In vitro fermentation of raffinose to unravel its potential as prebiotic ingredient

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ABSTRACT

Until now the prebiotic potential of pure trisaccharide raffinose on human health assessed through high-throughput sequencing remains poorly investigated. In this work, an *in vitro* model using human fecal inocula of two healthy volunteers (D1 and D2) was used to study the prebiotic potential of raffinose and compare it with the well-stablished and commercial prebiotic lactulose. The intestinal microbiota showed preference for raffinose as substrate showing the highest consumption value at 48 h ($96.0 \pm 0.9\%$ D1 and $95.3 \pm 0.7\%$ D2). The fermentation of raffinose decreased the medium pH, the ammonia concentration and the relative amount of *Proteobacteria*, while increasing the total production of lactate and short chain fatty acids (129.9 ± 2.6 mmol/L D1 and 179.6 ± 0.6 mmol/L D2), CO_2 (10.8 ± 0.8 mmol/L_{medium} D1 and 5.2 ± 0.3 mmol/L_{medium} D2) and the relative amount of *Bifidobacterium* and *Lactobacillus*. This study suggests that raffinose holds potential functional properties for human health.

1. Introduction

The loss of food functional properties due to the current industrial processing and the growing changes on people modern lifestyles associated with unhealthy eating behaviors are among the negative consequences of the globalization impact (Adamberg et al., 2014). As a result, the global epidemic of diet-related diseases had become a major public health issue (WHO - World Health Organization, 2018). However, the consumer awareness has increased towards the importance of using food as preventive medication, being willing to pay more for healthier eating options (Forbes, 2015). Thus, bioactive compounds produced from a natural source are currently preferred to modern therapeutic agents (Samanta et al., 2015). In particular, the use of bioactive compounds, such as prebiotics, has been shown to be very attractive given their role on modulating the gut microbiota and the subsequent beneficial effects on human and animal health over different physiological functions. The link between gut microbiota health and several different disorders, e.g. disbyosis caused by antibiotics intake or food poisoning, has been the recent focus of increased research (Kang et al., 2019; Yeung, Mocan, & Atanasov, 2018).

Recently updated by the International Scientific Association for Probiotics and Prebiotics (ISAPP), the current definition establishes a prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit", and the host can be either human or animal (Gibson et al., 2017). Prebiotic beneficial effects have been reported on the gastrointestinal tract, cardiometabolism, skeletal system, nervous system, among others (Gibson et al., 2017; Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013).

The global market demand of prebiotic is in expansion, registering 623.5 kton in 2015 and growing at a compound annual growth rate of 8.0% from 2016 to 2024 (Grand View Research, 2018). Prebiotic carbohydrates, particularly oligosaccharides, are the most widely studied and the best market established type of prebiotics. These compounds are also marketed beyond their traditional functional properties, being industrially used for instance as sugar and fat replacement, as well as taste and texture enhancement (Das, Choudhary, & Thompson-Witrick, 2019; Saremnezhad, Zargarchi, & Kalantari, 2020). Prebiotic oligosaccharides present favorable organoleptic properties, temperature and acidic stability which make them potential food ingredients (Zhao et al., 2017).

However, new potential prebiotic and less explored oligosaccharides have emerged as possible competitors, namely raffinose. Raffinose is an oligosaccharide composed by galactose, glucose, and fructose residues that can be hydrolyzed to D-galactose and sucrose by the enzyme α-galactosidase (α-GAL) (Tester & Karkalas, 2003). This trisaccharide is non-digestible to humans but can be metabolized by colon bacteria

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(Fernando et al., 2010), therefore being considered a prebiotic candidate. Furthermore, raffinose can be produced from cheap and abundant agro-residues such as soybean waste, holding a promising environmental and economic advantage.

Prebiotic oligosaccharides are used by the gut microbiota to growth and to produce short-chain fatty acids (SCFAs), lactate (a known precursor of SCFAs) (Flint, Duncan, Scott, & Louis, 2015), gases (mostly carbon dioxide and hydrogen), among other products (Topping & Clifton, 2001). The most common SCFAs being produced are acetate, propionate and butyrate, widely reported as key metabolites for intestinal health, benefiting also other body sites than the gut (Gibson et al., 2017).

Different approaches for testing the prebiotic potential of new compounds have been described. However, an *in vitro* model is considered a more suitable first-stage strategy to screen prebiotics candidates (Date et al., 2014), contrarily to in vivo studies which are expensive and time-consuming.

In agreement with the recent International Scientific Association for Probiotics and Prebiotics (ISAPP) guidelines, the *in vitro* models should include the use of fecal inocula and accurate high-throughput sequencing techniques to evaluate the microbial modulation. The prebiotic potential of the raffinose family of oligosaccharides has been reported using single or co-cultured microorganisms' models (Martínez-Villaluenga, Frías, Vidal-Valverde & Gómez, 2005; Ose et al., 2018). Nonetheless, in the light of ISAPP guidelines, these approaches cannot mimic the complex diversity of the intestinal microbiota (Gibson et al., 2017). Up to now, studies regarding the prebiotic effect of pure trisaccharide raffinose meeting the ISAPP requirements are scarcely reported. In this study, an *in vitro* batch culture model was used to evaluate the prebiotic potential of raffinose and compare it with commercial lactulose, using human fecal inocula of two adult healthy donors. Among the most widely studied and commercialized prebiotics, lactulose presents a chemical structure that better resembles raffinose, therefore comprising an interesting comparison model. Next generation sequencing techniques, namely Illumina MiSeq analysis was used to evaluate microbiota diversity. To the authors' knowledge, this is the first study exploring the prebiotic potential of trisaccharide raffinose, used as a pure substrate and evaluated under an experimental approach compatible with the ISAPP guidelines.

2. Experimental

2.1. Materials

All chemicals and media components used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO), including commercial raffinose, unless specified otherwise.

2.2. Fecal inoculum

Fecal samples were collected from two healthy human volunteers (Donor 1, coded D1 and donor 2, coded D2), both aged 26, who were on a non-specific Mediterranean diet, had no metabolic and gastro-intestinal diseases, were non-smokers and had not received any antibiotics, pre- or probiotic supplements for at least 3 months before the fecal donation. A voluntary informed consent was obtained from the two fecal donors prior to this study. The fecal samples were 1:10 (w/w) diluted in anaerobic phosphate-buffered saline (0.1 M) at pH 7.0 and stored at 4 °C overnight before the inoculation. The anaerobic conditions were maintained using 100% N_2 in the headspace.

2.3. In vitro fermentation of raffinose

Fermentation experiments were performed using an *in vitro* static batch culture model, as described by Amorim et al. (2020). Briefly, the fermentations were run in anaerobic conditions at 37 °C for 48 h. The

growth medium at pH 7.0 was composed of: peptone water (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), KH_2PO_4 (40 mg/L), K_2HPO_4 (40 mg/L), $MgSO_4.7H_2O$ (0.01 g/L), $NaHCO_3$ (2 g/L), $CaCl_2.6H_2O$ (0.01 g/L), Tween 80 (14.8 ml/L), bile salts (0.5 g/L), hemin (5 mg/L), cysteine HCl (0.5 g/L), vitamin K1 (74.1 μl/L), $Na_2S.9H_2O$ (0.8 mmol/L) and resazurine (1 mg/L). The raffinose solution was added at a final concentration of 10 g/L, when required.

The vitamin k1 and substrate solutions were filter-sterilized and added to the remaining medium components sterilized by autoclave at 121 °C during 20 min. The fecal inoculum was added to the growth medium at 11% (v/v). The anaerobic conditions were attained by pressurizing the bottles headspace with N_2 up to 170 KPa.

Samples of the headspace and the fermentation broth were collected at different time points. The latter were centrifuged at 4000 x g for 10 min and the supernatant was collected and stored at −20 °C for further analysis. At 48 h the final pH of the medium was measured and the biomass samples were centrifuged, washed and resuspended in PBS (0.1 M, pH 7.0), and stored at −20 °C for DNA isolation and further sequencing analysis. Fermentations assays were performed in duplicate, running a negative control as blank with no prebiotic addition.

2.4. Analytical techniques

The substrate consumption and the production of lactate and SCFAs were analyzed by high performance chromatography (HPLC) according to Amorim et al. (2020). The headspace samples were evaluated by gas chromatography (GC) as described by Arantes, Alves, Alfons, Alves, and Sousa (2017).

2.5. Microbial analysis

Liquid samples from fecal inocula and fermentation broth (48 h) were taken and used for DNA extraction using the FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH), according to the manufacturer's procedures. The extracted DNA samples were further submitted to high-throughput sequencing (16S rRNA gene) by Illumina MiSeq technology, as described in detail previously (Salvador et al., 2019). All the sequencing analysis were performed at RTL Genomics (Lubbock, TX) and all the samples were analyzed in duplicate. All the sequences were submitted as FASTQ files at the European Nucleotide Archive under the accession number: ERS3592391, ERS3592398, ERS3592399 and ERS3592411 (BioProject accession number: PRJEB33616).

2.6. Data analysis

The negative (blank) and the positive (lactulose) controls reported previously by the same authors (Amorim et al., 2020) were used in this study for comparison purposes. Paired and unpaired t-tests were performed when required. Significant differences were considered when p-value was < 0.05.

3. Results and discussion

3.1. Substrate intake and production of lactate and short-chain fatty acids (SCFAs)

The total production of lactate and SCFAs (acetate, propionate and butyrate), and the residual substrate present in the fermentation medium are shown in Fig. 1. The fermentation of lactulose and raffinose at 10 g/L increased significantly the total amount of lactate and SCFAs (*t*-test student, α = 0.05) for both donors, as consequence of the selective growth-stimulation of SCFAs-producing bacteria (detailed in section 3.4). The results presented in Fig. 1 are in accordance with previous evaluations of the prebiotic effect reported for lactulose, using human fecal inocula (Ito et al., 1997), and raffinose using single cultures of intestinal bacteria (Ose et al., 2018).
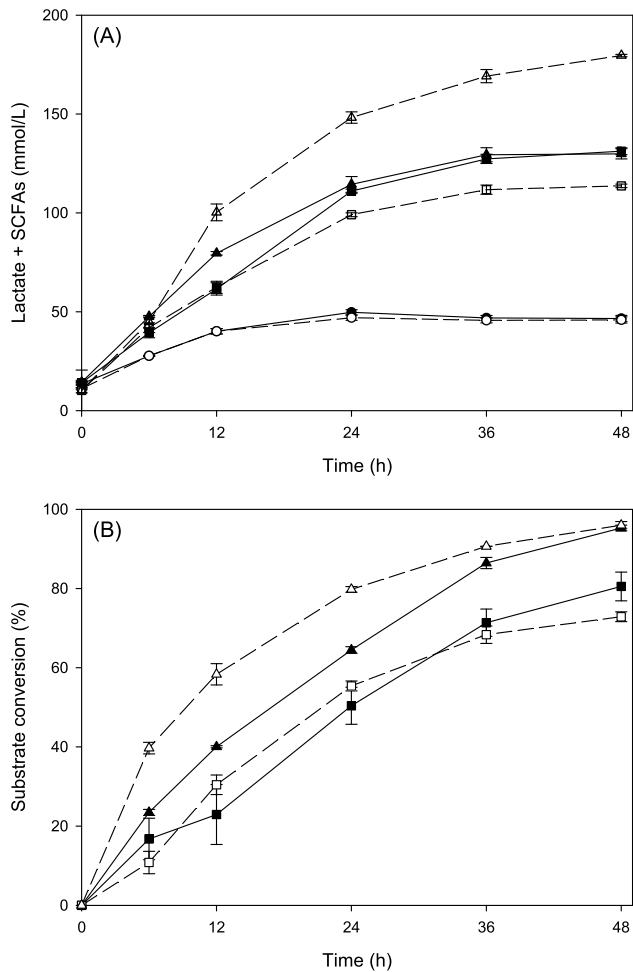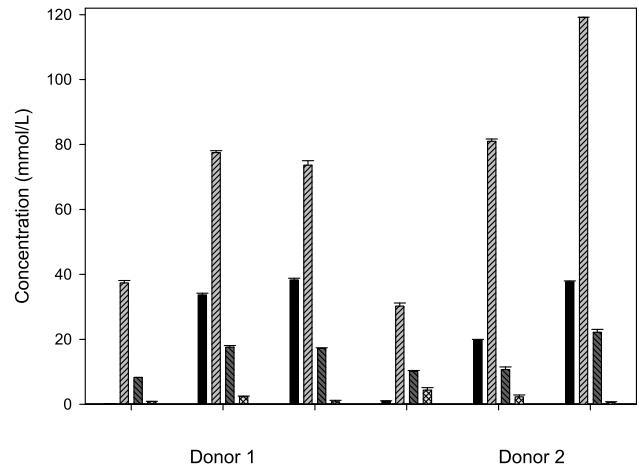
Fig. 1. Total production of lactate and short chain fatty acids (SCFAs) (**A**) and conversion of lactulose or raffinose (**B**) supplemented at a starting concentration of 10 g/L. The fermentations were run during 48 h, using human fecal inocula from donors D1 and D2. A negative control was performed in the absence of prebiotics (blank): Blank D1 (——●——); Blank D2 (— -○ —); Lactulose D1 (——■——); Lactulose D2 (— -◻ —; Raffinose D1 (——▲——); Raffinose D2 (— -△ —). Results represent the average of two independent fermentations and the analysis of each sample in triplicate ± standard deviation.

Fig. 2. Production profile of lactate and short chain fatty acids obtained after 48 h of fermentation, using human fecal inocula from donors D1 and D2. The medium was supplemented with lactulose or raffinose at a starting concentration of 10 g/L. A negative control was performed in the absence of prebiotics (blank): Lactate (■); Acetate (▨); Proprionate (▦); Butyrate (▩). Results represent the average of two independent fermentations and the analysis of each sample in triplicate ± standard deviation.

The maximum production of lactate and SCFAs was achieved at 24 h for the blank (49.7 ± 1.3 mmol/L D1, and 47.0 ± 1.3 mmol/L D2) and between 36 and 48 h for both lactulose (131.2 ± 1.7 mmol/L D1 and 113.6 ± 0.9 mmol/L D2) and raffinose (129.9 ± 2.6 mmol/L D1 and 179.6 ± 0.6 mmol/L D2). At this time point, the highest substrate consumption was found (lactulose: 80.5 ± 3.6% D1 and 72.9 ± 1.2% D2; raffinose: 95.3 ± 0.7% D1 and 96.0 ± 0.9% D2).

Raffinose was mainly consumed during the first 24 h of fermentation (64.5 ± 1.2 0.8% D1 and 79.8 ± 0.7% D2), leading to the faster accumulation of lactate and SCFAs (114.9 ± 4.0 mmol/L D1 and 148.2 ± 2.9 mmol/L D2) (Fig. 1). Additionally, when a 10 g/L mixture of lactulose and raffinose (1:1) was fermented by the fecal inocula of both donors, raffinose was firstly consumed, thus corroborating the preference of fecal microbiota for this substrate (*data not shown*).

Interestingly, for D2 the total amount of SCFAs and lactate obtained at 48 h with raffinose was 1.4-fold higher when compared with lactulose, while D1 presented no significant differences. This difference may be related with the specific microbiota diversity of each donor inoculum (Table A.1, Appendix A). Adamberg et al. (2018) also reported that fecal microbiota of normal and overweight children exhibited different compositions that responded in specific manners to

oligosaccharides and polysaccharides. Particularly, lactic acid was the main fermentation product for overweight children inocula, while the inocula of normal weight children resulted mostly on the production of acetate and butyrate. These facts highlight the need of testing a larger and more representative sample size of donors, including volunteers from different age, culture, health backgrounds and dietary habits, among others.

Besides the total production of SCFAs, the specific profile production, *i.e.* the proportions in which each SCFA is being produced, is also essential to evaluate the potential benefits on the host health related with the intake of these prebiotics (Gibson et al., 2017).

Fig. 2 shows the respective production profiles of lactate, acetate, propionate and butyrate, obtained at 48 h of fermentation, using human fecal inocula from D1 and D2.

Overall, acetate was the main SCFAs accumulated at 48 h (D1: 37.4 ± 0.7 mmol/L blank, 77.5 ± 0.6 mmol/L lactulose and 73.6 ± 1.4 mmol/L rafinose; D2: 30.2 ± 0.9 blank, 81.0 ± 0.7 lactulose and 119.1 ± 0.1 rafinose), with raffinose presenting the highest production of this metabolite that has been reported as playing an important role on colorectal cancer prevention (Casanova, Azevedo-Silva, Rodrigues, & Preto, 2018; Ferro et al., 2016; Oliveira et al., 2015).

On the other hand, lactate was the second most generated metabolite and it was only produced in the presence of a prebiotic substrate, with raffinose presenting the highest values (38.3 ± 0.5 mmol/L D1 and 37.8 ± 0.2 mmol/L D2). These results suggest that both raffinose and lactulose may stimulate the growth of lactate-producing bacteria. Lactate is a known precursor of SCFAs ant it has been reported as holding bioactive properties on human health, namely by being able to regulate critical functions of macrophages and dendritic cells of the immune system and to modulate inflammatory activation of epithelial cells (Garrote, Abraham, & Rumbo, 2015). Moreover, lactate and lactate-producing bacteria may exert an effect on extra-intestinal sites, such as vagina representing a key line defense against vaginal pathogens (Amabebe & Anumba, 2018). Thus, raffinose presents potential to be applied on prebiotic formulations for vaginal infections.

Ito et al. (1997) and Ose et al. (2018) also reported acetate and lactate as the most abundant SCFA resulting from the fermentation of lactulose and raffinose using fecal inocula and mono-culture models,

Table 1

pH and concentration of ammonia in the fermentation broth after 48 h of human fecal inocula growth from donors D1 and D2. The absence of prebiotics (blank) and the supplementation of lactulose or raffinose at 10 g/L were studied. Results represent the average of two independent fermentations and the analysis of each sample in triplicate $\pm$ standard deviation.

Assay	D1		D2	
	pH	Ammonia (mg/L)	pH	Ammonia (mg/L)
Blank	7.08 $\pm$ 0.02	152 $\pm$ 7	7.00 $\pm$ 0.03	162 $\pm$ 5
Lactulose	3.56 $\pm$ 0.03	59 $\pm$ 1	3.585 $\pm$ 0.05	49 $\pm$ 3
Raffinose	3.502 $\pm$ 0.002	49 $\pm$ 6	3.605 $\pm$ 0.005	42 $\pm$ 2

respectively.

The supplementation of lactulose or raffinose did not increase the production of butyrate. However, since lactate is an intermediary of other SCFAs, it would be possible that by increasing the fermentation time, a decrease of this metabolite and subsequent accumulation of SCFAs would be observed. Moreover, Bothe et al. (2017) reported a dose-dependent prebiotic effect of lactulose, which influenced the production of butyrate, using a computer-controlled *in vitro* model of the human large intestine. This highlights the importance of establishing the most effective prebiotic dose through the use of in vivo models.

3.2. Variation of pH and ammonia concentration

The increased production of SCFA due to the prebiotic fermentation causes a pH drop, which in turn promotes the reduction of intestinal pathogenic agents and ammonia production that is related with bad fecal odor and colon carcinogenesis (Visek, 1978).

The pH and the ammonia concentration values obtained after 48 h of fermentation are presented in Table 1. As expected, the supplementation with lactulose and raffinose led to an equivalent pH and ammonia reduction. Adamberg et al. (2018) also reported a reduction of the final pH values from 7.3 to 7.4 (control cultures) to pH 5.1 and 5.3 (cultures supplemented with a mixture of raffinose and melibiose).

Compared with in vivo and human studies, the *in vitro* models present some limitations, since they cannot replicate all the complex conditions occurring in the colon (Poeker et al., 2018). For instance, in an *in vitro* model without pH control, the fermentation medium mimics the distal colon pH at baseline. Nonetheless, the SCFAs formed during the fermentation are not absorbed, as it occurs using an in vivo model, thus, these metabolites cause significant variations of the medium pH (Pham & Mohajeri, 2018), which is a limitation of this type of models. On the other hand, the rapid absorption of SCFAs in in vivo models limits their measurement (Carlson, Erickson, Hess, Gould, & Slavin, 2017). Therefore, the *in vitro* models are an attractive alternative tool to study the kinetics of colonic fermentation (Tsitko, Mattila, Maukonen, & Saarela, 2019), but they should be regarded as semi-representative models of colonic fermentation.

3.3. Production of H_2 and CO_2

The prebiotic fermentation by human fecal inocula also exerts an effect on the production of gases. For both donors (D1 and D2), the addition of raffinose increased significantly the production of CO_2 when compared with the blank in particular for D1 (Fig. 3). The maximum production of CO_2 obtained with raffinose was 10.8 $\pm$ 0.8 mmol/L_{medium} at 48 h (D1) and 5.2 $\pm$ 0.3 mmol/L_{medium} at 36 h (D2).

Production of methane was not detected, which may suggest the absence of methanogenic bacteria in the original microbiota. In fact, it is known that only a fraction of the human population harbor methanogenic bacteria on their gut microbiota (Ghoddusi, Grandison,
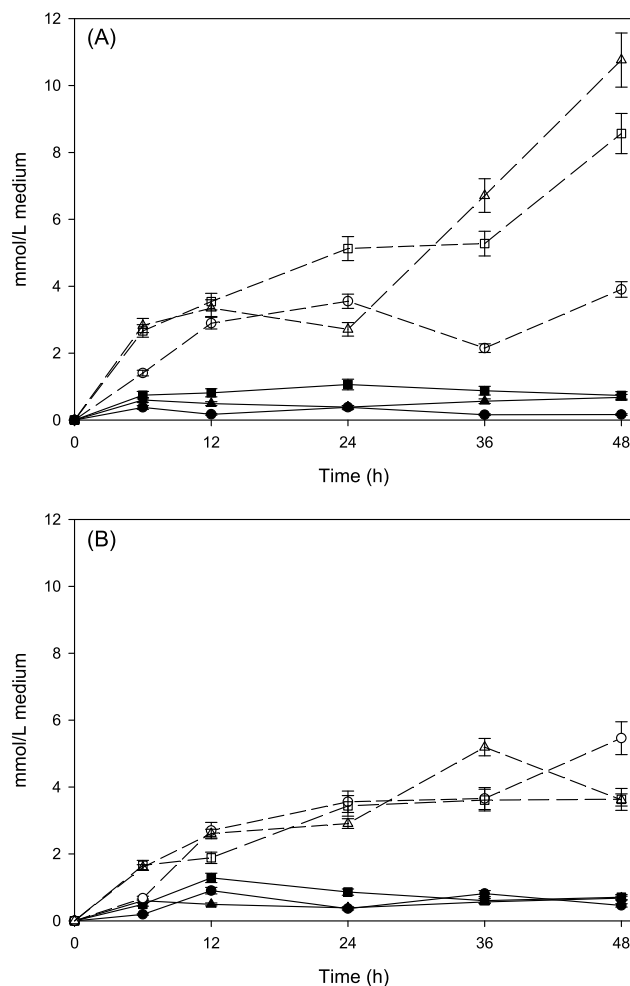
Fig. 3. Production of H_2 and CO_2 by human fecal inocula from donors D1 **(A)** and D2 **(B)** during 48 h. The absence of prebiotics (blank) and the supplementation of lactulose or raffinose at 10 g/L were studied: Blank H_2 (——●——); Blank CO_2 (— -○ —); Lactulose H_2 (——■——); Lactulose CO_2 (— -□ —); Raffinose H_2 (——▲——); Raffinose CO_2 (— -△ —). Results represent the average of two independent fermentations and the analysis of each sample in triplicate $\pm$ standard deviation.

Grandison, & Tuohy, 2007). However, the DNA profiles of the fermentation samples must be analyzed to support this hypothesis as further described in section 3.4.

Excessive production of gas resulting from raffinose fermentation may cause bloating and flatulence problems, which represents a clinical disincentive to its use (Cummings, Macfarlane, & Englyst, 2001). Dahl, Hanifi, Zello, and Tyler (2014) reported a slight increase of the flatulence and bloating rate due to the daily consumption of 5 g of raffinose by healthy adults, but no differences were found for diarrhea or abdominal pain when compared with the control. Suarez et al. (1999) verified an increase of the flatus rate in human subjects caused by the ingestion of 80 g of different soybean flours derived from beans. In particular, the flour containing higher amounts of raffinose (0.51%) resulted in the highest flatus rate. Therefore, it is essential to determine the proper dose of raffinose intake through in vivo models, consisting in the recommended dose that maximizes the prebiotic effect, while presenting minimal secondary effects.

3.4. Effect of prebiotics fermentation on intestinal microbiota

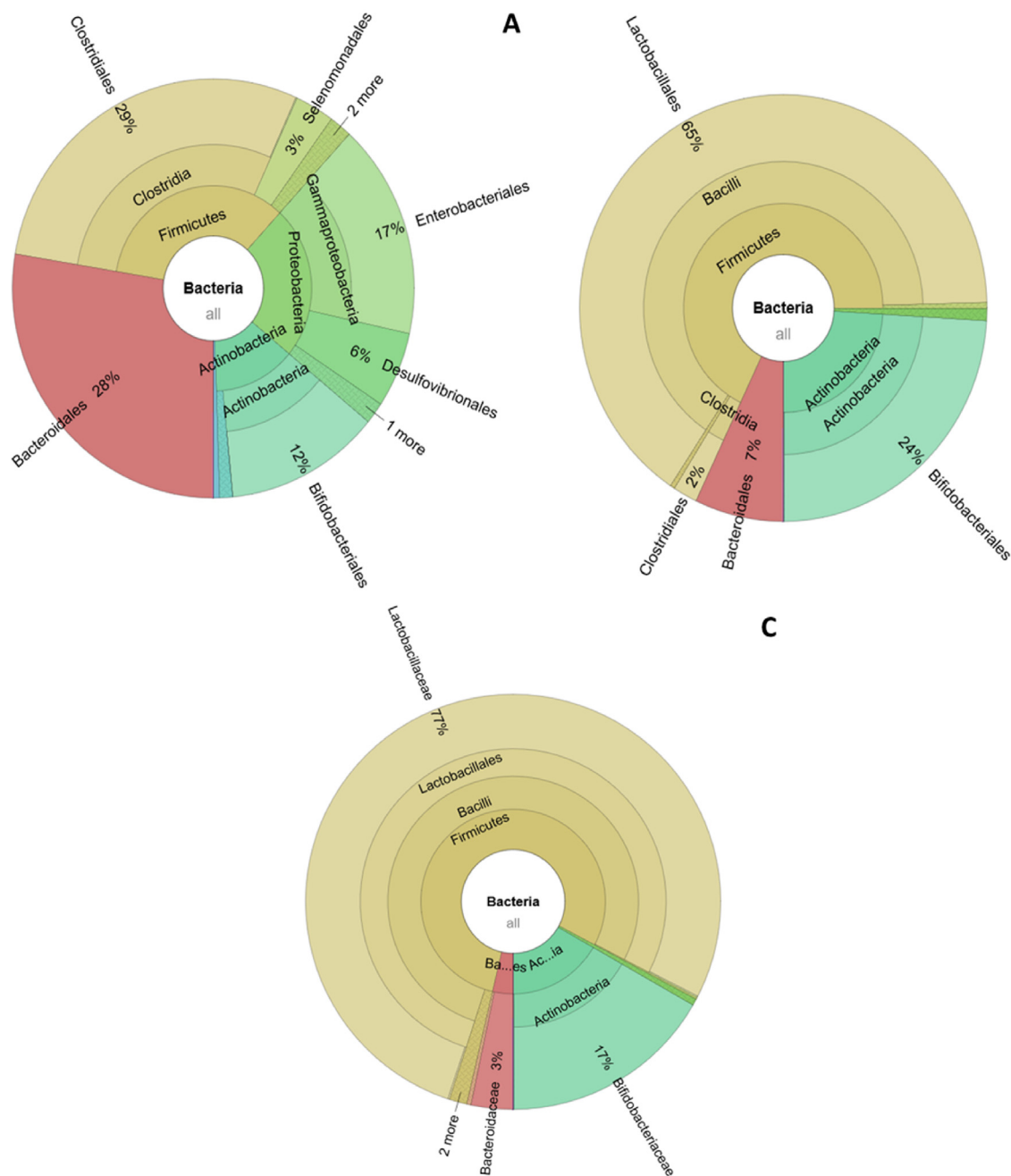The microbiota analysis of the fecal inocula showed the

Fig. 4. Relative abundance of different bacteria after 48h of *in vitro* fermentation by fecal inocula from donor1 in the absence of prebiotics (blank) **(A)** or enriched with a prebiotic solution of lactulose **(B)** or raffinose **(C)** at 10 g/L.

characteristic gut microbiota diversity of healthy human adults (Eckburg et al., 2005), including the presence of six bacterial phyla, of which Firmicutes dominate in terms of relative abundance, Table A.1, Appendix A.

The medium supplementation with lactulose or raffinose resulted in a similar modulation of the gut microbiota of both donors after 48 h of fermentation, Fig. 4 and Fig. 5. When compared with the blank, both prebiotics increased the relative abundance of well-known probiotics, namely organisms belonging to *Bifidobacteria* and *Lactobacillus*, with raffinose showing a 1.8 ± 0.8- and 4.1 ± 0.1-fold increase of *Bifidobacteria* and 725 ± 45- and 750 ± 0-fold increase of *Lactobacillus* for donors 1 and 2, respectively.

However, after the fermentation of lactulose or raffinose, microorganisms belonging to the order *Lactobacillales* presented the highest

relative abundance for D1, while *Bifidobacteriales* dominated in D2. Those differences could be explained by the intrinsic variations on the original inocula of the donors. Therefore, the specific prebiotic effect will greatly depend on the original inocula of the donors, although the main pattern of bacterial growth generated by the prebiotic fermentation were comparable between them. This fact highlights, as previously mentioned, the importance of performing *in vitro* and in vivo studies with a representative sample, in terms of size and diversity of subjects.

Lactobacillus and *Bifidobacterium* are widely known probiotic able to use complex carbohydrates and to mainly produce acetate and lactate, releasing CO_2, which corroborates the results presented in sections 3.1 and 3.2. However, these bacteria may also stimulate butyrate-producing microorganisms in case of cross-feeding effect, resulting in the production of this SCFA (Gibson et al., 2017).
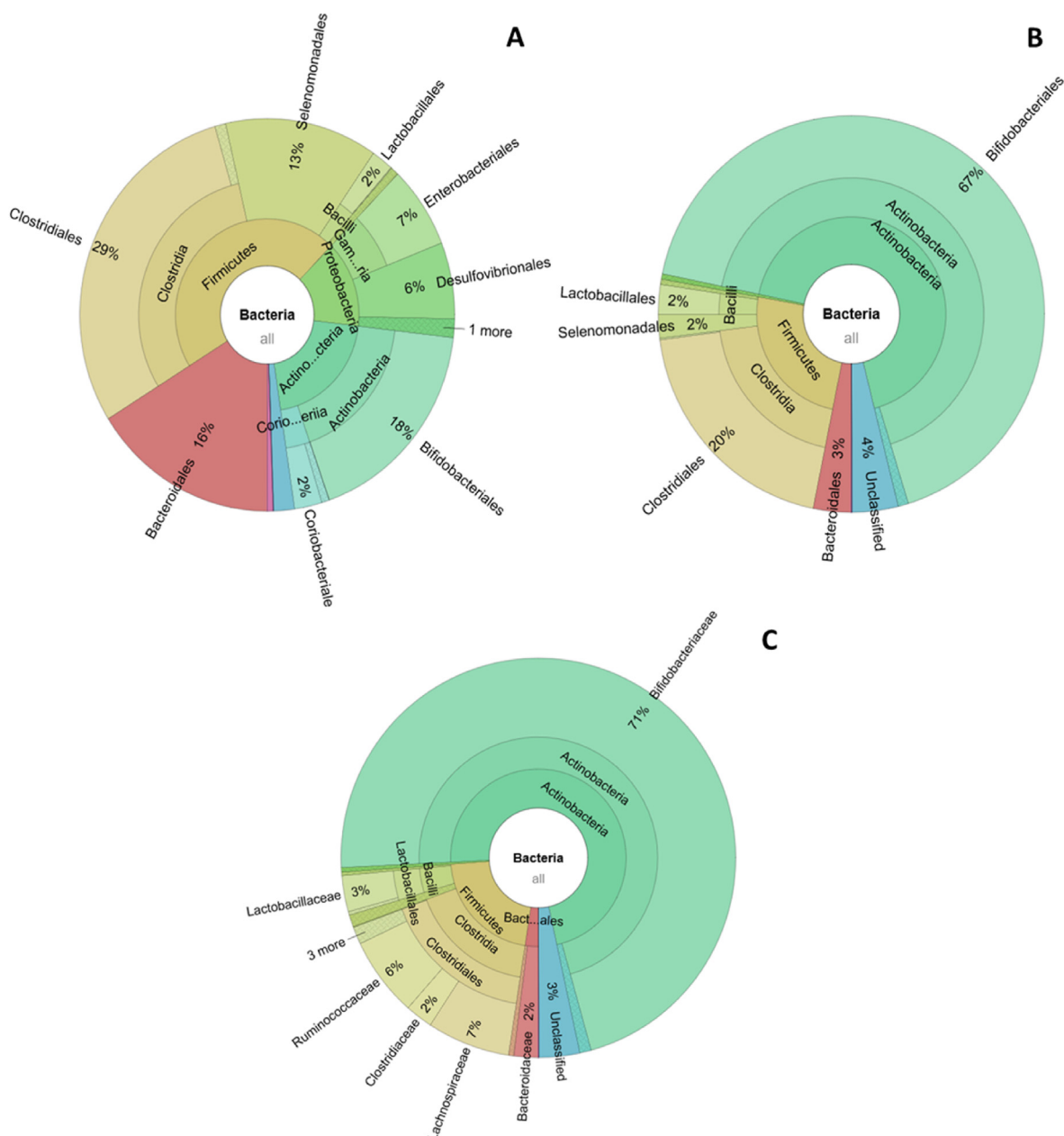
Fig. 5. Relative abundance of different bacteria after 48h of *in vitro* fermentation by fecal inocula from donor 2 in the absence of prebiotics (blank) **(A)** or enriched with a prebiotic solution of lactulose **(B)** or raffinose **(C)** at 10 g/L.

On the other hand, a significant reduction of relative amount of *Proteobacteria* was observed due to the fermentation of prebiotics. The highest reduction was achieved for raffinose (53 ± 7-fold D1, 43 ± 8-fold D2).

The abundance of *Proteobacteria* on gut microbiota is associated to several intestinal diseases. This phylum comprises several known human pathogens (Rizzatti, Lopetuso, Gibiino, Binda, & Gasbarrini, 2017), including *Escherichia coli* whose relative abundance was significantly reduced by de addition of lactulose (32.0 ± 5-fold D1, 1350 ± 650-fold D2) and raffinose (12 ± 4-fold D1, 28 ± 8-fold D2) (Table A.1, Appendix A). The reduction in the ammonia concentration (section 3.3) was confirmed by the reduction of ammonia producing bacteria including *E. coli* and *Clostridium* sp.

The absence of methanogenic archaea on the original inoculum of D1 could explain the lack of CH4 production (Table A.1, Appendix A). However, D2 presents Methanobacteriacea members on its original inoculum (0.85 ± 0.15%) which in turn decreased with the

fermentation of lactulose and raffinose, with raffinose presenting the most significant reduction (25 ± 5-fold). Nevertheless, the production of CH4 from D2 inoculum could be below the sensitivity limit of the GC method, since the relative abundance of these bacteria was significantly low on both inoculum and blank samples.

Taking into account the results herein gathered, raffinose presents a high prebiotic potential, comparable to the well-accepted lactulose, specially to act on *Bifidobacterium* and *Lactobacillus* reduced dysbiosis.

4. Conclusions

The addition of trisaccharide raffinose increased the production of SCFAs and CO_2, reducing the final pH of the medium and the ammonia concentration. Moreover, the presence of raffinose as substrate increased the relative abundance of *Bifidobacterium* and *Lactobacillus* species, while reducing *Proteobacteria*, including widely known pathogens, such as *E. coli*.

This study strongly suggests that raffinose holds potential prebiotic properties for human health and, subsequently, potential to be commercialized as functional food ingredient. Nevertheless, in vivo studies should be further carried out to access its prebiotic effect in a more realistic model and to establish a more accurate recommended daily dose of this compound.

CRediT authorship contribution statement

Cláudia Amorim: Investigation, Methodology, Writing - original draft. **Sara C. Silvério:** Investigation, Methodology, Writing - review & editing. **Beatriz B. Cardoso:** Investigation, Methodology. **Joana I. Alves:** Investigation, Methodology, Writing - review & editing. **Maria Alcina Pereira:** Funding acquisition, Resources, Validation, Writing - review & editing. **Lígia R. Rodrigues:** Funding acquisition, Resources, Validation, Supervision, Writing - review & editing, Project administration.

Declaration of competing interest

None.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.109322.

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