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Impact of functional flours from pineapple by-products on human intestinal microbiota

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ABSTRACT

Solid fractions from pineapple stems and peels are constituted by structural carbohydrates coupled with dietary fibre, simple sugars, but also vitamins and polyphenols, which together can have potential effects on human health. The present studies report for the first time the bioavailability and bioaccessibility of pineapple by-products fractions throughout simulated gastrointestinal tract, evaluates prebiotic potential and *in vitro* human microbiota fermentation. The pineapple flours promoted the human faeces fermentation through growth of beneficial strains, being corroborated by the decrease of simple sugars and the production of healthy organic acids (acetic, propionic and butyric acids) - well known short chain fatty acids. On the other hand, a high phenolic compounds content was release through flours digestion, developing an antioxidant environment within human gut. Thus, was possible to conclude that pineapple flour promoted a positive modulation in the overall system, proving a synergetic interaction of dietary fibre and polyphenols upon human microbiota.

1. Introduction

The by-products from agro-industrial processing are a rich source of numerous value-add compounds, with different biological properties (Ayala-Zavala et al., 2011). The recovery of such molecules have extreme importance, since allows the reuse of by-products, decreasing the amount of waste deposition and reducing the requeriment for primary raw materials (Chemat et al., 2019).

The fruit production and processing generates high amount of waste, and well oriented can be reused for different applications. Pineapple processing generates high quantity of by-products, and due to common structural base of fruits, have high content of carbohydrates, insoluble dietary fiber (IDF), which includes cellulose and hemicellulose. On the other hand, soluble dietary fiber (SDF) includes non-starch polysaccharides, such as pectin's, β-glucans, gums, mucilage's, oligosaccharides or inulin (Burton-Freeman, 2000; Quirós-Sauceda et al., 2014). Moreover, dietary fiber also enclose attached appreciable number of colorants and flavours, antioxidants, proteins and other substances with positive health effects (DeVries, 2004). Soluble dietary fiber have shown several technological and functional properties as food additives, such as water-holding capacity, swelling capacity, increasing viscosity or gel formation (Gowd, Xie, Zheng, & Chen, 2019) and as a promoter of prebiotic activity enhancing probiotic strains growth and metabolic activity (Holscher, 2017). The production of new foods with high dietary fiber content have been more searched and being more available in the market and fruit by-products can be suitable alternative to the food industry (Elleuch et al., 2011).

Phenolic compounds and dietary fiber are generally studied separately, due to differences in chemical structure, physicochemical and biological properties (Tabernero, Venema, Maathuis, & Saura-Calixto, 2011). However, there is scientific evidence suggesting that indigestible components of dietary fiber (polysaccharides) can be associated to other food constituents, such as phenolic compounds (Tabernero et al., 2011). Therefore, the dietary fibers associated with polyphenols make them multifunctional (combining both biological effects) and enhance the bioactive and technological properties of products (Quirós-Sauceda et al., 2014).

In fact, several studies have demonstrated the susceptibility of dietary polyphenols to the gastrointestinal digestion and microbiota fermentation, leading to different results in the interaction profile with human microbiota and absorption process in the human gut, since dietary polyphenols undergoes through a degradation process leading

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to structural modifications (Bao, Li, Xie, Jia, & Chen, 2019). Furthermore, studies have shown that the subsequential digestions leads to formation of the most bioactive metabolites, increasing bioavailability, bioaccessibility and intakes rates (Gowd, Bao, & Chen, 2019). Several studies have reported the effects of single dietary polyphenols or highly concentrated antioxidant extracts with the human gut interactions, nonetheless the understanding of digestion of functional fruit flours digestion was not yet studied.

An integrated valorization approach was applied to pineapple by-products based on a green chemistry approach, leading to production of two functional flours (press cake from peels and stems). Thus, in this work the potential prebiotic activity of functional flours were evaluated through gastrointestinal tract simulation, followed by *in vitro* human intestinal microbiota fermentation assay. Furthermore, a chemical characterization of polysaccharides and simple sugars profile was performed, as well as, a characterization of phenolic compounds before and after fermentation.

2. Materials and methods

2.1. Materials

The oligosaccharides standards containing D-xylose, xylobiose, xylotriose, xylotrehalose, xylopentaose were purchased from Megazymes (Bray, Ireland). The calibration curve of molecular weight of polysaccharides were purchased from Shodex™ (Munich, Germany). The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2′-azo-bis-(2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), α-amylase, bile salts, d-galactose, d-cellobiose, Folin-Ciocalteu reagent, fluorescein, hydrochloric acid (HCl), pancreatin, pepsin, sodium hydrogen carbonate ($NaHCO_3$) and sulphuric acid were purchase from Sigma-Aldrich (St. Louis, MA, USA). The potassium persulfate ($K_2S_2O_8$) was obtained from Merck (Kenilworth, NJ, USA).

All media, including de Man-Rogosa-Sharp (MRS) medium, Muller-Hinton (MH) medium were purchased from Biokar (Allonne, France). Cysteine was used to supplement the MRS medium and was obtained from Merck (Darmstadt, Germany), on the other hand, fructooligosaccharides (FOS) was purchased in Sigma-Aldrich (St. Louis, MA, USA).

2.2. Raw materials

Fresh ¾ stage of maturation pineapples (*Ananas comosus* Merr.) were purchased from Costa Rica, exported to Portugal and processed to produced cut-fresh fruit, by a national Portuguese company – NuviFruits S.A. In an industrial environment the pineapple fruit was processed automatically by detaching the crown and stem, and peeling off the skin. The by-products parts were frozen at −20 °C for a maximum period of 90 days until further use.

2.3. Samples preparation

The functional flours were prepared as described before Campos et al. (2019). Pineapple by-products (stems and peels) were separated into two fractions, using a juice machine (model: MES1020 of 380 W, Bosch), sorting out the solid fraction (press cake) from the remain fraction. The solid fraction of both by-products was dried at 50 °C in a oven with air circulation for 48 h, and grinded into a fine powder.

2.4. Gastrointestinal tract simulation

The effect of simulated gastrointestinal tract (GIT) upon to pineapple flours was previous described with some modifications by Madureira, Amorim, Gomes, Pintado, and Malcata (2011). The system simulated pH, temperature, peristaltic movements and specific enzymes juices at each stage. After passage through each section – mouth, stomach and small intestine (duodenal zone) – the total carbohydrates and polyphenols were characterized. The profile for simple sugars, as well as, soluble dietary fiber (polysaccharides) were analysed and quantified by HPLC. The total amount of phenolic compounds was quantified before and after each compartment of GIT by Folin-Ciocalteu method, and each phenolic compound was cquantified by HPLC. The antioxidant capacity (AA) was also determined using ABTS assay, as described below.

2.4.1. Mouth simulation

Samples were prepared in duplicate, 1 g of dried flour was added to 20 mL of distilled water, three independent experiments were performed. To start the mouth digestion the initial pH of samples was adjusted between 5.6 and 6.9 (1 M $NaHCO_3$ was used) and α-amylase at 100 U mL^{-1} was added at a rate of 0.6 mL min^{-1} of sample digestion. The enzymatic digestion was carried out during 2 min of mastication, at 37 °C and 200 rpm.

2.4.2. Stomach simulation

To start the stomach digestion the samples pH was adjusted to 2.0 using 1 M HCl. Pepsin at 25 mg mL^{-1} was added at rate of 0.05 mL mL^{-1} of sample to simulate the gastric juice. The digestion was carried out during 60 min, 37 °C and 130 rpm.

2.4.3. Small intestine simulation

To initiate the duodenal digestion the sample pH was adjusted to 6.0 (using 1 M $NaHCO_3$). Intestinal juice was simulated by dissolving 2 g L^{-1} of pancreatin and 12 g L^{-1} bile salts and the mixture was added at a rate of 0.25 mL mL^{-1} of sample. The samples were incubated during 120 min, at 37 °C and 45 rpm, to mimic a long intestine digestion. Aliquots of 4 mL were taken in each step and were evaluated for its biological capacities.

2.4.4. Dialysis of small intestine digestion

The resulting samples were submitted to dialysis membranes with molecular poor size of 3 kDa (Spectra/Pro, Spectrum Lab, Breda, Netherlands) to reproduce the natural absorption step in the small intestine, during 48 h, at room temperature, at water flow of 7 L h^{-1} (the water was propelled with a peristaltic pump). The final samples were centrifuged to separate supernatant from the digested solid fraction. The precipitates were dried at 40 °C during 24 h.

2.5. Determination of the antioxidant capacity

2.5.1. ABTS assay

The total antioxidant activity of its pineapple flours and sub-sequential fraction from GIT simulation were measured by the ABTS radical cation decolorization assay as described by Re et al. (1999). ABTS was dissolved in water at a final concentration of 7 mM. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (Merck) (final concentration) and kept in the dark at room temperature (25 ± 2 °C) for 12–16 h before use. The radical maintains a stable form for more than two days when stored in the dark. Before analysis, ($ABTS^{\cdot+}$) was filtered using 0.22 μm filter (Orange Scientific, Braine-l'Alleud, Belgium) and diluted with redistilled water to an absorbance of 0.700 ± 0.02 to 10 μL of sample, the absorbance was read exactly 6 min after initial mixture. Since the inhibition percentage (IP) must be between 20 and 80%, the samples were diluted when needed. These values were then calculated using the Eq. (1) and then converted into g/L of ascorbic acid equivalent, through a calibration curve prepared using standard solutions of ascorbic acid. All assays were performed in triplicate, considering three different replicates of the analysed sample.

$$IP = \frac{OD\ (diluted\ \text{ABTS}\bullet+) - OD\ sample}{OD\ (diluted\ \text{ABTS}\bullet+)} \times 100 \quad (1)$$

2.6. Quantification of total polyphenol

2.6.1. Folin-Ciocalteu method

The total polyphenol content of the pineapple flours were measured using a modified Folin-Ciocalteu colourimetric method described previously by Gao, Ohlander, Jeppsson, Björk, and Trajkovski (2000). Aliquots of 50 μL of samples and control (distilled water) were mixed with 50 μL of Folin-Ciocalteu reagent (0.25 N), 1 mL of sodium carbonate (1 N) and 1.4 mL of distilled water, added in this exact order. The total polyphenol content was determined after 1 h of incubation at room temperature (25 °C). Using an UVmini 1240 UV–Vis spectrophotometer (Shimadzu, Washington, Maryland, USA), the absorbance of the reulsting blue colour was measured at 750 nm by colourimetry. A standard curve was performed using different concentrations of gallic acid. For each experiment, all measurements were performed in triplicate.

2.6.2. Individual phenolic compound identification by HPLC

The phenolic profile of the pineapple flours were evaluated using a Waters e2695 separations module system interfaced with Photodiode array UV/Vis detector (PDA 190–600 nm), according to the method described previously by Campos, Madureira, Sarmento, Gomes, and Pintado (2015), changes in the original method was made to better separate the pineapple phenolic compounds, as describe by Campos et al. (2019). Three independent analyses were performed for each experiment. Separation was done in a C18 reverse-phase column coupled with a guard column (pore size 100 Å, particle size 5 μm, lengths 4.6 mm × 150 mm) containing the same stationary phase (Symmetry® C18, Waters, Milford, Massachusetts, USA). Chromatographic separation of phenolic compounds was carried out with mobile phase A - water, methanol (Panreac, Barcelona, Spain) and formic acid (Merck, Darmstadt, Germany) (92.5:5:2.5) and mobile phase B - methanol, water and formic acid (92.5:5:2.5) under the following conditions: gradient elution starts at 100% mobile phase A and ends at 55% mobile phase B after 55 min at a continuous flow 0.5 mL min^{-1}, between 50 and 55 min the mobile phase A returns to 100% and remains at this percentage for 4 min (until 59 min). The injection volume was 20 μL. Using a diode array detector (Waters, Milford, Massachusetts, USA) was possible to achieve the detection at wavelengths ranging from 200 to 600 nm measured in 2 nm intervals. The peaks were obtained at 280 nm (catechins/ procyanidins), 320 nm (phenolic acids), 330 nm (flavonols) and 520 nm (anthocyanins) and were analysed by comparison of retention time and spectra with pure standards. For each experiments, three independent analyses were performed.

2.7. Carbohydrates determination by HPLC

2.7.1. Determination of molecular weight distribution of oligosaccharides

The chromatographic analysis was performed using a Beckman & Coulter 168 series HPLC system with refracttive index - RI detector (Knauer, Berlin, Germany). Separation was done using columns packed with hydroxylated polymethacrylate-based gel, Waters Ultrahydrogel SEC Columns (Waters, Milford, Massachusetts, USA); two coupled columns UltraHydrogel 120 and 250 were used (pore size 120 Å and 250 Å, lengths 7.8 * 300 nm), allowing saccharides separation between 125 and 80.000 Da. Quantification of saccharides was carried out with ultrapure water (Milli-Q, Merck, Darmstadt, Germany) under an isocratic gradient – at a continuous flow of 0.5 mL min^{-1}. Data acquisition and analysis were accomplished using Karat32 software. Peaks were analysed and quantified using two calibration curves of pure monosaccharides and oligosaccharides, with different Mw (150, 180, 282, 342, 414, 547, 679 Da) and other using polysaccharides (5.9, 9.6, 21.1, 47.1, 107, 200, 344 and 708 kDa), the retention time was compared in both curves. Three independent analysis were performed for each experiment.

2.7.2. Determination of simple sugars and organic acids

Supernatants from the batch cultures were filtered through 0.20 μm cellulose acetate membranes. The chromatographic analysis was performed exactly as described in the Section 2.7.1. The separation was performed using Aminex HPX-87H column (BioRad, Hercules, CA) operated at 50 °C; mobile phase, 0.003 mol L^{-1} H_2SO_4; flow, 0.6 mL min^{-1}. Aliquots of the filtered samples were assayed for organic acids (lactic, formic, acetic, citric, succinic and malic acids) and for simple sugars (D-glucose, fructose, galactose, mannose) using an Agilent 1200 series HPLC system with an RI detector (Agilent, Germany) and with UV detector.

2.8. In vitro fermentation assays

2.8.1. Human faecal inocula

The human faeces were collected into sterile plastic vases and kept under anaerobic conditions, until further used (maximum of 2 h after collection). The samples were obtained fresh, from healthy human donors, with the permiss of not having any known metabolic and gastrointestinal disorder. Moreover, the donors confirmed not to be taking any probiotic or prebiotic supplements, as well as, any form of antibiotics for 3 months. The basal medium was prepared as described previously by Madureira, Campos, Gullon, et al. (2016), basically nutrient base medium comprised 5.0 g L^{-1} trypticase soya broth (TSB) without dextrose (BBL, Lockeysville, USA), 5.0 g L^{-1} bactopeptone (Amersham, Buckinghamshire, UK), 0.5 g L^{-1} cysteine-HCl (Merck, Germany), 1.0% (v/v) of salt solution A (100.0 g L^{-1} NH_4Cl, 10.0 g L^{-1} $MgCl_2{\cdot}6H_2O$, 10.0 g L^{-1} $CaCl_2{\cdot}2H_2O$) and a trace mineral solution, 0.2% (v/v) of salt solution B (200.0 g L^{-1} $K_2HPO_4{\cdot}3H_2O$) and 0.2% (v/v) of 0.5 g L^{-1} resazurin solution, prepared in distilled water and at adjusting the pH at 6.8. The basal medium was dispensed into airtight glass anaerobic bottles, sealed with aluminium caps before sterilization by autoclave. Stock solutions of Yeast Nitrogen Base (YNB) were sterilised with 0.2 μm syringe filters (Chromafils, Macherey-Nagel, Düren, Germany) and inserted into the bottles. The serum bottles were incorporated with pineapple by-products precipitate from *in vitro* GIT simulation (peels and stems) at a final concentration of 2% (w/v) and inoculated with faecal slurries of 2% (v/v) at 37 °C for 48 h without shaking. Samples were taken at 0, 12, 24 and 48 h of fermentation. All the experiments were carried out inside an anaerobic cabinet with 5% of H_2, 10% of CO_2 and 85% of N_2). All experiments were performed in compliance with the institutional guidelines.

2.9. Gut microbiota evaluation

2.9.1. DNA extraction

Genomic DNA was extracted and purified from stool samples using NZY Tissue gDNA Isolation Kit (Nzytech, Lisboa, Portugal) with some modifications and previous described Madureira, Campos, Gullon, et al. (2016). Briefly, the samples were centrifuged at 11,000*g* during 10 min, to separate the supernatant from the pellet. Around 170–200 mg of pellet was taken from control and test samples for all times. After, the pellets were homogenized in TE buffer (10 mM Tris/HCl; 1 mM EDTA, pH 8.0) and centrifuged again at 4000*g* for 15 min. Supernatant was discarded and the pellet was resuspended in 350 μL of buffer NT1. After an incubation step at 95 °C for 10 min, samples were centrifuged at 11 000*g* for 1 min. Then, 25 μL of proteinase K was added to 200 μL of supernatant for incubation at 70 °C for 10 min. The remaining steps followed the manufacturer's instructions. The DNA purity and quantification were assessed with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Real-time PCR for microbial analysis at stool: Real-time PCR was performed as described before by

Table 1
Primer sequences and real-time PCR conditions used for gut microbiota analysis.

Target group	Maximum growth rate (μ_{max}, h^{-1})			
	Primer sequence (5′-3′)	Genomic DNA standard	PCR product size (bp)	AT (°C)
Universal	AAA CTC AAA KGA ATT GAC GG CTC ACR RCA CGA GCT GAC	*Bacteroides vulgatus* ATCC 8482 (DSMZ 1447)	180	45
Firmicutes	ATG TGG TTT AAT TCG AAG CA AGC TGA CGA CAA CCA TGC AC	*Lactobacillus gasseri* ATCC 33323 (DSMZ20243)	126	45
Clostridium leptum	GCA CAA GCA GTG GAG T CTT CCT CCG TTT TGT CAA	*Clostridium leptum* ATCC 29065 (DSMZ 753)	239	45
Lactobacillus spp.	GAG GCA GCA GTA GGG AAT CTT C GGC CAG TTA CTA CCT CTA TCC TTC TTC	*Lactobacillus gasseri* ATCC 33323 (DSMZ 20243)	126	55
Bacteroidetes	CAT GTG GTT TAA TTC GAT GAT AGC TGA CGA CAA CCA TGC AG	*Bacteroides vulgatus* ATCC 8482 (DSMZ 1447)	126	45
Bacteroides spp.	ATA GCC TTT CGA AAG RAA GAT CCA GTA TCA ACT GCA ATT TTA	*Bacteroides vulgatus* ATCC 8482 (DSMZ 1447)	495	45
Actinobacteria *Bifidobacterium* spp.	CGC GTC YGG TGT GAA AG CCC CAC ATC CAG CAT CCA	*Bifidobacterium longum* subsp. *Infantis* ATCC 15697 (DSMZ 20088)	244	50

Abbreviations: AT, annealing temperature; bp, base pairs; PCR, polymerase chain reaction.

Madureira, Campos, Gullon, et al. (2016), in sealed 96-well microplates using a LightCycler FastStart DNA Master SYBR Green kit and a Light Cycler instrument (Roche Applied Science, Indianapolis, ID, USA). PCR reactions mixtures (total of 10 μL) contained 5 μL of 2 × Faststart SYBR Green (Roche Diagnostics Ltd), 0.2 μL of each primer (final concentration of 0.2 μM), 3.6 μL of water and 1 μL of DNA (equilibrated to 20 mg). Primer sequences (Sigut microbiota-Aldrich, St. Louis, MO, USA) used to target the 16S rRNA gene of the bacteria and the conditions for PCR amplification reactions are reported in Table 1. To verify the specificity of the amplicon, a melting curve analysis was performed via monitoring SYBR Green fluorescence in the temperature ramp from 60 to 97 °C. Data were processed and analysed using the LightCycler software (Roche Applied Science). Standard curves were constructed using serial tenfold dilutions of bacterial genomic DNA, according to the following webpage http://cels.uri.edu/gsc/cbdna.html. Bacterial genomic DNA used as a standard (Table 1) was obtained from DSMZ (Braunschweig, Germany). Genome size and the copy number of the 16S rRNA gene for each bacterial strain used as a standard was obtained from NCBI Genome database (http://www.ncbi.nlm.nih.gov). Data are presented as the mean values of duplicate PCR analyses. The F:B ratio was obtained by dividing the number of copies of Firmicutes divisions by the number of copies of Bacteroidetes divisions. Moreover, the relative differences to negative control percentage (only faeces fermentation) was calculated using the following Eq. (2):

$$\textit{Relative difference to control } \% = \frac{(SMC - CMC)}{CMC} \times 100 \quad (2)$$

where SMC is the mean copy numbers of the sample at a certain time (12 or 24 or 48 h) and CMC is the mean copy numbers of the control sample at the same time as SMC. Positive % values mean the occurrence of an increase in the number of copies relative to the control sample at that certain time. Higher the value, the higher increase.

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism, v. 500 software (GraphPad Software, San Diego, CA, USA). The homoscedasticity assumption was met, hence analysis of variance (ANOVA), with a 95% confidence interval, was applied to every dependent parameter, to assess differences between the different media tested. The Tukey test, with a 95% confidence interval, was used for the pair-wise comparisons. Correlation between the parameters was determined by Spearmańs rho, with a 99% confidence interval.

3. Results and discussion

3.1. Effects of simulated GIT upon pineapple solid fractions – Antioxidant activities and phenolic compounds

The antioxidant activity was measured by ABTS assays before and after the GIT simulation and results are despicted in the Table 2 for pineapple flours. Differences of antioxidant activity before and after digestion were found, being statistically significant ($P < 0.05$). The antioxidant capacity values increased for both pineapple flours after the GIT simulation. The results obtain for Folin-Ciocalteu analysis were different (Table 2) from those obtained for ABTS assay. The total content of polyphenols decreased throughout the system, which means the loss of polyphenols throughout the GIT compartments, probably due to polyphenols structure degradation by drastic pH changes and by gastrointestinal enzymes, as described by Bao et al. (2019). And the differences found between the total content of polyphenols and consequent antioxidant activity, it was an expected result.

Pineapple flours are constituted by a high content of structural carbohydrates, which are complexed with dietary soluble fiber that can embed different compounds, such as polyphenols (Saura-Calixto & Goñi, 2006), therefore the continuous action of GIT conditions led to cleavage of linkage between dietary fiber and polyphenols. Furthermore, polyphenols have hydrophobic aromatic rings and hydrophilic hydroxyl groups that can be linked to polysaccharides and some of them can exert antioxidant capacity once they are hydrolysed and released by an enzymatic reaction (Blancas-Benitez et al., 2015). Thus, the pineapple flours had an initial free content of polyphenols, and the mouth digestion by the action of amylase led to a release of new polyphenols, which proves the existence of phenolic compounds

Table 2
Evaluation of antioxidant capacity (ABTS assay) and total phenolic compounds (Folin-Ciocalteu method) of pineapple by-products solid fraction (stems and peels) in the simulated GIT. All results expressed in mg/100 g on dry basis.

Pineapple by-product	GIT stage	ABTS assay (mg AAE/100 g DB)	Folin-Ciocalteu (mg GAE/100 g DB)
Peel flour	T_0	341.6 ± 7.7	492.3 ± 3.9
	After intestine	390.1 ± 20.0	235.4 ± 15.7
Stem flour	T_0	285.7 ± 4.7	482.5 ± 6.9
	After intestine	385.4 ± 10.0	194.1 ± 6.4

Abbreviations: Ascorbic acid equivalent, AAE; Gallic acid equivalent. GAE; GIT – Gastrointestinal tract; T_0 – before gastrointestinal tract; DB – dry basis.

complexed with the dietary fiber. The sequential stomach digestion led to loss of the free polyphenols, due to absence of protection during exposure to drastic changes, such as pH and enzymatic hydrolyses, leadin to total reduction of the total content of polyphenols. Blancas-Benitez et al. (2015), have studied the bioaccessibility, bioavailability and bioactions of polyphenols throughout GIT simulation and described the initial release of phenolic acids, apparently linked to soluble dietary fiber, which enables the sustained release during different steps of digestion process. However, other compounds, such as hydroxycinnamic acids, which showed higher molecular weight and strongest linkage to dietary fiber, presented a delay in their release during digestion. At the same time, authors noticed an increase in the antioxidant capacity, concluding a direct connection between release of certain phenolic compounds and antioxidant capacity (Blancas-Benitez et al., 2015). Thus, different stages, different digestion rates; the initial digestion step lead to phenolic compounds release of smaller molecular weight and of weakest linkages to dietary fiber (e.g. galic acid), which have associated smaller antioxidant capacity. On the other hand, the higher molecular weight polyphenols with strongest linkages with dietary fiber happen leaded to released only in the duodenal phase, where the linkage between both molecules were cutted, increasing antioxidant capacity, even though the total content of polyphenols decreased (Table 2), polyphenols degradation throughout GIT digestion (Blancas-Benitez et al., 2015). Campos et al. (2015) have previously described the loss of polyphenols in contact with simulated GIT, justifying the encapsulation of such molecules to be delivered direct in the duodenal zone to promote a beneficial effect of polyphenols.

A statistical analysis was performed before and after GIT simulation to understand if the pineapple flour presented differences between stem and peel, and results of Folin-Ciocalteu showed that differences were not statistically significant between pineapple flours before the GIT digestion ($P > 0.05$), while after digestion the differences were statistically different ($P < 0.05$). Regarding the antioxidant activity and comparing flours, statistically significant differences were only found before digestion ($P < 0.05$). Furthermore, as can be seen at Table 2, there was an increase of the antioxidant capacity of both pineapple by-product flours. The digestion of pineapple by-products flours through GIT was studied for the first time, thus the lack of data for comparision with pineapple matrix. But, Blancas-Benitez et al. (2015) described the loss of antioxidant capacity of mango peel flour during the simulation of GIT and the results presented were not in accordance with the ones presented in this work.

A HPLC analysis was performed to identify and quantify specific pineapple polyphenols potentially responsible for biological activities. Samples were taken after each GIT stage and evaluated by HPLC. Results are described in the Table 3 for both pineapple flours.

In the initial samples of pineapple peel and stem flours two phenolic compounds, caffeic and ferulic acids, were quantified. The pineapple stem flour presented similar concentrations of caffeic and ferulic acids, ca. 0.19 mg/mL, while pineapple peel flour presented higher concentration of caffeic acid, ca. 0.63 mg/mL and residual concentration of ferulic acid, ca. 0.05 mg/mL. These results were in accordance to the ones obtained for total phenolic content and antioxidant activity, before digestion a slight lower antioxidant activity was found for pineapple flours. But after duodenal stage, though the total content of phenolic compounds decreased due to loss of activity by the free phenolic compounds, there was a release mainly of coumaric and ferulic acids, well known by the high antioxidant power, leading to the increase of the total antioxidant activity.

After mouth simulation for stem flour, three more molecules were identified, chlorogenic acid, coumaric acid and N-[(Benzyloxy)carbonyl]leucyileucinamide, being only possible to quantify chlorogenic acid and coumaric acid (N-[(Benzyloxy)carbonyl]leucyileucinamide was not quantifed due to absence of standard compound). The caffeic acid present in the initial sample disappeared after mouth digestion, while the amount of ferulic acid increased after mouth and throughout the GIT.

In the peel flour, a similar behaviour was found for the digestion and for polyphenols profile, the only difference was the initial concentration of caffeic acid present in the sample, which allowed to detected this phenolic compound until the stomach phase. A sustained decrease of caffeic acid concentration throughout the GIT simulation was visualized, totally disappearing after duodenal digestion.

The present results suggest that the free polyphenols (caffeic acid) decreased throughout the GIT system, which means that this molecule was losing activity due to structure degradation. On the other hand, the polyphenols that were being released due to linkage rupture formed between pineapple carbohydrates and/ or proteins with polyphenols, showed to have a gradual increase during the GIT system (Campos et al., 2015; Madureira, Campos, Oliveira, et al., 2016).

It has been reported that polyphenols, generally hydroxycinnamic acids derivatives, such as, coumaric and ferulic acids, can be found in the fiber fractions forming crosslinks with the polysaccharides of the cell wall (Gorinstein et al., 2011). However, the compounds usually related to insoluble dietary fiber, such as, vanillic, hydroxycinnamic and *p*-hydroxybenzoic acids were not found during the GIT simulation, which means that GIT conditions did not released them from the food matrix, and are not apparently absorbed in the small intestine (Blancas-Benitez et al., 2015), therefore the only compounds found are those from degradation of soluble dietary fiber throughout GIT.

These compounds linked to soluble dietary fiber of the peel and stem flours, which were not accessible before, could be now relased and bioaccessible for absorption in the small intestine to promote positive effects.

3.2. *Effects of simulated GIT upon pineapple solid fractions – Simple sugars and complex carbohydrates*

The simple sugars were identified and quantified by HPLC throughout GIT simulation, results in the Fig. 1 shows the content of glucose and fructose for both pineapple flours. Similar content of glucose was quantified for both flours, ca. 50 mg/mL, while for fructose ca.

Table 3
Identification and quantification of phenolic compounds present in pineapple by-products solid fractions (stems and peels) in the simulated GIT, analysis performed by HPLC. All results expressed in mg/100 g on dry basis.

Pineapple by-product	GIT stage	Chlorogenic acid (mg/ 100 g DB)	Caffeic acid (mg/ 100 g DB)	Coumaric acid (mg/ 100 g DB)	Ferulic acid (mg/ 100 g DB)
Peel flour	T_0	0.00 ± 0.00	12.56 ± 0.48	0.00 ± 0.00	1.02 ± 0.24
	After mouth	0.00 ± 0.00	3.56 ± 0.04	67.90 ± 0.54	3.68 ± 0.06
	After stomach	0.00 ± 0.00	2.74 ± 0.42	60.20 ± 2.00	3.78 ± 0.10
	After intestine	0.00 ± 0.00	0.00 ± 0.00	113.12 ± 0.94	69.68 ± 4.68
Stem flour	T_0	0.00 ± 0.00	3.82 ± 0.40	0.000 ± 0.00	3.76 ± 0.34
	After mouth	17.22 ± 0.68	0.00 ± 0.00	45.80 ± 1.40	5.70 ± 0.06
	After stomach	15.48 ± 1.52	0.00 ± 0.00	33.10 ± 0.70	68.18 ± 0.20
	After intestine	5.44 ± 0.38	0.00 ± 0.00	67.86 ± 0.78	94.72 ± 0.10

Abbreviations: GIT – Gastrointestinal tract; T_0 – before gastrointestinal tract; DB- dry basis.

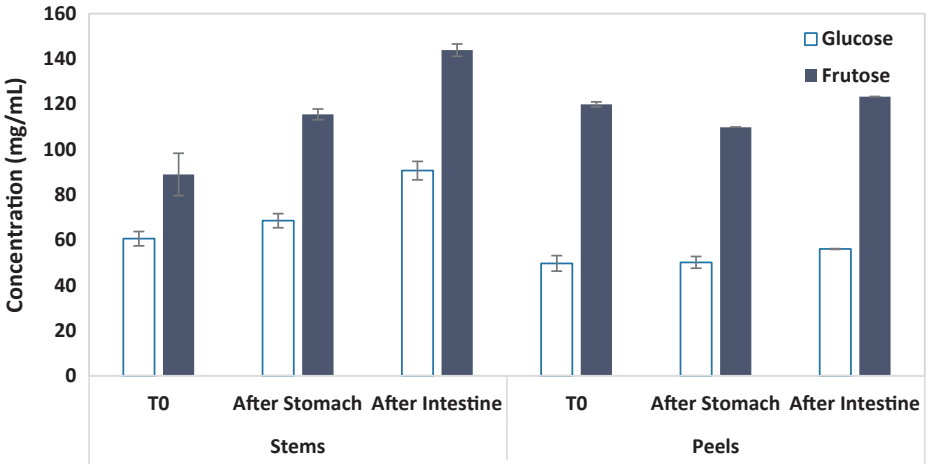

Fig. 1. Content evaluation of simple sugars, D-glucose and fructose (mean ± standard deviation) along the GIT simulation. Samples were taken after mouth (data not shown), stomach and small intestine (duodenal) stages for both studied pineapple flours (stems and peels).

90 mg/mL were quantified for stem flour and ca. 120 mg/mL peel flour. Throughout the GIT simulation the amount of both simple sugars gradually increased. In general, the stem flour presented higher final content of both sugars than the peel flour. Difonzo et al. (2019) have described the characterization of both pineapple pulp and peels and showed that the pulp part had higher concentration of total sugars, when compared with peel flour, which are in accordance with the results obtained in this work. After statistical analysis it was possible to conclude that differences between both flours were statistically significant ($P < 0.05$) after each stage, as well as between glucose and fructose, with exception of initial concentration of glucose for both samples.

The pineapple flours were also evaluated for their polysaccharides profile in terms of molecular weight throughout the GIT (Fig. 2). Before GIT digestion, the stem flour presented high molecular weight polysaccharides, one peak at ca. 8141 Da and other ca. 1660 Da. In this fraction di- and monosaccharides were also identified, being in accordance with the results previously obtained for simple sugars analysis. After mouth digestion, the average of high molecular weight polysaccharide decreased from ca. 8141 Da to ca. 5577 Da, with the maintenance of di- and monosaccharides. Throughout the stomach and duodenal digestion there was a sequential decrease of molecular weight of soluble carbohydrates, where only oligosaccharides were observed. Although, there were differences between all stages, the most important on carbohydrates cleavage was the mouth and the duodenal stages, due to the presence of specific enzymes responsible for carbohydrates digestion (Sanchez et al., 2009).

The peel flour presented a similar behaviour on the digestion of polysaccharides throughout the GIT simulation. High molecular weight polysaccharides were found for the peel flour before digestion where a peak of ca. 2579 Da was found, together with di- and monosaccharides. After total simulation of digestion only small carbohydrates molecules, oligo-, di- and monosaccharides were identified, as can be seen in Fig. 2.

These results are in accordance with the ones described by Quirós-Sauceda et al. (2014), which described the digestion of dietary fiber and polyphenols of different vegetable sources and Blancas-Benitez et al. (2015), which studied the digestion of dietary fiber from mango peels. Moreover, the presented results were also in accordance with the ones discussed by phenolic compounds release throughout GIT simulation, which shows the sustained release of phenolic compounds throughout the simulated GIT systems by the enzymatic degradation of soluble dietary fiber present in pineapple by-product flours.

3.3. Human gut microbiota fermentation

3.3.1. Evaluation of the gut microbiota profile groups

After GIT simulation, two digested pineapple flours (after driying) were applied to human faeces fermentation during 48 h, to study the effect upon the human microbiota. To understand the microorganisms growth and metabolic activities modulation during fermentation, samples at 0, 12, 24 and 48 h were taken and analysed. In the Table 4 are depicted the compositional average of copy numbers obtained by

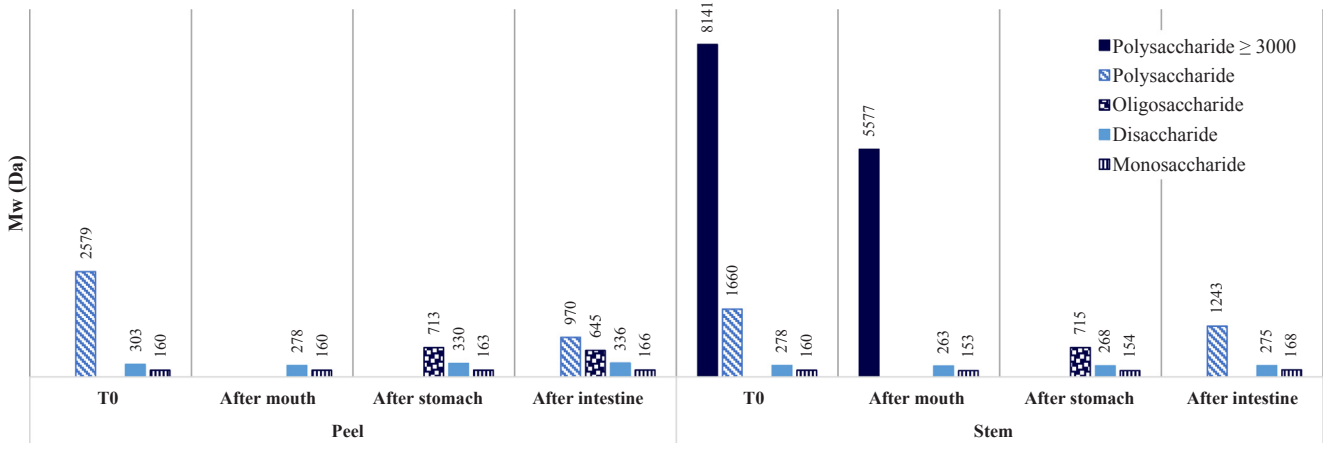

Fig. 2. Evaluation of molecular weight of dietary soluble carbohydrates (mean ± standard deviation) along the simulation of pH and gastrointestinal enzymes, throughout digestion. Samples were taken after mouth, stomach and intestine for both studied pineapple flours (peels and stems).

Table 4
Faecal microbiota composition of volunteer participants.

Division (genus)	Number of copies (n = 5)[a]
Universal	7.726 ± 0.614
Firmicutes	6.865 ± 1.418
Clostridium leptum	7.686 ± 1.397
Lactobacillus spp.	2.754 ± 0.530
Bacteroidetes	7.799 ± 0.761
Bacteroides spp.	6.925 ± 1.482
Actinobacteria	
Bifidobacterium spp.	4.950 ± 0.502
F:B ratio	0.880

[a] Values are presented as mean ± SD and expressed as log10 16S rRNA gene copies per 20 ng of DNA.

PCR real time of the main groups of human gut microbiota as described by Madureira, Campos, Gullon, et al. (2016). Three of the four dominant phyla in the human gut were evaluated, viz. *Firmicutes, Bacteroidetes* and *Actinobacteria* (Guarner & Malagelada, 2003). Numbers were in accordance with the ones found in healthy human volunteers' faeces, *e. g. Lactobacillus* spp. which is usually found in lower numbers in normal gut microbiota (Guarner & Malagelada, 2003; Madureira, Campos, Gullon, et al., 2016). In Fig. 3 are represented the relative differences percentages between the microbiota groups of the tested samples and control faeces during 12, 24 and 48 h of fermentation. Frutooligosaccharides (FOS) were used as positive control, a well-known and recognized prebiotic. In this work, the FOS promoted a growth enhancement effect in all phyla, with exception of *Bacteroidetes* after 48 h, and almost no effect on *Clostridium*.

The peel flour showed a positive effect upon *Lactobacillus* spp. and *Bifidobacterium* spp. phyla, which are bacterial groups associated to beneficial gut microbiota, as described by Guarner and Malagelada (2003). So the the pineapple peel promoted the growth of the probiotic microorganisms, which means that they may act as prebiotic. On the other hand, the peel flour presented a negative effect for *Firmicutes*. This phyla represents several microorganisms, being the most important *Clostridium leptum* and *Lactobacillus* spp. Through these results it was possible to conclude that the negative effect upon *Clostridium leptum* and other potential groups was higher than the positive effect upon *Lactobacillus* spp.

The pineapple stem flour also presented a positive effect for the same groups identified for peel flour, but also for *Firmicutes* and *Bacteroidetes*, which are representatives of a healthy microbiota. Moreover, the stem flour presented a higher positive effect (ca. 30% after 48 h) then the positive control and than the peel flour, which means that stem flour promoted in higher extent the growth of healthier microbiota. The most evident initial growth was obtained for *Lactobacillus* spp., which after 12 h showed an effect of ca. 60%, which slightly decrease after 48 h of fermentation up to ca. 45%, however this was the same trend as for the positive control. Moreover, a manifest result was obtained for *Bifidobacterium* spp. after 48 h, much higher than the positive control.

The relative differences to control gives an excellent prediction of general microbiota upon to certain samples, so the previous discussed results demonstrate that pineapple flour promotes a positive effect on growth.

The F:B ratio calculation involves two of the most abundant phyla in the human microbiota comprising 90% of total microbiota (Fig. 4), therefore, such ratio was calculated using the number of *Firmicutes* and *Bacteroidetes* (Eckburg et al., 2005; Madureira, Campos, Gullon, et al., 2016). Therefore, the proportion of such microorganisms will give an idea of initial amount present in the donors and how the positive control (FOS), as well as, the pineapple flours performed upon gut microbiota. The positive control presented the higher ratios for all hours, when compared with peel and stem flours, as expected. Higher the values obtained, higher the content of *Firmicutes* relative to the content of *Bacteroidetes*. Therefore, both flours presented high ratios values, which means higher content of potential beneficial microbiota (Guarner & Malagelada, 2003).

The digested flours were able to promote the growth of phyla of good microbiota, and inhibit the growth of undesirable phyla and strains, such as, *Bacteroides* spp., being an ingredient with functional properties promoting, healthier gut microbiota, and relevant to be applied in functional foods

3.4. Statistical analysis of microbiota fermentation with pineapple flours

Since the data obtained for the response variables are recorded more than once on the same experimental unit, the analysis is considered as "repeated measeures", applying then the procedure of Generalized Linear Models, GLM (Coscueta, Campos, Osório, Nerli, & Pintado, 2019; Vittinghoff, Glidden, Shiboski, & McCulloch, 2011). Adjusted GLMs were related to bacterial growth in number of DNA copies with three categorical predictive factors: experimental units (subject), substrate type (FOS control, negative control, Stem flour, Peel flour) and fermentation time.

3.4.1. Bacteroidetes

The *Bacteroidetes* growth enhancement showed differences between stem flour, peel flour and controls, but controls were not different from each other. Therefore, the stem flour enhanced bacterial growth, while peel flour tend to slightly reduce the bacterial population with respect to controls. However, from the statistical point of view, given that the P-value of the GLM ANOVA analysis this difference was $P < 0.05$, so there was a statistically significant relationship between bacterial growth of *Bacteroidetes* for both flour and the predictor variables. The coefficient of determination (R^2) indicated that the model, thus adjusted, explained 48.06% of the variability of the data, so there was a great variability that escaped the factors considered. In addition, the Sum of Squares Type III demonstrated a marginal significance of the substrate type ($P < 0.05$). This means that at least one of the substrates caused a different behaviour, which, statistically (HSD Tukey, $P < 0.05$) and graphically (Fig. 5), it is deduced to be the stem flour.

3.4.2. Firmicutes

The pineapple flours enhanced the growth of *Firmicutes*, mainly during the first 12 h of fermentation. In the case of the negative control it seemed to stabilize after this first period, while the FOS control seemed to continue to grow, which was to be expected. The stem flour had an initial growth (0–12 h) likewise FOS, which decreased between the range of 12–24 h. On the other hand, peel flour seemed to have a lower enhancement effect than the negative control during the first 12 h, which intensified between 12 and 24 h reaching the levels of this control. From the statistical point of view, using the same analysis described previously, results have shown a statistically significant relationship between the bacterial growth of *Firmicutes* and the predictor variables. The coefficient of determination (R^2) indicated that the model, thus adjusted, explained 47.35% of the variability of the data, so there was a great variability that escapes the factors considered. In addition, the Sum of Squares Type III demonstrated a marginal significance of the fermentation time ($P < 0.05$). This indicates that, as also inferred from the graphic analysis, there was a significant general trend of change in the bacterial population with the course of the fermentation, being in this case an increasing trend, i.e. growth promotor (Fig. 5). However, statistically it cannot be said that there is a differentiating effect between the different substrates nor with/ between the controls.

3.4.3. Bifidobacterium spp.

Bifidobacterium spp. showed an initial growth for positive control (FOS). Stem flour showed no differences from negative control, while

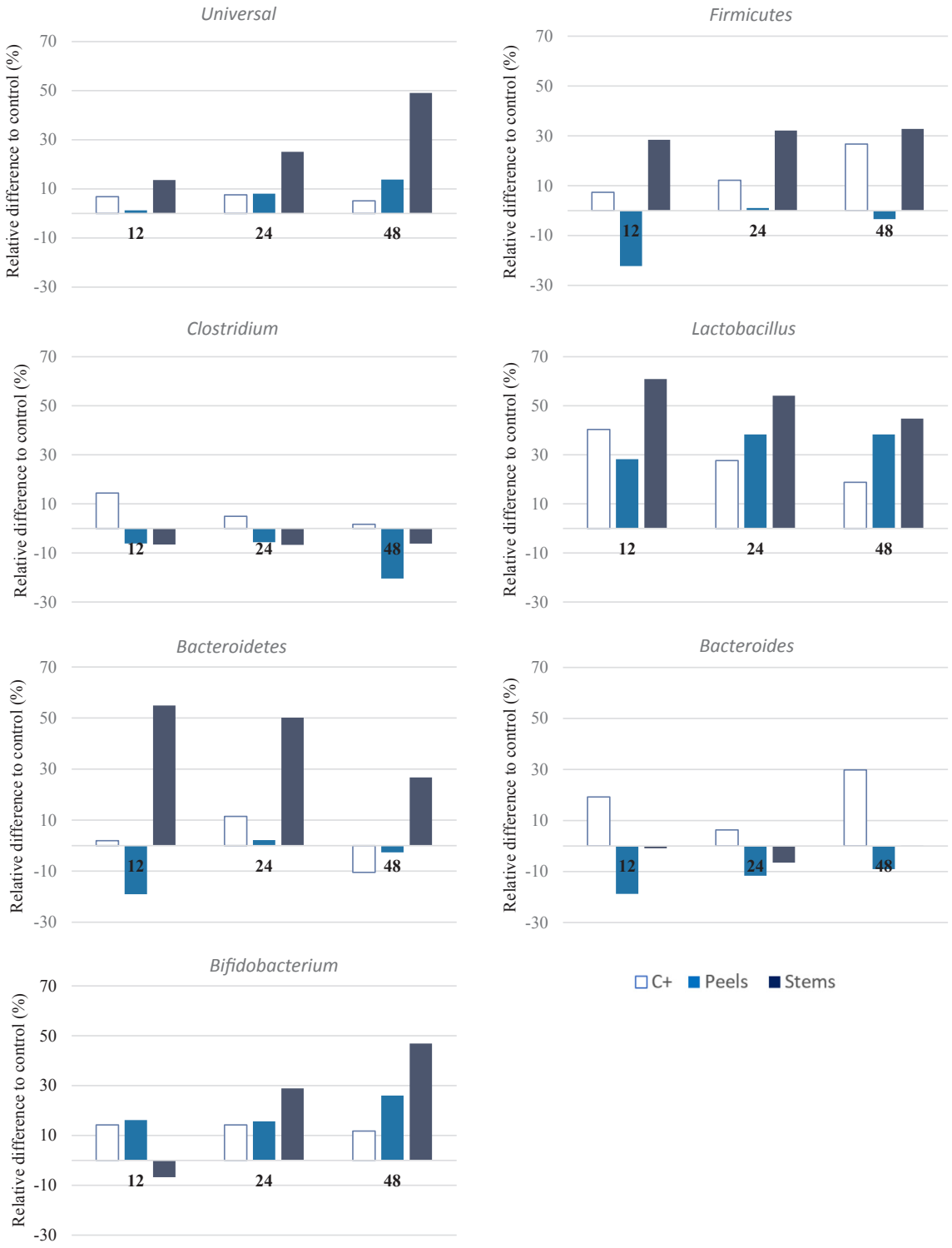

Fig. 3. Relative differences to negative control of human faeces fermentation. Data presented in percentage during fermentation times of 12, 24 and 48 h.

peel flour appeared to have a markedly higher proliferation during the first 12 h, exceeding the other substrates in bacterial population levels, followed by a decrease between 24 and 48 h. From the statistical point of view when the P-value was less than 0.05, there was a statistically significant relationship between the bacterial growth of *Bifidobacterium spp*. and the predictor variables. The coefficient of determination (R^2) indicated that the model, thus adjusted, explained 43.73% of the variability of the data, so there was a great variability that escaped the factors considered. In addition, the Sum of Squares Type III showed a marginal significance of the fermentation time ($P < 0.05$). This indicates that there was a significant general trend of change in the bacterial population with the course of fermentation, being in this case an increasing, i.e. proliferative, trend (Fig. 5). However, statistically it cannot be said if there is a discriminating effect between the different substrates nor with/ between the controls.

3.4.4. *Lactobacillus spp.*

As shown in Fig. 5, *Lactobacillus* spp. had a positive growth with FOS

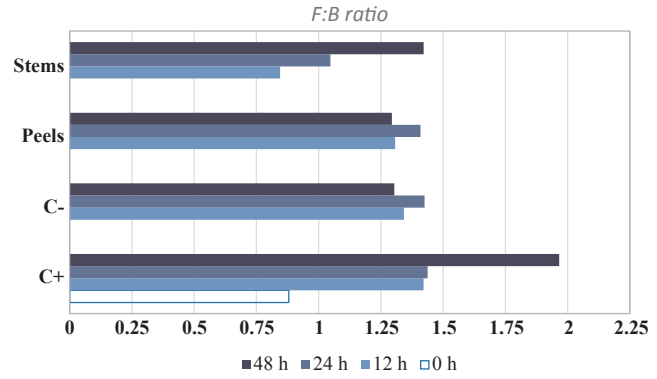

Fig. 4. F:B (*Firmicutes*: *Bacteroidetes*) ratio values evaluation during fermentation with human faeces. Samples fermented with pineapple peel flour, pineapple stem flour and frutooligosaccharides (FOS).

and maintained the initial levels of bacterial population during the first 12 h for both flours. Both FOS and peel flour promoted proliferation of *Lactobacillus spp*. in a similar way. On the other hand, for stem flour the bacterial growth normalise between 12 and 24 h and then increased until 48 h.

From the statistical point of view, the growth enhancement for *Lactobacillus* spp. given the P-value was greater than 0.05, there was no statistically significant relationship between the bacterial growth of *Lactobacillus* spp. and the predictor variables. In addition, the Sum of Squares Type III indicated a marginal significance of the subject random factor ($P < 0.05$). This means that there was too much variability in the response among the subjects analysed, which does not allow us to infer a population behaviour for *Lactobacillus* spp.

3.4.5. *Bacteroides spp.*

The Fig. 5 showed that the only substrate that appeared to lead to *Bacteroides spp*. proliferation was FOS. On the other hand, the negative control and the stem flour seemed to cause a slight reduction of the bacterial population throughout the 48 h of faeces fermentation, while for the peel flour this reduction seemed to be much higher, especially during the first 12 h. However, from the statistical point of view, given that the P-value for *Bacteroides* spp. was greater than 0.05, there was no statistically significant relationship between the bacterial growth of *Bacteroides* spp. and the predictor variables. On the other hand, the Sum of Squares Type III indicated a marginal significance of the type of substrate ($P < 0.05$). This means that at least one of the substrates

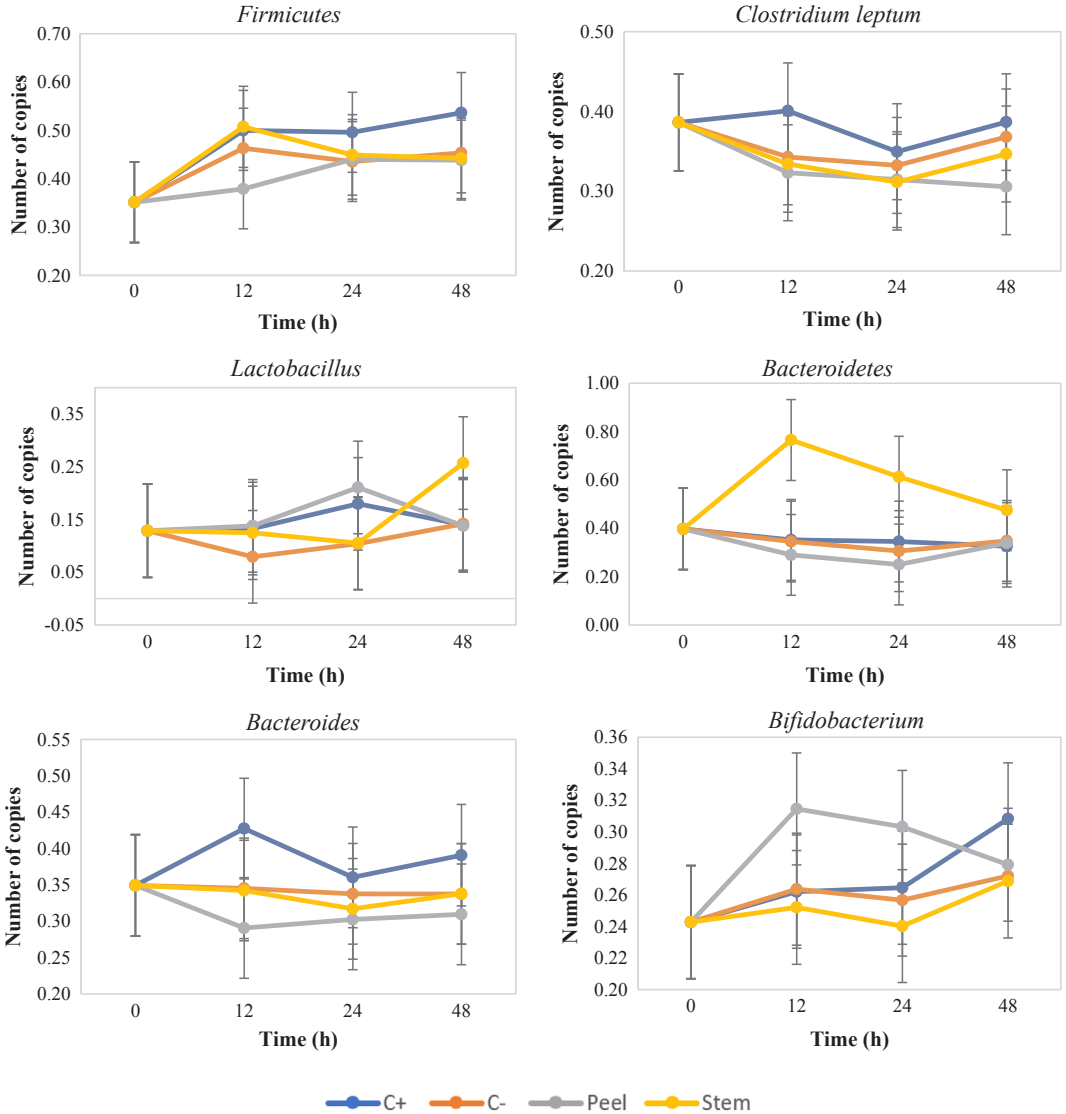

Fig. 5. Number of DNA copies of PCR real time, throughout fermentation time of pineapple peel flour and pineapple stem flour, as well as, fermentation of positive control, FOS at 2% (w/v). Results were statistically analysed and adjusted generalized linear model are presented.

caused a different effect, which occurred between the FOS and the peel flour (HSD Tukey, $P < 0.05$).

3.4.6. *Clostridium leptum*

Results showed that booth pineapple flours, as well FOS did not promote the *Clostridium leptum* growth (Fig. 5). The growth for positive control was maintained until 12 h of fermentation and then started to reduced, which shows that *Clostridum leptum* did not use FOS as energy source. Moreover, pineapple flours showed a similar to the negative control, which means that does not promote the bacterial growth of the studied strain. However, from the statistical point of view, there was a statistically significant relationship between the bacterial growth of *Clostridium leptum* and the predictor variables. The coefficient of determination (R^2) indicated that the model, thus adjusted, explained 41.71% of the variability of the data, so there was a great variability that escaped the factors considered. In addition, the Sums of Squares Type III showed marginal significance ($P < 0.05$) of the subject random factor and the time of fermentation. This means that there was a significant variability of the response between the different subjects analysed, and in turn there was a general tendency of change in the bacterial population with the course of the fermentation, being in this case a decreasing tendency (*Clostridium leptum* growth was not promoted by pineapple flours).

3.5. Sugars and organic acids profile

The sugar and organic acids profile of faeces samples were evaluated through HPLC and results are despicted in Table 5. The human faces of the five donors were supplement with FOS, as a positive control, and high concentrations of the main sugars (galactose, glucose, mannose and fructose) were found throughout fermentation. Moreover, residual amounts of sugars were found for the negative control (no supplementation).

Regarding the pineapple flours, the presence and release of glucose was very different before fermentation. The peel flour presented ca. 0.1 mg mL^{-1} of glucose, while stem flour presented 0.4 mg/mL. These results were in accordance with the ones obtained for final samples after GIT simulations. For stem flour at initial stage and after 24 h of fermentation there was an increase of glucose concentration ca. 0.5 mg mL^{-1}, which was metabolized between the 24 h and 48 h of fermentation.

Galactose concentration was very similar between FOS and pineapple flours, before human microbiota fermentation. After 12 h of fermentation, it was noticed a slight increase in galactose content, which was a result of metabolization of more complex soluble sugars into simple sugar, followed by a consumption during the microbiota fermentation.

Generally, the digested pineapple flours presented different profiles on sugar concentrations, stem flour presented higher concentrations of all sugars than the peel flour, being the main sugars D-glucose and galactose.

Regarding the organic acids in the human faeces' fermentation, six organic acids were identified throughout all samples and times, propionic, butyric, succinic, formic, lactic and acetic acids.

The succinic, propionic and butyric were the organic acids in higher concentrations for FOS and pineapple samples. Also, the lactic and acetic acids were not detected before fermentation, which means that they were produced during fermentation through metabolization of of carbohydrates. In both acids was possible to see a gradual increase between 0 h until 24 h, followed by a decrease until the 48 h.

The statiscal analysis on sugars and organic acids content showed that the results between positive control and pineapple flours were not statistically significant for acetic and succinic acids ($P > 0.05$), while for the remain organic acids were statiscally significant.

The SCFA are generally produced by intestinal bacteria through fermentation of non-digestible carbohydrates. The most common SCFA are acetate, propionate and butyrate, but also can be produced formate, isobutyrate, valerate and isovalerate (Wong, De Souza, Kendall, Emam, & Jenkins, 2006).

As expected, the SCFA accumulation was visualized for positive control, as well as, for pineapple peel and stem flours. Such behaviour did not occurred for negative control. The SCFA concentration profiles determined in samples containing carbon sources were in agreement with the decrease of pH, which occurred for positive control and pineapple flours, on the other hand the pH for the negative control increased (Table 5). The higher content of total SCFA was obtained for stem pineapple flour, only after 12 h of fermentation reached ca. 3.5 mg mL^{-1} of total SCFA. The total SCFA concentrations after 48 h varied between 2.95 and 3.35 mg mL^{-1}, confirming that the degree of metabolic modulation varied depending on the carbohydrate type as it was previously established for other carbohydrates by several authors (Cardelle-Cobas et al., 2012; Gullón et al., 2014; Rivas, Gullón, Gullón, Alonso, & Parajó, 2012; Salazar et al., 2009).

The most important SCFA are propionic, acetic and butyric acids and several studies have shown the beneficial effects of such molecules in the human gut health, thus the high importance of identify and characterize prebiotics that are able to promote the bacterial growth to produce selectively butyrate and propionate.

Usually, propionic acid is produced by *Bacteroides* and an important ratio can be calculed with acetic/ propionic acids. This ratio was linked to inhibition of cholesterol synthesis both in the liver, as well as, intestinal biosynthesis (Delzenne & Kok, 2001). The pineapple flour, after 48 h of fermentation, showed to have higher ratios than the positive control (FOS). Clearly, FOS promoted acetic acid profile, since presented a ratio of 0.68, while average ratio of acetic/ propionic for peel flour was 2.18 and for stem flour was 1.15, which shows that pineapple flour promoted a propionic acid environment. However, the ratio found were minor than the ones found by Rivas et al. (2012) which used galactoglucomannns derived from wood to ferment human faecal inoculum and obtained an acetic/propionic ration of 4.9. Connolly, Lovegrove, and Tuohy (2010) studied the effect of fermented konjac gluomannan hydrosaltes and results were in accordance with the ratios obtained in this research. The ratios values obtained for pineapple flours, were somewhat expected, due to the presence of bifidobacteria, as well as, their growth promotion by pineapple flours. The bifidobacteria pathway following fermentation by human faecal suspensions produces in majority acetic acid (Salazar et al., 2009), furthermore acetic acid is also formed by many anaerobic bacteria from the human gut. Hence, the fermentation with faecal inoculum in the presence of pineapple by-product flours promotes in general the propionic acid SCFA profile, as well as, the growth of beneficial strains, such as *Bifidobacteria*.

The same microorganisms responsible to produce acetate and propionate, also are responsible for the production of succinate (Miller & Wolin, 1979). The succinic acid was present in the samples even before the fermentation started, and decreased during fermentation time, which means that the microbiota metabolize this organic acid somewhow. Miller and Wolin (1979) have described a pathway for propionate formation by some strains present in the human gut, that uses succinic acid to produces propionate and energy.

Another very important SCFA is butyric acid, is produced in the human gut by the eubacteria and some clostridia, and it has been reported as a healthy metabolite, since influences positively the cell growth and differentiation, exerts anti-inflammatory effects and has been linked to reduction of colon cancer (Laparra & Sanz, 2010). In the present work, the butyric acid accumulation was more pronunced for positive control than the pineapple flours. Similar values were values by Gullón et al. (2014), which found higher concentrations of butyric acid for FOS, when compared with arabinooligosaccharides.

Finally, the formic acid was detected in the negative control, which means that was a product of the fermentation of the microbiota, but was not found for positive control. Although was found for pineapple

Table 5
Concentration variation (mean ± standard deviation) of sugars (glucose, galactose, fructose, mannose) and organic acids (lactic, succinic, acetic acids, propionic and butyric) and pH for *in vitro* fermentation of human microbiota.

Sugars and organic acids	Incubation time (h)	Negative control (w/v)	Positive control (w/v)	Pineapple by-products flours (w/v)	
			FOS 2%	Peel 2%	Stem 2%
pH	0	6.2	6.2	6.2	6.2
	12	6.8	4.5	5.2	4.1
	24	7.5	4.0	5.5	4.3
	48	7.2	4.0	5.0	4.3
Glucose	0	0.11 ± 0.04	0.11 ± 0.04	0.11 ± 0.04	0.11 ± 0.04
	12	0.09 ± 0.01	4.05 ± 0.85	0.11 ± 0.02	0.36 ± 0.09
	24	0.00 ± 0.00	4.20 ± 0.91	0.00 ± 0.00	0.46 ± 0.09
	48	0.03 ± 0.00	1.12 ± 0.18	0.12 ± 0.02	0.23 ± 0.03
Fructose	0	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.12 ± 0.02
	12	0.04 ± 0.00	0.04 ± 0.01	1.35 ± 0.09	0.30 ± 0.02
	24	0.00 ± 0.00	1.86 ± 0.45	0.00 ± 0.00	0.27 ± 0.09
	48	0.00 ± 0.00	1.17 ± 0.26	0.08 ± 0.01	0.05 ± 0.01
Galactose	0	0.66 ± 0.10	0.66 ± 0.10	0.66 ± 0.10	0.66 ± 0.10
	12	0.37 ± 0.05	1.46 ± 0.80	0.74 ± 0.04	0.87 ± 0.22
	24	0.42 ± 0.06	1.95 ± 0.00	0.55 ± 0.01	0.50 ± 0.02
	48	0.28 ± 0.04	0.97 ± 0.20	0.35 ± 0.05	0.50 ± 0.06
Mannose	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	12	0.08 ± 0.02	0.28 ± 0.06	0.08 ± 0.01	0.28 ± 0.05
	24	0.00 ± 0.00	1.69 ± 0.32	0.25 ± 0.03	0.45 ± 0.05
	48	0.12 ± 0.02	0.28 ± 0.05	0.10 ± 0.01	0.04 ± 0.00
Propionic acid	0	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.01
	12	1.22 ± 0.57	0.71 ± 0.18	0.86 ± 0.07	1.54 ± 0.59
	24	0.47 ± 0.20	0.72 ± 0.15	1.15 ± 0.38	0.76 ± 0.15
	48	0.88 ± 0.19	0.73 ± 0.23	0.76 ± 0.15	1.28 ± 0.40
Succinic acid	0	2.61 ± 0.58	2.61 ± 0.58	2.61 ± 0.58	2.61 ± 0.58
	12	1.18 ± 0.35	1.49 ± 0.53	1.62 ± 0.73	2.36 ± 0.57
	24	1.39 ± 0.27	1.93 ± 0.66	1.93 ± 0.25	3.08 ± 1.35
	48	0.56 ± 0.11	0.77 ± 0.01	1.27 ± 0.02	0.82 ± 0.09
Butyric acid	0	0.18 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.18 ± 0.02
	12	0.29 ± 0.11	0.30 ± 0.14	0.20 ± 0.09	0.24 ± 0.04
	24	0.44 ± 0.06	0.31 ± 0.14	0.40 ± 0.07	0.24 ± 0.08
	48	0.36 ± 0.12	0.20 ± 0.03	0.26 ± 0.06	0.22 ± 0.06
Lactic acid	0	0.01 ± 0.00	0.05 ± 0.03	0.05 ± 0.03	0.05 ± 0.03
	12	0.66 ± 0.19	2.59 ± 0.12	1.74 ± 0.83	1.42 ± 0.50
	24	1.27 ± 0.25	5.69 ± 2.11	2.07 ± 0.56	2.26 ± 1.01
	48	0.59 ± 0.05	3.59 ± 0.48	1.34 ± 0.46	1.61 ± 0.56
Acetic acid	0	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00
	12	0.97 ± 0.16	0.97 ± 0.05	1.40 ± 0.07	1.04 ± 0.05
	24	2.33 ± 0.69	2.30 ± 0.74	1.85 ± 0.00	1.66 ± 0.01
	48	1.11 ± 0.10	2.02 ± 0.01	1.82 ± 0.42	1.47 ± 0.30
Formic acid	0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	12	0.32 ± 0.04	0.00 ± 0.00	0.22 ± 0.03	0.68 ± 0.00
	24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.43 ± 0.05
	48	0.15 ± 0.07	0.00 ± 0.00	0.31 ± 0.05	0.38 ± 0.03

Abbreviations: FOS – frutooligossacharides. Sugars and organic acids are presented in mg mL^{-1}.
[a]The differences between the means in the same row labelled with same superscript are not statistically significant ($P > 0.05$). Analysis of variance was used to estimate the effects of each carbohydrate percentage in the microbial growth of different probiotic strains. Tukey test was used as post-test. The expressed values are the equation slope, which (m), means maximum growth rate.

flours during fermentation.

Through evaluation of organic acids and SCFA of fermentation human faecal inoculum was possible to confirm that the pineapple flours were used as carbon source for the metabolization by microbiota, leading to generation of positive metabolites.

3.6. Phenolic compound profile during fermentation

The digested samples after GIT simulation were submitted to dialysis membranes, allowing the transfer of free molecules from de digested to the permeate side, mimicking the absorption that occurs after duodenal zone. Therefore, the inner part of dialysis membranes were submitted to *in vitro* fermentation with human microbiota. Thus, for the samples at initial time it was not detected substancial content of any polyphenols.

The phenolic compounds were evaluated by HPLC for 0, 12, 24 and 48 h of fermentation, as described at Table 6. Results in general allowed

Table 6
Identification and quantification of phenolic compounds present in pineapple by-products solid fraction (stems and peels) after human gut fermentation, analysis performed by HPLC. All results expressed in mg/ 100 g on dry basis.

Pineapple by-product	Fermentation stage (h)	Caffeic acid (mg/ 100 g DB)	Coumaric acid (mg/ 100 g DB)	Ferulic acid (mg/ 100 g DB)
Peel solid flour	T_0	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.01
	T_{12}	1.15 ± 0.16	21.47 ± 0.34	3.63 ± 0.11
	T_{24}	2.11 ± 0.12	19.36 ± 1.09	10.05 ± 0.19
	T_{48}	1.78 ± 0.04	19.22 ± 0.28	11.87 ± 0.05
Stem solid flour	T_0	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.01
	T_{12}	0.00 ± 0.00	7.48 ± 0.16	24.88 ± 0.05
	T_{24}	0.00 ± 0.00	7.33 ± 0.36	30.17 ± 0.17
	T_{48}	4.81 ± 0.00	7.46 ± 0.39	27.29 ± 0.05

Abb.: T_0 – before human gut fermentation; DB – dry basis.

the quantification of three main compounds, caffeic, coumaric and ferulic acids, and the identification of other three compounds, caffeoyl and feruloyl aldarates and N-[(Benzyloxy)carbonyl] leucyileucinamide. The compounds that were only identified was due to the absence of specific standards.

After 12 h of fermentation, the pineapple peel flour presented high concentration of coumaric acid and smaller concentrations of caffeic and ferulic acids, while for stem flour only were identified two phenolic compounds, coumaric and ferulic acids. These phenolic compounds are hydroxicinnamic acids, which generally are trapped to insoluble dietary fiber, thus the human microbiota was able to break the linkage between insoluble fibers and other molecules, leading to release of these compounds. Moreover, three typical pineapple glycosylated polyphenols were also identified, caffeoyl and feruloyl aldarates and N-[(Benzyloxy) carbonyl] leucyileucinamide, which shows that part of these molecules were also complexed with strucutures from insoluble dietary fiber (Blancas-Benitez et al., 2015), since are molecules belonging to the caffeic and ferulic acids family. The caffeic acid was only detected for pineapple stem flours after 48 h of fermentation. Usually, caffeic acid it is associated to cell wall of vegetable, and only released from such structure by enzymatic hydrolysis, as described before by Blancas-Benitez et al. (2015).

The main differences between the both flours was found in the profile of phenolic compounds found. The main phenolic content found for peel flour was coumaric acid which has been more associated to hemicellulose, while for stem flour the phenolic compound at higher concentrations was found ferulic acid, which has been reported to be complexed with lignin of insoluble fiber (Dey, Chakraborty, Jain, Sharma, & Kuhad, 2016). Campos et al. 2020, have described the fiber characterization of both pineapple by-product flours. The stem flour was constituted by ca. 11% of insoluble dietary fiber, while the peel flour was constitute by ca. 7%, moreover the total amount of phenolic compounds quantified at 48 h of fermentation was also higher for stem flour.

The previous results show that after GIT simulation of pineapple solid fractions, still there are complexed phenolic compounds with soluble and insoluble dietary fiber, which could be metabolized by the human microbiota. Positive aspects can be taken out of these experiments, such as the maintenance of antioxidant potential throughout all the simulated GIT system, which could be thought as a controlled system of sustained release of polyphenols with high antioxidant capacity. This proposal is corroborated by Quirós-Sauceda et al. (2014) that described the interaction of dietary fiber with the intestinal microbiota, leading to release of phenolic compounds, being gradually released in the intestinal lumen and partially absorbed by gut epithelial cells. Therefore, the non-absorbable phenols and phenolic compounds remain in colonic tissue scavenging free radical and counteracting the effects of dietary fiber pro-oxidants (Quirós-Sauceda et al., 2014; Saura-Calixto & Goñi, 2006)

Several works have associated the positive effects of dietary fiber and polyphenols to other possible positive effects. Saura-Calixto (2010) described the action of antioxidant dietary fiber as having positive effects on lipid metabolism, total cholesterol, LDL-cholesterol, and triacylglycerides, as well as, increasing the antioxidant activity in the large intestine and cecum. These results can have a positive effect in different areas, such as cardiovascular disease prevention and gastrointestinal health, including prevention of colon cancer and the prevention of chronic diseases, such as syndrome of inflammatory bowl and chron's diseases. Also, the modulation of the lower gastrointestinal tract could lead to prevention of some degenerative diseases (Knasmüller, DeMarini, Johnson, & Gerhäuser, 2009).

Studies on this topic have been growing, mainly with animal models given a prediction of future possible results, but studies focused on the impact of the human gut microbiota and how the fermentation will specifically modelate the gut environment in a positive way should be performed.

4. Conclusions

The effect simulated GIT on bioactive compounds of pineapple peel and stem flours and the impact of digested flours on human fecal microbiota modulation was studied for the first time.

Both flours presented an initial digestion after the mouth stage due to the action of salivar amylase on small polysaccharides, oligossacharides and simple sugars, leading to the release of smaller phenolic compounds, with low antioxidant impact, which were complexed with such structures. The free phenolic compounds that reached the stomach phase were unprotected being affected by the action of stomacal pepsin and drastic decrease of pH. This behaviour led to the reduction of total antioxidant activity on this stage. Nonethelss, after duodenal digestion a new release of phenolic compounds was reached by a second carbohydrates digestion, leading to cleavage of soluble dietary fiber, releasing bigger phenolic compounds with higher impact on the antioxidant activity.

On the other hand, the pineapple flours showed to have a very polydisperse molecular weight of polysaccharides and oligosaccharides that also suffer digestion by GIT digestion. A clear decrease on the molecular weight of soluble carbohydrates was visualized and these results were corroborated by the quantification of glucose and fructose throughout all GIT stages, wich increased for both simple sugars.

The digested pineapple flours were used by human gut bacteria assuring the maintainence or enhancement the growth of specific microorganisms and modulate ther metabolism. The pineapple stem flour was able to positive promote the growth of different bacteria phylo, being *Lactobacillus* spp. and *Bifidobacterium* spp., the ones showing most relevant growth. On the other hand, the pineapple peel flour, only promoted the growth of *Lactobacillus* spp. and *Bifidobacterium* spp and with lower performance, probably due to the lower content of soluble dietary fiber and simple sugars. Moreover, the content of phenolic compounds was also evaluated throughout human gut microbiota fermentation, and a high release of derivatives of hydroxycinnamic acids, such as, caffeic, coumaric and ferulic, showing the connection between such molecules with insoluble dietary fiber. The non-digestable insoluble dietary fiber suffered digestion by fermentation of human gut microbiota, as well as, by microbial enzymes, which allows the hydrolyses of these carbohydrates, leading to release of phenolic compounds with and antioxidant potential.

Therefore, in basis of such results it is possible to conclude that both pineapple by-product flours are a natural controlled release system of phenolic compounds with high antioxidant capacity, as well as, due to the presence of all content of dietary fiber can be applied as a technological ingredient in foods, which helps the food formulation. Moreover, with high functional and biological properties, enables the positive modulation in each stage of the upper gastrointestinal tract, as well as, in the human gut.

Ethics statements

The authors indicate that this research did not include any human subjects and animal experiments.

CRediT authorship contribution statement

Débora A. Campos: Writing - original draft, Visualization, Investigation. **Ezequiel R. Coscueta:** Data curation, Formal analysis. **Ana A. Vilas-Boas:** Data curation, Validation, Formal analysis. **Sara Silva:** Data curation, Validation. **José A. Teixeira:** Supervision, Validation. **Lorenzo M. Pastrana:** Supervision, Validation. **Maria Manuela Pintado:** Conceptualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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