Lean and obese microbiota: differences in *in vitro* fermentation of food-by-products

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Abstract

The aim of the study was to investigate the potential prebiotic effects of food-by-products (cassava bagasse (n=3), orange bagasse (n=2) and passion fruit peel (n=3)) using an *in vitro* model simulating the proximal colon, and to assess possible differences in fermentation when using faecal microbiota from lean or obese people. Fermentation of the by-products was compared to a control medium and the prebiotic inulin. The effects of the by-products on the dynamics of the gut microbiota differed according to the type of microbiota, as well as the type of by-product used. Principal Coordinate Analysis of the microbiota showed evidence of a clear separate clustering of lean and obese microbiota before the addition of substrates, which disappeared after fermentation, and instead, distinct clusters due to primary carbohydrate composition of the by-products (starch, fructan and pectin) were present. This is evidence that the substrates drove the obese microbiota to a healthier profile, more similar to that of the lean microbiota. Cassava bagasses enriched the beneficial genus *Bifidobacterium* in the obese microbiota. The production of total SCFA by cassava bagasses by the obese microbiota was higher than for control medium and inulin. Orange bagasses stimulated the growth of the butyrate-producing genus *Coprococcus*. Passion fruit peels were poorly fermented and generated negligible amounts of intermediate metabolites, indicating slow fermentation. Nevertheless, passion fruit peel fermentation resulted in a microbiota with the highest diversity and evenness, a positive trait regarding host health. In conclusion, the use of food-by-products could be an important step to tackle obesity and decrease the waste of valuable food material and consequently environmental pollution. They are an inexpensive and non-invasive way to be used as a dietary intervention to improve health, as they were shown here to drive the composition of the obese microbiota to a healthier profile.

Keywords: cassava bagasse, orange bagasse, passion fruit peel, gut microbiota, prebiotic

1. Introduction

The problem of overweight and obesity has reached an incredible dimension worldwide, and some of the consequences are the increased risk for several diseases, such as cardiovascular disease, different types of cancer and more recently, as a factor for disease severity of the novel coronavirus (COVID-19) (Simonnet *et al.*, 2020; WHO, 2020).

The gut microbiota is emerging as a possible tool to be used in this battle against obesity. In this context, host diet has a direct influence on microbiota composition (Shin *et al.*,

2019), and is considered an important way to modulate the gut microbiota (Scott *et al.*, 2013). Indigestible products that reach the colon are the main substrates for the gut microbiota, and among them, dietary fibres constitute an important source of energy for these microorganisms (Martens *et al.*, 2014; Martinez *et al.*, 2015). Fibres that can selectively be used by the host gut microbiota and confer health benefits to the host are the so-called prebiotics (Gibson *et al.*, 2017).

The end products of fibre fermentation by the gut microbiota include the beneficial metabolites short chain fatty acids (SCFA), mainly acetate, propionate and butyrate,

as well as lactate (Kovatcheva-Datchary *et al.*, 2009). One of the proposed mechanisms by which SCFA may protect against obesity is through the activation of free fatty acid receptor (FFAR) 2 and 3 (Nohr *et al.*, 2013) by SCFA. FFAR 2 and 3 are responsible for triggering the release of the satiety hormones glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) by L-cells (Nohr *et al.*, 2013; Samuel *et al.*, 2008; Tolhurst *et al.*, 2012), and in that way contribute to energy balance. Nowadays, however, the food industry is producing more and more ultra-processed foods by which several components from the raw material are removed and discarded, generating by-products, such as fruit and vegetable peels. These by-products are mostly underused and their inappropriate disposal in the environment contributes to pollution (Pandey *et al.*, 2000). The food-by-products are chemically treated and then disposed in the environment without any type of cleaning, and consequently they are a source of pollution (in the case of cassava bagasse there is leaching of cyanides), and considered an environmental hazard, unlike composting. At the same time, the consumption of ultra-processed foods that lack important nutrients, like fibres, but instead are filled with high density energy ingredients (simple sugars/fats), is contributing to the increased incidence of diseases related with bad eating habits, especially obesity (Hall *et al.*, 2019; Louzada *et al.*, 2015).

A proper utilisation of these by-products, such as to incorporate them back into food products, for example as functional foods, could represent one step to decrease their wastage and increase fibre intake. This interconnected scenario of lack of fibre consumption, modulation of the gut microbiota through ingestion of fibres and the use of fibre-rich by-products from the food industry seems durable with regards to a favourable human health and environmentally friendly reuse of wasted food. However, before incorporating these by-products into food, it is necessary to characterise them and to study their possible health effects. We have previously characterised the by-products from the Brazilian food industry used here – cassava bagasse, orange bagasse and passion fruit peel – regarding their chemical composition and digestibility (Bussolo de Souza *et al.*, 2018). Interestingly, the results showed differences in composition of the same by-product from different suppliers, which can have an influence on the health effects when consuming these fibres.

The aim of the present study was to investigate the potential prebiotic effects of selected food-by-products using an *in vitro* model that closely simulates the proximal colon (TNO *in vitro* model of the colon [TIM-2]) and assess possible differences in fermentation when using a gut microbiota from lean or obese people.

2. Material and methods

Substrates

In total, 8 substrates (plus 2 controls) were tested for the fermentation experiments. Three different cassava bagasses (CB) were tested, CB1 and CB2 were from the same supplier but different batches (Grazimara, Biguaçu, Brazil), CB3 was from Lorenz (Indaial, Brazil), all in the form of powder. Two samples of orange bagasses (OB) were tested, both in the form of powder – OB1 from Chá&Cia (Jacareí, Brazil), OB2 from a store with bulk sale (Florianópolis, Brazil). Three passion fruit peels (PFP) were tested, all in the form of powder – PFP1 from Chá&Cia, PFP2 from Phytomare (Governador Celso Ramos, Brazil) and PFP3 from the same store as OB2 (Florianópolis). According to the manufacturers, only the peels were used, seeds were disposed. Inulin from chicory root was provided by its manufacturer (Orafti, Amsterdam, the Netherlands) and it was used as positive control (purity $\geq 90\%$, degree of polymerisation >10). Simulated ileal effluent medium (SIEM) was used as control and it mimics the indigestible fibres from western diet (Sáyago-Ayerdi *et al.*, 2020). More detailed information about the substrates was described elsewhere (Bussolo de Souza *et al.*, 2018) and summarised in Supplementary Table S1.

Dynamic *in vitro* model of the colon (TIM-2)

TIM-2 is a validated, dynamic *in vitro* model that closely mimics the proximal colon, extensively described by Minekus *et al.* (1999) (Figure 1). Briefly, this computer-controlled system simulates several features from the human large intestine, such as temperature (kept at 37°C), pH (kept at 5.8) and anaerobic environment (by constant flushing with gaseous nitrogen). Water and fermentation products are removed through a dialysis system, and the speed of the dialysis fluid was set at 1.5 ml/min. This prevents accumulation of microbial metabolites which would otherwise lead to inhibition or even death of the microbiota. Mixing and transport of intestinal contents is achieved through the simulation of peristaltic movements.

Origin of the gut microbiota

The gut microbiota used for the TIM-2 experiments consisted of active pooled faecal material from (1) 8 healthy lean volunteers (body mass index (BMI) = $20 \pm 1.48 \text{ kg/m}^2$, male: $n=4$, female: $n=4$, age range 25–42 years) and referred to as 'lean microbiota', or (2) 7 obese but otherwise healthy volunteers (BMI = $32 \pm 1.17 \text{ kg/m}^2$, male: $n=3$, female: $n=4$, age range 29–68 years) denoted as 'obese microbiota'. Lean volunteers were recruited from TNO (Zeist, the Netherlands) and obese volunteers from Maastricht University Medical Centre (Maastricht, the Netherlands). Volunteers received oral and written explanation about

Figure 1. Schematic representation of the TIM-2 system: (a) peristaltic compartments with a dialysis membrane inside, (b) pH sensor, (c) NaOH secretion, (d) dialysate system (d1=dialysate in, d2=dialysate out), (e) level sensor, (f) gaseous N₂ inlet, (g) gas outlet, (h) sampling port, (i) feeding syringe with test compound, and (j) temperature sensor. Adapted from Rehman *et al.* (2012).

the study and how to proceed with faeces collection. Prior to their participation, each person provided an informed consent. The exclusion criteria for both groups included the use of antibiotics, probiotics, prebiotics and laxatives in the 3 months preceding donation. Faecal samples were self-collected: volunteers were instructed to defecate in a bag and immediately place it into a gastight plastic jar containing anaerobic packs (AnaeroGen™, Oxoid, Cambridge, UK). Faeces were mixed in an anaerobic cabinet (80% N₂, 10% CO₂, 10% H₂) as described by Aguirre *et al.* (2015). The faecal material was subsequently aliquoted, snap-frozen in liquid nitrogen and stored at -80 °C. This inoculum was

thawed by 1 h in a 37 °C water bath before its introduction to the TIM-2 system (Aguirre *et al.*, 2016).

Experimental protocol and study design

Before the addition of the standardised microbiota to the system, TIM-2 was flushed with gaseous nitrogen for 3 h to create a complete anaerobic environment. Afterwards, the system was inoculated with approximately 30 ml of the standardised microbiota and 90 ml of dialysate (described by Aguirre *et al.*, 2015), yielding a total of 120 ml (total volume of the system). The microbiota was fed with SIEM (2.5 ml/h) and adapted to the system for 16 h. After the adaptation period, the feeding system was stopped and a 2 h starvation period allowed the microbiota to ferment all remaining carbohydrates present in the model. Subsequently, samples were collected at time zero (t0 h) after which the substrates were added. From this point onwards, a 3-day experimental period started, and the microbiota received the standard feeding (SIEM, referred to from now on as control), or this medium but without the carbohydrate sources, which was substituted by the substrates – inulin, cassava bagasse, orange bagasse or passion fruit peel. SIEM contained 7.5 g/day of carbohydrates and the same amount of substrates was added. Samples of simulated lumen and spent dialysate (dialysate out) were taken at the following times: 0 (t0 h), 24 (t24 h), 48 (t48 h) and 72 h (t72 h) and stored (-80 °C) until analysis. Four TIM-2 units were run in parallel. The experimental set-up can be viewed in Figure 2.

SCFA, BCFA and organic acids quantification in simulated lumen and dialysate out samples

Simulated lumen and dialysate out samples from TIM-2 were analysed by Brightlabs (Venlo, the Netherlands) to quantify SCFA (acetate, propionate and butyrate), branched chain fatty acids (BCFA) (*iso*-butyrate and *iso*-valerate), as well as organic acids (valerate, caproate, lactate, succinate and formate), described in detail elsewhere (Larsen *et al.*, 2019b).

Figure 2: Study design with timeline for TIM-2 experiments.

Energy extraction

Energy extraction in the form of SCFA was calculated using the following values for acetate, propionate, butyrate and lactate, respectively: 209, 367, 524 and 326 kcal/mol (Van der Kamp *et al.*, 2010).

Microbiota composition characterisation

Genomic DNA isolation from TIM-2 samples (simulated lumen, t0 h and t72 h) was performed using standard molecular biology kits from ZYMO (ZYMO Research, Co., Irvine, CA, USA) by Baseclear (Leiden, the Netherlands). PCR amplification of the 16S rRNA gene (V3-V4 region), barcoding and library preparation were also performed by Baseclear. Short paired-end sequence reads were generated using the Illumina MiSeq system (San Diego, CA, USA) and converted into FASTQ files using the BCL2FASTQ pipeline version 1.8.3. Quality trimming was applied based on Phred quality scores. Microbial analyses were performed with Quantitative Insight Into Microbial Ecology (QIIME) software package (version 1.9.0) (Caporaso *et al.*, 2010). The sequences were classified using Greengenes (version 13.8) as a reference 16S rRNA gene database.

Data analyses

Experiments were performed in duplicate ($n=2$) per substrate and per microbiota (lean or obese), except for control ($n=3$ with lean microbiota and $n=4$ with obese microbiota) and inulin ($n=3$). Results are displayed as average. R (version 3.1.3) was used in RStudio for statistical analyses for gut microbiota composition. For SCFA analyses, comparison between the two groups (lean and obese) and between substrates and control was performed using t-test with significance $P<0.05$. For microbiota composition analyses, differences in multiple categories were tested with Kruskal-Wallis, with P -values $P<0.05$ set to be significantly different. Dunn post hoc test was used to find differences between groups. Spearman correlation was calculated between the relative abundance of operational taxonomic units (OTUs) and continuous variables. Multiple comparisons were corrected using the false discovery rate (FDR), and q -values (adjusted P -values) were considered significantly different at a stringent cut-off of <0.05 . Permutational multivariate analysis of variance (PERMANOVA; (Anderson, 2017)) was performed to test the significance of beta diversity (weighted and unweighted UniFrac) between lean and obese microbiota for t0 h, and between primary substrates for t72 h in QIIME. Changes in microbial composition were assessed through the difference of their initial relative abundance and final abundance (t72 h – t0 h), and compared to control. Linear discriminant analysis effect size (LEfSe) (Segata *et al.*, 2011) was used to find biomarkers between groups using relative abundances from the OTU tables generated in QIIME.

3. Results and discussion

Obese microbiota do not always produce more total SCFA and consequently do not extract more energy than the lean microbiota

The amount of SCFA produced by the lean and obese microbiota was dependent on the substrate tested, and demonstrated that the obese microbiota not always generated more SCFA than the lean microbiota (Figure 3 and Supplementary Figure S1). The same was the case for energy extraction in the form of SCFA that was also dependent of the test compound, and not always related to the type of microbiota (Supplementary Figure S2). This was unexpected, since a pioneering study showed that the gut microbiota from obese individuals extracted more energy than lean individuals (Turnbaugh *et al.*, 2006). For 5 out of 10 substrates the obese microbiota was able to produce more total SCFA than the lean, although when comparing the total amount of SCFA produced by the lean and obese microbiota (from all substrates), the differences were not significant ($P>0.05$). Similar results are available in the literature. *In vitro* fermentation of diverse types of fibres showed that production of SCFA and consequently energy extraction was dependent of the test compound used, not the type of inoculum (lean or obese) (Aguirre *et al.*, 2014), which is in accordance with our results.

The role of SCFA in obesity, either protective or causative, remains to be elucidated. However, it is worth noting that when detrimental aspects are related to SCFA, this is generally present when there is a dysbiosis – related to a certain disease or inappropriate life style. In turn, these aspects affect normal microbiota functioning – compositionally and metabolically speaking (Turnbaugh *et al.*, 2006). Under ‘normal’ conditions (healthy state), it has to be acknowledged that SCFA are essential in maintaining the equilibrium in host health through regulation and participation in several metabolic pathways. The effects of individual SCFA are described in the sections of each test compound.

Primary composition of substrates drive important changes in microbiota composition

Principal coordinate analysis (PCoA) showed a clear clustering of lean and obese microbiota before the addition of substrates (t0 h), in both weighted and unweighted UniFrac analyses (Figure 4A and 4B). PERMANOVA analysis demonstrated that this difference was significant between lean and obese (weighted: $P=0.002$; unweighted: $P=0.001$). At t72 h, this clustering disappeared, and instead, distinct clusters by primary composition of the substrates (starch as present in cassava bagasses and control, fructan in inulin or pectin present in orange bagasses and passion fruit peels), and no longer a distinction between lean and obese

Figure 3. Cumulative production of short chain fatty acids (acetate, propionate and butyrate) by the lean (A) and obese (B) microbiota at t72 h with different substrates. CB = cassava bagasse; OB = orange bagasse; PFP = passion fruit peel.

Figure 4. Principal coordinate analysis plots of the microbiota after 72 h fermentation period in TIM-2. Samples are plotted on the first three Principal Coordinates (PC1, PC2 and PC3). (A) Weighted and (B) unweighted UniFrac showing 2 different clusters among lean and obese samples at t0 h, which disappeared after 72 h fermentation period. (C) Weighted and (D) unweighted UniFrac showing that at t72 h, samples were clustered according to their primary substrate (fructan, pectin or starch).

microbiota, was present (Figure 4C and 4D for weighted and unweighted UniFrac). At t72 h, the unweighted UniFrac between lean and obese was still significantly different ($P=0.001$), but not significantly different in the weighted ($P=0.235$).

For t72 h, the overall difference for ‘primary substrate’ was significant (weighted: $P=0.001$; unweighted: $P=0.001$). For the individual substrates: fructan vs the others (weighted: $P=0.001$; unweighted: $P=0.001$), pectin vs the others (weighted $P=0.001$; unweighted: $P=0.001$); starch vs the others (weighted: trend for significance $P=0.053$; unweighted: $P=0.001$). This is evidence that the substrates drove the obese microbiota to a healthier profile, that is more similar to that of the lean microbiota, with the stimulation of commensals in the simulated lumen, among them, health promoting bacteria (see below). The Shannon index showed that fermentation of PFP3, OB1 and PFP1

stimulated the growth of more bacterial species (abundance and evenness) (Supplementary Figure S3). The effects that fermentation of each test compound had on the microbial composition will be discussed below for some selected genera for which effects were observed.

Cassava bagasses stimulated the beneficial genus *Bifidobacterium* in the obese microbiota

In our study, for the lean microbiota, all tested compounds (except inulin) caused a decrease in *Bifidobacterium* when compared to the control (Figure 5A). LEfSe analysis showed that *Bifidobacterium* is a discriminate feature for fructan (present in inulin), in the lean microbiota (Figure 6). In the obese microbiota, nonetheless, CB1 and CB2 were the only substrates (apart from inulin) that were capable of stimulating the growth of this genus (Figure 5B). Even though *Bifidobacterium* ferments mostly mono- and

Figure 5. Heatmap of percentage of changes of bacterial taxa at t72 h, compared to control in the lean (A) and obese (B) microbiota. Green colours represent an increase in abundance, whereas red colours represent a decrease. CB = cassava bagasse; OB = orange bagasse; PFP = passion fruit peel.

Figure 6. LefSe with the different primary carbohydrate components showing which bacterial groups were significantly different among samples (lean microbiota).

oligosaccharides, some species are capable of degrading complex carbohydrates like starch (Rajilić-Stojanović and De Vos, 2014), producing mainly acetate and lactate (De Graaf and Venema, 2008). Spearman correlation analyses indicated a positive association between bifidobacteria and the presence of glucose ($\rho=0.68$; $q\text{-value}=0.00021$), the monomer that composes starch. The genus *Bifidobacterium* is known by its positive correlation with health status of the host and several studies have shown that the genus *Bifidobacterium* is decreased in several different disease states (Wong *et al.*, 2020).

Interestingly, in our previous study with cassava bagasse, although fermentation of cassava bagasse by the obese microbiota produced an increase in the abundance of *Bifidobacterium*, a stronger bifidogenic effect was observed by fermentation of the lean microbiota (Bussolo de Souza *et al.*, 2014). Despite some differences between the two studies, still cassava bagasses (CB1 and CB2) were shown here to exert a beneficial effect on the obese microbiota regarding the stimulation of the genus *Bifidobacterium*.

Cassava bagasses were the substrates that, when used by the obese microbiota, produced similar amounts of acetate compared to our controls, and higher amounts than PFPs ($P<0.05$) (Figure 3). It was demonstrated in mice that the consumption of vinegar lowered body weight and body fat (Beh *et al.*, 2017). Another study showed a positive

effect of acetate related to obesity in overweight and obese men who received infusions of acetate in the distal and proximal colon. Distal infusions of acetate, but not proximal, increased fasting fat oxidation and fasting peptide YY (PYY), when compared to the placebo group (Van der Beek *et al.*, 2016). Another study, nevertheless, showed that increased acetate production by an altered microbiota was found to increase ghrelin secretion, causing hyperphagia and obesity in rats fed with high fat diet (Perry *et al.*, 2016). As mentioned earlier, it seems that the presence of an altered microbiota results in negative effects related to SCFA.

Fermentation of the three tested cassava bagasses enriched the abundance of *Ruminococcus* in the lean microbiota (Figure 5A). In the obese microbiota, however, fermentation of CB1 and CB2 caused a large decrease in *Ruminococcus* (~20%). CB3, instead, stimulated it by 7% (Figure 5A), and at t72 h, the microbiota was composed by 30.2% of the genus *Ruminococcus* (Figure 7). Some species of *Ruminococcus* were found to degrade resistant starch (Rajilić-Stojanović and De Vos, 2014). We have demonstrated in an *in vitro* model of the upper gastrointestinal tract (TIM-1) that CB1, CB2 and CB3 were composed on average of 12% of resistant starch (Bussolo de Souza *et al.*, 2018), which may explain the reason this genus was enriched upon addition of cassava bagasses. *Ruminococcus* has been inversely correlated with pro-inflammatory markers, such as interleukin-6 (IL-6) and C-reactive protein (CRP) (Van den Munckhof *et al.*, 2018).

Figure 7B. Microbiota composition after fermentation period with the different substrates (% of bacterial abundance), in obese (B) microbiota. CB = cassava bagasse; OB = orange bagasse; PFP = passion fruit peel.

Obesity is a metabolic disease characterised by a low-grade inflammation, which contributes to an increased risk to develop insulin resistance and type 2 diabetes (Cani *et al.*, 2007). A prebiotic which can enrich the abundance of anti-inflammatory species that can contribute to counteracting the negative effects of obesity is highly warranted.

It is very intriguing to observe that cassava bagasses (and passion fruit peels) (Figure 5) were only effective in increasing the abundance of *Ruminococcus* in lean microbiota. Apart from CB3, all other compounds decreased *Ruminococcus* abundance of the obese microbiota (Figure 5). It remains unclear what caused such differences.

The butyrate-producing genus *Coprococcus* was stimulated upon orange bagasses fermentation

Fermentation of orange bagasses led to the production of SCFA, which levels were comparable to our controls (Figure 3), showing the potential of this by-product to be used as substrate by the gut microbiota.

In the lean microbiota, fermentation of OB2 produced comparable amounts of butyrate (44.9 mmol) to our positive control – inulin (43.4 mmol). In the obese microbiota, fermentation of both orange bagasses – OB1 and OB2 (36.9 mmol and 38.5 mmol, respectively) resulted in similar amounts of butyrate production as inulin (37.2 mmol). Similar results were found when citrus pectin was used in an *in vitro* model inoculated with microbiota from obese individuals (Bianchi *et al.*, 2018). Butyrate is by far the most well studied SCFA, and with regard to obesity, it has an important role in keeping the integrity of the intestinal barrier by acting on the mucosal layer and tight junctions (Hatayama *et al.*, 2007; Willemsen *et al.*, 2003). Obesity is characterised by low grade inflammation that might be caused by the translocation of bacterial lipopolysaccharides due to a leaky gut (Cani *et al.*, 2007). It was also demonstrated that acute butyrate administration decreased food intake, while chronic supplementation prevented diet-induced obesity in mice (Li *et al.*, 2018). In our study, butyrate-producing bacteria from the genus *Coprococcus* were stimulated upon OB1 and OB2 addition in the lean microbiota, and by OB2 in the obese microbiota (Figure 5).

A robust increase in abundance of *Collinsella* upon fermentation of OB2 was found (increase of 40.5%) with a final abundance of 41% (Figure 7A), in the lean microbiota. A similar effect of orange bagasses on *Collinsella* abundance was observed with the obese microbiota. OB1 enriched the abundance of this genus by 26%, and at the end of the fermentation, *Collinsella* accounted for 28% of the total abundance (Figure 7B). Although the orange bagasses tested here were rich in small sugars (22.8% in OB1 and 33.4% in OB2) and a positive correlation between *Collinsella* and

small sugars was detected ($\rho=0.52$; q -value=0.03), our previous study with the isolated fibre from orange bagasse (and therefore without small sugars) also found an increase in the genus *Collinsella* (Bussolo de Souza *et al.*, 2019). Another correlation that may explain such robust increase in this genus due to orange bagasse addition was not found, requiring further research.

A correlation between *Collinsella* and lipid and glucose metabolism was found in some studies (Gomez-Arango *et al.*, 2016; Lambeth *et al.*, 2015), and *Collinsella* was found to be increased in subjects that followed a fast food diet (Zhu *et al.*, 2020). The exact mechanism by which *Collinsella* affects host lipid/glucose metabolism is not completely understood, but it is known that this genus has the ability to deconjugate bile acids (Rajilić-Stojanović and De Vos, 2014). A 'normal' gut microbiota is required for the production of colonic bile acids with higher amounts of secondary bile acids (Tran *et al.*, 2015). Secondary bile acids are ligands of the cell membrane receptor G-protein-coupled bile acid receptor (TGR5), which also triggers the release of the satiety hormones GLP-1 and PYY. These satiety hormones are also recognised for their effects on gut barrier function (Cani *et al.*, 2013).

Lower production of SCFA and intermediate organic acids may be an indication of slow fermentation of passion fruit peels

PFP1 and PFP2 were the substrates that compared to control and inulin, had the poorest fermentation, yielding smaller amounts of SCFA, although this difference was only significant in the lean microbiota ($P<0.05$). PFP3, which contained higher amounts of starch among the three passion fruit peels (38.9%), yielded comparable amounts of total SCFA than control and inulin (Figure 3 and Supplementary Figure S1). Overall, passion fruit peels were the substrates that led to the highest cumulative amounts of BCFA, both in experiments with the lean and obese microbiota (Table 1). PFP1 fermentation by the lean microbiota produced the highest cumulative amount of *iso*-butyrate (1.92 mmol), whilst in the obese microbiota PFP3 fermentation was responsible for the highest amount of *iso*-valerate (3.35 mmol).

The passion fruit peels contained higher amounts of proteins, which may explain the production of BCFA, as these are the end-products of bacterial fermentation of proteins and peptides (Flint *et al.*, 2009). Fermentation of passion fruit peels generated negligible amounts of lactate and succinate (Supplementary Table S2), indicating that these by-products were not fermented at a high speed. Lactate and succinate are intermediate metabolites and their accumulation is a sign of fast fermentation (Koenen *et al.*, 2016).

Table 1. Cumulative production of branched chain fatty acids (*iso*-butyrate and *iso*-valerate) by the lean and obese microbiota after 72 h fermentation with the different substrates.¹

	<i>i</i> -butyrate		<i>i</i> -valerate		Total	
	Lean	Obese	Lean	Obese	Lean	Obese
Control	0.2±0.2	0.5±0.8	0.4±0.1	1.3±1.2	0.6±0.3	1.9±2.0
Inulin	0.1±0.0	0.0±0.0	0.2±0.1	0.0±0.0	0.2±0.1	0.0±0.0
CB1	0.7±0.1	0.7±0.3	2.1±0.2	1.5±0.4	2.7±0.1	2.2±0.7
CB2	0.6±0.2	0.3±0.0	1.3±0.2	0.7±0.0	1.9±0.5	1.0±0.1
CB3	1.7±0.1	0.5±0.4	1.5±0.1	0.8±0.3	3.2±0.0	1.3±0.7
OB1	1.2±0.0	0.2±0.1	2.2±0.3	1.2±0.3	3.4±0.3	1.4±0.5
OB2	0.9±0.1	0.2±0.2	2.1±0.0	0.2±0.0	2.9±0.1	0.4±0.2
PFP1	1.9±0.2	1.8±0.3	3.3±0.3	3.0±0.2	5.2±0.5	4.8±0.5
PFP2	1.4±0.3	0.6±0.1	2.3±0.4	1.6±0.0	3.8±0.7	2.2±0.1
PFP3	1.7±0.1	1.7±0.7	2.9±0.4	3.4±1.0	4.6±0.5	5.1±1.8

¹ CB = cassava bagasse; OB = orange bagasse; PFP = passion fruit peel.

Our previous study with isolated fibre from passion fruit peels showed similar results, with lower cumulative production of SCFA and negligible amounts of intermediate metabolites, reinforcing our hypothesis that this by-product was fermented at a slow pace (Bussolo de Souza *et al.*, 2019). This might be a positive feature regarding distal colon health, since most of the fibres are consumed by the gut microbiota in the proximal and transverse part of the colon, and due to the lack of carbohydrates, fermentation of proteins become quantitatively more important distally, where for instance, the majority of colorectal cancers occur (De Graaf and Venema, 2008). Complex composition of passion fruit peels resulted in a microbiota with the highest diversity and evenness (Supplementary Figure S3) among all tested compounds, a positive trait regarding host health.

The genus *Bacteroides* was stimulated upon fermentation of passion fruit peels, either when the lean or obese microbiota was used. The effects were more prominent for the obese microbiota (Figure 5B), which at the end of the experiment led to higher abundances of *Bacteroides* than in the lean microbiota (Figure 7). Despite that growth of *Bacteroides* has been shown to be affected by dilution rate, pH and other factors (Adamberg and Adamberg, 2018; Adamberg *et al.*, 2020), we show here a positive association between *Bacteroides* and the presence of xylose ($\rho=0.79$; $q\text{-value}=7.09\times10^{-6}$) and the presence of fucose ($\rho=0.63$; $q\text{-value}=0.003$), both sugars present in PFPs (Supplementary Table S1). Xylose and fucose are sugars present in non-starch polysaccharides, such as hemicellulose and pectin. However, there was no significant association between *Bacteroides* and pectin. Other studies demonstrated that *Bacteroides* was enriched with pectin availability (Aguirre *et al.*, 2014; Tian *et al.*, 2016). It is worth to highlight, however,

that pectins are complex plant hetero-polysaccharides, with major structural differences between various sources (Larsen *et al.*, 2019a). Other characteristics of pectins, like composition of neutral sugars and distribution of homogalacturonan and rhamnogalacturonan fractions might have an effect on the enrichment of diverse bacterial groups. Another *in vitro* study by us showed that *Bacteroides* in general was enriched with pectin feeding, but specific species (*Bacteroides uniformis*, *Bacteroides plebeius* and *Bacteroides ovatus*) demonstrated an inconsistent modulation among all different pectins tested (Larsen *et al.*, 2019b). This is hypothesised to be due to the highly competitive environment, or even specific structural preferences by bacterial groups. This may explain why *Bacteroides* were only enriched with passion fruit peels and not orange bagasses, although the latter contained higher amounts of pectin (Supplementary Table S1). It appears that other characteristics of pectins are crucial for it to be utilised by *Bacteroides*. We also found that *Bacteroides* was positively associated with degree of acetylation (DA) ($\rho=0.71$; $q\text{-value}=0.0003$), and orange bagasses had low DA (Supplementary Table S1).

The genus *Faecalibacterium* in the lean microbiota only increased in abundance upon PFP2 fermentation, with a final abundance of 4%. In the obese microbiota PFP1 and PFP2 fermentation caused a small increase in *Faecalibacterium* (~1.5%) and the final abundances were 3.7% and 3.3%, respectively (Figure 7B). Its only known species, *F. prausnitzii*, is an anti-inflammatory commensal bacterium. Many studies showed inverse correlations between inflammatory bowel disease (IBD) and *F. prausnitzii* (Machiels *et al.*, 2014; Miquel *et al.*, 2013).

4. Conclusions

Our results showed that fermentation of different types of food-by-products, with different primary carbohydrates, were metabolised differently by the lean and obese microbiota. Regarding microbiota composition, a clear separated cluster at the beginning of the experiments between lean and obese microbiota was found, and at the end of the fermentation period, the clustering within lean and obese samples disappeared, and instead presence of primary carbohydrate in the tested food-by-products drove a new clustering. This new clustering showed the potential of food-by-product in driving the obese microbiota into a healthier profile, similar to lean, with the stimulation of commensal and beneficial bacteria.

The results found in this study show that food-by-products have the potential to be used as a tool to manipulate not only the microbiota from obese individuals, but also from e.g. healthy individuals. The use of food-by-products and the fibres they contain may represent a way to give a better destination to these by-products, such as to incorporate them back into food products as functional ingredients, at the same time increasing fibre intake, which is in general lower than the recommendation. The results of the present mechanistic study show promise that these by-products could be an important step forward to tackle obesity and at the same time decrease the waste of valuable food material and consequently environmental pollution. Moreover, they are inexpensive and a non-invasive way to be used as a dietary intervention to improve health. Further experiments, either *in vitro* or *in vivo* are suggested to confirm our findings and contribute to elucidate the interactions between gut microbiota, obesity and utilisation of complex polysaccharides. For instance, combinations of the food-by-products could be tested to study their synergistic or antagonistic effect.

Supplementary material

Supplementary material can be found online at <https://doi.org/10.3920/BM2020.0151>.

Figure S1. Kinetics and total cumulative production of short chain fatty acids by the lean and obese microbiota during 72 h fermentation in TIM-2 with different test compounds.

Figure S2. Total energy production by the lean and obese microbiota after fermentation with the different test compounds.

Figure S3. Shannon index at t72 h (lean and obese combined) with test compounds.

Table S1. Chemical composition of cassava bagasses, orange bagasses and passion fruit peels.

Table S2. Cumulative production of organic acids (succinate, lactate, formate and caproate) by the lean and obese microbiota after 72 h fermentation with the different substrates.

Conflict of interest

The authors declare that there are no conflicts of interest.

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