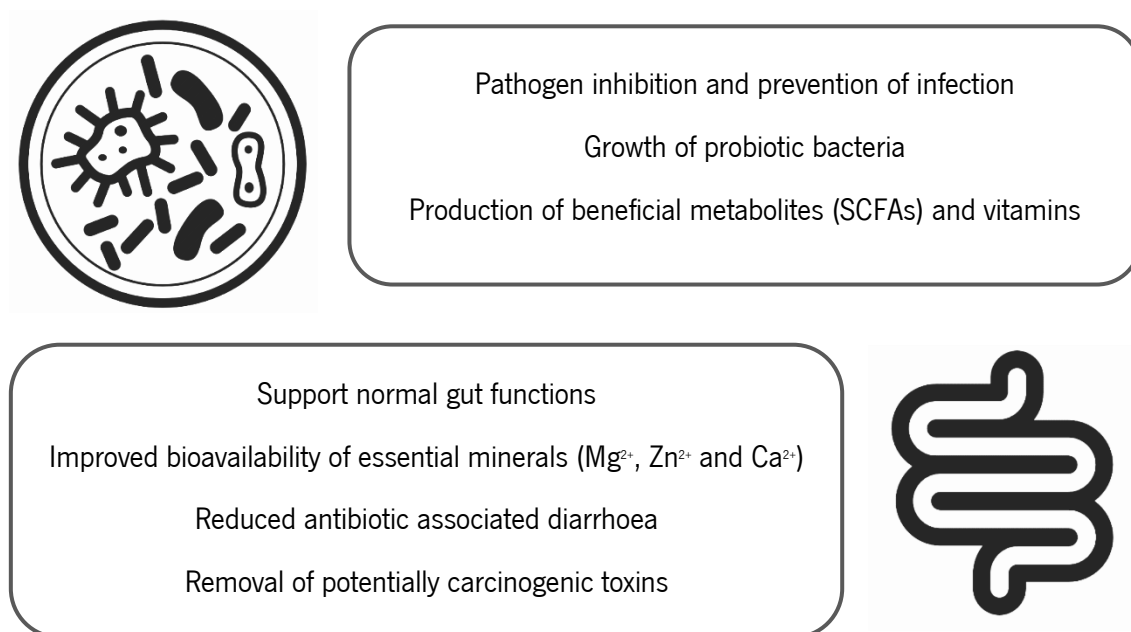
Figure 1: Main health benefits to the GI tract associated with prebiotic supplementation.

Besides the prebiotic potential, FOS and GOS possess interesting functional properties, such as low sweetness, humectancy and acid stability, thus making them attractive to the functional food industry.

1.4. Prebiotic Potential and Health Benefit

According to the most recent prebiotic definition, the health benefits linked to the prebiotic activity need to be an outcome of microbiota modulation and metabolism. Moreover, the prebiotic potential must be confirmed on the target host for its designated use (humans and other animals) (Gibson et al. 2017).

In order to verify the effectiveness of novel oligosaccharides as prebiotics, experiments to demonstrate that the prebiotic is selectively metabolized by non-pathogenic groups of microorganisms naturally present in the colon is required. These include *Bifidobacterium, Eubacterium, Lactobacillus* and *Roseburia*, which increase in number, while reducing the number of potentially pathogenic bacteria, e.g. *Escherichia coli*. These changes occur because different species of bacteria are, genetically, more capable of using different substrates as energy sources (Gibson et al. 2017; Power et al. 2014). Apart from the adaptability of different microorganisms, the ability to ferment certain oligosaccharides depends on the degree of polymerization and branching, as well as on the glycosidic linkages and synergy between bacteria during fermentation (Al-Sheraji et al. 2013).

To establish the prebiotic potential of a substrate both *in vivo* and *in vitro* studies must be carried out. *In vitro* studies are a cheap and effective screening tool to initially assess the prebiotic potential of different compounds. However, the use of pure cultures, or mixed cultures with a limited number of bacterial genera does not constitute a reliable method to determine the interactions between gut microbiota, as well as their changes in response to prebiotics. Therefore, for a more accurate representation of colonic bacteria, faecal samples should be used as inoculum on batch culture fermentations supplemented with the substrate being tested (Roberfroid 2007). The modulation of gut microbiota and the metabolites' production profiles should be evaluated to establish if the prebiotic fermentation was selective.

Microbial changes can be assessed by molecular and metagenomic techniques, including next generation 16S rRNA sequencing and whole genome shotgun sequencing (Valdes et al. 2018). 16S rRNA gene sequencing allows taxonomical analysis whether by genus identification or species identification. Genome shotgun sequencing involves the sequencing and the computer assembly of DNA fragments until the information contained in chromosomes is compiled, granting the understanding of the full genetics of different organisms including the genes encoding enzymes (Green 2001; Power et al. 2014; Valdes et al. 2018).

The gut microbiota modulation previously mentioned can occur due to the production of antimicrobial compounds, decrease in luminal pH, competition for nutrients and prebiotics, competitive

exclusion, production of growth substrate, production of SCFAs and inherent composition of the host's microbiota. (Flint et al. 2015).

As previously mentioned, *in vivo* studies should be conducted afterwards on the intended target host to confirm the suggested health benefits, including benefits to the GI tract (e.g. immune stimulation), on cardiometabolism (e.g. insulin resistance), bones (e.g. mineral bioavailability), among others. These studies must be randomized control trials in order to positively relate changes in health markers/symptoms to the prebiotic at study, and account for interindividual variations on microbiota as a modulator of possible outcomes. From these studies the appropriate dosage to achieve the prebiotic effect while preventing negative side effects such as non-selective utilization, gas accumulation and discomfort or diarrhoea, is expected (Gibson et al. 2017).

Tuohy and collaborators (Tuohy et al. 2001) developed an *in vivo* double-blind randomize clinical trial (31 participants) to establish the prebiotic effect of biscuits containing FOS. Such experiment revealed that the ingestion of 6g/day of prebiotics, for a controlled period, resulted in the increase of *Bifidobacteria* in faecal samples, and individuals with lower initial number of *Bifidobacteria* showed the most significant increase.

Regarding the GI tract and the cardiometabolism, prebiotics have been associated with prevention of bacterial infections, cancer, diabetes and metabolic syndrome (Di Bartolomeo, Startek, and Van den Ende 2012; Genta et al. 2009; Griffin et al. 2003).

Bacterial infection prevention with prebiotics occurs as a result of mucin production and direct immune stimulation. Mucin is one of the main components of the gut epithelium protective mucus layer which forms a gel-like barrier that hinders the colonization by pathogens such as *Salmonella* spp and *Vibrio cholerae.* SCFAs production also mediate intestinal epithelial cells defence mechanisms by regulating inflammatory responses. Among the SCFAs, butyrate is the most important for colonic cell's proliferation and injury repair, since it is the main energy supplier for such cells (Di Bartolomeo et al. 2012).

Colorectal cancer causes thousands of deaths worldwide every year and is associated with wrong dietary habits, among other factors. Prebiotics, via the enhanced production of SCFAs are suggested to neutralize colon carcinogenesis, adjusting tumour cells' gene expression or reducing the activity of cancer promoting bacteria. The latest can be achieved due to decreased colonic pH that promotes preferential growth of beneficial bacteria to the detriment of pathogenic ones (Di Bartolomeo et al. 2012).

Prebiotic consumption can also contribute to the relief of conditions associated to metabolic syndrome. Metabolic syndrome includes ailments such as high blood pressure, obesity, hyperglycaemia and high triglycerides levels, which result in increased risk for cardiovascular diseases and type 2 diabetes. Studies performed in animal models revealed that prebiotic supplementation improved body weight of diabetic rats and increased numbers of *Bifidobacteria* and *Lactobacilli* and reduced pH values. Increased SCFAs production resulting from bacterial modulation is known to influence blood glucose and cholesterol levels and lipid metabolism (Di Bartolomeo et al. 2012). Prebiotic supplementation in animal models has been found to modulate gut microbiota and to improve the reduction of body weight, fat and adipocyte size via appetite and food intake modulation, as well as diminished ghrelin production and fatty acid storage. Such supplementation also results in improved gut barrier integrity, glucose tolerance and insulin sensitivity, with similar results attained in human trials. A study performed with oligofructose supplementation during 120 d on obese-dyslipidaemic women revealed reduced body weight, body mass index and low-density lipoprotein cholesterol levels (Genta et al. 2009).

Bone health, among others, is inherently dependent on mineral availability since it is a rigid organ made up of a flexible matrix and minerals such as calcium. Since calcium absorption is mainly gut-regulated, dietary alterations such as the supplementation of prebiotics can improve bone health. Acidic colon pH impairs calcium linkage to negatively charged metabolites increasing mineral absorption which results in increased bone mineralization. Several clinical trials have been conducted on the influence of prebiotic supplementation on mineral absorption, bone health outcomes and intestinal parameters. For example, Van der Heuvel and co-workers (van den Heuvel, Schoterman, and Muijs 2000), in a placebo controlled trial, studied the effect of 20 g/day GOS supplementation, for 5 d, on postmenopausal women and determined a direct increase in calcium absorption. The same pattern was observed in a placebo-controlled study on girls aged 10-15 years after supplementation with 8 g/day of inulin for 3 weeks (Griffin et al. 2003).

1.5. Functional Food

In the past years, food has been seen not only for body nutrition, but also to improve physical well-being and to prevent nutrition related diseases like obesity upon regular consumption. Functional food was described as food which typical appearance and is consumed as part of the daily diet but has demonstrated physiological benefits beyond basic nutrition, of which it can be highlighted the influence on the intestinal microbiota (Sangwan et al. 2011).

Functional foods include foods with natural occurring bioactive substances, such as dietary fibre, foodstuffs that have been made functional due to the addition or improvement of the bioavailability of a particular component and derived food ingredients, such as prebiotics. Such food products can be consumed by the general population or can target specific subgroups such as infants, children, women and the elderly (Al-Sheraji et al. 2013; Sangwan et al. 2011).

Prebiotics can be added to dairy products, beverages, baked goods and clinical nutrition with the aim of producing functional foods but also as a fat, sugar and calorie replacement, and to improve the organoleptic properties of said products (Lamsal 2012; Sangwan et al. 2011). FOS and GOS are examples of prebiotics that have been added to a variety of products due to their technological properties since they are low calorie, low sweeteners, hygroscopic, highly soluble, thermo and acid stable and prevent the colouration of foods by Maillard reactions. These properties make FOS and GOS attractive food ingredients to be incorporated on (Dominguez et al. 2014; Nobre et al. 2015; Sangwan et al. 2011; Torres et al. 2010):

- low glycaemic index, low calorie foods, clinical nutrition;
- infant nutrition and growing up milk;
- dairy products and beverages (fruit juices);
- bakery products;
- frozen foods and meat products;
- fruit jams and confectionary products.

Since neither FOS nor GOS have a prominent taste, their addition to food has been highly accepted. Due to their characteristics, prebiotics can be added to dairy products either before or after fermentation, since only a limited number of microorganisms can metabolize them, resulting in smooth and creamy products. In baked goods, since they are heat stable, the addiction of prebiotics allows the incorporation of fibre while maintaining the moisture of low sugar products and preventing browning due to Maillard reactions (Kovács et al. 2013; Nobre et al. 2015; Sangwan et al. 2011).

The market for functional foods and beverages is rapidly growing and is expected to reach €252.079 million worldwide by 2025 (Grand View Research 2019).

1.5.1 Sensory testing

The modifications performed to the traditional recipe of a food product, either by incorporating novel components or by changing the amount of specific ingredients, may affect its characteristics and, therefore, its market acceptability. Sensory evaluation methods, developed by sensory science, can be used to analyse differences between product's characteristics. Sensory science has been described as: "a scientific method used to evoke, measure, analyse and interpret those responses to products perceived through the senses of sight, smell, touch, taste and hearing"(Lawless and Heymann 2010). Appearance, aroma, taste and texture are examples of the properties usually evaluated by a group of panellists when analysing food products. The panellists can be experts, trained panellists or consumers, depending on the sensory testing being used (Teixeira 2009; Yang and Lee 2019).

Sensory evaluation relies on two types of methods: (1) analytical, which includes discriminatory, and descriptive methods and (2) hedonic. Quantitative descriptive analysis aims to provide descriptions of products based on the perception of a group of trained panellists and can result to compare, for example, physical and chemical analysis, products formulations and preferences. Hedonic methods rely on consumers as members of the group of panellists. These methods often use acceptance tests applying scales to translate consumer's opinion (e.g. scale 1-7, with 1 meaning very bad and 7 meaning excellent) (Canavari et al. 2009; Teixeira 2009).

2. Project aims

Due to an increased consumers' awareness and interest on healthy lifestyle, there are more products available marketed as low calorie and functional food. However, the pastry industry does not offer healthy options for consumers with dietary restrictions due to medical conditions or consumers who follow the healthy lifestyle or trend. Thereby, novel pastry products containing functional ingredients and sugar reduction would improve the industry's competitiveness in the current market.

This thesis aimed to incorporate functional ingredients into a traditional Portuguese cake, resulting in a healthier option to the current pastry options available. The specific goals were:

- *In vitro* determination of the prebiotic potential of different mixtures of commercial oligosaccharides;
- Selection and optimization of a traditional Portuguese rice cake recipe;
- Incorporation of the oligosaccharide mixture with the best prebiotic potential in the selected Portuguese rice cake recipe while reducing the refined sugar content;
- Sensory profile evaluation of the Portuguese rice cake recipes.

3. Materials and Methods

3.1. Materials

FOS from agave inulin was purchased from Entelees® (Mexico) and GNC Prebiotic GOS from GNC® (USA). All chemicals and media components were of analytical grade and obtained from Sigma-Aldrich Chemical LTD.

3.2. Faecal human microbiota

Faecal microbiota samples were collected from one healthy human volunteer who did not present any metabolic or gastrointestinal diseases and who did not take any antibiotics, pre- or probiotic supplements for 3 months prior to the study, as well as during the study. The female volunteer, aged 23 was a non-smoker who consumed a non-specific Mediterranean diet. Each sample was collected on site, diluted to a final concentration of 10% (w/w) in phosphate-buffered saline (PBS), 0.1M, pH 7.0, and kept at 4 °C overnight, until inoculation (Gannasin et al. 2015).

3.3. *In vitro* batch culture fermentation of oligosaccharides using faecal human microbiota

Static batch culture fermentations were performed during 48h at 37 °C, in anaerobic culture bottles (70mL) sealed with a rubber stopper and a metal cap. The culture bottles were filled with 31.6 mL of growth medium (2 g/L peptone water, 2 g/L yeast extract, 0.1 g/L NaCl, 0.04 g/L K_2HPO_4, 0.01 g/L $MgSO_4.7H_2O$, 0.01 g/L $CaCl_2.6H_2O$, 2 g/L $NaHCO_3$, 14.8 mL/L Tween-80, 0.05 g/L haemin, 74.1 μL/L vitamin K1, 0.5 g/L L-cysteine HCl, 0.5 g/L bile salts, 0.001 g/L resazurin (oxygen indicator) and Na_2S 0.1 M (oxygen reducer)). The solutions of prebiotics GOS, FOS and mixtures composed of GOS and FOS in proportions of 1:1, 3:1 and 1:3 were added when required at a final concentration of 10 g/L (4 mL).

With the exception of the filter-sterilized solution of vitamin K1, the medium and the oligosaccharides solutions were sterilized by autoclave (121 °C, 1 atm for 20 min). Each sterilized medium-filled bottle was inoculated with 4.4 mL of faecal human microbiota previously prepared (section 3.2)

Anaerobic conditions were attained by sparging the bottles with oxygen-free nitrogen gas using a manifold equipment, performing 2 injections of 8 cycles each and reaching a final pressure of 1.6 atm.

Medium samples (1 mL) were collected at different time points (0, 12, 24, 36 and 48h) and, centrifuged at 4000 x g for 10 min. The supernatant was further used for HPLC analysis (section 3.4). Headspace samples (0.5 mL) were collected at different time points (12, 24, 36, 48h) and used to determine the gas production profile (section 3.5).

The pH was measured at 48h using a pH electrode and the fermentation broth was drawn from the bottles, centrifuged, washed and resuspended in PBS (0.1 M pH 7.0) and stored at 4°C for future DNA sequencing analysis (assays to be conducted after this project completion).

Fermentations were run in duplicates using a negative control with no added prebiotics.

3.4. Metabolites analysis

The production of SCFA (acetate, propionate and butyrate) and lactate were analysed using an HPLC fitted with a Jasco-RI detector, a Knauer UV detector and an Aminex HPX 87H column (300 mm x 7.8; Biorad, USA). A 40 μL volume of the supernatant sample was eluted using 5 mM H_2SO_4 as the mobile phase, at a flow rate of 0.7 mL/min and a temperature of 60 °C.

Ammonia concentration was determined using an enzymatic kit (LCK 304) from Hach Lange (Düsseldorf, Germany).

3.5. Gas production analysis

Gaseous samples were analysed via gas chromatography (GC) using a Bruker Scion 456-GC (Billerica, MA, USA) equipped with a thermal conductivity detector and two columns: a BR-QPLOT column (30 m length, 0.53 mm internal diameter; film thickness, 20 μm) and a Molsieve packed column (30 x 80/100, 2 m length, 2.1 mm internal diameter). The Molsieve column allowed the measurement of H_2 and CH_4, using argon as the carrier gas, and the BR-QPLOT column allowed the measurement of CO_2, at a set flow rate of 30 mL/min. The temperatures in the injector, column and detector were 100, 35 and 130 °C, respectively (Arantes et al. 2017).

3.6. Selection of a Portuguese rice cake "bolo-de-arroz" recipe

A preliminary search in Portuguese rice cakes recipes online and in culinary books revealed the existence of wide variety of recipes. Therefore, three recipes containing the same ingredients in different proportions, except for milk, were selected (Table 1).

Table 1: Ingredients and proportions used in the 3 recipes selected for sensory testing.

	Recipe 1	Recipe 2	Recipe 3
Ingredients		%	
Sugar	23.4	23	22.6
Butter	9.4	15.3	12.7
Eggs	19.1	23.4	27.7
Milk	14	6.9	-
Wheat flour	28	15.3	9
Rice flour	5.6	15.3	27.1
Baking soda	0.5	0.8	0.9

Cakes from each recipe were baked at 150 °C for 20 min and left to cool overnight at room temperature (20-25 °C) prior to the sensory testing (section 3.8).

3.7. Sugar reduction and prebiotic addition to the rice cake recipe

New cakes were produced based on the selected recipe (section 4.6), with two modifications:

1. 25% sugar reduction (recipe 4);
2. 25% sugar reduction and supplementation with 1 % of the selected prebiotic or prebiotic mixtures (section 4.4) (recipe 5).

Cakes from the original and modified recipes were prepared as described in section 3.6. The ingredients for the modified recipes are presented in Table 2.

The addition of 1% of prebiotic mixture took into account the recommended daily dose provided by the manufacturer, assume that an individual would consume up to 3 cakes a day.

Table 2: Ingredients and proportions used in the modified recipes selected for sensory testing.

	Recipe 4	Recipe 5
Ingredients	%	
Sugar	18.63	18.48
Butter	9.94	9.85
Eggs	20.27	20.10
Milk	14.90	14.78
Wheat flour	29.81	29.56
Rice flour	5.96	5.91
Baking soda	0.50	0.49
Prebiotic	-	0.81
Contaminants	-	0.72

3.8. Sensory testing

The samples baked according to the recipes described in sections 3.6 and 3.7 were evaluated using acceptance hedonic sensory tests.

In section 3.6 untrained panellists were asked to evaluate appearance, aroma, taste, texture and overall impression of each sample using a hedonic scale with 5 levels: 1 distaste, 2 dislike, 3 neither like nor dislike, 4 like and 5 like very much. The panellists were also asked to indicate which of the samples best resembled a traditional Portuguese rice cake (Appendix II).

In section 3.7 untrained panellists were asked to evaluate appearance, aroma, taste, texture and overall impression of each sample using a hedonic scale with 5 levels: 1 distaste, 2 dislike, 3 neither like nor dislike, 4 like and 5 like very much (Sensory test 2, Appendix III). This sensory test (Sensory test 2, Appendix III) and a second sensory test (Sensory test 3, Appendix IV) also assessed the acceptability of the different recipes before and after the recipe disclaim (Appendix IV).

3.9. Statistical analysis

Results are presented as mean values ± standard deviation (SD). The statistical significance of differences was evaluated by two-way ANOVA test ($p < 0.05$) using the software GraphPad Prism version 6.

4. Results and Discussion

The *in vitro* fermentations were performed with human faecal inoculum to assess and compare the prebiotic potential of commercially available prebiotics, FOS (inulin) and GOS individually and in different mixtures. Both FOS and GOS are well-establish prebiotics with a wide variety of applications for humans and animals (Gibson et al. 2017).

The prebiotic potential was determined through the evaluation of lactate, SCFAs, gas and ammonia production, as well as pH variation.

4.1. Short-chain fatty acids (SCFAs) production

The fermentation of prebiotic oligosaccharides by human gut microbiota results in the production of lactate and SCFAs, such as acetate, propionate and butyrate, among other metabolites (Gill et al. 2018). The production of lactate and the SCFAs was analysed via HPLC for samples taken every 12h from fermentations supplemented with FOS (section 4.1.1.), GOS (section 4.1.2) and mixtures of GOS and FOS in different proportions, namely 1:3, 3:1 and 1:1 (section 4.1.3). The results of each assay are presented below and discussed in detail in section 4.1.3.

4.1.1. Fructooligosaccharides (FOS)

The production of lactate and SCFAs obtained from the fermentation supplemented with FOS is illustrated in Figure 2 together with the negative control fermentation (control 1, no prebiotic addition).

The results showed a significant increase in the accumulation of acetate after 48h (24.00 ± 0.06 mM for control 1 and 47.72 ± 1.75 mM for FOS, $p<0.0001$) and lactate (1.77 ± 0.01 mM for control 1 and 28.29 ± 0.15 mM for FOS, $p<0.0001$) for prebiotic supplementation, corresponding to a $99\pm7\%$ and $1498\pm8\%$ increase, respectively, when compared to the control fermentation. Propionate showed less statistically significant changes throughout the fermentation period (11.49 ± 1.03 mM for Control 1 and 16.58 ± 1.31 mM for FOS at 48h, $p<0.05$), with an increase of $44\pm11\%$ relative to the control. The final accumulation of butyrate for the different conditions revealed no significant variations.
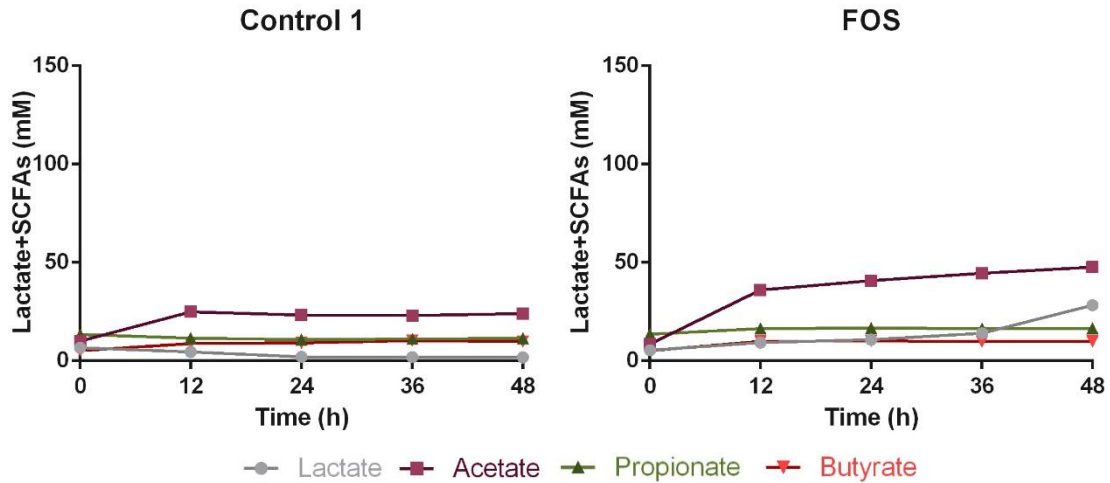
Figure 2: Lactate and SCFAs' production during a period of 48h of faecal human microbiota growth from one donor, in the absence of prebiotics (Control) and in a medium supplemented with a prebiotic solution of 10g/L FOS. The results are the average of two independent fermentations ± standard deviation.

4.1.2. Galactooligosaccharides (GOS)

The results obtained for the production of lactate and SCFAs from the fermentation supplemented with GOS and the negative control (no prebiotic addition) are presented in Figure 3.
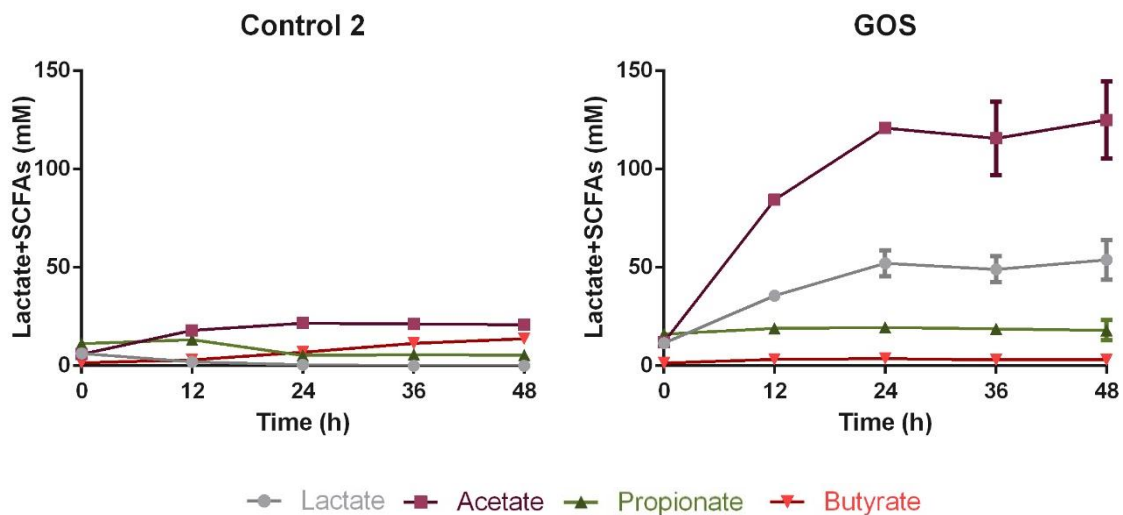
Figure 3: Lactate and SCFAs' production during a period of 48h of faecal human microbiota growth from one donor, in the absence of prebiotics (Control) and in a medium supplemented with a prebiotic solution of 10g/L GOS. The results are the average of two independent fermentations ± standard deviation.

The GOS supplementation resulted in a higher accumulation of acetate (20.96±0.66 mM for control 2 and 125.10±19.62 mM for GOS, $p<0.0001$) when compared to the control, corresponding to a 496±93% increase. The production of lactate was also observed in a total amount of (53.94±10.12

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mM, $p < 0.0001$). Similarly to FOS, in the control fermentation the consumption of lactate leading to a null accumulation after 48h was observed. The differences of the final concentration of propionate and butyrate were also statistically significant when compared to the control. The final concentration propionate increased in comparison with the control by 230±93% (5.49±0.30 mM for control 2 and 18.09±5.08 mM for GOS, $p < 0.001$) while the concentration of butyrate decreased by 77±10% (13.80±0.089 mM for control 2 and 3.05±1.41 mM for GOS, $p < 0.0001$).

4.1.3. Mixtures of FOS and GOS (GF)

The supplementation with different GOS and FOS mixtures resulted in different lactate and SCFA production profiles, as shown in Figure 4.
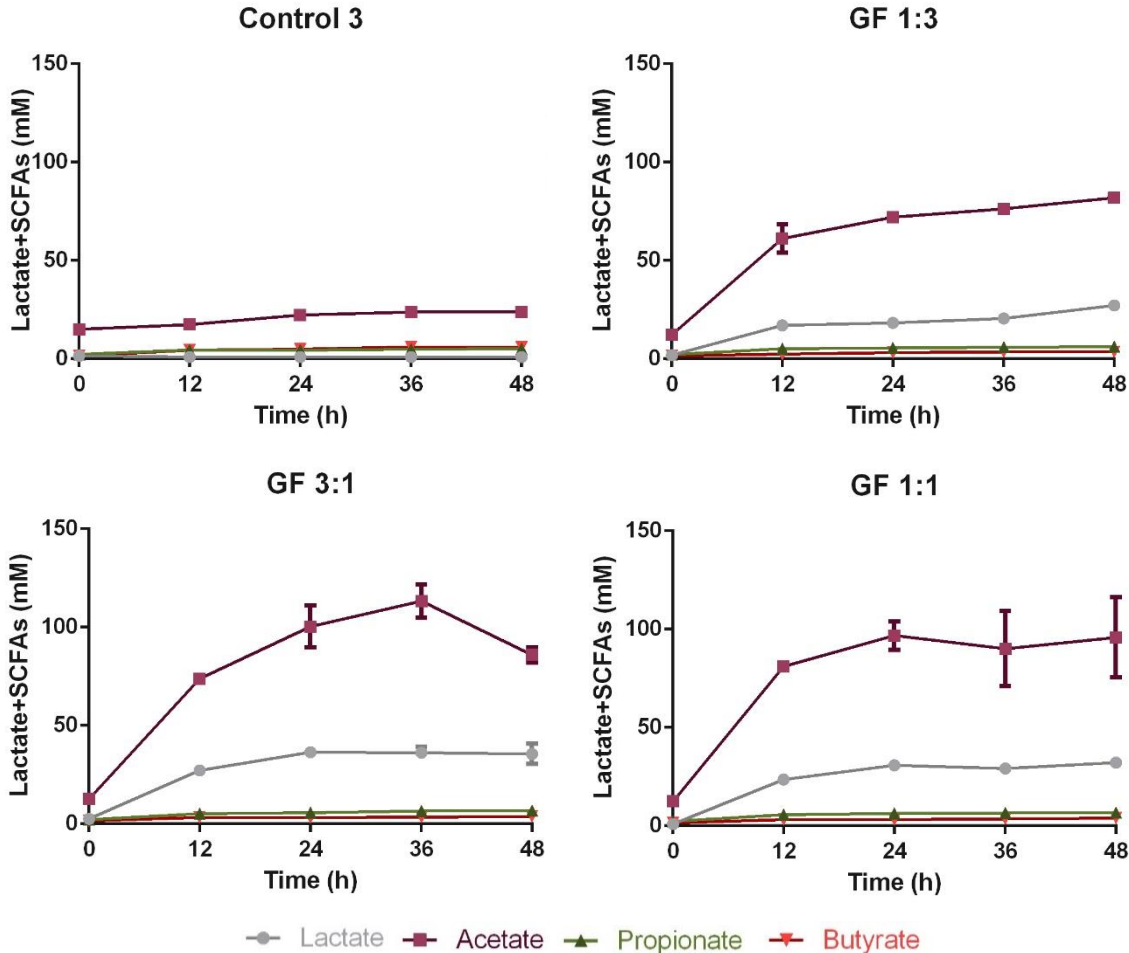
Figure 4: Lactate and SCFAs' production during a period of 48h of faecal human microbiota growth from one donor, in the absence of prebiotics (Control 3) and in a medium supplemented with a prebiotic solution of 10g/L of either GF 1:3, GF 3:1 or GF 1:1. The results are the average of two independent fermentations ± standard deviation.

In comparison with the control, the highest lactate accumulation was found for the mixture GF 3:1, with an increase of $3509 \pm 520\%$ (0.985 mM for control 3, 35.57 ± 5.12 mM for GF 3:1, $p < 0.0001$) after 48h. However, when compared to the other mixtures the lactate production of GF 3:1 was only significantly higher than GF 1:3 (27.15 ± 0.64 mM for GF 1:3, $p < 0.001$, and 32.13 ± 1.79 mM for GF 1:1, non-significant). In agreement with previous results (section 4.2.1 and 4.2.2), acetate was the major SCFA accumulated after 48h for all mixtures tested, presenting no significant difference amongst the mixtures. Butyrate accumulation slightly increased over time with and without supplementation, however, the final concentrations for each mixture (3.55 ± 0.17 mM for GF 1:3, $p < 0.01$, 3.79 ± 0.17 mM for GF 3:1, $p < 0.01$, and 3.91 ± 0.68 mM for GF 1:1, $p < 0.01$) were lower than the control (5.95 ± 0.51 mM). Furthermore, the propionate accumulation showed no significant differences comparing to the control.

Lactate and SCFAs production profiles presented in sections 4.1.1, 4.1.2 and 4.1.3, showed that acetate was the main SCFAs being produced regardless of the supplementation, followed by the organic acid lactate, with propionate and butyrate being the least SCFAs produced.

Lactate and acetate production were significantly higher in experiments using GOS rather than FOS. Rycroft and co-workers (Rycroft et al. 2001) reported comparable results with increased production of acetate, propionate and lactate resulting from GOS supplementation when compared to inulin, both added at a concentration of 10 g/L.

Since lactate and SCFAs production is related to bacterial metabolism, it is comprehensible that acetate was the most produced metabolite since its production pathways are present in many bacterial groups, while lactate, propionate and butyrate production pathways exhibit a more conserved distribution (Louis et al. 2007; Morrison and Preston 2016). It is also known that FOS and GOS promote the growth of *Bifidobacteria* which are known to be non-butyrogenic bacteria (Gibson et al. 2017; Louis et al. 2007).

The three experiments were carried in three different occasions, with different faecal samples from the same donor, and exhibited the similar lactate and SCFAs production profiles.

4.2. pH modification and ammonia accumulation

As previously discussed, *in vitro* fermentations also allow a better understanding of ammonia and pH variations comparing to *in vivo* studies. Even though the culture medium was designed to reproduce the distal colon pH at the baseline, the continuous production and accumulation of SCFAs causes an

abrupt decrease in pH which would not occur *in vivo* due to swift absorption (Macfarlane, Steed, and Macfarlane 2007).

SCFAs accumulation decreases the medium pH, promoting the reduction of pathogenic bacteria and allowing the increase of the population of beneficial bacteria. The shift in microbial population should also lead to alterations in the metabolic pathways, namely the prevalence of saccharolytic fermentation over proteolytic fermentation, which results in the diminished production of ammonia. Ammonia contributes to foul faecal odour and, being a toxic compound, can lead to colon carcinogenesis (Kolida and Gibson 2007; Louis et al. 2007; Power et al. 2014). Table 3 presents the final pH and ammonia concentration after 48h fermentation for the control and the supplemented fermentations.

Table 3: pH and ammonia concentration after 48h of faecal human microbiota growth from one donor in the absence of prebiotics (control) and in a medium supplemented with one of the following prebiotic mixtures: FOS, GOS, GF 1:3, GF 3:1 or GF 1:1 (10 g/L). The results are the averages of two independent fermentations ± standard deviation.

Assay	Control	Sample	*p* value
FOS			
pH	7.01±0.03	3.69±0.02	<0.0001
Ammonia (mg/L)	408.98±0.00*	97.75±3.18	<0.0001
GOS			
pH	6.87±0.04	3.61±0.02	<0.0001
Ammonia (mg/L)	397.49±4.28	73.60±2.40	<0.0001
GF 1:3			
pH	7.18±0.06	3.93±0.01	<0.0001
Ammonia (mg/L)	436.0±16.97	78.9±1.98	<0.0001
GF 3:1			
pH	7.18±0.06	3.78±0.014	<0.0001
Ammonia (mg/L)	436.0±16.97	47.85±2.05	<0.0001
GF 1:1			
pH	7.18±0.06	3.85±0.00*	<0.0001
Ammonia (mg/L)	436.0±16.97	61.85±0.64	<0.0001

* Both replicas presented the same experimental measurements, according to the equipment's precision

The results showed a consistent decrease in the pH value of all supplemented cultures, as well as a decrease in ammonia concentration. The highest pH reduction comparing to the control, was achieved by the mixture GF 3:1, with a difference of 3.4. Moreover, the same mixture presented the lowest concentration of ammonia, with a 9-fold reduction in comparison to the control fermentation.

Pompei et al. (2008) reported similar results with a significant decrease of pH value in *in vitro* static batch fermentations of faecal microbiota supplemented with oligofructose, a well-established prebiotic, in comparison to the control fermentations. These authors also described a lower concentration of ammonia as a result of oligosaccharide supplementation (Pompei et al. 2008).

Moreover, colonic microbiota will favour saccharolytic fermentation over proteolytic fermentation, causing acidification of the proximal colon where it is possible to find non-digested carbohydrates. In the distal colon, where pH is close to neutral, it occurs the majority of proteolytic fermentation due to depletion of fermentable carbohydrates (Korpela 2018). The availability of fermentable carbohydrates and acidic pH resulting from *in vitro* models, where distal colon conditions are mimicked, promote saccharolytic fermentation and subsequently, the reduction of the ammonia concentration.

4.3. Gas production

The fermentation of prebiotic oligosaccharides by faecal human microbiota can influence the production of gases such as carbon dioxide (CO_2), hydrogen (H_2) and methane (CH_4). CH_4 is only produced in individuals who harbour methanogenic bacteria in their gut microbiota (Ghoddusi et al. 2007). Gas production was analysed by GC of samples taken every 12h during the 48h fermentations supplemented with FOS (section 4.3.1.), GOS (section 4.3.2) and mixtures of GOS and FOS in different proportions, 1:3, 3:1 and 1:1 (section 4.3.3), with a final concentration of 10 g/L. The results of these analysis are presented below and discussed in section 4.3.3.

4.3.1. Fructooligosaccharides (FOS)

Figure 5 presents the CO_2, H_2 and CH_4 production profile during the 48h of fermentation supplemented with FOS. As expected, larger concentrations of CO_2 were obtained when comparing to the control fermentation with an increase of $85.91 \pm 12.12\%$ (6.15 ± 0.30 mmol/L for Control 1 and 11.43 ± 0.75 mmol/L for FOS, $p<0.0001$). The concentration of CH_4 also increased in comparison to the control by $48 \pm 9\%$ (0.952 ± 0.05 mmol/L for Control 1 and 1.41 ± 0.08 mmol/L for FOS, $p<0.001$). For H_2

production, no significant differences were observed comparing to the control fermentation with the supplemented fermentation. Overall, the CO_2 production was significantly higher than the other gases analysed.
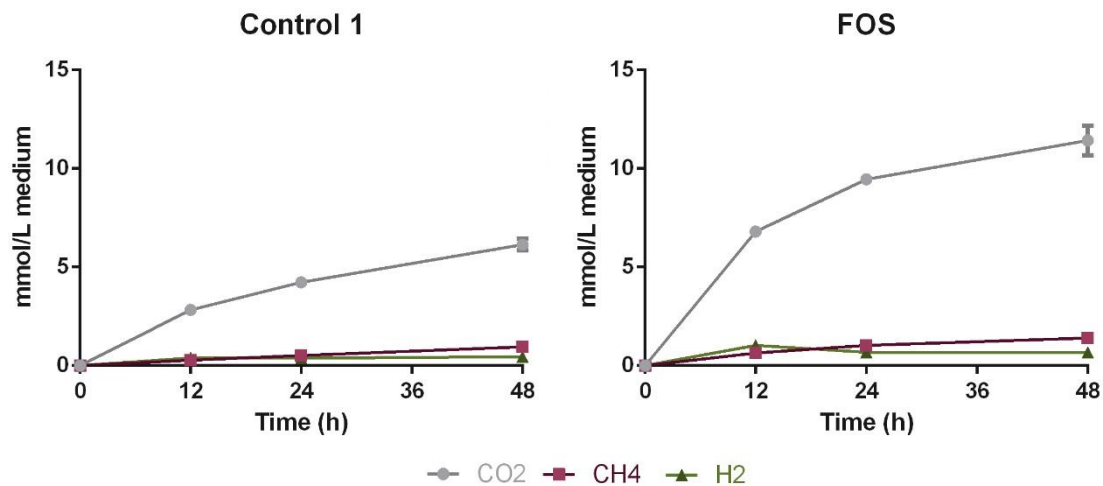
Figure 5: CO_2, H_2 and CH_4 production during a period of 48h of faecal human microbiota growth from one donor, in the absence of prebiotics (control) and in a medium supplemented with a prebiotic solution of FOS (10g/L). The results are the average of two independent fermentations and triplicate analysis of each sample ± standard deviation.

4.3.2. Galactooligosaccharides (GOS)

Figure 6 illustrates the CO_2, H_2 and CH_4 production profile during the 48h of fermentation supplemented with GOS. Similarly to FOS, larger concentrations of CO_2 were produced, with the maximum production occurring after 24h of fermentation (7.54±0.16 mmol/L, $p<0.0001$), having no significant differences when compared to the 48h, while the CO_2 produced in the control fermentation achieved its maximum at 48h (4.67±0.004 mmol/L, $p<0.0001$).

CO_2 production after 24h resulted in an increase of 151.51 ± 5.47%, comparing to the control, which decreased to a 67.38 ± 2.96% net difference at the last sample time of 48h. The production of H_2 showed no significant alterations throughout the fermentation times comparing to the control fermentation.

Interestingly, the GOS supplementation led to a decrease of 78.76% in the production of CH_4 when compared to the control. Furthermore, the supplementation with GOS resulted in no significant alterations in CH_4 concentration from 12h to 48h (0.14 ± 0.02 mmol/L and 0.19 ± 0.04 mmol/L, respectively), while in the control increased amounts of CH_4 were produced (0.33±0.04 mmol/L at 12h and 0.91±0.13 mmol/L at 48h, $p<0.0001$)

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H_2 production showed no significant differences as compared to the control fermentation with the supplemented fermentation.

Figure 6: CO_2, H_2 and CH_4 production during a period of 48h of faecal human microbiota growth from one donor in the absence of prebiotics (control) and in a medium supplemented with a prebiotic solution of GOS (10g/L). The results are the average of two independent fermentations and triplicate analysis of each sample ± standard deviation.
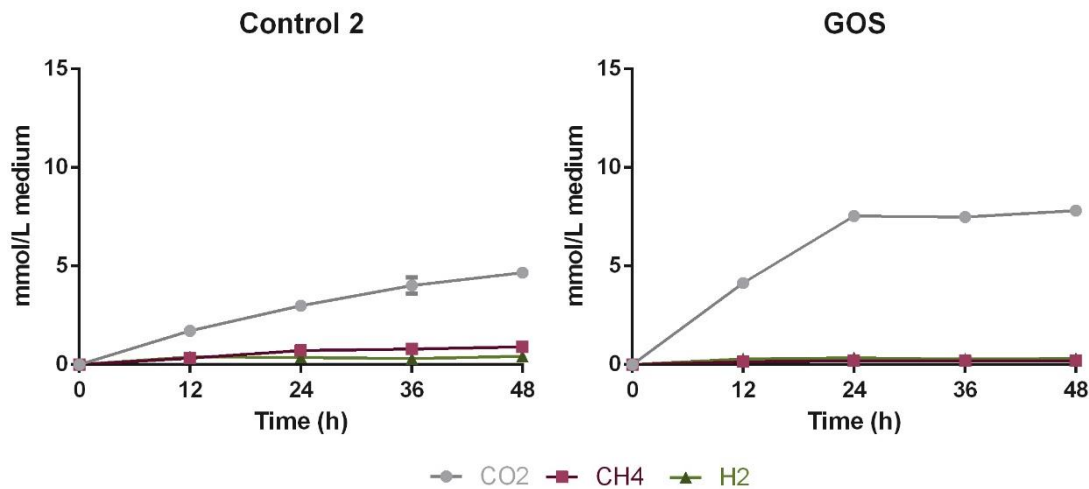
4.3.3. Mixtures of FOS and GOS (GF)

In accordance with the previous results (section 4.3.1. and 4.3.2.), the supplementation with FOS and GOS mixtures also resulted in a gas production profile with CO_2 being the predominant gas produced regardless of the ratio, as shown in Figure 7. After 48h of fermentation, the highest CO_2 production was achieved with GF 3:1 supplementation (9.82±0.16 mmol/L, $p<0.0001$) when compared to the control fermentation (4.33±0.33 mmol/L), yielding an increase of 126.73 ± 3.77%. Amongst the mixtures, statistical significance only allowed for the distinction between GF 3:1 and GF 1:1, with GF 3:1 being higher than the GF 1:1 (7.41 ± 1.40 mmol/L, $p<0.05$).

H_2 concentrations showed no significant differences after 48h when compared to the control and amongst the mixtures.

As for CH_4, the supplementation with the different mixtures led to significant reductions, when compared to the control fermentation after 48h (0.781±0.07 mmol/L for control 3, 0.43±0.05 mmol/L for GF 1:3, $p<0.05$, 0.33±0.02 mmol/L for GF 3:1, $p<0.01$ and 0.27±0.08 mmol/L for GF 1:1, $p<0.01$). Even though no significant differences amongst the mixtures were found, there was an overall reduction of 56.26 ± 10.61% when comparing the final concentration of CH_4 produced by the supplemented fermentations to the control fermentation.
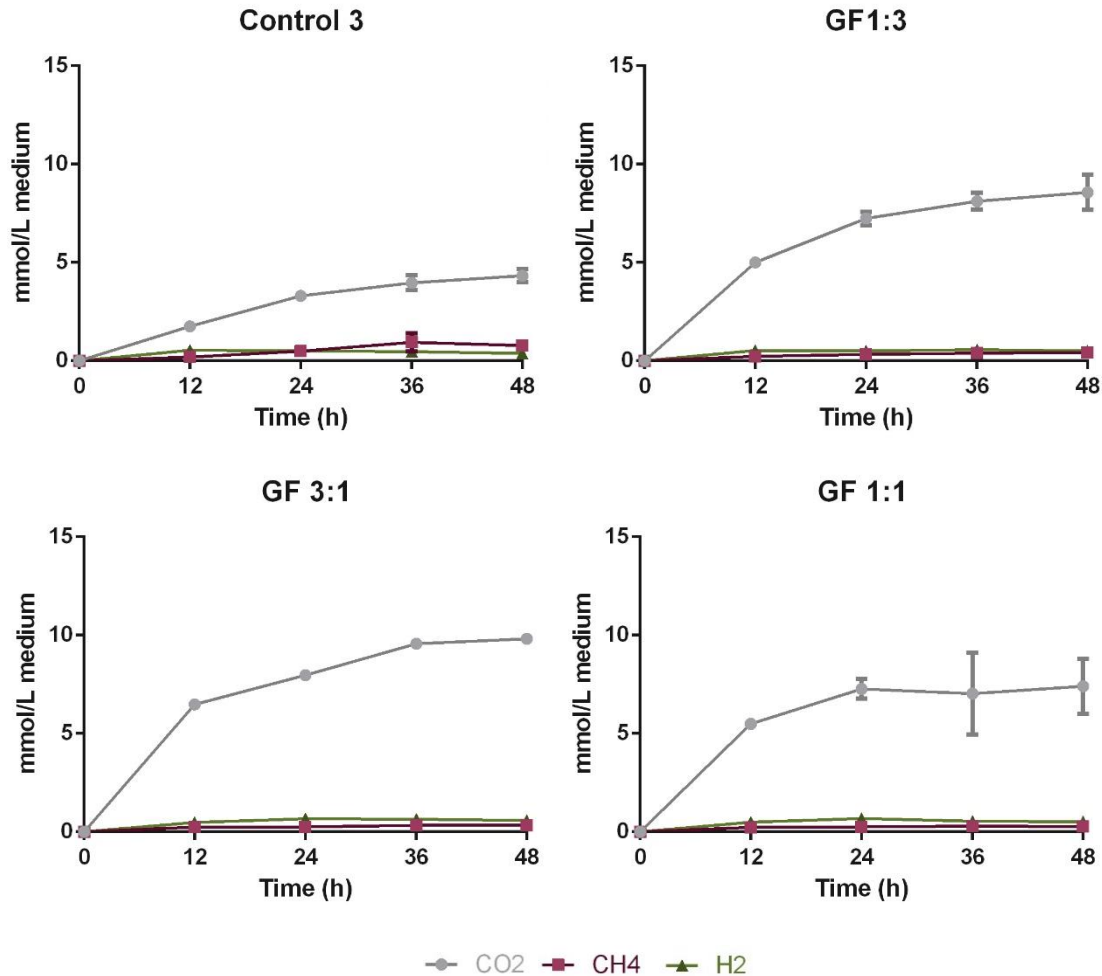
Figure 7: CO_2, H_2 and CH_4 production during a period of 48h of faecal human microbiota growth from one donor in the absence of prebiotics (control) and in a medium supplemented with a prebiotic solution 10g/L of either GF 1:3, GF 3:1 or GF 1:1. The results are the average of two independent fermentations and triplicate analysis of each sample ± standard deviation.

The gas production profiles presented in sections 4.2.1, 4.2.2 and 4.2.3 relative to the prebiotic supplementation revealed that overall CO_2 was the most produced gas, H_2 production was not affected by none of the studied supplementations and CH_4 production was significantly reduced in the presence of GOS supplementation.

The highest total gas production occurred as a result of FOS supplementation while GOS supplementation led to lower overall gas production. Similar results were achieved by Hernot and co-workers (Hernot et al. 2009) and Rycroft and co-workers (Rycroft et al. 2001) that compared the effect of different oligosaccharides. Hernot and co-workers, after evaluating the gas production profile of fructans with different chain lengths and DP and other oligosaccharides, such as GOS, concluded that due to its branched structure, agave inulin was more rapidly fermented yielding the highest amounts of gas, while GOS yielded more moderate gas production (Hernot et al. 2009). Similarly, in the results reported by

Rycroft and co-workers, the gas production profiles obtained revealed that FOS and inulin produced the highest amounts of total gas, while GOS resulted in the lowest production. GOS are considered more competent in increasing number of *Bifidobacteria* than FOS, which could explain the differences found here in gas production, since these bacteria usually generate lower gas volumes.

As mentioned in section 1.3, only some individuals harbour methanogenic bacteria in their gut microbiota. Elevated CH_4 production has been associated with obesity, colorectal cancer, irritable bowel syndrome and chronic constipation. In fact, said gas has been shown to reduce intestinal transit time by 59% (Gaci et al. 2014; Lurie-Weinberger and Gophna 2015). Therefore, further studies on the ability of GOS and GF mixtures to reduce methane production should be carried out.

The three experiments herein performed were carried in three different occasions, with different faecal samples from the same donor, and exhibited the similar gas production profiles.

4.4. Selection of the prebiotic supplementations with higher potential

To select the supplementations with higher prebiotic potential, lactate, SCFA, ammonia and gas production were used as criteria.

Figure 8 presents the concentration of lactate and total SCFAs overtime in relative percentages compared to the control. The fermentations supplemented with GOS, GF 1:3, GF 3:1 or GF 1:1 showed an increased production of lactate and SCFA at an early time point (12h), having reached maximum values at 24h (570.51 ± 29.02% for GOS, 302.89 ± 6.12% for GF 1:3, 445.72 ± 37.31% for GF 3:1 and 418.97 ± 21.84% for GF 1:1). The supplementation with FOS resulted in non-significant changes in lactate and SCFA production overtime, thus being considered as less promising. The production at 24h with single GOS supplementation was higher than with GF mixtures ($p < 0.0001$ when compared with GF 1:3, $p < 0.01$ when compared with GF 3:1 and GF 1:1). Concerning the mixture supplementation, GF 3:1 showed the highest production (444.67±35.17% at 36h). However, it was only significantly higher when compared to GF 1:3 (296.38±9.75% for GF 1:3, $p < 0.01$, 360.60±60.61% for GF 1:1, non-significant).

The production of lactate and SCFA led to a significant acidification of all culture media regardless of the supplementation.
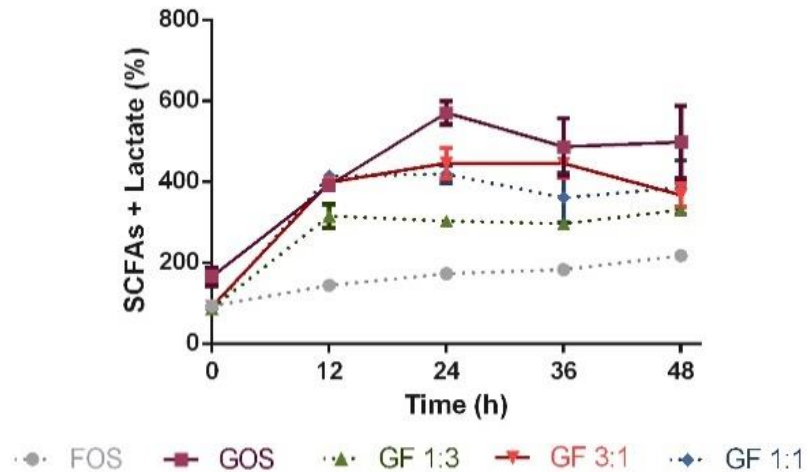
Figure 8: Lactate and short-chain fatty acids (SCFAs) production during a period of 48h of faecal human microbiota growth from one donor, in the absence of prebiotics (control) and in a medium supplemented with a prebiotic solution of either FOS, GOS, GF 1:3, GF 3:1 or GF 1:1 (10g/L). The results presented in relative percentages compared to the control of each experiment, are the average of two independent fermentations ± standard deviation.

Figure 9 shows the reduction in ammonia concentration (in relative percentages compared to the control) due to prebiotic supplementation. All supplemented fermentations resulted in significantly lower ammonia concentrations (24.39±0.09% for FOS, 18.52±0.61% for GOS, 18.10±0.45% for GF 1:3, 10.98±0.47% for GF 3:1 and 14.19±0.15% for GF 1:1). The supplementation with single GOS presented lower percentage of ammonia concentration than single FOS ($p<0.0001$). While for the supplementation with mixtures, GF 3:1 showed the lowest concentration of ammonia ($p<0.0001$ compared with FOS, GOS and GF 1:3 and $p<0.01$ compared with GF 1:1).
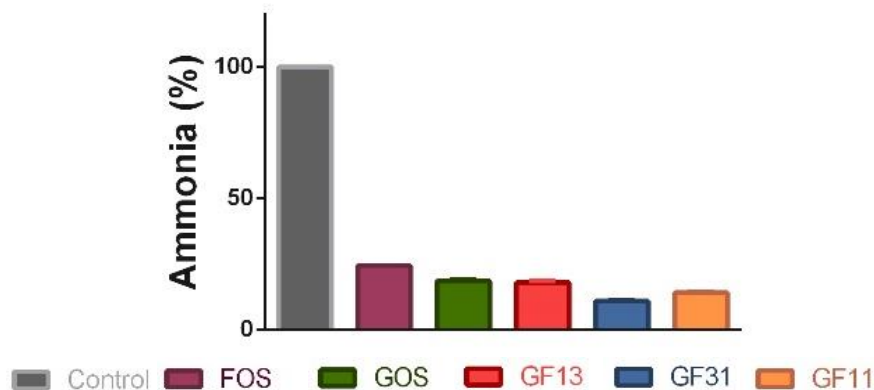
Figure 9: Ammonia concentration after 48h of faecal human microbiota growth from one donor in the absence of prebiotics (control) and in a medium supplemented with a prebiotic solution of either FOS, GOS, GF 1:3, GF 3:1 or GF 1:1 (10g/L). The results presented in relative percentages compared to the control of each experiment, are the

The total gas (CO_2, CH_4 and H_2) production profile during the 48h fermentations is represented in Figure 10 in relative percentages compared to the control. After 12h of fermentations a production peak was observed for the majority of the prebiotics (178.74±10.98% for FOS, 187.49±7.93% for GOS, 230.78±3.87% for GF 1:3, 288.08±9.87% for GF 3:1 and 249.03±1.45% for GF 1:1). FOS, GF 1:3, GF 1:1 and GF 3:1 supplementation showed similar gas production profiles. The supplementation with single GOS and GF 3:1 led to the lowest and the highest concentration of total gas, respectively, however, both conditions showed the ability to reduce methane production (Appendix I).
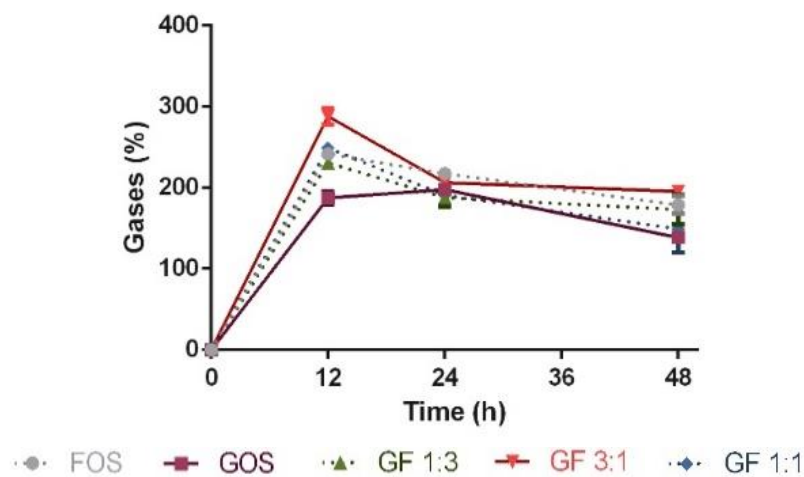
Figure 10: CO_2, H_2 and CH_4 production during a period of 48h of faecal human microbiota growth from one donor in the absence of prebiotics (control) and in a medium supplemented with a prebiotic solution of either FOS, GOS, GF 1:3, GF 3:1 or GF 1:1 (10g/L). The results presented in relative percentages compared to the control of each experiment, are the average of two independent fermentations and triplicate analysis of each sample ± standard deviation.

Considering the lactate and SCFAs production, pH reduction, ammonia concentration and gas production parameters, GOS and GF 3:1 were selected as the most promising prebiotic individual oligosaccharide and mixture to be incorporated in a food matrix (section 3.6).

4.5. Selection of a recipe of the traditional Portuguese rice cake ("bolo-de-arroz")

The food matrix studied in this work was the traditional Portuguese cake, "bolo-de-arroz". This matrix was previously selected under the scope of the "NewFood" project that aims to innovate traditional Portuguese foods. The selection of the original cake recipe for subsequent modification, was performed through sensory testing of three different formulations of rice cake. All recipes were available online in cooking websites. Comparatively, recipe 1 possessed more milk and wheat flour, while recipe 2 possessed more butter and recipe 3 (a recipe from the margarine brand "Vaqueiro") possessed more eggs and rice flour (Table 1, section 3.6). The percentage of sugar in the three recipes was similar. Figure

11 represents the sensory profiles obtained for each recipe, from the answers to questionnaire 1 (Appendix II) regarding appearance, aroma, flavour, texture and overall impression.

The panellists showed low acceptance of the recipe 3, which presented significant lower scores in all the evaluated parameters. No significant differences where observed between recipes 1 and 2.

Based on the overall evaluations recipe 3 was considered the less marketable attending the goal of the project.
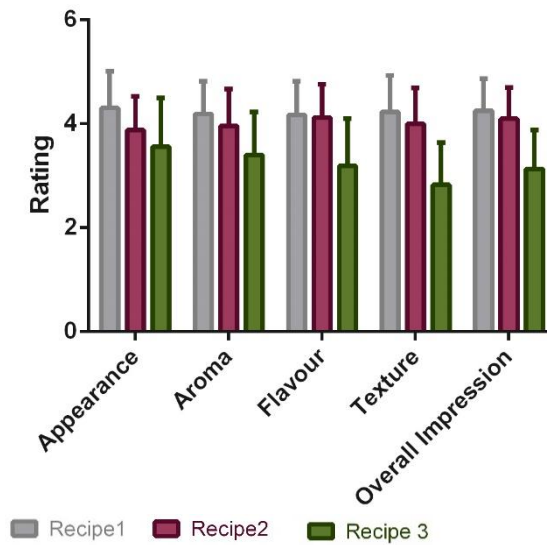
Figure 11: Sensory profiles of the 3 recipes regarding appearance, aroma, flavour, texture and overall impression, rated from 1 to 5 by 33 female panellists and 19 male panellists. The results are presented in average± standard deviation.

Since the sensory parameters were inconclusive for the selection of a single recipe, the question "which of the samples best resembled a traditional Portuguese rice cake?" was taken into account as a decision-maker parameter. Thus, the most scored recipe was recipe 1 (Figure 12).

75% Recipe 1   25% Recipe 2
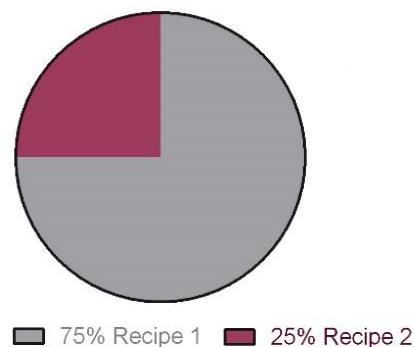
Figure 12: Evaluation of the similarity between recipes 1 and 2 cakes and the traditional Portuguese rice cake. The results are presented in percentages.

Different rice cakes were produced based on modifications to the recipe 1 (section 3.6) and presented to 56 untrained panellists (36 females and 20 males). Cakes obtained from recipe 1 were used as control, previously described in section 3.7, while three modified recipes were presented: a 25% sugar reduction (recipe 4), a 25% sugar reduction and addition of GOS (6% w/w) (recipe 5) and 25% sugar reduction and addition of GF 3:1 (6% w/w) (recipe 6). Panellists evaluated the presented recipes in two different stages by answering questionnaires 2 (Appendix III) and 3 (Appendix IV).

The aim of the questionnaire 2 was to determine the sensory differences between the recipes. During this taste test, the panellists had no knowledge of the composition of each recipe.

The panellists were asked to evaluate the appearance, aroma, flavour, texture and overall impression from 1 to 5. Figure 13 represents the sensory profiles obtained for each recipe.

Regarding appearance, recipe 4 was more accepted than recipe 1 ($p<0.0001$) and recipe 5 ($p<0.001$), however showing no significant differences when compared to recipe 6. Recipe 6 was also more accepted comparing to recipes 1 ($p<0.0001$) and 5 ($p<0.01$). The aroma and flavour did not show significant differences among recipes, meaning that the reduction of sugar and addition of prebiotics did not influence the sensory profiles of the cakes. Therefore, those who eat the regular recipe would also accept the modified ones. In terms of texture, recipes 4 and 5 showed slightly higher rating than recipe 6 ($p<0.5$), although none of the modified recipes revealed clear differences. The overall impression only allowed the discrimination between recipes 1 and 6, with recipe 1 being preferred ($p<0.5$).
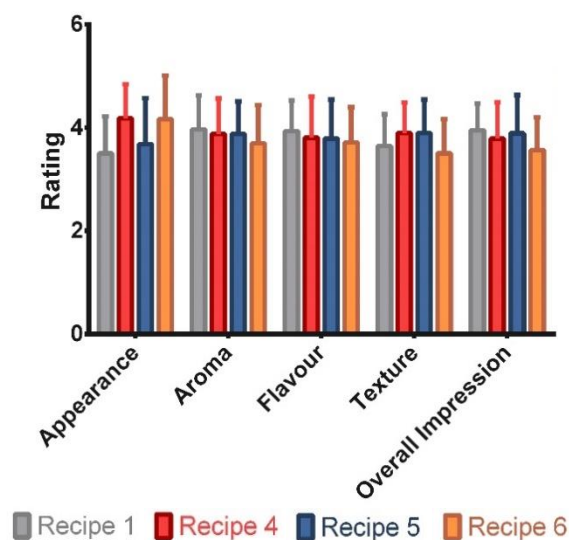
Figure 13: Sensory profiles of the recipes 1, 4, 5 and 6, regarding appearance, aroma, flavour, texture and overall impression, rated from 1 to 5 by 56 panellists. The results are presented in average± standard deviation.

Overall, the only conclusive factor was appearance. As seen in Figure 14, the cake baked according to recipe 1 presented a browner colour compared to the other samples, which probably resulted from Maillard reactions during the baking process. Maillard reactions occur due to condensation of amino acids with carbonyl groups on reducing sugars, such as glucose and galactose, which ultimately leads to the formation of melanoidins and browning. These reactions have implications on food colour and taste with their rate depending on different factors such as temperature/time combination (Lund and Ray 2017). Since among the recipes presented for sensory testing, recipe 1 contained the highest amount of sugar, it is understandable the development of a stronger brown colour comparing to the sugar reduced recipes (4, 5 and 6). Furthermore, in a study performed by Zhang et al. (2019) the incorporation of GOS in a food matrix led to reduced browning during heating (Zhang et al. 2019) which is also in accordance to the results obtained, since the sample from recipe 5 (Figure 14 C) presented the lightest colour of the group.
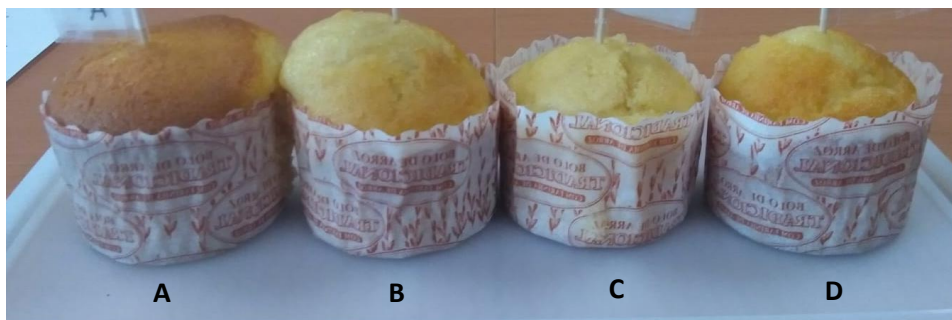
Figure 14: Representative samples of the traditional Portuguese rice cakes baked according to recipe 1 (A), recipe 4 (B), recipe 5 (C) and recipe (D), under the conditions described in section 3.6.

The remaining sensory factors revealed that a 25% sugar reduction and the addition of the prebiotic mixtures exhibited no influence on the global organoleptic characteristics comparing to the original recipe.

Also in questionnaire 2, panellists were asked to refer whether they would refrain from buying any of the samples provided and to explain the reason based on the sensory parameters. 48% of the panellists indicated they would buy cakes from any recipe if available on the market and 52% would reject one or more of the recipes presented, basing their choice on texture and flavour. The rejection rates of each recipe are presented in Figure 15.

Upon analysis of Figure 15, it was possible to conclude that recipe 6 was not approved by 21% of panellists followed by recipe 1, with 14% of rejection. Without knowing the composition of each cake,

the most accepted recipes were recipe 4 and 5 which had a 25% sugar reduction and 25% sugar reduction and GOS addition, respectively.
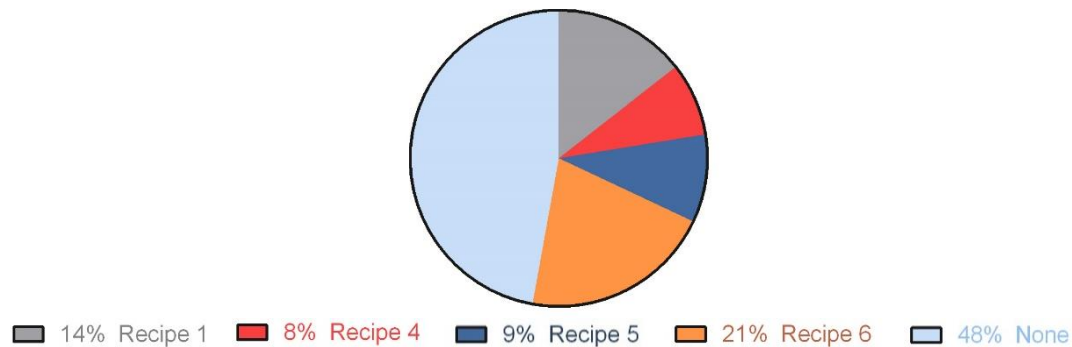
Figure 15: Representation of the rejection rates for recipes 1, 4, 5 and 6 upon sensory testing, without the knowledge of the composition of each recipe. The results are presented in percentages.

The goal of questionnaire 3 was to establish the panellist's perception of lifestyle and functional food consumption. In this questionnaire, panellists were informed of the composition of the cakes tested in order to establish if the knowledge of the dietary benefits would influence their opinion when buying such products.

In general, panellists who participated in the taste tests lived healthy lifestyles with only 14% perceiving to have unhealthy eating habits. The functional food consumption, however, did not reflect the trend in healthy lifestyle with 61% admitting consuming such products. This result may suggest that there is still room for expansion in this industry namely improving foods that are not necessarily considered as healthy options (such as cakes) to reach a great number of consumers.

After disclosing the modifications applied to each recipe, only 28% would buy cakes from any recipes, meaning that the knowledge of the composition of each recipe reduced the acceptance of one or more recipes. Of those who would reject some recipes (Figure 16), 41% would reject recipe 1, here the unhealthiest option, meaning the panellists would value, when available, healthier options. These results also revealed that, even though the panellists favoured healthier options, the benefits brought by the additions of the prebiotics were not totally valued (recipe 4, with 25% sugar reduction, presented lower rejection than recipes 5 and 6 with prebiotic addition).

Based on the results obtained, recipe 4 was found to be the preferred by the panellists. Nonetheless, considering recipes with prebiotic addition, recipe 5 (25% sugar reduction and 6% addition of GOS) was the most accepted (only 10% rejection).
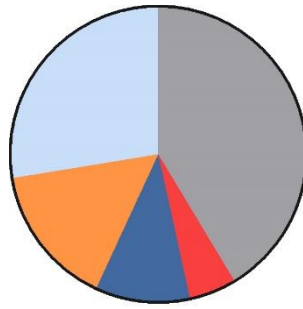
Figure 16: Representation of the rejection rates for recipes 1, 4, 5 and 6, upon disclosure of the modification applied to each one. The results are presented in percentages.

Since sugar reduction and prebiotic addition did not seem to affect the sensory characteristics evaluated, the low acceptance of recipes containing prebiotics may be associated to the lack of information easily available to the consumers, which possibly impairs their judgement. Although functional foods are widely available in the market, e. g. probiotic rich yogurts and vitamin supplemented milks, the consumer seems not to be yet familiarized with the prebiotic concept. Therefore, in order to successfully introduce foods supplemented with prebiotics into the Portuguese market, the consumer should be clearly informed and educated on the subject. Specific information on the product label and advertising campaigns about the benefits of prebiotics may help to increase the consumption of functional foods containing prebiotics.

5. General Conclusions and Future Perspectives

The main aim of this project was to determine the prebiotic potential of commercial FOS and GOS, individually and in mixtures, to be further incorporated in a food matrix. To fulfil those purposes, static batch fermentations were performed to select the substrates with the highest prebiotic potential, and sensory tests were conducted to determine the most accepted food matrix.

Static *in vitro* batch fermentations inoculated with human faecal served as a preliminary assessment of the prebiotic potential of FOS and GOS. The fermentation medium simulated the distal colon conditions, and it was supplemented with oligosaccharides. The SCFAs and gas production profiles, as the ammonia generation and pH variation were evaluated for 48h.

The increased SCFAs and gas production together with the reduction of ammonia concentration and the final pH value suggest that both FOS and GOS present prebiotic potential, as expected, since these oligosaccharides have had functional properties recognized for over 20 years.

The production of lactate, a SCFAs precursor, was also evaluated. High concentrations of lactate were obtained with the supplementation of oligosaccharides, in particular GOS. Overall, acetate was the most produced SCFAs followed by propionate with the highest accumulation resulting from GOS supplementation.

CO_2 was the main fermentation gas produced as a result of all supplementations. This result highlights the importance of determining a recommended daily dose to prevent gas related discomfort. Interestingly, GOS were found to significantly reduce the CH_4 production. Since methane production has been associated with diseases such as obesity, colorectal cancer and irritable bowel syndrome, further studies on this topic should be performed in order to establish new treatments or strategies to minimize the effects of methane in the organism.

GOS and the mixture GF 3:1 were selected for further incorporation in the food matrix, since they presented high lactate and SCFAs production profiles, lowest accumulation of ammonia and presented the ability to reduce methane production.

The traditional Portuguese rice cake was the food matrix previously chosen for this study under the scope of the NewFood project. Three traditional cake recipes were presented to the untrained panellists who selected recipe 1 as the one that most resembled the traditional recipe.

Recipe 1 was modified with sugar reduction and oligosaccharide addition and presented to untrained panellists for sensory testing, in order to determine the market acceptability. The panellists elected recipes 4 (25% sugar reduction) and 5 (25% sugar reduction and 6% w/w GOS addition) as the most accepted recipes. In addition to the sensory test, panellists were also asked to indicate whether they lived healthy lifestyles and if they consumed functional foods. It was found that 86% of the panellists have healthy lifestyles 61% consume functional products. Even though healthier options are valued by the panellists, who rejected the original recipe by 41% after the recipes' composition was disclosed, the addition of oligosaccharides with prebiotic potential was met with resistance with recipe 6 (25% sugar reduction and GF 3:1 (6% w/w)) being the most rejected of the modified recipes. It can be assumed that this resistance is due to lack of knowledge about prebiotics, since 61% consume functional products, including foods with probiotics, the consumption of food products enriched with prebiotics was not expected to be undervalued.

Further research on the assessment of the prebiotic potential of the studied oligosaccharides should include a more complex *in vitro* model able to reproduce different parts of the gut, namely the proximal colon, with pH monitoring during the fermentation period. Given the interindividual gut microbiota diversity, to better established the prebiotic potential of oligosaccharides, a more heterogenous sample should be tested, including individuals from different age and sex groups, presenting varied dietary habits and health backgrounds. New studies should include *in vitro* assessment of microbiota modulations by metabolomic and metagenomic techniques such as high-throughput sequencing analysis, and determination of the recommended daily dose, especially for the new GF 3:1 mixture.

Further tests should also be carried out to study the effect of prebiotic addition on organoleptic properties, as well as on how to manipulate those properties while maintaining health promoting benefits. *In vivo* studies should be further performed to possibly introduce the developed cake on the market under the status of a functional food product with health benefits. To make these products appealing to consumers, efforts should be made to clearly inform and educate potential consumers on the benefits associated to the consumption of functional foods.

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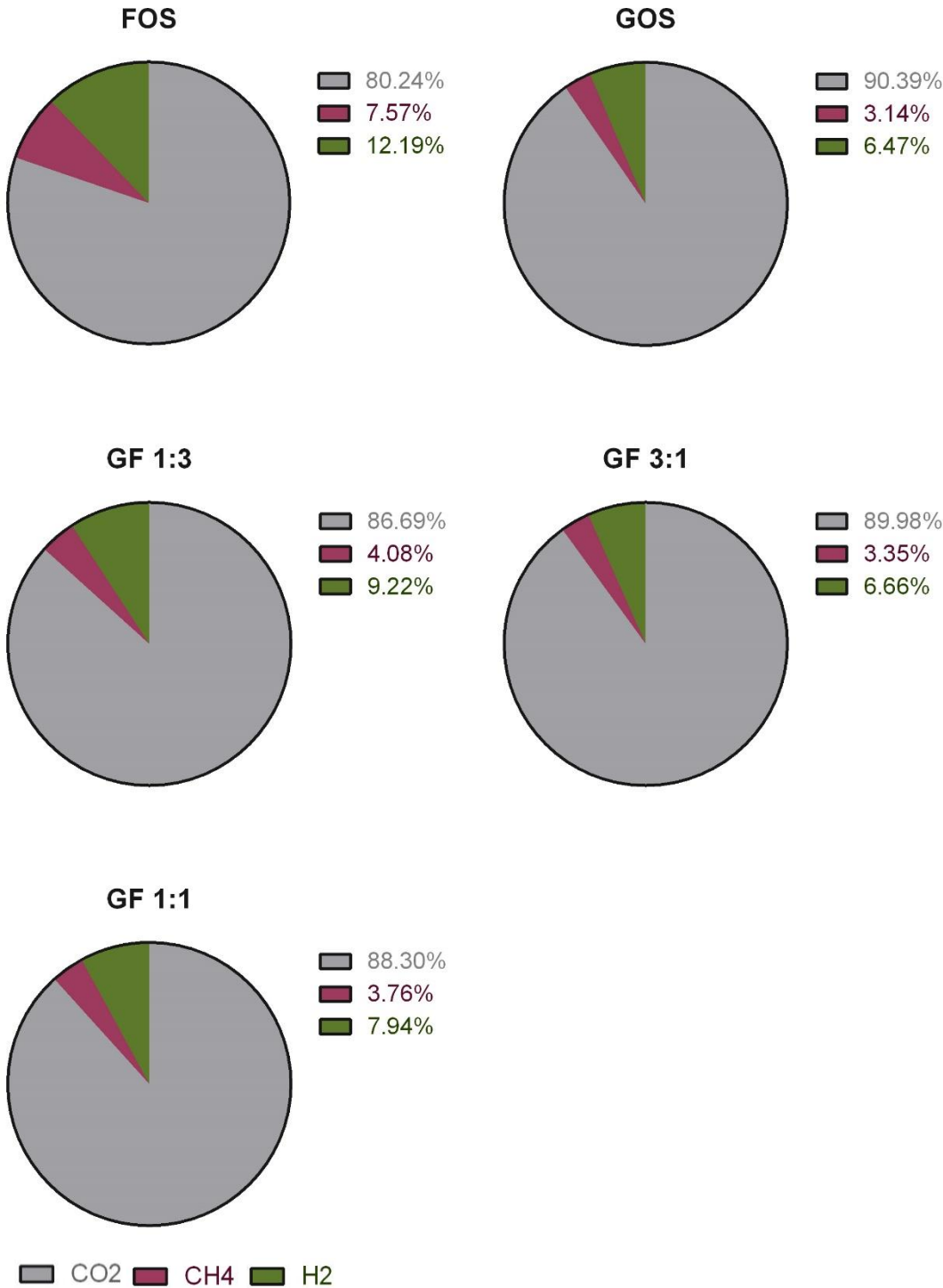
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FOS

- 80.24%
- 7.57%
- 12.19%

GOS

- 90.39%
- 3.14%
- 6.47%

GF 1:3

- 86.69%
- 4.08%
- 9.22%

GF 3:1

- 89.98%
- 3.35%
- 6.66%

GF 1:1

- 88.30%
- 3.76%
- 7.94%

CO2 CH4 H2

Figure S 1: CO_2, H_2 and CH_4 accumulation after 12h of faecal human microbiota growth from one donor, in a medium supplemented with either FOS, GOS, GF 1:3, GF 3:1 or GF 1:1 (10g/L). The results presented in relative percentages compared to the control of each experiment, are the average of two independent fermentations and triplicate analysis of each sample.

Universidade do Minho
Escola de Engenharia
Departamento de Engenharia Biológica

Prova sensorial: Produto alimentar

Género: F ☐ M ☐   Idade: _____

Estamos a efetuar uma pesquisa sobre a preferência do consumidor para o produto apresentado. Prove as amostras e classifique-as de acordo com a sua preferência.

Classificação:
1. Detestei
2. Não gostei
3. Gosto pouco
4. Gostei
5. Adorei

Amostra	Aparência	Aroma	Sabor	Textura	Impressão global

Qual a amostra que mais se assemelha ao bolo de arroz tradicional? _____

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Teste de comparação múltipla

Esta prova faz parte da dissertação de mestrado da aluna Jessica Silva, e do projeto NewFood.

As amostras apresentadas contêm apenas ingredientes comerciais seguros e validados, disponíveis no mercado para consumo público. Provadores com disbiose intestinal ou doenças autoimunes estão excluídos da prova por precaução.

Alergénios: Leite, ovos, trigo

Género: _____   Idade: _____≤25   _____≥25

Caro provador, avalie cada uma das amostras apresentadas e classifique-as de acordo com a sua preferência segundo a classificação apresentada, entre cada amostra beba um pouco de água:

Classificação:

1.Detestei   2.Não gostei   3.Gosto pouco   4.Gostei   5.Adorei

Amostra	Aparência	Aroma	Sabor	Textura	Impressão global

Deixaria de comprar alguma(s) amostra(s)?

_____

Se sim, qual/quais e quais os parâmetros da tabela anterior que considera relevantes para a sua exclusão?

_____

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Género: _____   Idade: _____≤25   _____≥25

É consumidor de produtos funcionais que promovem a saúde? _____

Segue um estilo de vida saudável? _____

       Prebióticos são substratos utilizados seletivamente por microrganismos presentes num hospedeiro, conferindo benefícios à saúde do mesmo como redução de colesterol, aumento da absorção mineral e estimulação do crescimento de bactérias benéficas em detrimento de bactérias patogénicas. Na confeção das amostras foram utilizados 2 tipos de prebióticos, fruto-oligossacáridos (FOS) e galacto-oligossacáridos (GOS).

       Da esquerda para a direita, as amostras apresentadas no seu prato possuem:

100% Açúcar (A);   -25 % de açúcar (B);   -25 % de açúcar e adição de GOS (C) e   – 25 % de açúcar e adição de GOS e FOS na proporção de 3:1 (D).

Atendendo à informação prestada e ao teste realizado anteriormente, compraria alguma das amostras, se disponível no mercado? Quais? _____

Qual a amostra que não compraria novamente? _____