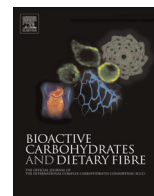Contents lists available at ScienceDirect

Bioactive Carbohydrates and Dietary Fibre

journal homepage: www.elsevier.com/locate/bcdf

Kiwifruit fermentation drives positive gut microbial and metabolic changes irrespective of initial microbiota composition

Paul Blatchford [a,b], Halina Stoklosinski [a], Gemma Walton [b], Jonathan Swann [b], Glenn Gibson [b], Richard Gearry [c], Juliet Ansell [d,*]

[a] The New Zealand Institute for Plant & Food Research Limited, Private Bag 11600, Palmerston North 4442, New Zealand
[b] Department of Food and Nutritional Sciences, The University of Reading, Reading RG6 6AP, United Kingdom
[c] Department of Medicine, University of Otago, Christchurch, New Zealand
[d] Zespri International Limited, 400 Maunganui Road, PO Box 4043, Mt Maunganui 3149, New Zealand

ABSTRACT

It is well established that individuals vary greatly in the composition of their core microbiota. Despite differing ecology, we show here that metabolic capacity converges under the pressure of kiwifruit substrates in a model gut system. The impact of pre-digested green and gold kiwifruit on the human colonic microbiota and their metabolic products was assessed using *in vitro*, pH-controlled, anaerobic batch culture fermenters. Phylogenetic analyses revealed that bacterial composition changed over time, irrespective of whether a substrate was added or not, indicating a natural adjustment period to the gut model environment. Adding kiwifruit substrate caused additional changes in terms of growth of specific bacterial groups, bacterial diversity and metabolite profiles. Relative abundance of *Bacteroides* spp. increased with both green and gold kiwifruit substrate while *Bifidobacterium* spp. increased only with green kiwifruit. NMR spectroscopy and GC demonstrated an increase in organic acids (primarily acetate, butyrate, and propionate) and a concomitant decrease in several amino acids and oligosaccharides following addition of green and gold kiwifruit substrate. The experiments demonstrated that despite markedly different baseline profiles in individual donor inoculum, kiwifruit components can induce substantive change in microbial ecology and metabolism which could have consequences for human health.

1. Introduction

The gut microbiota is a highly diverse collection of trillions of microbes comprised of hundreds of species (Gill et al., 2006). In the densely colonized large intestine, the microbiota can reach numbers of up to 10^{12} cfu/g colon contents (Gueimonde & Collado, 2012). Such vast numbers of symbionts can have a considerable impact on the health of the host. The gut microbiota has evolved with humans to a complex inter-dependant state, where their genome in addition to our own generates a profound ability to metabolise the diverse array of substrates in the human diet (Xu et al., 2007). Predominant phyla in the human gut are Bacteroidetes and Firmicutes, making up over 90% of all resident colonic bacteria with the two other subdominant phyla being Actinobacteria and Proteobacteria (Eckburg et al., 2005; Ley, Turnbaugh, Klein, & Gordon, 2006). There have been numerous studies

conducted recently on the use of purified and processed foods or food additives to modify bacterial composition. It is clear that diet has an effect on microbiota and this in turn affects health; as many as a third of all diseases, including cardiovascular disorders such as coronary heart disease and hypertension, type 2 diabetes, functional bowel problems and cancer, are lifestyle related and their risk may be mitigated through dietary means (Johnson, Chua, Hall, & Baxter, 2006; Shahidi, 2009; Tuomilehto et al., 2001). Prebiotic supplementation is commonly used to treat gastrointestinal dysfunction. These are a class of non-digestible food ingredients such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and xylo-oligosaccharides (XOS) (Gibson, 2004) that confer a health benefit to the host through selectively modulating bacterial composition (Gibson et al., 2010). Prebiotic molecules are often sourced from plants, where specific oligosaccharides are isolated and concentrated in order to be used as a supplement to a regular diet. An alternative to prebiotic supplementation is the use of whole fruits and vegetables as health promoting foods, which are easier to implement into a dietary routine (Lipsky, Cheon, Nansel, & Albert, 2012). In addition to the health benefits of whole foods,

* Corresponding author. Fax: +64 7 574 8031.
  E-mail address: juliet.ansell@zespri.com (J. Ansell).

they make for a more marketable product. An absence of processing maintains the natural structure of nutrients which are potentially more bioavailable in whole foods (Chandrasekara, Naczk, & Shahidi, 2012; van der Sluis, Dekker, Skrede, & Jongen, 2002).

The most commonly sold kiwifruit are from the species *Actinidia deliciosa* (typically green fleshed e.g. 'Hayward') and *Actinidia chinensis* (typically yellow fleshed e.g. 'Gold3'). Kiwifruit are rich in vitamin C, potassium, folate, and phytochemicals (Ferguson & Ferguson, 2003). The principal carbohydrate found in kiwifruit is starch, with non-starch polysaccharides (NSP) such as pectic polysaccharides, hemicelluloses and celluloses amounting to 2–3% of total kiwifruit constituents (Carnachan, Bootten, Mishra, Monro, & Sims, 2012; Dawson & Melton, 1991; Ferguson & Ferguson, 2003; Seager & Haslemore, 1993). NSP are essentially resistant to digestion by enzymes encountered in the human stomach and small intestine. Therefore, they reach the colon largely intact where pectic polysaccharides and, to a lesser extent, hemicelluloses and celluloses are fermented by the gut microbiota (Cummings & Englyst, 1987). Prebiotic effects, namely beneficial changes to the composition of the existing microbiota and colonic metabolites, may subsequently be observed. Several studies have examined kiwifruit fibre digestion *in vitro,* finding a chemically unaltered structure with only minor modifications to galacturonic acid residues and molecular weight profiles in the soluble fibre fraction (Carnachan et al., 2012; Dawson & Melton, 1991). In a recent study, upper gastrointestinal tract digestion had little effect on either green or gold kiwifruit in an *in vivo* porcine model, with the dietary fibre fraction being completely undigested at the terminal ileum (Henare et al., 2012).

Changes in bacterial composition can lead to a modified metabolite profile which can have direct consequences for host health. Recent research has shown that the observed metabolic profile can be altered by changing the substrates available for fermentation. Substrates that can induce changes in metabolic profiles include: carbohydrates such as resistant starch, unabsorbed sugars, non-starch polysaccharides, gums and cellulose; and proteins from the diet and endogenous sources such as mucin (Cummings & Englyst, 1987; Cummings & Macfarlane, 1991; Louis, Scott, Duncan, & Flint, 2007). Some of the main end products of fermentation are short chain fatty acids (SCFA), branched chain fatty acids (BCFA) and gases like hydrogen, carbon dioxide and methane (Blaut, 2002; Rosendale, Cookson, Roy, & Vetharaniam, 2011). This study determined the effect of whole kiwifruit components that escape gastric and small intestinal digestion on the colonic microbiota and metabolites in an *in vitro* batch culture gut model.

2. Materials and methods

2.1. Simulated gastrointestinal digestion (SGD)

Two kiwifruit substrates were used in the batch culture models: Green kiwifruit (*A. deliciosa*) 'Hayward' and Gold kiwifruit (*A. chinensis*) 'Hort16A'. The compositional data of these two kiwifruit are outlined in Table S1. As a control, no exogenous substrate was added. Green and gold kiwifruit were peeled, chopped and mashed finely. The samples were subjected to the simulated gastric digestion procedure as detailed by Mills et al. with minor modifications (Mills et al., 2008). Briefly, 60 g of sample was weighed and added to 150 mL of autoclaved distilled water in a stomacher bag where it was homogenised (Stomacher 400) for 5 min at normal speed (460 paddle beats/min). After addition of 0.001 mol/L salivary α-amylase the solution was incubated for 30 min on a shaker at 37 °C. The pH was adjusted to 2.0 using 6 M HCl. Pepsin solution was added to the mixture which was incubated at 37 °C

gently shaking for 2 h. The pH was adjusted to 7.0 following addition of a pancreatin/bile mixture (P8096/B8631 Sigma) and the solution was incubated at 37 °C for 3 h. Samples were then transferred to a 500 Da dialysis membrane (Spectra/Por, Spectrum Laboratories Inc.) to remove most di- and mono-saccharides. This was dialysed for 15 h against a 10 mM NaCl solution at 4 °C. The dialysis fluid was replenished and the samples dialysed for a further 2 h. Samples were then frozen at -80 °C and freeze-dried.

2.2. pH controlled anaerobic faecal batch cultures

Batch culture systems allow the study of microbial fermentation in a simulated colonic environment. The apparatus was set up the day before the experiment and sterilised by autoclaving. The basal culture medium used for the batch cultures contained (per L): 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 6H_2O$, 2 g $NaHCO_3$, 2 mL Tween 80, 0.05 g haemin (dissolved in a few drops of NaOH), 10 μl vitamin K, 0.5 g cysteine HCl, 4 mL resazurin solution (0.025 g/100 mL) and 0.5 g No.3 bile salts. The solution was made up to 1 L with distilled water and sterilised by autoclaving. All chemicals were obtained from Sigma. One hundred and thirty-five millilitres of freshly autoclaved medium was aseptically poured into 280 mL capacity water-jacketed batch culture vessels. The medium was continually mixed using a magnetic stirrer and maintained at 37 °C with a circulating waterbath. Oxygen free N_2 gas was bubbled through the media overnight to establish an anoxic environment. Excess gas was vented outside through a 0.22 μm filter.

On the morning of the experiment, calibrated pH electrodes were inserted into each vessel. A freshly voided stool sample was obtained from a healthy volunteer who had not taken any supplemental probiotics, prebiotics or antibiotics for 6 months prior. The stool was diluted 1:10 in sterile PBS, stomached for 2 min and 15 mL was added to the vessels, yielding a total volume in each vessel of 150 mL. Then 1.5 g (1% w/v) of each kiwifruit substrate was added to the vessels (excluding the control). Approximately 5.5 mL of sample was taken from each vessel immediately upon addition of substrate representing the 0 h time point. Samples were then taken at 5, 10, 24 and 48 h time points. Each sample was placed on ice, dispensed into aliquots and stored at -80 °C for metabolomics and -20 °C for all other samples. The batch culture systems were monitored throughout the 48 h run, with any adjustments of stirrer speed, N_2 flow rates or temperature carried out as required. This initial batch culture experiment was repeated twice with different faecal donors giving a total of three biological replicates.

2.3. Nuclear magnetic resonance (NMR) spectroscopy

One millilitre of fermenta was taken and centrifuged at 16,200*g* for 10 min; then the supernatant was decanted and frozen at -80 °C until analysis. Samples were then defrosted, vortexed and 400 μL transferred into a sterile eppendorf. Two hundred microlitres of phosphate buffer (containing 1 mM of the internal standard TSP (3-(trimethylsilyl)-[2,2,3,3-d4]-propionic acid sodium salt), the bacteriostatic sodium azide in 100% D_2O) was added to the samples which were then vortexed and centrifuged at 10,000*g* for 10 min. The supernatant (550 μL) was then transferred to a 5 mm glass NMR tube. All samples (and a batch culture medium only control) were run on a Bruker Avance III 700 MHz NMR spectrometer. Initial spectral processing was conducted using Bruker's Topspin software. Spectra were baseline corrected to remove systemic offsets, phased to yield accurate peak integration and peak shape and the TSP (internal chemical shift standard) adjusted to 0 ppm. Further data processing was carried out using

MATLAB 7.8.0 R2013b. The spectral regions containing the resonances from residual water and polyethylene glycol were removed to minimise the effects of baseline distortions. Principal Component Analysis (PCA) was performed on the metabolic profiles in Matlab using scripts provided by Korrigan Sciences Ltd. This unsupervised approach was used to determine inherent changes between batch culture samples of different substrates or at different time-points.

2.4. Gas chromatography

Concentrations of acetate, butyrate, formate, heptanoate, hexanoate, isobutyrate, isovalerate, lactate, propionate, succinate and valerate were quantified by gas chromatography equipped with a flame ionisation detector (GC-FID) following a modified method of Richardson et al. (Richardson, Calder, Stewart, & Smith, 1989). In brief, 1.5 mL of sample was centrifuged at 16,200g for 10 min. The supernatant was diluted in 0.01 M phosphate buffered saline with 2-ethylbutyric acid (5 mM) as an internal standard. The sample was then centrifuged at 3000g for 5 min (4 °C). The clarified supernatant was acidified with concentrated hydrochloric acid and diethyl ether added, and following vortexing, was centrifuged at 10,000g for 5 min (4 °C). The upper diethyl ether phase was collected and derivatised with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (MTBSTFA+TBDMSCI, 99:1; Sigma-Aldrich) by heating to 80 °C for 20 min. To allow complete derivatisation, the samples were left for 48 h at room temperature before analysis. Standards containing 2-ethylbutyric acid (5 mM) as an internal standard were prepared alongside the samples. Analysis was performed on a Shimadzu gas chromatograph system (GC-17A, Kyoto, Japan) equipped with a flame ionisation detector and fitted with a HP-1 column (10 m × 0.53 mm ID × 2.65 μm) (Agilent Technologies). The carrier gas was helium with a total flow rate of 37 mL/min and pressure of 7 kPa. The temperature profile began at 70 °C, increasing to 80 °C at 10 °C/min, with a final increase to 255 °C at 20 °C/min, holding for 5 min. The pressure programme was set to 7 kPa, increasing to 15 kPa at 0.8 kPa/min, holding for 4 min. Injector and detector temperatures were set at 260 °C. Samples were injected (1 μL) with a splitless injection. The instrument was controlled and chromatograms acquired using GC Solution Chromatography Data System software, Version 2.3 (Shimadzu). The acquired GC data were used to plot standard curves and calculate compound response factors in relation to the internal standard, enabling a sample result of μmol SCFA/mL fermentation supernatant to be calculated.

2.5. DNA extraction, 16S rRNA gene sequencing and bioinformatics

A 1.5 mL sample aliquot was centrifuged at 16,200g for 10 min. The pellet was resuspended in 500 μL sterile 50% glycerol in PBS (v/v) and frozen at −20 °C until analysis. This sample was centrifuged at 16,200g for 5 min and the pellet washed in 1 mL of PBS. The sample was centrifuged again at 16,200g for 5 min and the pellet resuspended in 500 μL of TES buffer pH 8.0 (Trizma HCl 0.254 g, Trizam base 0.048 g, NaCl 0.116 g, EDTA 0.068 g, and sterile distilled water 400 mL). The samples were placed on ice and 8 μL lysozyme (10 mg/mL) and 2 μL mutanolysin (1 mg/mL) were added. The cell suspension was vortexed and incubated at 37 °C for 30 min. The samples were removed and placed on ice where 10 μL proteinase K (20 mg/mL) and 10 μL RNase (10 mg/mL) were added. The cell suspension was vortexed and incubated at 65 °C for 1 h. Then 100 μL of 10% SDS was added and the tubes were gently mixed by inversion. The samples were then incubated for a further 15 min at 65 °C. The samples were cooled on ice for 30 min and then 620 μL phenol/chloroform/water mix was added to the samples. The tubes were gently mixed by inversion for 2 min and then centrifuged at 4100g for 10 min. The upper (aqueous) layer was transferred to a clean eppendorf tube and 1 mL of ice-cold ethanol was added. The samples were left on ice for 30 min or stored overnight at −20 °C. The samples were centrifuged for 5 min at 16,200g, the supernatant was carefully removed and the pellet allowed to air dry for 2–3 h or overnight. The pellet was resuspended in 50 μL sterile H_2O, mixed well and DNA purity and concentration was assessed by running 2 μL on the NanoDrop ND-100 spectrophotometer.

DNA was used as a template to amplify variable regions V2-V3 of the 16S rRNA gene (position 336-535 in the *Escherichia coli* rRNA gene) using primers HDA-1 (cgtatcgcctccctcgcgccatcagACTCCTAC-GGGAGGCAGCAGT) and HDA-2 (ctatgcgccttgccagcccgctcagNNN-NNNNNNNGTATTACCGCGGCTGCTGGCAC) (Rosendale et al., 2012) where the sequences of the forward and reverse primers are shown in lower case, the four base library "key" sequence is underlined, the letter N denotes the 10 base barcode sequence and the remaining capital letters the template-specific HDA primers (Tannock et al., 2000). Twenty-five microlitres of HotStarTaq master mix (QIAGEN, 203443) was mixed with 1 μL template DNA and 100 nM of each primer (total reaction volume 50 μL). PCR conditions were as follows: Initial denaturation 95 °C for 15 min then 30 cycles of 1 min 95 °C denaturation, 45 sec 65 °C annealing, 1 min 72 °C extension. The PCR products were gel purified using the QIAquick PCR purification kit, (QIAGEN, 28104), quantified using the Qubit 2.0 fluorometer (Life Technologies), pooled in equimolar quantities, and submitted for sequencing on the Roche 454 GS FLX Titanium platform (Macrogen Inc., Korea).

QIIME software V1.8.0 was used to analyse the 454 sequencing data (Caporaso et al., 2010b). Reads were clustered into operational taxonomic units (OTUs) based on a 97% identity threshold value. Alignment of the sequences was carried out using PyNAST (Caporaso et al., 2010a) with reference to the Greengenes core reference database (version 13_8) (DeSantis et al., 2006). Taxonomic assignment was made using the RDP Naive Bayesian classifier (Wang, Garrity, Tiedje, & Cole, 2007). Chimeric sequences were removed from the reads using the ChimeraSlayer algorithm (Claesson et al., 2012; Haas et al., 2011). Alpha rarefaction was calculated using the Phylogenetic Diversity (PD) whole tree, Chao1, Observed Species and Shannon diversity metrics. Beta diversity was determined using UniFrac distances as input and EMPeror to visualise relationships in three dimensions (Vazquez-Baeza, Pirrung, Gonzalez, & Knight, 2013).

2.6. Statistical analysis

The non-parametric two-sample *t*-test (Monte Carlo permutation) from QIIME was used for determining alpha diversity significance. The ADONIS test for determining statistical significance from between sample groups was conducted using the compare _categories.py script from QIIME. All other statistical calculations were conducted in R Studio using the vegan and made4 packages (Culhane, Thioulouse, Perriere, & Higgins, 2005; Oksanen et al., (2013); RStudio, 2012). The Mann–Whitney *U* test was conducted to assess differences between taxa at the phylum, family and genus level. A *P*-value of less than 0.05 was deemed significant and the False Discovery Rate (FDR) method was used for correcting for multiple comparisons.

3. Results

### 3.1. 16S rRNA gene sequencing

The V2-V3 hypervariable region of the 16S rRNA gene sequencing results yielded 253,852 reads that passed the quality filters at an average of 5903 ± 1703 reads per sample. Reads were
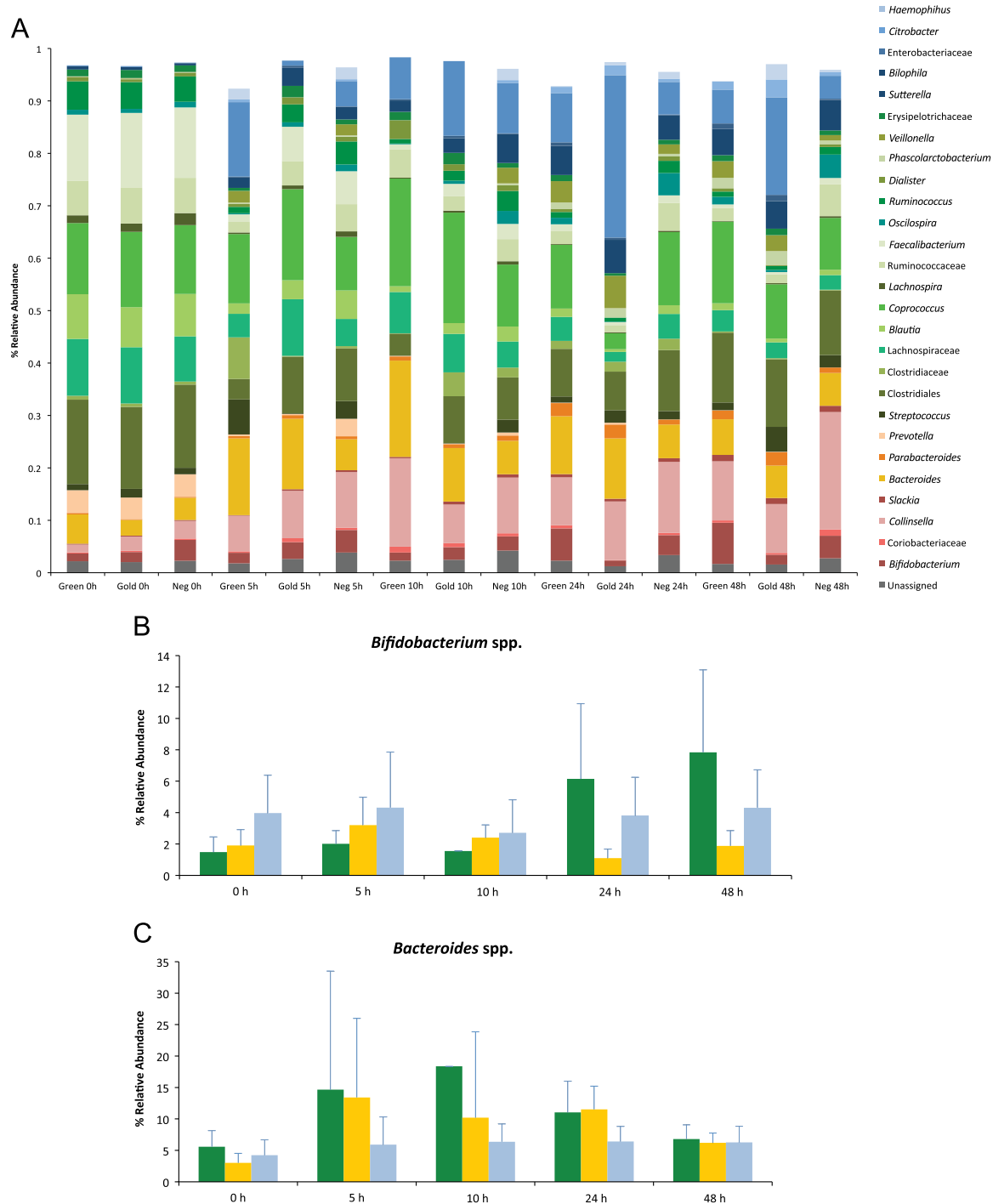
Fig. 1. Microbial composition of samples at all time points as determined by 16S rRNA gene sequencing. (A) Mean ($n=3$). The four most abundant phyla are depicted as four colours – *Actinobacteria* (red), *Bacteroidetes* (yellow), *Firmicutes* (green) and *Proteobacteria* (blue) with family and genus level taxonomy portrayed as differing shades of those colours within each phylum. Only genera of greater than 1% total read composition were included in this graph. (B) Relative abundance of *Bifidobacterium* spp. and (C) *Bacteroides* spp. Green, gold and blue bars represent green kiwifruit, gold kiwifruit and the control respectively. Error bars are the SEM of the three fermentations. SEM are displayed as error bars ($n=3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

clustered into non-chimeric OTUs based on a 97% identity threshold value. Inspection of the relative abundance data at phylum and genus levels (Fig. 1) indicated that the substrates had distinct effects on the microbial ecology within the *in vitro* fermentations. Bacterial profiles at time 0 for both substrates and the control were very similar and form a baseline from which changes over time caused by fermentable substrate were measured. Several bacterial groups increased in abundance in all three vessels over the course of the experiment including *Enterobacteriaceae,*

Sutterella spp., *Veillonella* spp., *Collinsella* spp., and *Citrobacter* spp. Genera which decreased in abundance, irrespective of vessel, over time were *Faecalibacterium* spp., *Blautia* spp., *Prevotella* spp., *Lachnospira* spp., and *Bifidobacterium* spp. increased when the vessel was supplemented with green kiwifruit digesta (Fig. 1B). This 5-fold increase was observable despite the *P*-value falling short of significance after FDR correction (*P* – 0.2 for 0 h *vs* 24 h and *P* – 0.4 for 0 h *vs* 48 h). This is most likely due to the variation in the donor faecal inoculum composition, which is quite common
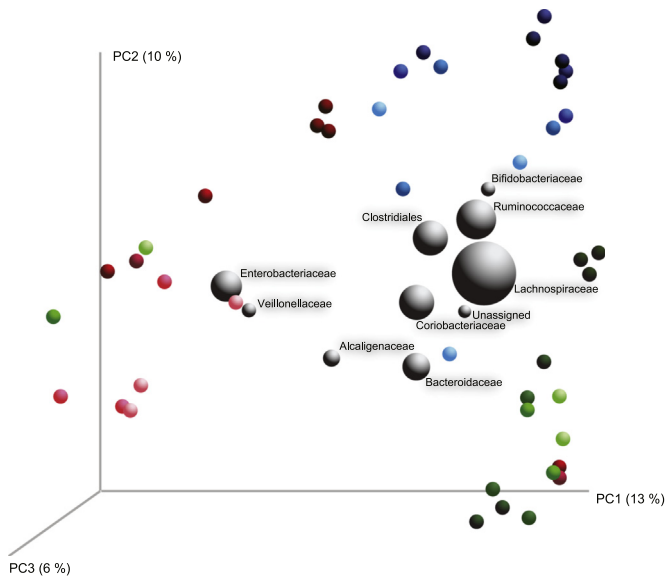
Fig. 2. Jackknifed beta diversity PCoA biplots showing unweighted UniFrac distances. Plotted by faecal donor (fermentation), where donor 1, 2 and 3 are shown with blue, green and red spheres, respectively. Within the three colours, the fermentations are plotted by time, with darker hues moving to lighter hues from 0 h, 5 h, 10 h, 24 h, and 48 h. Vector loadings by bacteria at the family level are shown with grey spheres, the size of which corresponds to their contribution to differentiation in the plot. Separation by faecal donor ($P – 0.001$, $R^2 – 15\%$) and time ($P – 0.005$, $R^2 – 13\%$) are statistically significant based on the ADONIS statistical test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and is also demonstrated in the beta diversity biplots (Fig. 2). *Bacteroides* spp. multiplied in the green and gold kiwifruit vessels after 5 to 10 h and then dropped back by 48 h, whereas *Bacteroides* spp. in the control vessel remained relatively constant (Fig. 1C). Although barely detectable in the kiwifruit vessels, *Oscillospira* spp. became prominent in the control vessel, reaching almost 5% of total abundance. At species-level, it was found that *Faecalibacterium prausnitzii* accounted for the majority of the *Faecalibacterium* species which dropped from about 13% of total abundance at time 0 to less than 2% by 24 h in all 3 vessels. Conversely, *Collinsella aerofaciens* increased in all three vessels and was the predominant *Collinsella* species.

Alpha diversity analysis was calculated by time, fermentation and substrate using four rarefaction metrics (PD whole tree, Chao1, Observed species and Shannon diversity). No significant associations were evident when plotted by time or fermentation, but a clear trend towards higher diversity in the control and lower diversity in the gold kiwifruit vessel was observed when plotted by substrate (see Fig. S1). For the PD whole tree metric, gold kiwifruit had significantly lower species richness than the control ($P – 0.003$) at an average of 15.84 ± 1.59 (gold kiwifruit) *vs* 17.56 ± 0.93 (control) as assessed by the nonparametric two-sample *t*-test (Monte Carlo permutation).

When the diversity of the bacterial populations between vessels was examined (beta diversity), it was evident that separation was greatest when plotted by fermentations on a principal coordinates analysis (PCoA) plot (Fig. 2); this demonstrated how samples were grouped by donor and time. In the PCoA plots an ordination method was used to plot variance against orthogonal axes. When the PCoA was overlaid with the ten family level bacterial groups most responsible for causing variance using the biplot function, it showed that *Coriobacteriaceae*, *Clostridiales*, *Ruminococcaceae* and *Lachnospiraceae* were the core taxa changing in the vessels. When plotted by time (Fig. 2), a trend emerged that as time progressed the points moved from the upper right space of each of the three fermentations

towards the bottom left area of the plot. This direction of migration indicates an increase in the *Bacteroidaceae*, *Alcaligenaceae*, *Veillonellaceae* and *Enterobacteriaceae*. When comparing samples as a function of substrate, there was a tendency for the control to cluster close to the time 0 point (data not shown).

3.2. NMR spectroscopy

Principal components analysis (PCA) was applied to visualise the metabolic evolution of the fermentation supernatant over time (Fig. 3). Principal components 1 (PC1) and 2 (PC2) collectively accounted for 77% of the variability of the data. A time-dependant shift was observed in the scores from PC1 (horizontal movement in the scores plot; Fig. 3A) and the loadings for PC1 (Fig. 3B) indicate that oligosaccharides of various chain lengths are the main variables contributing to this shift. At the 0 h timepoint, the metabolic profiles from vessels containing kiwifruit contained a greater amount of oligosaccharides than the control. At this time point substrate has been added, indicating that kiwifruit contain a higher abundance of oligosaccharides than the control. Over time the metabolic profiles of the green and gold kiwifruit fermentations followed similar trajectories (negative PC1 and positive PC2 movement) with increased production of acetate (in agreement with the organic acid data shown in Fig. 4). Propionate were also produced as the fermentation progressed, whereas lactate, leucine, alanine, succinate, and histidine decreased over time. In contrast, a minimal time-dependant shift was observed in the metabolic profiles of the control fermentation.

3.3. Gas chromatography

Concentrations of acetate and propionate increased throughout the batch culture runs in the kiwifruit vessels and to a lesser extent in the control vessel (Fig. 4). Butyrate also increased over the course of the experiment, but at a similar rate for all substrates including the control. Formic acid, succinic acid and lactic acid were liberated at the 5 and 10 h time points, and concentrations decreased thereafter (Fig. 4). Interestingly, the vessel that was not supplemented with any additional carbohydrate (control) had a higher production of valerate and the BCFAs isobutyrate and isovalerate (Fig. 4).

4. Discussion

This study demonstrated that non-digestible components from green and gold kiwifruit can be utilised as fermentable substrates, effecting change to both bacterial composition and metabolism. The microbial profiles at time 0 for all three vessels were relatively similar but after 5–10 h *Enterobacteriaceae* and *Collinsella* spp. begin to markedly increase while *Faecalibacterium* spp. and *Blautia* spp. decreased, irrespective of the presence or absence of substrate. As these changes were also observed in the control, it is more likely a function of the gut model system, rather than a substrate response. There were un-fermented substrates present within the faecal inoculum and the medium to a small extent, which may have contributed to the initial changes in fermentation seen in the model. We note that early proliferation of *Enterobacteriaceae* was also observed by a group running the simulator of the intestinal microbial ecosystem (SHIME) models to measure the impact of polyphenols on the human gut microbiota (Kemperman et al., 2013). Despite these background changes, the green and gold kiwifruit substrates exerted an additional powerful influence. *Bifidobacterium* spp. increased in abundance after 24 h of fermentation following exposure to green kiwifruit polysaccharides. *Bacteroides* spp. increased in relative abundance in
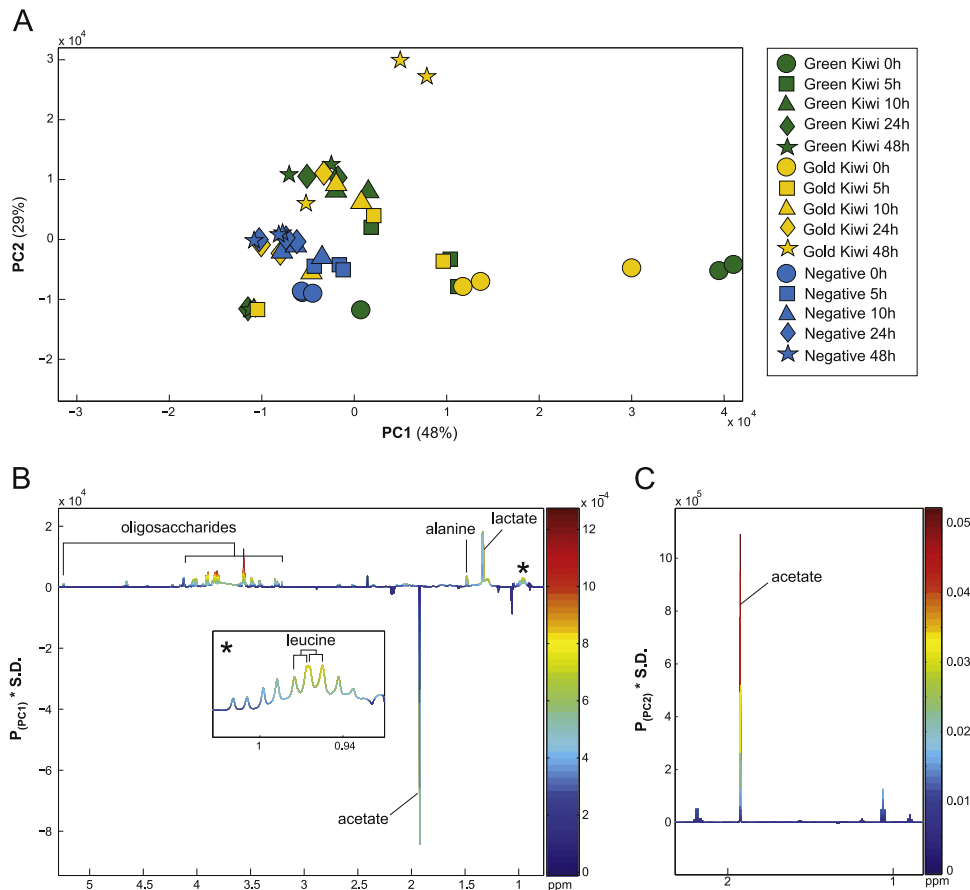
Fig. 3. Principal Component Analysis (PCA) model of the metabolic profiles of all batch culture supernatants. (A) Scores plot for PC1 *vs* PC2 (% variance explained in parenthesis). Product of PC loadings with standard deviation of the entire data set is plotted and coloured by the square of the PC for (B) PC1 and (C) PC2. The contribution of each variable (metabolite) to sample classification was visualised by back-scaling, which is the PC loading multiplied by the standard deviation (S.D.) of the variable. The coloured bar represents the contribution of each metabolite to variation in the plot, with red indicating high significance and blue indicating low significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

response to the kiwifruit substrates prior to reverting to control levels. The genus *Bacteroides* include species that have a diverse array of substrate utilisation machinery encoded on polysaccharide utilisation loci (PUL) (Martens, Chiang, & Gordon, 2008). Potentially, *Bacteroides* spp. were able to engage PULs to utilise recalcitrant substrates, thereby increasing their relative abundance before exhausting their supply. These increases in *Bifidobacterium* spp. and *Bacteroides* spp. are consistent with previously published green and gold kiwifruit fermentation results (Parkar et al., 2012).

Interestingly, the alpha diversity was lower in the vessel with the gold kiwifruit than the vessel containing the green substrate or the control vessel. However, this occurrence is most likely due to the large bloom of *Enterobacteriaceae* in the gold kiwifruit vessel which were unable to be taxonomically classified below the family level. The beta diversity analyses showed a clear partitioning when calculated by donor and a distinct pattern of movement as the fermentation progressed. It is clear that different faecal donors had a considerable impact on the starting point and successive fermentation direction. This effect has been observed in experiments conducted with kiwifruit polysaccharides previously (Rosendale et al., 2012). It is not unexpected that the diversity and composition between donors varies considerably as has been shown in many studies of gut microbial ecology (Arumugam et al., 2011; Eckburg et al., 2005; Ley, Peterson, & Gordon, 2006; Rosendale et al., 2012). However, despite the variability between donors, there were still evident trends in the modulation of the microbiota.

Acetate was the most prevalent SCFA produced in all three fermentations. Acetate is absorbed into the bloodstream and used by peripheral tissue and muscle (Wong, de Souza, Kendall, Emam, & Jenkins, 2006) and is a major metabolite of bifidobacteria (Wolfe, 2005). Propionate, butyrate and ethanol were also seen to increase over time. Organic acids are quantitatively the most abundant end-products of microbial fermentation in the human colon and their production lowers pH and directly inhibits the growth of pathogens (Blaut, 2002; Cummings & Englyst, 1987). The accumulation of propionate and butyrate over time was expected as these cannot be used by cross-feeding bacteria (Louis et al., 2007). Although acetate can be metabolised, it tends to accrue over time, the generation of this oxidised molecule being an energy favourable reaction (Macfarlane & Macfarlane, 2003). Given the comparable rates of butyrate production in the kiwifruit vessels compared with the control, it can be concluded that kiwifruit polysaccharides are not particularly butyrogenic. Green and gold kiwifruit substrates stimulated an increase in succinate and lactate production in the first several hours post inoculation before dropping back by the 24 h and 48 h time points. These intermediates can serve as substrate for other bacteria and may have been further converted to acetate or propionate (Louis et al., 2007). Like succinate and lactate, formate also behaved in this intermediate manner. This could be explained by the onward conversion of formate to methane which can be performed by methanogens such as Methanobrevibacter smithii or to acetate by acetogens such as Blautia hydrogenotrophica (Flint, Scott, Louis, & Duncan, 2012).
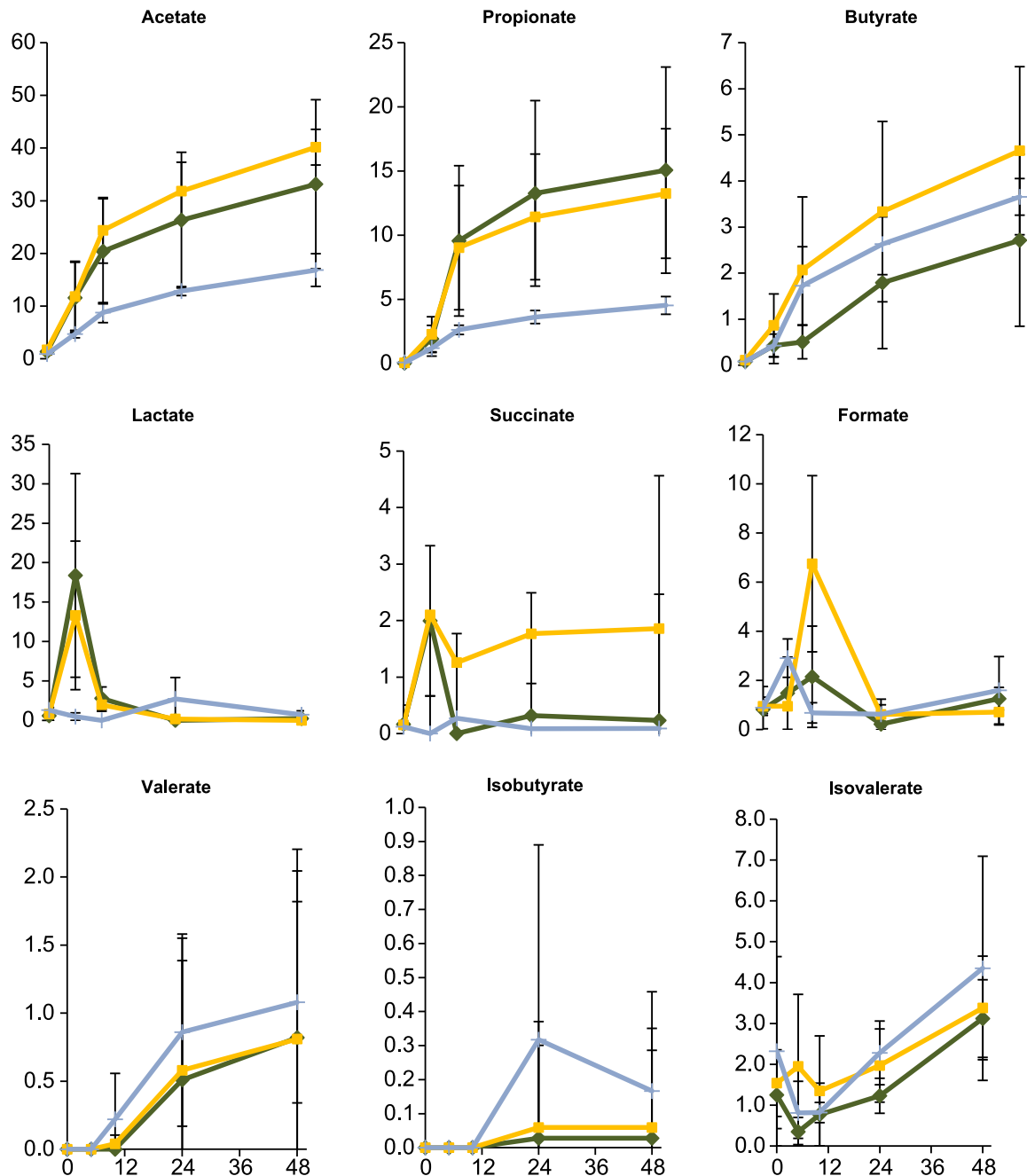
Fig. 4. Short-chain fatty acid and organic acid production as determined by GC-FID analysis. The units of the vertical axes are concentration (μmol/mL fermenta) and the horizontal axes are time (hours). Green kiwifruit (green line), gold kiwifruit (gold line), control (blue line). SEM are displayed as error bars ($n=3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Protein only accounts for about 1% of the edible portion of green kiwifruit and a considerable proportion of this would be unable to make it to the colon without being digested or absorbed in the small intestine (Ferguson & Ferguson, 2003). However, small amounts of protein are included in the medium, plus low levels of protein may have been introduced along with the faecal inoculum and turnover of microbes could also add to amino acids available as substrates. The BCFAs, isovalerate and isobutyrate are products of the fermentation of branched chain amino acids such as leucine, isoleucine and valine which are potentially available in the fermentation medium (Macfarlane & Macfarlane, 2003). The control produced more BCFA than the kiwifruit vessels: this could indicate that the microbiota were scavenging any available substrate as they would in the carbohydrate-deficient distal colon. Given that

protein fermentation is an undesirable phenomenon which can lead to detrimental health effects, the lower level of BCFA production associated with kiwifruit fermentations is a positive outcome (Mortensen, Clausen, Bonnen, Hove, & Holtug, 1992; Nyangale, Mottram, & Gibson, 2012). It is not clear exactly which components of kiwifruit NSP were responsible for the changes observed in this study. Certain differences exist between green and gold kiwifruit constituents; for example, gold kiwifruit is higher in hemicellulosic polysaccharides and lower in pectic polysachharides than green kiwifruit (Sauvageau, Hinkley, Carnachan, & Sims, 2010). The greater amount of hemicellulose in gold kiwifruit may be responsible for stimulating the Enterobacteriaceae, resulting in a lower abundance of bifidobacteria. More complex investigations are warranted that take into account

the different microbial niches encountered in the large bowel and the fermentation of other sources of carbohydrates such as host mucin (Macfarlane, Woodmansey, & Macfarlane, 2005).

5. Conclusions

In summary, we have shown that kiwifruit non-digestible polysaccharides have a number of effects on gut microbial ecology. The starting population of bacteria in the inoculum varies greatly between donors and has a considerable impact on the subsequent fermentation trajectory. However, notwithstanding the initial composition, kiwifruit NSP fermentation facilitated similar changes in microbial composition and metabolite production. These changes included increasing *Bifidobacterium* spp. and *Bacteroides* spp. and beneficial metabolites such as organic acids, which may have positive consequences for human health.

Conflict of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at: http://dx.doi.org/10.1016/j.bcdf.2015.07.001.

References

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., & Meta, H. I. T. (2011). Consortium enterotypes of the human gut microbiome. *Nature*, *473*(7346), 174–180. http://dx.doi.org/10.1038/nature09944.

Blaut, M. (2002). Relationship of prebiotics and food to intestinal microflora. *European Journal of Nutrition*, *41*(Suppl. 1), 11–16.

Caporaso, J. Gregory, Bittinger, Kyle, Bushman, Frederic D., DeSantis, Todd Z., Andersen, Gary L., & Knight, Rob (2010). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*, *26*(2), 266–267.

Caporaso, J. Gregory, Kuczynski, Justin, Stombaugh, Jesse, Bittinger, Kyle, Costello, Elizabeth K., Bushman, Frederic D., Costello, Elizabeth K., Gordon, Jeffrey I., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336.

Carnachan, S. M., Booten, T. J., Mishra, S., Monro, J. A., & Sims, I. M. (2012). Effects of simulated digestion in vitro on cell wall polysaccharides from kiwifruit (Actinidia spp.). *Food Chemistry*, *133*(1), 132–139. http://dx.doi.org/10.1016/j.foodchem.2011.12.084.

Chandrasekara, Anoma, Naczk, Marian, & Shahidi, Fereidoon (2012). Effect of processing on the antioxidant activity of millet grains. *Food Chemistry*, *133*(1), 1–9. http://dx.doi.org/10.1016/j.foodchem.2011.09.043.

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S., & O'Toole, P. W. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature*, *488*(7410), 178–184. http://dx.doi.org/10.1038/nature11319.

Cummings, J. H., & Englyst, H. N. (1987). Fermentation in the human large-intestine and the available substrates. *American Journal of Clinical Nutrition*, *45*(5), 1243–1255.

Cummings, J. H., & Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology*, *70*(6), 443–459. http://dx.doi.org/10.1111/j.1365-2672.1991.tb02739.x.

Culhane, A. C., Thioulouse, J., Perriere, G., & Higgins, D. G. (2005). MADE4: an R package for multivariate analysis of gene expression data. *Bioinformatics*, *21*(11), 2789–2790. http://dx.doi.org/10.1093/bioinformatics/bti394.

Dawson, D. M., & Melton, L. D. (1991). 2 pectic polysaccharides from kiwifruit cell-walls. *Carbohydrate Polymers*, *15*(1), 1–11. http://dx.doi.org/10.1016/0144-8617(91)90015-5.

van der Sluis, A. A., Dekker, M., Skrede, G., & Jongen, W. M. F. (2002). Activity and concentration of polyphenolic antioxidants in apple juice. 1. Effect of existing production methods. *Journal of Agricultural and Food Chemistry*, *50*(25), 7211–7219. http://dx.doi.org/10.1021/jf020115h.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., & Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science*, *308*(5728), 1635–1638. http://dx.doi.org/10.1126/science.1110591.

Flint, Harry J., Scott, Karen P., Louis, Petra, & Duncan, Sylvia H. (2012). The role of the gut microbiota in nutrition and health. *Nature Reviews Gastroenterology & Hepatology*, *9*(10), 577–589.

Gibson, G. R. (2004). Fibre and effects on probiotics (the prebiotic concept). *Clinical Nutrition Supplements*, *1*(Suppl. 2), 25–31. http://dx.doi.org/10.1016/j.clnu.2004.09.005.

Ferguson, A.R., Ferguson, L.R., 2003. Are kiwifruit really good for you? In: Huang, H.W. (Ed.), *Proceedings of the Fifth International Symposium on Kiwifruit*. pp. 131–138.

Gibson, G. R., Scott, K. P., Rastall, R. A., Tuohy, K. M., Hotchkiss, A., Dubert-Ferrandon, A., & Buddington, R. (2010). Dietary prebiotics: current status and new definition. *Food Science and Technology Bulletin: Functional Foods*, *7*(1), 1–19.

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., & Birren, B. W. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Research*, *21*(3), 494–504. http://dx.doi.org/10.1101/gr.112730.110.

Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., & Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science*, *312*(5778), 1355–1359. http://dx.doi.org/10.1126/science.1124234.

Gueimonde, M., & Collado, M. C. (2012). Metagenomics and probiotics. *Clinical Microbiology and Infection*, *18*, 32–34. http://dx.doi.org/10.1111/j.1469-0691.2012.03873.x.

Oksanen, Jari, Blanchet, Guillaume, F., Kindt, Roeland, Legendre, Pierre, Minchin, Peter R., O'Hara, R.B., Simpson, Gavin L., Solymos, Peter, Henry, M., Stevens, H., Wagner, Helene, 2013. *Vegan: Community Ecology Package*. R package version 2.0-10. ⟨http://CRAN.R-project.org/package=vegan⟩.

Johnson, S. K., Chua, V., Hall, R. S., & Baxter, A. L. (2006). Lupin kernel fibre foods improve bowel function and beneficially modify some putative faecal risk factors for colon cancer in men. *British Journal of Nutrition*, *95*(2), 372–378.

Henare, S. J., Rutherford, S. M., Drummond, L. N., Borges, V., Boland, M. J., & Moughan, P. J. (2012). Digestible nutrients and available (ATP) energy contents of two varieties of kiwifruit (Actinidia deliciosa and Actinidia chinensis). *Food Chemistry*, *130*(1), 67–72. http://dx.doi.org/10.1016/j.foodchem.2011.06.055.

Kemperman, R. A., Gross, G., Mondot, S., Possemiers, S., Marzorati, M., Van de Wiele, T., & Vaughan, E. E. (2013). Impact of polyphenols from black tea and red wine/grape juice on a gut model microbiome. *Food Research International*, *53*(2), 659–669. http://dx.doi.org/10.1016/j.foodres.2013.01.034.

Ley, Ruth E., Turnbaugh, Peter J., Klein, Samuel, & Gordon, Jeffrey I. (2006). Microbial ecology-human gut microbes associated with obesity. *Nature*, *444*(7122), 1022–1023. http://dx.doi.org/10.1038/nature4441022a.

Ley, R. E., Peterson, D. A., & Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, *124*(4), 837–848. http://dx.doi.org/10.1016/j.cell.2006.02.017.

Lipsky, L. M., Cheon, K., Nansel, T. R., & Albert, P. S. (2012). Candidate measures of whole plant food intake are related to biomarkers of nutrition and health in the US population (National Health and Nutrition Examination Survey 1999–2002). *Nutrition Research*, *32*(4), 251–259. http://dx.doi.org/10.1016/j.nutres.2012.03.005.

Louis, P., Scott, K. P., Duncan, S. H., & Flint, H. J. (2007). Understanding the effects of diet on bacterial metabolism in the large intestine. *Journal of Applied Microbiology*, *102*(5), 1197–1208. http://dx.doi.org/10.1111/j.1365-2672.2007.03322.x.

Macfarlane, S., & Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. *Proceedings Nutrition Society*, *62*(1), 67–72. http://dx.doi.org/10.1079/PNS2002207.

Macfarlane, S., Woodmansey, E. J., & Macfarlane, G. T. (2005). Colonization of mucin by human intestinal bacteria and establishment of biofilm communities in a two-stage continuous culture system. *Applied and Environmental Microbiology*, *71*(11), 7483–7492. http://dx.doi.org/10.1128/aem.71.11.7483-7492.2005.

Martens, E. C., Chiang, H. C., & Gordon, J. I. (2008). Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. *Cell Host Microbe*, *4*(5), 447–457. http://dx.doi.org/10.1016/j.chom.2008.09.007.

Mills, D. J. S., Tuohy, K. M., Booth, J., Buck, M., Crabbe, M. J. C., Gibson, G. R., & Ames, J. M. (2008). Dietary glycated protein modulates the colonic microbiota towards a more detrimental composition in ulcerative colitis patients and non-ulcerative colitis subjects. *Journal of Applied Microbiology*, *105*(3), 706–714. http://dx.doi.org/10.1111/j.1365-2672.2008.03783.x.

Mortensen, P. B., Clausen, M. R., Bonnen, H., Hove, H., & Holtug, K. (1992). Colonic fermentation of ispaghula, wheat bran, glucose, and albumin to short-chain fatty acids and ammonia evaluated in vitro in 50 subjects. *Journal Parenteral and Enteral Nutrition*, *16*(5), 433–439.

Nyangale, E. P., Mottram, D. S., & Gibson, G. R. (2012). Gut Microbial Activity, Implications for Health and Disease: The Potential Role of Metabolite Analysis. *Journal of Proteome Research*, *11*(12), 5573–5585. http://dx.doi.org/10.1021/Pr300637d.

Parkar, S. G., Rosendale, D., Paturi, G., Herath, T. D., Stoklosinski, H., Phipps, J. E., & Ansell, J. (2012). In vitro utilization of gold and green kiwifruit oligosaccharides by human gut microbial populations. *Plant Foods for Human Nutrition*, *67*(3), 200–207. http://dx.doi.org/10.1007/s11130-012-0293-1.

Rosendale, D. I., Blatchford, P. A., Sims, I. M., Parkar, S. G., Carnachan, S. M., Hedderley, D., & Ansell, J. (2012). Characterizing kiwifruit carbohydrate utilization in vitro and its consequences for human faecal microbiota. *Journal of Proteome Research*, 11(12), 5863–5875. http://dx.doi.org/10.1021/pr300646m.

Rosendale, Douglas, Cookson, Adrian, Roy, Nicole, & Vetharaniam, Indrakumar (2011). Opportunities for predictive modelling and gut health. Conceptually exploring an in silico tool for quantifying the benefits of dietary fibre consumption. *Agro Food Industry Hi-Tech*, 22(6), 18–22.

Richardson, A. J., Calder, A. G., Stewart, C. S., & Smith, A. (1989). Simultaneous determination of volatile and non-volatile acidic fermentation products of anaerobes by capillary gas-chromatography. *Letters in Applied Microbiology*, 9(1), 5–8. http://dx.doi.org/10.1111/j.1472-765X.1989.tb00278.x.

RStudio, 2012. *RStudio: Integrated Development Environment for R* (*Version 0.97.551*) [*Computer software*]. Boston, MA. ⟨http://www.rstudio.org/⟩.

Sauvageau, Janelle, Hinkley, Simon F., Carnachan, Susan M., & Sims, Ian M. (2010). Characterisation of polysaccharides from gold kiwifruit (Actinidia chinensis Planch. 'Hort16A'). *Carbohydrate Polymers*, 82(4), 1110–1115. http://dx.doi.org/10.1016/j.carbpol.2010.06.039.

Seager, N. G., & Haslemore, R. M. (1993). Rapid estimation of fruit starch and soluble sugar concentrations in kiwifruit. *Hortscience*, 28(9), 948–950.

Shahidi, F. (2009). Nutraceuticals and functional foods: whole versus processed foods. *Trends in Food Science & Technology*, 20(9), 376–387. http://dx.doi.org/10.1016/j.tifs.2008.08.004.

Tannock, G. W., Munro, K., Harmsen, H. J., Welling, G. W., Smart, J., & Gopal, P. K. (2000). Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Applied and*

Environmental Microbiology, 66(6), 2578–2588.

Tuomilehto, J., Lindstrom, J., Eriksson, J. G., Valle, T. T., Hamalainen, H., Ilanne-Parikka, P., & Finnish Diabetes Prevention Study, Group (2001). Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *New England Journal of Medicine*, 344(18), 1343–1350. http://dx.doi.org/10.1056/NEJM200105033441801.

Vazquez-Baeza, Y., Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: a tool for visualizing high-throughput microbial community data. *Gigascience*, 2(1), 16. http://dx.doi.org/10.1186/2047-217X-2-16.

DeSantis, Todd Z., Hugenholtz, Philip, Larsen, Neils, Rojas, Mark, Brodie, Eoin L., Keller, Keith, Andersen, Gary L., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, 72(7), 5069–5072.

Wang, Qiong, Garrity, George M., Tiedje, James M., & Cole, James R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, 73(16), 5261–5267.

Wolfe, A. J. (2005). The acetate switch. *Microbiology and Molecular Biology Reviews*, 69(1), 12–50. http://dx.doi.org/10.1128/MMBR.69.1.12-50.2005.

Wong, J. M. W., de Souza, R., Kendall, C. W. C., Emam, A., & Jenkins, D. J. A. (2006). Colonic health: Fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology*, 40(3), 235–243.

Xu, Jian, Mahowald, Michael A., Ley, Ruth E., Lozupone, Catherine A., Hamady, Micah, Martens, Eric C., & Gordon, Jeffrey I. (2007). Evolution of symbiotic bacteria in the distal human intestine. *PLoS Biology*, 5(7), 1574–1586. http://dx.doi.org/10.1371/journal.pbio.0050156.