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Sugarcane polyphenol and fiber to affect production of short-chain fatty acids and microbiota composition using *in vitro* digestion and pig faecal fermentation model

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ABSTRACT

This study aimed to examine the effects of sugarcane polyphenol and fiber (Phytolin + Fiber) on gut microbiota, short-chain fatty acids (SCFAs) production and phenolic metabolites production using *in vitro* digestion and fermentation model. Microbial profiling by 16S rRNA sequencing was used to analyze the pig faecal microbiota profile. SCFAs were identified and quantified by GC-FID, and phenolic metabolites were characterized by LC-ESI-QTOF-MS/MS. The results showed that Phytolin + Fiber exert synergistic effects on the pig gut microbiota by increasing the relative abundances of *Lactobacillus* and *Catenibacterium*, and decreasing the relative abundances of *Mogibacterium*, *Dialister*, and *Escherichia-Shigella*. Phytolin + Fiber also significantly increased the total SCFAs production, particularly the propionic and butyric acids. Production of phenolic metabolites related to major polyphenols in Phytolin were tentatively identified. These results suggest that Phytolin + Fiber could be beneficial to human colon health given the similarities between pig and human intestine in terms of physiology and microbiome.

1. Introduction

The gut microbiota plays an important role in human health. There is a complicated relationship between diet, the gut microbiota, and human health. Undernourished diet and dysfunctional gut microbiota have been associated with the elevated risk of having noncommunicable diseases such as cardiovascular disease, obesity, inflammatory bowel disease, diabetes, and cancers (Danneskiold-Samsøe et al., 2019). Diet is crucial in shaping and modulating the gut microbiota composition and functionality. Plant-based diets are associated with gut microbiota enterotypes that may confer health benefits, as opposed to protein- and animal fat-based diets (De Filippis et al., 2016). The updated definition of the term "prebiotics" as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" has extended the concept of prebiotics to include non-carbohydrate compounds such as polyphenols, and addresses their impact beyond the gastrointestinal tract (Gibson et al., 2017). Dietary fiber and polyphenols are widely present in plant food and can be metabolized by bacteria to produce health-promoting short-chain fatty acids (SCFAs) and phenolic metabolites, respectively. However, the potential synergistic effects between fiber and phenolic compounds with the gut microbiota and their subsequent health impacts remain unclear (Loo, Howell, Chan, Zhang, & Ng, 2020).

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Sugarcane (*Saccharum officinarum*) molasses is an undervalued by-product in sugar mill industry. It was found to regulate carbohydrate metabolism and be beneficial in protection against metabolic disorders *in vitro* (Ji, Yang, Flavel, Shields, & Kitchen, 2019), and showed inhibitory effects on mutation and nitric oxide production that suggested anti-inflammatory activity (Wang et al., 2011). Antioxidant activity and polyphenol composition of sugarcane molasses extract were investigated in a previous study (Deseo, Elkins, Rochfort, & Kitchen, 2020). Sugarcane fiber is also postulated to act as a natural carrier of phenolic compounds to target the colon and bring potentially beneficial health effects (Loo et al., 2020). However, to our knowledge, little is known about the interaction between sugarcane fiber and polyphenols extract, their reciprocal effects with the gut microbiota, and their significance to gut health.

Among animal models, pigs (*Sus scrofa*) are the best non-primate model for studying human nutrition and digestion (Miller & Ullrey, 1987). In terms of the microbes found in the colon of the pig gut, comparable composition and diversity were found in intestines of pigs and humans, suggesting pigs can be used as a suitable model for understanding complex interactions with foods (Leser et al., 2002). Hence, this study aimed to investigate the effects of sugarcane polyphenols and fiber on pig gut microbiota in the hope that we can infer their health effects in humans. We hypothesize that the combination of sugarcane polyphenols and fiber would result in a synergistic effect on the pig gut microbiota profile that would result in the alteration of the productions of SCFAs and phenolic metabolites.

2. Material and methods

2.1. Chemicals and reagents

The following chemicals and reagents were analytical grade and were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia): calcium chloride, sodium chloride, acetic acid, sodium hydroxide, potassium chloride, monopotassium phosphate, sodium bicarbonate, magnesium chloride, ammonium carbonate, porcine pepsin, pancreatin, sodium phosphate dibasic, sodium phosphate monobasic, bile salts, peptone, yeast extract, casein, L-Cysteine, Tween-80, guar, soluble starch, tryptone, pectin, mucin, sodium bicarbonate, magnesium sulfate, dipotassium phosphate, formic acid, *ortho*-phosphoric acid, 4-methylvaleric acid, acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid, and hydrocholoric acid (HCl). The following chemicals and reagents were LCMS grade and were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia): methanol, formic acid, 3-phenylpropionic acid, 3-(3-hydroxyphenyl)-propanoic acid, 3-hydroxy-4-methoxybenzoic acid, 3-(3,4-dihydroxyphenyl)-propionic acid, and luteolin.

2.2. Sample preparation

Polyphenol-rich sugarcane extract (Phytolin®), sugarcane fiber (Fiber), and sugarcane polyphenol-fiber mix (Phytolin + Fiber) are provided by The Product Makers (TPM) (Keysborough, Victoria, Australia). Phytolin is a bio-active natural extract from sugarcane which is a dark brown syrup, with total phenolic content (TPC) of 18 mg gallic acid equivalents (GAE)/mL and total flavonoids content of 4.2 mg catechin equivalents/mL, prepared as the ethanol extract (ME) sugarcane molasses according to Deseo et al. (2020). The Fiber was prepared from sugarcane bagasse according to Pluschke, Feng, Williams, and Gidley (2019). Briefly, sugarcane bagasse was extracted with water to remove sugars and other water-soluble materials, and the residue which constitutes sugarcane insoluble fiber is then dried at low-heat (<40 °C) and dried materials milled to produce the Fiber powder < 100 μm in size with a final dietary fiber content of 0.84 mg/g sample. Phytolin + Fiber is produced by mixing liquid Phytolin with Fiber (v/w) and the water component removed by oven-drying for 72 hr at 60 °C into a fine powder with a TPC of 13.7 mg GAE/ g sample.

2.3. Experimental design

To examine the effects of continuous digestion on colonic metabolites production, Phytolin, Fiber, and Phytolin + Fiber mixture were subjected to simulated *in vitro* oral, gastric, and intestinal digestion followed by *in vitro* colonic fermentation. The samples subjected to the digestion were 1 g of Phytolin, Fiber or Phytolin + Fiber. All samples and control (without added sample) were subjected to the continuous stage digestions and fermentation in triplicate (n = 3).

2.3.1. Simulated gastric and intestinal digestion

The simulated gastrointestinal digestion process was performed according to Minekus et al. (2014) with minor modifications as described in the sections 2.3.1.1 to 2.3.1.3. Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as previously described (Minekus et al., 2014).

2.3.1.1. Oral phase. 1 g of each sample was mixed with 2 mL of SSF, 3 mL of water, and 12.5 μL of 0.3 mol/L $CaCl_2$ in a 50 mL centrifuge tube. Salivary α-amylase was left out of the mix as the mixture will be subjected to gastric digestion immediately. Controls were made up without adding any samples. Tubes were then vortexed to mix well the mixture.

2.3.1.2. Gastric phase. The oral-phase mixture was mixed with 3.75 mL of SGF, 0.8 mL porcine pepsin stock solution (25000 U/mL), 2.5 μL of 0.3 mol/L $CaCl_2$ and 347.5 μL of water. The pH of the mixture was adjusted to around 3.0 by adding around 0.1 mL of 1 mol/L HCl. Tubes were then shaken at 150 rpm and 37 °C for 2 hrs in shaking incubator. Samples were carried forward to intestinal digestion.

2.3.1.3. Intestinal phase. The gastric-phase mixture was mixed with 5.5 mL of SIF, 2.5 mL of freshly prepared pancreatin solution (800 U/mL), 1.25 mL of fresh fed state bile solution (40 mg/mL), 20 μL of 0.3 mol/L $CaCl_2$ and 655 μL of water. The pH of the mixture was adjusted to around 7.0 by adding around 75 μL of 1 mol/L NaOH. Tubes were then shaken for 2 hrs at 37 °C by shaking incubator at 150 rpm. After this phase, samples were collected, snap frozen using liquid nitrogen, and stored at −20 °C for further analysis.

2.3.2. Colonic fermentation

2.3.2.1. Basal media preparation. Basal media was prepared as previously described (Sirisena, Ajlouni, & Ng, 2018) 5 g soluble starch, 5 g peptone, 5 g tryptone, 4.5 g yeast extract, 4.5 g NaCl, 4.5 g KCl, 2 g pectin, 4 g mucin, 3 g casein, 1.5 g $NaHCO_3$, 0.8 g L-Cysteine HCl, 1.23 g $MgSO_4{\cdot}7H_2O$, 1.0 g guar, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.4 g bile salts, 0.11 g $CaCl_2$ and 1 mL Tween-80 were dissolved and made up to 1 L with MilliQ water. The basal media was adjusted to pH 7.0 using 1 mol/L HCl or 1 mol/L NaOH at 25 °C and sterilised at 121 °C for 20 min with autoclave.

2.3.2.2. Faecal slurry preparation. Freshly defecated pig faeces from 3 healthy female pigs aged 18 weeks (average weight of 80 kg), that maintained a standard commercial grower diet (14% digestible energy, 18% crude protein, 2.7% fat, 2.38% fibre, 56.3% starch), was collected for faecal slurry preparation. These pigs were control group in another pig-feeding study with ethical approval obtained from the Veterinary and Agricultural Sciences Human Ethics Advisory Group, University of Melbourne (Ethics Approval ID: 1914753.1). 20 g faeces were weighed into a stomacher bag and 80 g sterilised pre-N_2 flushed 0.1 M phosphate buffer (pH = 7.0) was added to make 20% w/w faecal slurry. It was then homogenised for 5 min in a stomacher mixer and filtered through sterile muslin cloth to remove particulate matter. Faecal slurry was then

transferred to 50 mL sterile, pre-N_2 flushed tubes with 5 mL aliquots using sterile pipette. The tubes were used for experiment on the same day. All work involving faecal samples were carried aseptically under biosafety chamber (Sirisena et al., 2018).

2.3.2.3. In vitro colonic fermentation. The *in vitro* colonic fermentation process was performed according to Sirisena et al. (2018) with modifications. The intestinal-phase mixture was thawed and warmed to 37 °C before centrifugation at 5000 rpm for 15 min at 4 °C to obtain the precipitate fractions for colonic fermentation process. Previously prepared faecal slurry and basal media were pre-warmed at 37 °C. 5 mL of faecal slurry and basal media were added to the tubes containing the intestinal-phase precipitate. Tubes were then flushed with nitrogen gas, and the tubes capped tightly with the lids and sealed with parafilm. All capped tubes were then placed in a shaking incubator maintained at 100 rpm, 37 °C for 24 hrs. Samples were collected at 7 different time points (0, 2, 4, 8, 12, 18, and 24 h) from different tubes throughout the colonic fermentation phase, snap frozen using liquid nitrogen, and stored at −20 °C for further analysis.

2.4. Determination of total phenolic and antioxidant activity

2.4.1. Sample extracts preparation

Raw samples (Phytolin, Fiber, and Phytolin + Fiber) were extracted with 80% acidified methanol (0.1% formic acid) by mixing at room temperature and 150 rpm for 24 hr, and followed by centrifugation at 5000 rpm and 4 °C for 20 min. The resulted supernatant was filtered with 0.45 μm syringe filter and stored at – 20 °C before analysis.

In vitro digesta collected from gastric and intestinal phases were separated by centrifugation at 5000 rpm and 4 °C for 20 min into supernatant and precipitate for determination of total phenolic content (TPC) and Trolox Equivalent Antioxidant Capacity (TEAC). The digesta precipitates were extracted with 5 mL of 80% acidified methanol (0.1% formic acid) by mixing at room temperature and 150 rpm for 24 hr and followed by centrifugation at 5000 rpm and 4 °C for 20 min to obtain the resulted supernatant for the chemical assays.

The digesta supernatants were treated with two different ways for determination of TPC and TEAC. Firstly, the digesta supernatants used for the determination of TPC were treated with Carrez precipitation method to remove interfering substances from the *in vitro* digestive fluids. Briefly, 500 μL of sample was mixed with 100 μL of 15% (w/v) $K_4[Fe(CN_6)]\cdot 3H_2O$ solution and 100 μL of 30% (w/v) $ZnSO_4\cdot 7H_2O$ solution. Mixture was then centrifuged at 10,000 g and 4 °C for 20 min. 500 μL of the resulted supernatant was mixed with 500 μL methanol and centrifuged again at 10,000*g* and 4 °C for 20 min. The final supernatant was collected and used for the determination of TPC. Secondly, the digesta supernatants used for the quantification of TEAC were prepared by mixing the *in vitro* digested supernatant with acidified methanol (0.1% formic acid) at 1:1 ratio (v/v) by vortex for 10 min, and then centrifuged at 10,000 g and 4 °C for 10 min to obtain the final supernatants for TEAC assays.

2.4.2. Total phenolic content (TPC)

The TPC of the sample extract was analyzed using a modified Folin-Ciocalteu reagent (FCR) method (Singleton & Rossi, 1965). All samples were determined in triplicate and the TPC was derived from the gallic acid standard curve and expressed as mg gallic acid equivalents (mg GAE).

2.4.3. Trolox equivalent antioxidant capacity (TEAC)

The TEAC of the sample extract was analyzed using 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as the free radical as previously described (Re et al., 1999) All samples were determined in triplicate and the TEAC was derived from the Trolox standard curve and expressed as mg Trolox equivalents (mg TE).

2.4.4. Intestinal bio-accessibility and colonic availability

The intestinal bio-accessibility (IB) represents the percentage of TPC or TEAC after simulated gastrointestinal digestion that are soluble, thus could be available for absorption into the systemic circulation. The colonic availability (CA) represents the percentage of TPC or TEAC carried over to the *in vitro* colonic fermentation after *in vitro* digestion that could be accessible to the gut microbiota. These parameters were calculated as follows:.

$$\text{Intestinal bio} - \text{accessibility (IB) (\%)} = \frac{A}{A+B} \times 100$$

$$\text{Colonic availability (CA) (\%)} = \frac{B}{A+B} \times 100$$

where:

A = TPC or TEAC of digesta supernatant after *in vitro* digestion;
B = TPC or TEAC of digesta precipitate after *in vitro* digestion.

2.5. 16S rRNA extraction and sequencing

Each replicate from every sample collected at different time points throughout the *in vitro* colonic fermentation were thawed, washed three times in ice-cold phosphate buffered saline with 2% polyvinylpolypyrrolidone and centrifuged at 10,000×*g* for 10 min to remove DNA-extraction-interfering phenolic substances. The obtained pellets were stored in 2 mL screw cap tubes covering with DNA/RNA shield liquid (Zymo Research, California, U.S.A.), and sent for DNA extraction and 16S rRNA sequencing services provided by Australian Genome Research Facility Ltd (Australia). The bacterial DNA was extracted using the DNeasy® PowerSoil® Pro Kit (QIAGEN GmbH, Hilden, Germany). The 16S rRNA gene from V1 to V3 regions were amplified by PCR using the 27F-519R primers. The sequencing was conducted on an Illumina MiSeq (San Diego, CA, USA) with a V3, 600 cycle kit (2 × 300 base pairs paired-end).

2.6. SCFAs extraction and analysis of in vitro colonic fermentation samples

Sample preparation method was according to Gu et al. (2019) with slight modifications. Samples collected at each time points throughout the colonic fermentation phase were centrifuged at 5000 rpm for 15 min at 4 °C. 1.5 mL of the resulting supernatant was transferred into a new 10 mL plastic centrifuge tube and mixed well with 3.5 mL of dilute acid (1% formic acid and 1% orthophosphoric acid) and 8.0 μmol 4-methylvaleric as the internal standard. 1.5 mL of the mixture was then transferred into GC vial for GC-FID analysis. Standard curves of the analytical standards were prepared by serial dilutions of acetic, propionic, butyric, isobutyric, valeric and isovaleric acid solutions using the dilute acid. All the prepared standards and reagents were stored at 4 °C before GC-FID analysis.

The analysis of SCFAs was performed as previously described Gu et al. (2019), using gas chromatography (GC) (7890B Agilent, CA, USA) coupled with a flame ionization detector (FID), an autosampler (7693 Agilent, CA, USA) and an autoinjector (G4513A Agilent, CA, USA). A SGE BP21 capillary column (12 × 0.53 mm internal diameter (ID) with 0.5 μm film thickness, SGE International, Ringwood, VIC, Australia, P/N 054473) and a retention gap kit (including a 2 × 0.53 mm ID guard column, P/N SGE RGK2) were attached. The carrier gas was helium with a flow rate at 14.4 mL/min. The GC conditions were as follows: oven temperature initiated at 100 °C for 30 s, increasing to 180 °C at a rate of 6 °C/min, and held for 1 min, increasing again to 200 °C at a rate of 20 °C/min, and held for 10 min; FID temperature setting at 240 °C; injection port temperature setting at 200 °C; makeup gases of nitrogen, hydrogen and air at the flow rates of 20, 30, and 300 mL/min, respectively; and sample injection volume of 1 μL. All results were converted to

mM per digesta for subsequent statistical analysis.

2.7. Analysis of phenolic metabolites from in vitro colonic fermentation

Samples collected at each time points throughout the *in vitro* colonic fermentation were centrifuged at 5000 rpm for 15 min at 4 °C. 1 mL of the resulting supernatant was transferred into a new 5 mL plastic centrifuge tube and vortexed with 1 mL of acidified LCMS-grade methanol (0.1% formic acid) for 5 min. The mixture was centrifuged at 10000 rpm and 4 °C for 15 min. The resulted supernatant was filtered by 0.45 μm syringe filter and stored at −20 °C before the LC-ESI-QTOF-MS/MS analysis.

Characterization of phenolic metabolites was performed using an Agilent 1200 series HPLC (Agilent Technologies, CA, USA) equipped with an Agilent 6520 Accurate-Mass Q-TOF LC/MS (Agilent Technologies, CA, USA). The separation was performed by using a Synergi Hydro-RP 80A, LC column 250 × 4.6 mm, 4 μm (Phenomenex, Torrance, CA, USA). Mobile phase A consisted of 0.1% formic acid in MilliQ water, and mobile phase B consisted of 0.1% of formic acid in acetonitrile/water (95:5, v/v). Elution was performed using the following gradient program: 10–35% B (0–30 min); 35–40% B (30–35 min); 40–55% (35–40 min); 55–75% B (40–50 min); 75–95% B (50–55 min); 95–100% B (55–57 min); 100–10% B (57–60 min). At the end of each injection, the column was equilibrated using the following gradient elution: 10–50% B (0–5 min); 50–100% B (5–10 min); 100% B (10–20 min); 100–10% B (20–22 min); 10% B (22–30 min). The flow rate was set at 0.6 mL/min and the injection volume was 20 μL.

Dual electrospray ionization (ESI) was used as the ionization source operating in negative mode. Mass spectra in the *m/z* range from 90 to 1000 were obtained. The mass spectrometry conditions were set as the following: nitrogen gas temperature at 325 °C with flow rate of 7 L/min, and the sheath gas was set at 11 L/min at 250 °C, nebulizer gas pressure of 40 psi. The capillary and nozzle voltages were set at 3500 V and 500 V, respectively. MS/MS analysis was performed in automatic mode using collision energy of 15 and 30 eV for fragmentation. Data acquisition and analysis were performed using MassHunter workstation software (Qualitative Analysis, version B.03.01). Peak identification was performed by comparison of retention time, precursor *m/z* and product ions with reference standards.

2.8. Data analysis

Differences in SCFAs concentration among different samples were analyzed with one-way analysis of variance (ANOVA) and Tukey's HSD test performed by IBM® SPSS statistics software 27 (SPSS Inc., Chicago, IL, USA). The p value for significance was validated by Bonferroni adjustment and reported as $p < 0.007$ with 7 multiple comparisons (total SCFAs and 6 specific SCFAs) among 4 different treatments (the $p < 0.05$ value was divided by 7).

Microbiome bioinformatics were performed with QIIME 2 2020.11 (Bolyen et al., 2019). Raw sequence data were demultiplexed and quality filtered using the q2-demux plugin followed by denoising with DADA2 (via q2-dada2) (Callahan et al., 2016). Alpha diversities of bacterial communities were calculated using the Shannon's diversity index and Pielou's evenness index. The factorial Kruskal–Wallis sum-rank test ($\alpha = 0.05$) was used to determine the statistically significant differences in the alpha-diversity. Non-parametric microbial interdependence test (NMIT) was performed within the QIIME 2 environment to examine the differences of gut microbiota composition between different treatments by determining the longitudinal sample similarity as a function of temporal microbial composition (Zhang, Han, Cox, & Li, 2017). Principial coordinate analysis (PCoA) plot was generated using R (R Core Team, 2021) with the "qiime2R" package (Bisanz, 2018). Significant taxonomic differences of faecal bacteria after 24 h *in vitro* fermentation between treatments were examined by linear discriminant analysis (LDA) effect size (LEfSe) analysis (https://huttenhower.sph.harvard.edu/galaxy/) (Segata et al., 2011). The taxa with significant differential abundances between treatments (all-against-all comparisons) were identified by the factorial Kruskal–Wallis sum-rank test ($\alpha = 0.05$), and the effect size of each discriminative feature was then estimated by the logarithmic LDA score (threshold = 2.0). The identified significant taxa were used to plot the taxonomic cladograms demonstrating differences between treatments.

Differences in the relative abundances of selected major genera (relative abundance > 0.1%) at specific fermentation time points among different treatments were analyzed with one-way analysis of variance (ANOVA) and Tukey's HSD test performed by IBM® SPSS statistics software 27 (SPSS Inc., Chicago, IL, USA). The p value for significance was validated by Bonferroni adjustment and reported as $p < 0.0015$ with 32 multiple comparisons among 4 different treatments (the $p < 0.05$ value was divided by 32). The relative abundances plot was generated using R (R Core Team, 2021) with the "ggplot2" (Wickham, 2011).

Spearman's rank correlation coefficient was calculated using R (R Core Team, 2021) to estimate the association between gut microbiota composition and SCFAs concentration and visualized using the "corrplot" package (Wei & Simko, 2021). The Benjamini–Hochberg false-discovery rate-corrected p value (q value) was calculated using the "p.adjust" function to correct for multiple comparisons in the calculation of Spearman's rank correlation coefficient. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) pipeline was used for functional prediction based on 16S rRNA sequencing data (Douglas et al., 2020).

3. Results and discussion

3.1. Total phenolic content and antioxidant activity of Phytolin in intestinal phase were affected by the inclusion of Fiber

To investigate the availability of phenolic materials to the colon, samples were subjected to *in vitro* sequential oral-gastric-intestinal digestion, and partitioning of phenolics into the supernatant and precipitate fractions from low-speed centrifugation in the intestinal digesta (Table 1).

The retention of FCR reactive materials, as measured by the TPC assay, in the intestinal digesta after oral and gastric digestions was between 51.5 and 72.9% which might indicate degradation of these materials. But the near to complete recovery of antioxidant activity in the intestinal digesta that ranged from 92.3% to 112% suggested that the antioxidant materials were stable through the digestion. The results suggested that TEAC was a more reliable indicator in tracking the movement of phenolic compounds of the digesta throughout the *in vitro* digestion.

The phenolic compounds in Phytolin were largely soluble in the intestinal digesta as shown by high TPC (96.2%) detected in the supernatant of the intestinal digesta (Table 1). However, solubility is lower in the Phytolin + fiber sample, possibly due to interaction of phenolic compounds with sugarcane fiber. The colonic availability (CA) in comparing Phytolin and Phytolin + Fiber was increased from 3.8% to 34.8% as measured by the TPC assay, and from 1.8% to 13.8% as measured by the TEAC assay. For the Fiber sample which contain residue phenolic materials, the CA was 100% with both assays since all the phenolic materials were associated with the precipitate. These results show that phenolic compounds in Phytolin and Fiber could be delivered to the colon after digestion, but more importantly the results also showed addition of Phytolin to Fiber substantially increased the level of phenolic compounds that can be carried over to the colonic compartment after *in vitro* digestion in the small intestine.

It is suggested that dietary fiber can be utilized as a carrier of dietary antioxidants, particularly polyphenols (Loo et al., 2020). Polyphenols and dietary fibers can associate and interact with each other through hydrogen bonding, Van der Waals forces and hydrophobic interactions, depending on their structural characteristics (Jakobek & Matić, 2019).

Table 1
TPC and TEAC of undigested and digested samples.

Analysis	Undigested Sample (1 g)	Digested Sample (intestinal digesta)			**IB (%)	***CA (%)
		Supernatant	Precipitate	Total (supernatant+precipitate)		
TPC (mg GAE)	Phytolin (18.00 ± 0.03)	8.93 ± 0.25	0.35 ± 0.04	9.28 ± 0.30 (51.5%)*	96.2	3.8
	Fiber (3.47 ± 0.09)	n.d.	2.24 ± 0.12	2.24 ± 0.12 (64.5%)*	0	100
	Phytolin + Fiber (13.70 ± 0.25)	6.51 ± 0.15	3.47 ± 0.17	9.99 ± 0.03 (72.9%)*	65.2	34.8
TEAC (mg TE)	Phytolin (25.7 ± 2.6)	23.30 ± 0.65	0.43 ± 0.03	23.73 ± 0.65 (92.3%)*	98.2	1.8
	Fiber (2.27 ± 0.07)	n.d.	2.15 ± 0.29	2.15 ± 0.29 (94.7%)*	0	100
	Phytolin + Fiber (14.50 ± 0.45)	14.05 ± 2.11	2.28 ± 0.04	16.3 ± 2.1 (112%)*	86.2	13.8

*Percentage recovery compared to undigested sample.
**Intestinal Bio-accessibility (IB) (%) = A/(A + B) × 100 and ***Colonic Availability (CA) (%) = B/(A + B) × 100, where: A = TPC or TEAC of digesta supernatant after *in vitro* digestion; B = TPC or TEAC of digesta precipitate after *in vitro* digestion.

Subsequently, the associations of polyphenols with fibers can enhanced the availability of polyphenols that can be delivered to the colon. This is, indeed, the case as observed with polyphenols from the Phytolin product added to the sugarcane fiber.

3.2. Microbiota profile was altered when Phytolin and Fiber were applied to the fermentation

To elucidate the effects of Phytolin, Fiber, and Phytolin + Fiber on the microbiota profile throughout the *in vitro* colonic fermentation, we assessed the bacterial composition by analysing the 16S rRNA amplicon of the samples collected at different time points (0, 2, 4, 8, 12, 18, and 24 h) throughout the fermentation process. Alpha diversity indices (Pielou's evenness and Shannon) were used to estimate the distribution of abundances of the taxonomic groups and the richness of these groups, respectively, in the bacterial community within each treatment group (Fig. 1a). In term of Pielou's evenness, control and Phytolin exhibited significant increase in bacterial community evenness starting from 4 h and maintained the significance up to 24 h of fermentation, as compared to their own initial bacterial community at 0 h. However, Fiber and Phytolin + Fiber showed different patterns of changes in Pielou's evenness during the fermentation process when comparing to their respective initial microbiota composition at 0 h. Microbiota evenness in Fiber decreased significantly from 2 h to 8 h of fermentation and showed significant increases at 18 h and 24 h of fermentation. In the case of Phytolin + Fiber, significant increase in microbiota evenness were only observed from 8 h to 18 h of fermentation. In term of Shannon diversity, control and Fiber had no significant alteration throughout the 24 h fermentation process. Phytolin showed significant increases in bacterial community richness starting from 4 h to 12 h and at the end of 24 h fermentation as compared to the initial condition, whereas Phytolin + Fiber also significantly increased the bacterial community richness from 8 h to 12 h of fermentation. These results showed that Phytolin + Fiber combined the significant effects observed with Phytolin or Fiber alone based on the alpha diversity indices, where both the Pielou's evenness and Shannon diversity of Phytolin + Fiber increased in the middle of 24 h *in vitro* colonic fermentation.

To evaluate how the interdependencies of OTUs within the microbiota community might differ over time between treatment groups, NMIT was performed to examine the longitudinal sample similarity as a function of temporal microbial composition (Zhang et al., 2017). It showed that Phytolin + Fiber exhibited the most significant different bacterial community among the 3 samples as compared to the control after 24 h of fermentation (Fig. 1b). Clear clustering of the microbial communities for different treatment groups was observed. Different bacterial community patterns between samples were related to the significant associations (LEfSe; Kruskal–Wallis sum-rank test, $\alpha < 0.05$) between bacterial taxa and samples after 24 h of fermentation (Fig. 1c). These results agree with previous findings where plant-based polysaccharides, polyphenols or the combinations of both components were showed to regulate the gut microbiota profiles to different extents, for example, sweet potato polyphenols combined with cellulose or inulin, were shown to significantly affect the alpha- and beta-diversities of swine faecal microbiota, depending on their differences in fermentability (Kilua et al., 2019).

To determine the alteration of specific bacterial taxa throughout the 24 h of *in vitro* colonic fermentation, relative abundance of the most abundant microbial genera (>0.1%) across all samples were compared using ANOVA and Tukey's HSD test to identify the significant differences ($p < 0.0015$) among the bacterial community between different treatments at genus level. Phytolin, Fiber, and Phytolin + Fiber modulated the microbiota composition differently by altering the relative abundances of different microbial genera at specific fermentation time points throughout the *in vitro* colonic fermentation (Fig. 2 and Supplementary Table 1).

Among these alterations, Phytolin + Fiber retained the potentially beneficial effects caused by Phytolin and Fiber alone, where similar significant changes in the relative abundances of specific genera were observed. For instance, at 4 h and 12 h of fermentation, Fiber and Phytolin + Fiber significantly increased the relative abundance of *Lactobacillus*, which is recognised as a positive member of the human gut microbiota (Sánchez et al., 2017), while reduction of *Lactobacillus* was observed at the same fermentation time in the Phytolin-only sample. This suggested that the Phytolin + Fiber was more effective in promoting the growth of *Lactobacillus*. Additionally, Phytolin and Phytolin + Fiber also caused significant increment in the relative abundance of *Catenibacterium* from 2 h to 18 h of fermentation, while Fiber-only showed no significant impact on this genus. In previous studies, *Catenibacterium* was associated with improvement of gut health by increasing the fermentation of fiber and resulting in higher SCFA production (He et al., 2018). However, the increments in these specific genera did not persist up to 24 h of fermentation, which might be due to the insufficiency of substrate to support the growth of these specific genera. It was also postulated that short-term dietary alterations were not enough to induce significant changes to the gut microbiota profile due to the resilience of human gut microbiota (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). Therefore, long-term intervention study using similar samples with different dosages can be conducted in the future to investigate the potential beneficial effects of the combined Phytolin + Fiber preparation on the gut microbiota.

Furthermore, Phytolin + Fiber resulted in significant reduction of relative abundances of *Mogibacterium*, *Dialister*, and *Echerichia-Shigella* up to 24 h of fermentation. Similar reductions were also observed in Phytolin or Fiber, nevertheless, Phytolin + Fiber caused the most significant effect in these modulations. *Mogibacterium* abundance was positively correlated with helminth infection in low socioeconomic status children in Indonesia (Amaruddin et al., 2020). *Dialister* was positively correlated with the risk factor of developing spondyloarthritis (Tito et al., 2017). In term of *Echerichia-Shigella*, it was suggested to be opportunistic pathogenic due to its association with gut microbiota

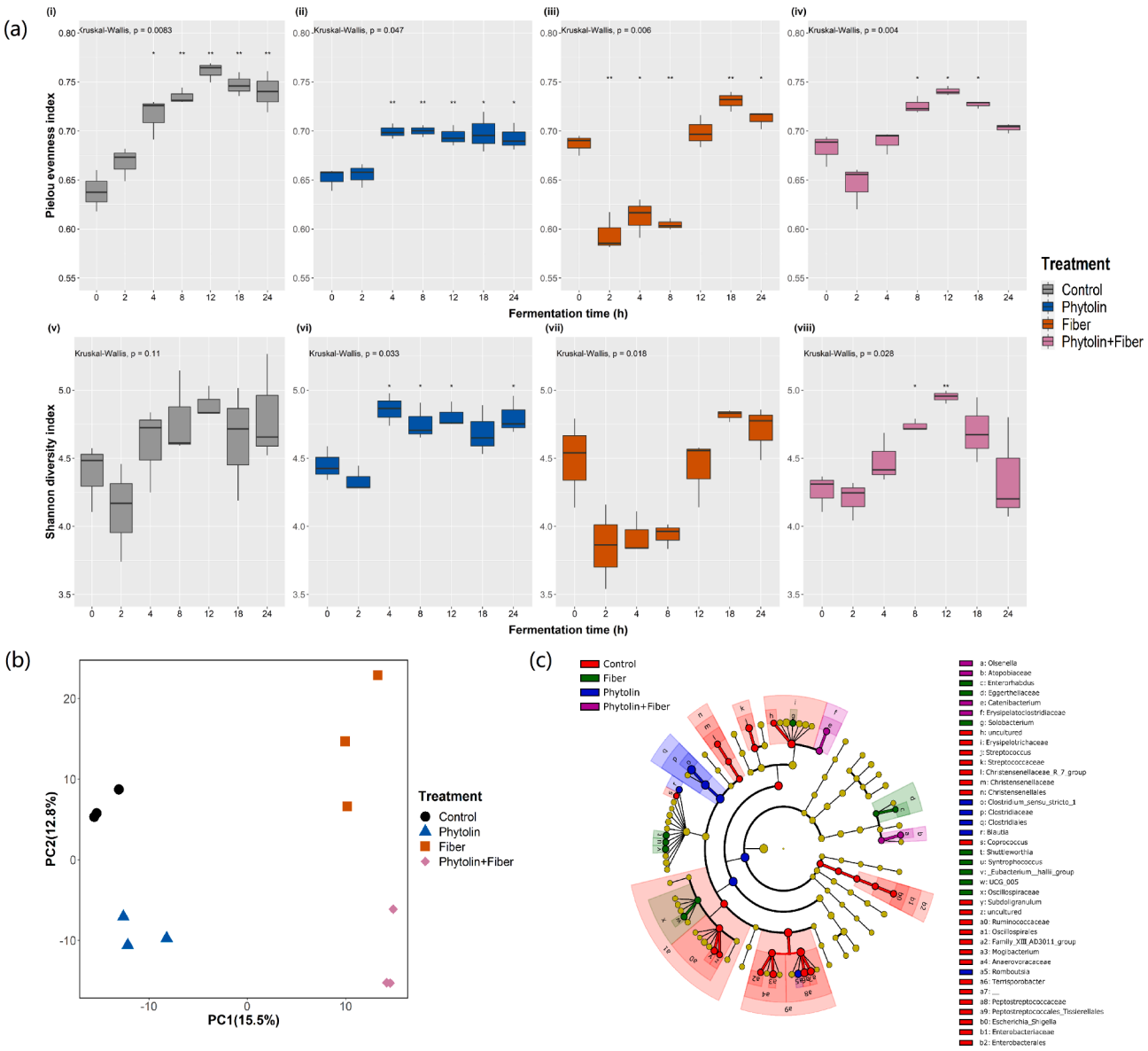

Fig. 1. Shifts in the faecal microbiota in different treatments (a) Alpha diversity analysis of faecal microbiota throughout the *in vitro* colonic fermentation at different time points (0 h, 2 h, 4 h, 8 h, 12 h, 18 h and 24 h). Panel (i) control, (ii) Phytolin, (iii) Fiber, and (iv) Phytolin + Fiber indicated the Pielou evenness index of different treatments from 0 h to 24 h of fermentation; while panel (v) control, (vi) Phytolin, (vii) Fiber, and (viii) Phytolin + Fiber indicated the Shannon diversity index of different treatments from 0 h to 24 h of fermentation. Statistical significance between different time points (2–24 h) to the reference time point 0 h within each treatment group were analyzed using the Kruskal-Wallis test and denoted as significant with one asterisk (* = $p < 0.1$) or two asterisks (** = $p < 0.05$). (b) PCoA plot based on the distance calculated by the nonparametric microbial interdependence test (NMIT) at genus level taxa to determine the longitudinal sample similarity as a function of temporal microbial composition between different treatments. The percent of dataset variability explained by each principal coordinate is shown in parentheses in axis titles. (c) Linear discriminant analysis (LDA) effect size (LEfSe) taxonomic cladogram identifying significantly discriminant (Kruskal–Wallis sum-rank test $\alpha < 0.05$; LDA score > 2.00) taxa associated with different treatments. The different colour shading represents bacterial taxa that were significantly higher in particular treatment group, as indicated. The yellow circles on the cladogram represent bacterial taxa that were not significantly changed.

dysbiosis in patients with colorectal cancer (Gao, Guo, Gao, Zhu, & Qin, 2015).

3.3. *Immediate production of SCFAs occurred when Phytolin and Fiber were applied*

The production of SCFAs during fermentation is an important indicator of positive microbial activity. We measured the SCFAs throughout the 24 h *in vitro* colonic fermentation period. Phytolin, Fiber, and Phytolin + Fiber contributed to alterations in total SCFAs production throughout the 24 h *in vitro* colonic fermentation (Fig. 3), compared to the control fermentation with faecal materials only that went through the same digestion phases. However, Phytolin, Fiber and Phytolin + Fiber, respectively, were observed to show significant differences ($p < 0.007$) (Supplementary Table 2) in the production of SCFAs at specific time points.

As compared to control, production of total SCFAs from Phytolin + Fiber was significantly higher at an earlier fermentation time of 8 h and maintained its significance throughout the fermentation up to 24 h. Production of total SCFAs from Fiber and Phytolin, respectively, were significantly higher only after 18 h of fermentation compared to control.

Effects on the production of SCFAs from the different samples were further investigated by looking into the production of specific SCFAs. Among the 3 samples, there were minimum changes in acetic acid

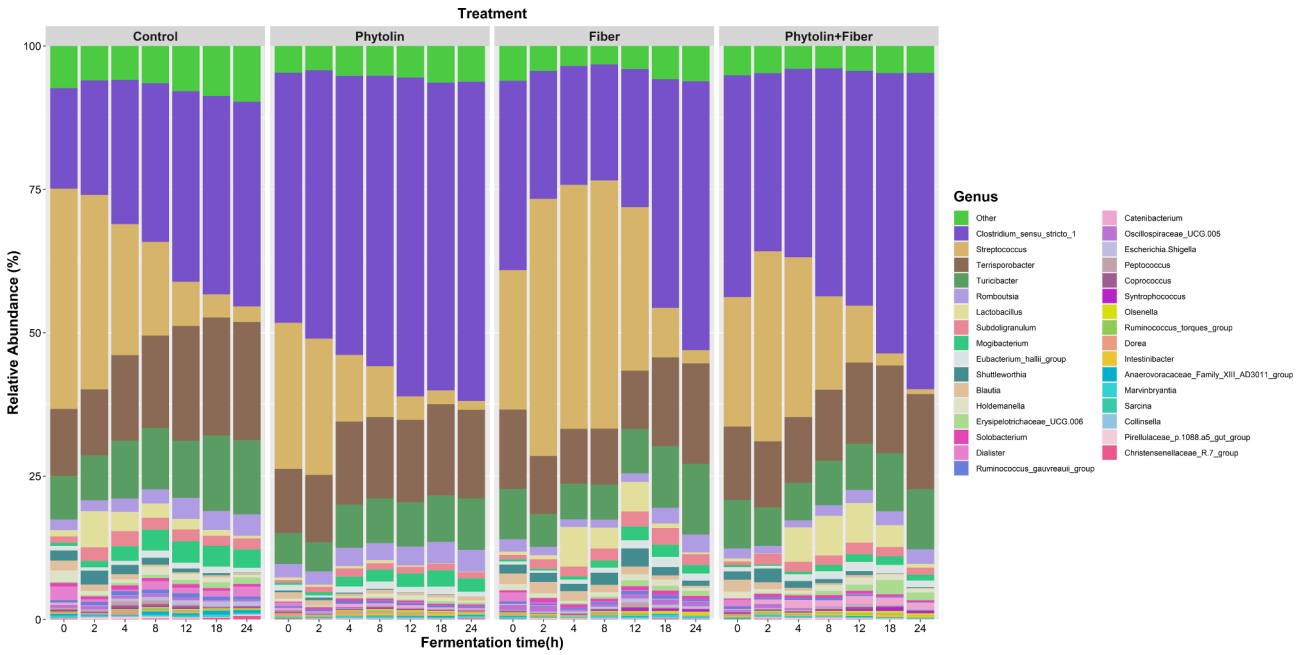

Fig. 2. Alterations in the relative abundances of the faecal microbiota at genus level in different treatments throughout 24 h of *in vitro* colonic fermentation Relative abundances plot of the most abundant microbial genera (>0.1%) across all treatment groups (control, Phytolin, Fiber, and Phytolin + Fiber). Each column shows a community derived from an independent microbiota-based fermentation of specific treatment at particular fermentation time points (0 h, 2 h, 4 h, 8 h, 12 h, 18 h or 24 h).

production throughout the 24 h incubation period compared to control, but a noticeable lower production for the Phytolin + Fiber sample between the period of 2 h to 12 h of fermentation (Fig. 3b). In contrast, production of propionic acid was significantly higher for all 3 samples compared to control starting at 4 hr and maintained this significance up to the 24 h of fermentation, especially for Phytolin + Fiber that significantly produced higher amount of propionic acid than all other samples (Fig. 3c). Production of butyric acid showed different patterns among the three different samples as compared to control. For Phytolin + Fiber, production of butyric acid was significantly higher starting at 8 h maintaining this significance up to the 24 h of fermentation (Fig. 3d). For Fiber, production of butyric acid showed significantly lower amount at the earlier stage between 2 h and 4 h of fermentation (Fig. 3d). For Phytolin, production of butyric acid was significantly higher between the period of 4 h to 8 h, and 18 h to 24 h of fermentation, respectively (Fig. 3d). Regarding the production of isobutyric and isovaleric acids, similar trends were observed within each sample comparing to the control. For Phytolin, no significant alteration was observed, whereas for Fiber and Phytolin + Fiber, significant reductions were observed in isobutyric acid production between 2 h and 8 h of fermentation (Fig. 3e), and isovaleric acid production between 2 h and 12 h of fermentation (Fig. 3g). Regarding the production of valeric acid, Phytolin caused significantly higher production from 4 h to 12 h of fermentation while Phytolin + Fiber maintained this similar significance up to 18 h of fermentation, as compared to the control respectively. For Fiber, production of valeric acid showed significantly lower amount starting at 2 h, 8 h, 12 h and 24 h of fermentation (Fig. 3f).

Amongst the SCFAs measured, acetic, propionic, and butyric acids were the major SCFAs produced by the faecal microbiota with higher concentrations ranging from approximately 30 mM to 90 mM, while the isobutyric, valeric and isovaleric acids were the minor SCFAs produced with relatively lower concentrations ranging from approximately 5 mM to 19 mM. This result is in line with the results from earlier *in vitro* and *in vivo* fermentation studies using cranberry extract and jackfruit pulp, which showed acetic, propionic, and butyric acids as the major SCFAs produced and isobutyric, valeric, and isovaleric acids as the minor SCFAs produced (Tamargo, Cueva, Taladrid, Khoo, Moreno-Arribas, Bartolomé, & de Llano, 2022; Zhu et al., 2021).

Microbial fermentation of carbohydrates and production of SCFAs in the colon is increasingly recognised in contributing to optimal health. For example, acetic acid is known to act as a signalling molecule in the metabolic pathways of gluconeogenesis and lipogenesis, and serves as an energy source for gut peripheral cells and the liver (Wang et al., 2019). Propionic acid was reported to exert immunosuppressive actions, improve tissue insulin sensitivity, and lower fatty acids concentration in liver and plasma, thus, could be an important effector in obesity and type 2 diabetes management (Sa'ad, Peppelenbosch, Roelofsen, Vonk, & Venema, 2010). Butyric acid has been demonstrated to serve as an important colonocytes energy source and play pivotal roles in the prevention of inflammatory bowel diseases by regulating colonocyte proliferation and apoptosis, gastrointestinal tract motility and anti-inflammatory activity (Załęski, Banaszkiewicz, & Walkowiak, 2013).

Although acetic acid production in Phytolin + Fiber was lower than control between 2 h and 12 h of fermentation, it was recovered to the same level as that of control by the end of 24 h fermentation. Additionally, the productions of propionic and butyric acids in Phytolin + Fiber were statistically significantly higher than the other treatments including the control. The supply of Fiber to the gut microbiota provided more substrate for the bacterial community to produce SCFAs while the supply of Phytolin might stimulated the bacterial metabolic capacity related to the production of SCFAs. These suggested a synergistic effect between Phytolin and Fiber interacting with the faecal microbiota in their reciprocal interactions in improving the microbiota fermentative activity for specific SCFAs production. Similar observation was also obtained with oat bran polyphenols and fibers (Kristek et al., 2019). Thus, the combinations of polyphenols and dietary fibers may better improve specific SCFAs production by gut microbiota than fiber alone.

3.4. *Production of phenolic metabolites occur throughout the in vitro fermentation*

Based on the previous study by Deseo et al. (2020), diosmin, chlorogenic acid, and orientin were found to be the major polyphenol composition of Phytolin. Thus, we focused on the characterization of

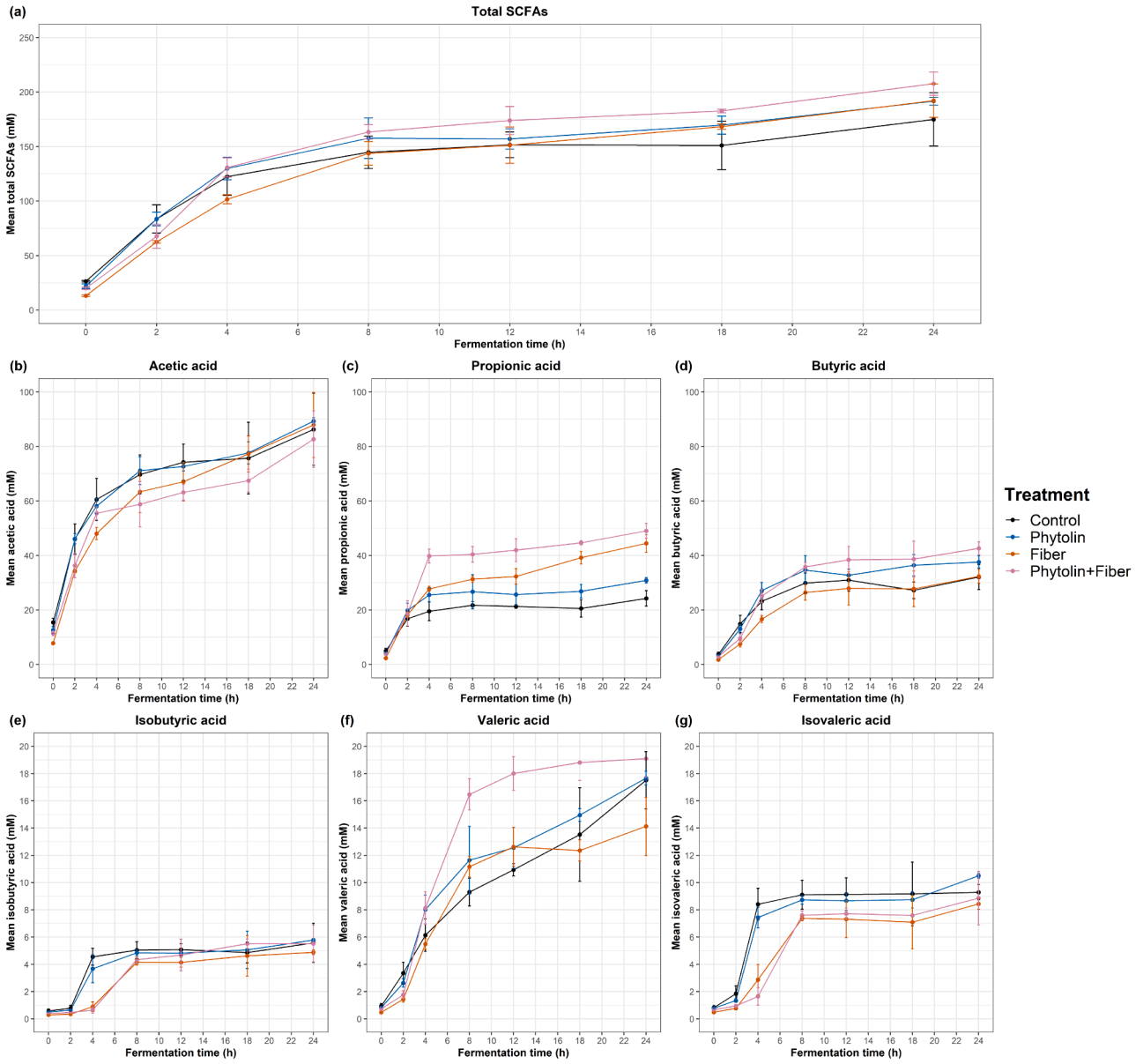

Fig. 3. Production of short chain fatty acids during colonic fermentation. Samples were subjected to the digestion phases and undigested insoluble materials from the small intestinal digestion stage were carried forward to the colonic fermentation stage. (a) Total SCFAs, (b) acetic acid, (c) propionic acid, (d) butyric acid, (e) isobutyric acid, (f) valeric acid and (g) isovaleric acid content (mM) of control, Phytolin, Fiber, and Phytolin + Fiber throughout the *in vitro* colonic fermentation at different time points (0 h, 2 h, 4 h, 8 h, 12 h, 18 h and 24 h). Results are expressed as means ± SD (n = 3).

Table 2
Qualitative characterization of phenolic metabolites in Phytolin and Phytolin + Fiber after *in vitro* colonic fermentation by LC-ESI-QTOF-MS/MS.

No.	Compounds	Molecular Formula	Retention Time (min)	Mode of Ionization (ESI-)	Molecular Weight	Theoretical *m/z*	Observed *m/z*	Product ions	Samples [name: fermentation time (h)]
1	3-(3,4-dihydroxyphenyl)-propionic acid	$C_9H_{10}O_4$	17.1	$[M - H]^-$	182.17	181.0501	181.0522	109, 121, 137	Phytolin + Fiber: 2,4,12,18,24 Phytolin: 2,4
2	3-hydroxy-4-methoxybenzoic acid	$C_8H_8O_4$	20.2	$[M - H]^-$	168.15	167.0344	167.0360	108, 123, 152	Phytolin + Fiber: 2,18,24
3	3-(3-hydroxyphenyl)-propanoic acid	$C_9H_{10}O_3$	25.5	$[M - H]^-$	166.17	165.0550	165.0543	106, 119, 121	Phytolin + Fiber: 2,4,8,18,24 Phytolin: 4,8,12,18,24
4	Luteolin	$C_{15}H_{10}O_6$	39.9	$[M - H]^-$	286.24	285.0398	285.0641	285	Phytolin + Fiber: 2,4,8,12,18,24 Phytolin: 4,8,12,18,24

phenolic metabolites of these polyphenols contained in Phytolin and Phytolin + Fiber after *in vitro* fermentation. A qualitative analysis of the phenolic metabolites was performed by LC-ESI-QTOF-MS/MS with comparison to reference standards. 4 major phenolic compounds were tentatively identified as the major phenolic metabolites of diosmin, chlorogenic acid and orientin (Table 2). 3-(3-hydroxyphenyl)-propanoic acid, 3-(3,4-dihydroxyphenyl)-propionic acid and luteolin were tentatively identified in Phytolin and Phytolin + Fiber samples after *in vitro* fermentation, while 3-hydroxy-4-methoxybenzoic acid was only found in Phytolin + Fiber samples after fermentation. 3-(3-hydroxyphenyl)-propanoic acid was previously identified as a phenolic metabolite of diosmin and chlorogenic acid (Cova, De, Giavarini, Palladini, & Perego, 1992; Tomas-Barberan et al., 2014). Luteolin was previously found to be a gut microbial metabolite of orientin and diosmin in different studies (Currò, 2018; Xu et al., 2014), while 3-(3,4-dihydroxyphenyl)-propionic acid was previously identified as the microbial metabolite of chlorogenic acid and luteolin (Schoefer, Mohan, Schwiertz, Braune, & Blaut, 2003; Tomas-Barberan et al., 2014). Lastly, 3-hydroxy-4-methoxybenzoic acid was also identified as a metabolite of diosmin in previous study (Cova et al., 1992). In short, this result suggested that the major polyphenols in Phytolin were carried over to the colonic phase and metabolized by the gut microbiota which led to the production of their phenolic metabolites found in the *in vitro* fermentation samples.

3.5. Abundance of faecal microbiota correlated with SCFAs production

The gut microbiota is known to play an essential role in the catabolism of plant fibers resulted in the production of SCFAs. Thus, we further examined the correlations between the faecal microbiota and SCFAs production by correlating the values of differences in the bacterial genera relative abundances and SCFAs concentrations between two consecutive time points over the 24 h of *in vitro* colonic fermentation. Spearman's correlation was performed in this analysis and the result was visualized using heatmap, where positive correlation indicated likelihood of co-occurrence while negative correlation indicated no relationship, not necessarily a negative correlation (Fig. 4).

When different samples were introduced, positive correlation patterns between the faecal bacteria and SCFAs production changed (Fig. 4). This indicated that different samples might distinctively modify the metabolic capacity of specific groups of microbiotas based on their specific preferences on different substrates, and the reciprocal effects between polyphenols and fiber with the bacterial community would further influence on the metabolic profile of the gut microbiota. Eventually, these modifications had led to different changes of SCFAs production in each treatment. To better understand the metabolic relationship between different faecal bacteria and the production of SCFAs, PICRUSt2 analysis was performed to obtain the prediction of metagenome functions from each genus. In this analysis, we only reported the functional compositions of all the OTUs which taxonomic annotation matches with the bacterial genera that were significantly

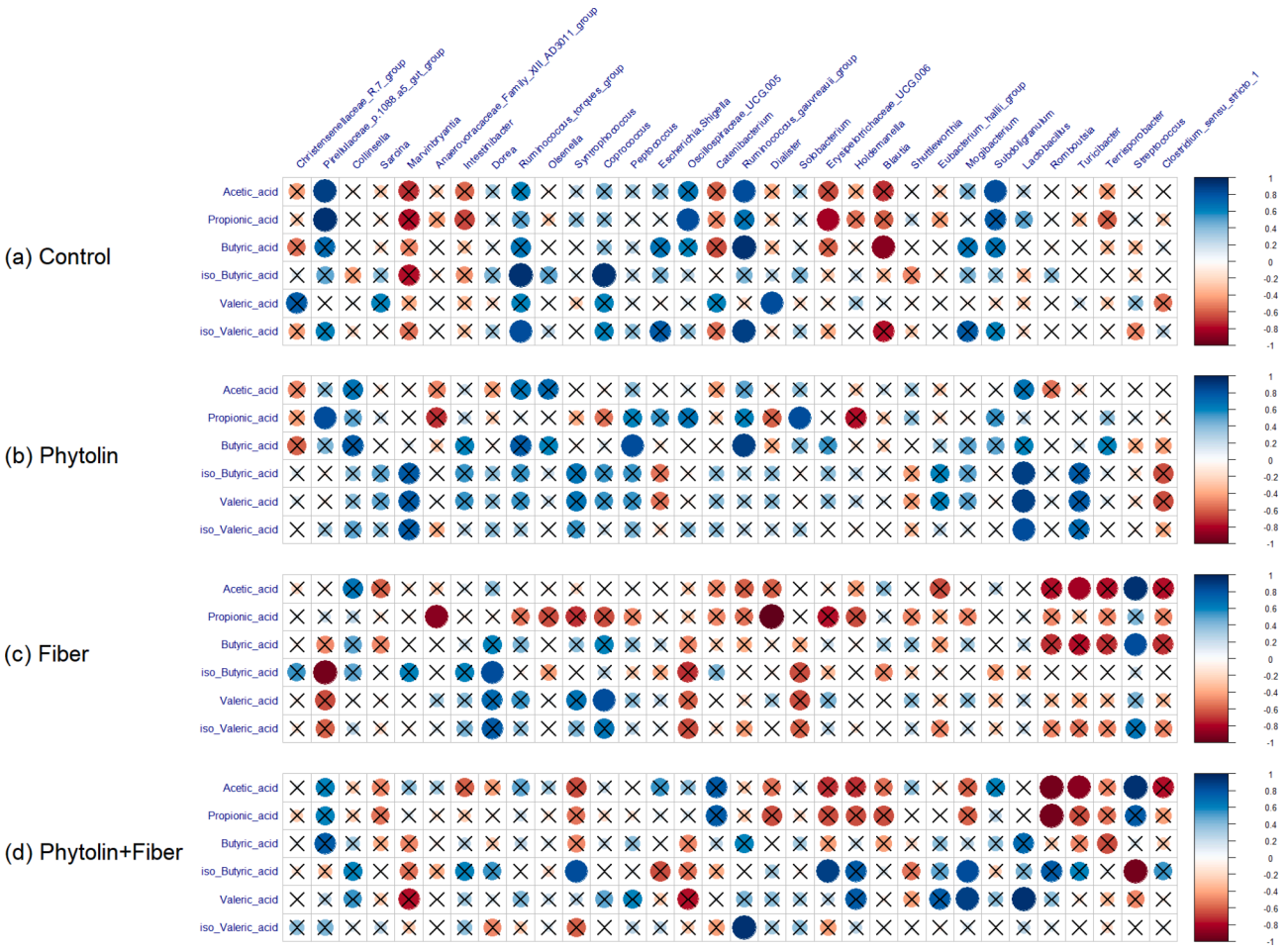

Fig. 4. Spearman's correlation heatmap between bacterial genera (relative abundance > 0.1%) and SCFAs in treatment (a) Control, (b) Phytolin, (c) Fiber, and (d) Phytolin + Fiber. Spearman's correlation was performed using the values of differences in the bacterial genera relative abundances and SCFAs concentrations between two consecutive time points over the 24 h of *in vitro* colonic fermentation. Blue dots (positive *r* values) indicate likelihood of co-occurrence while red dots (negative *r* values) indicate no relationship, not necessarily a negative correlation. Dots without X represent statistically significant (FDR adjusted $p < 0.05$) result.

correlated with individual SCFAs based on the result obtained from Spearman's correlation test (Supplementary Table 3). 10 metabolic pathways involved in the production of acetic acid in the control group were predicted for *Subdoligranulum*, *Ruminococcus gauvreauii group* and *Pirellulaceae p-1088-a5 gut group*, while 3 pathways were predicted for *Pirellulaceae p-1088-a5 gut group* and *Oscillospiraceae UCG-005* to produce propionic acid, and *Ruminococcus gauvreauii group* to produce butyric acid, respectively. In the case of Phytolin, 3 metabolic pathways were predicted for *Solobacterium* with propionic acid production, and 1 pathway was predicted for *Ruminococcus gauvreauii group* and *Peptococcus* with butyric acid production. Regarding the Fiber and Phytolin + Fiber groups, similar metabolic pathways were predicted, showing that 9 pathways were involved in the production of acetic acid by *Streptococcus*. Besides, 4 pathways were predicted to produce butyric acid by *Streptococcus* in the Fiber group. This suggested that these bacterial genera were responsible for the production of each of these SCFAs in different treatments.

SCFAs produced by the gut microbiota from fibers play a critical role in connecting microbiota composition and the numerous physiological effects mediated by SCFAs (Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016). These alterations in correlations between specific genera with SCFAs production might be due to that the interaction between different samples with the faecal bacterial community that lead to changes in activity of certain bacteria groups which their metabolic capacities were enhanced, and thus, resulting in higher ability to metabolize the substrates supplied. As shown in the PICRUSt2 result, distinct metabolic pathways were involved in different bacteria that were significantly correlated with specific SCFAs. Similar findings were also reported in other studies when different treatment groups were applied, for example, consumption of different diets was found to differently correlate with specific SCFAs production and bacterial genera (De Filippis et al., 2016).

However, due to the limited information related to the productions of isobutyric, valeric and isovaleric acids in the MetaCyc metabolic pathway database, only pathways related to the acetic, propionic, and butyric acids were predicted. This limited the capacity to explain the metabolic co-relationship between particular genera with the productions of minor SCFAs detected in the present study.

4. Conclusion

The associations of sugarcane polyphenols with sugarcane fiber enhanced the availability of polyphenols that can be delivered to the colon. This has led to the observed synergistic effects on the pig gut microbiota which eventually regulated the microbial community towards a profile that combines the potential beneficial effects shown by application of the Phytolin or Fiber alone. Phytolin + Fiber contributed to significant changes in the pig faecal microbiota profile compared to the faecal control; in particular, the relative abundances of *Lactobacillus*, and *Catenibacterium* were increased, while the relative abundances of *Mogibacterium*, *Dialister*, and *Escherichia-Shigella* were decreased. Combining Phytolin with Fiber also resulted in significantly higher production of total SCFAs, and specifically of propionic and butyric acids. The variations in associations between specific faecal bacterial genera with SCFAs production suggested that the interaction between different samples with the faecal bacterial community would lead to changes in activity of certain bacteria groups which their metabolic capacities were enhanced, and thus, resulting in higher ability to metabolize the substrates supplied. Production of phenolic metabolites related to the major polyphenols in Phytolin suggested that the pig gut microbiota is also involved in the metabolism of polyphenols during fermentation. Therefore, we can infer that the delivery of both sugarcane fiber and sugarcane polyphenols from the Phytolin product could be beneficial to human colon health from their reciprocal interaction with the human gut microbiota, based on similarities between pig and human gut physiology and microbiota population.

CRediT authorship contribution statement

Yit Tao Loo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Kate Howell:** Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Hafiz Suleria:** Formal analysis, Data curation, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Pangzhen Zhang:** Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Chunhe Gu:** Formal analysis. **Ken Ng:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Writing – review & editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132665.

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