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

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Paul Blatchford,[1,2] Halina Stoklosinski,[1] Gemma Walton,[2] Jonathan Swann,[2] Glenn Gibson,[2] Richard Gearry,[3] Juliet Ansell,[4]

[1]*The New Zealand Institute for Plant & Food Research Limited, Private Bag 11600, Palmerston North 4442, New Zealand*

[2]*Department of Food and Nutritional Sciences, The University of Reading, Reading RG6 6AP, United Kingdom*

[3]*Department of Medicine, University of Otago, Christchurch, New Zealand*

[4]*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, 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 polysaccharides can induce substantive change in microbial ecology and metabolism which could have consequences for human health.

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 colonised 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-dependent 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 *et al.*, 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 (Tuomilehto *et al.*, 2001, Johnson *et al.*, 2006, Shahidi, 2009). 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 *et al.*, 2012). In addition to the health benefits of whole foods, 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 (van der Sluis *et al.*, 2002, Chandrasekara *et al.*, 2012).

The most commonly sold kiwifruit are from the species *Actinidia deliciosa* (typically green fleshed e.g. ‘Hayward’) and *A. 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 (Dawson & Melton, 1991, Seager & Haslemore, 1993, Ferguson & Ferguson, 2003, Carnachan *et al.*, 2012). 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 (Dawson & Melton, 1991, Carnachan *et al.*, 2012). 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 *et al.*, 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 *et al.*, 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.

Materials and Methods

Simulated Gastrointestinal Digestion (SGD)

Two kiwifruit substrates were used in the batch culture models: Green kiwifruit (*Actinidia deliciosa*) 'Hayward' and Gold kiwifruit (*Actinidia chinensis*) 'Hort16A'. As a negative 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, Seward, West Sussex, UK) 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, Poole, Dorset, UK) 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., UK) 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.

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 (Poole, Dorset, UK). 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 negative 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 appropriately. 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.

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., United Kingdom. This unsupervised approach was used to determine inherent changes between batch culture samples of different substrates or at different time-points.

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 ionization detector (GC-FID) following a modified method of Richardson *et al.* (Richardson *et al.*, 1989). In brief, 1.5 mL of sample was centrifuged at 16,200 *g* 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 3000 *g* for 5 min (4 °C). The clarified supernatant was acidified with concentrated hydrochloric acid and diethyl ether added, and following vortexing, was centrifuged at 10,000 *g* 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 ionization detector and fitted with a HP-1 column (10 m × 0.53 mm ID × 2.65 µm) (Agilent Technologies, Santa Clara, CA, USA). 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 program 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.

DNA Extraction, 16S rRNA gene sequencing and bioinformatics

A 1.5mL sample aliquot was centrifuged at 16,200 *g* 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 16,200 *g* for 5 min and the pellet washed in 1 mL of PBS. The sample was centrifuged again at 16,200 *g* 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, 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 4,100 *g* 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 16,200 *g*, 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 (cgtatcgcctccctcgcgccatcagACTCCTACGGGAGGCAGCAGT) and HDA-2 (ctatgcgccttgccagcccgctcagNNNNNNNNNNGTATTACCGCGGCTGCTGGCAC) (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, Melbourne, Australia) was mixed with 1 µL template DNA and 100 nM of each primer (total reaction volume 50µL). PCR conditions were as follows: Initial denaturation 95 °C for 15 min then thirty 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, Melbourne, Australia), 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.*, 2010). 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.*, 2010) 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 *et al.*, 2007). Chimeric sequences were removed from the reads using the ChimeraSlayer algorithm (Haas *et al.*, 2011, Claesson *et al.*, 2012). 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 *et al.*, 2013).

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239 *Statistical Analysis*

240 ANOVA for determining differences in OTU frequencies between sample groups was
241 conducted in QIIME using the *group_significance.py* script. The non-parametric two-sample
242 t-test (Monte Carlo permutation) from QIIME was used for determining alpha diversity
243 significance. All other statistical calculations were conducted in R Studio using the vegan
244 and made4 packages (Culhane *et al.*, 2005, RStudio, 2012, Jari Oksanen, 2013). The
245 Wilcoxon/Mann-Whitney test was conducted to assess differences between taxa at the
246 phylum, family and genus level. A *P*-value of less than 0.05 was deemed significant and the
247 False Discovery Rate (FDR) method was used for correcting for multiple comparisons.

Results

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 clustered into non-chimeric OTUs based on a 97% identity threshold value. Inspection of the relative abundance data at phylum and genus levels (Figure 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 control were very similar (Figure S1) and form a baseline from which changes over time caused by fermentable substrate can be 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. and *Lachnospira* spp.. *Bifidobacterium* spp. increased when the vessel was supplemented with green kiwifruit digesta (Figure 1B). This enrichment 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 response to the treatment between donors, which is quite common and is also demonstrated in the beta diversity biplots (Figure 2). For example, donor 1 had a significantly higher occurrence of bifidobacterial OTU *denovo*1575 than donors 2 and 3 (*P* - <0.001). *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 negative vessel remained relatively constant (Figure 1C). Although barely detectable in the kiwifruit vessels, *Oscillospira* spp. became prominent in the negative 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) at 2800 reads rarefaction depth. No significant associations were evident when plotted by time or fermentation, but a clear trend towards higher diversity in the negative control and lower diversity in the gold kiwifruit vessel was observed (see Figure S2). For the PD whole tree metric, gold kiwifruit had significantly lower species richness than the negative control (*P* – 0.003) at an average of 15.84 ± 1.59 (gold kiwifruit) vs 17.56 ± 0.93 (negative) 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 (Figure 2); this demonstrated how samples were grouped by donor. 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 (Figure 2), it was clear 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 negative control to cluster close to the time 0 point (data not shown).

NMR Spectroscopy

Principal components analysis (PCA) was applied to visualise the metabolic evolution of the fermentation supernatant over time (Figure 3). Principal components 1 (PC1) and 2 (PC2) collectively accounted for 77% of the variablility of the data. A time-dependent shift was observed in the scores from PC1 (horizontal movement in the scores plot; Figure 3A) and the loadings for PC1 (Figure 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 negative control. At this time point substrate has been added, indicating that kiwifruit contain a higher abundance of oligosaccharides than the negative 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 Figure 4). Propionate were also produced as the fermentation progressed, whereas lactate, leucine, alanine, succinate, and histidine decreased over time. In contrast, a minimal time-dependent shift was observed in the metabolic profiles of the negative control fermentation.

Gas Chromatography

Concentrations of acetate and propionate increased throughout the batch culture runs in the kiwifruit vessels and to a lesser extent in the negative control vessel (Figure 4). Butyrate also increased over the course of the experiment, but at a similar rate for all substrates including the negative control. Formic acid, succinic acid and lactic acid were liberated at the 5 and 10 h time points, and concentrations decreased thereafter (Figure 4). Interestingly, the vessel that was not supplemented with any additional carbohydrate (negative control) had a higher production of valerate and the BCFAs isobutyrate and isovalerate (Figure 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 negative 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 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 *et al.*, 2008). Potentially, the easily fermentable substrate was metabolised in the first few hours, after which the *Bacteroides* were able to engage PULs to utilise the more recalcitrant substrates, thereby increasing their relative abundance. 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 negative 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 (Eckburg *et al.*, 2005, Ley *et al.*, 2006, Arumugam *et al.*, 2011, Rosendale *et al.*, 2012).

However, despite the variability between donors, there were still consistent 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 *et al.*, 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 (Cummings & Englyst, 1987, Blaut, 2002). 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 negative 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 *et al.*, 2012).

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 negative control produced more BCFA than the kiwifruit vessels: this could indicate that 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 *et al.*, 1992, Nyangale *et al.*, 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 *et al.*, 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 *et al.*, 2005)..

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, consistent changes in microbial composition and metabolite production were facilitated by kiwifruit NSP fermentation. These changes included increasing *Bifidobacterium* spp. and *Bacteroides* spp. and beneficial metabolites such as organic acids, which may have positive consequences for human health.

Acknowledgements

Simone Rampelli for advice and help with bioinformatics, Adele Costabile for help with the batch culture gut models and Doug Rosendale for advice on interpretation of results. Authors have no conflict of interest to declare.

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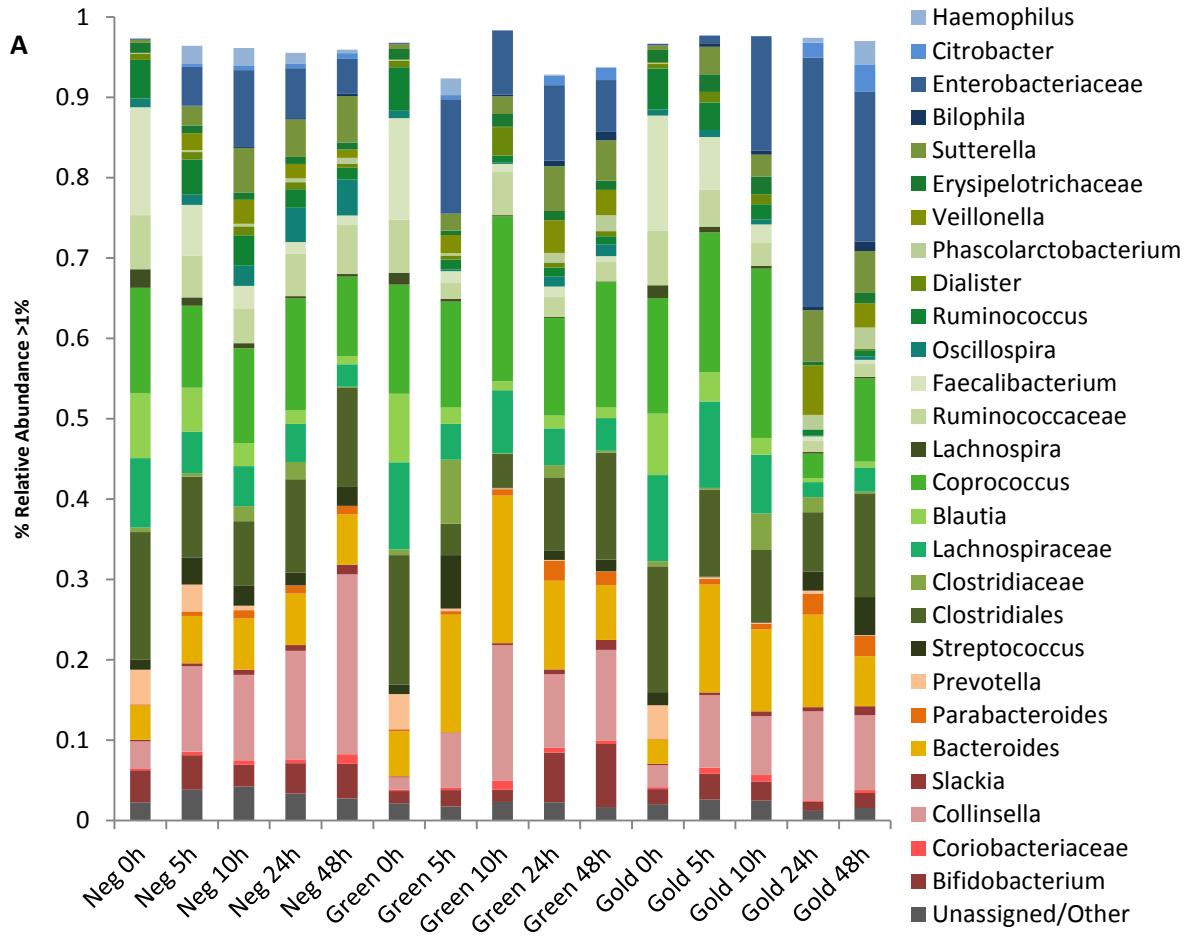
Figures:


A
% Relative Abundance >1%
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Neg 0h
Neg 5h
Neg 10h
Neg 24h
Neg 48h
Green 0h
Green 5h
Green 10h
Green 24h
Green 48h
Gold 0h
Gold 5h
Gold 10h
Gold 24h
Gold 48h
Haemophilus
Citrobacter
Enterobacteriaceae
Bilophila
Sutterella
Erysipelotrichaceae
Veillonella
Phascolarctobacterium
Dialister
Ruminococcus
Oscillospira
Faecalibacterium
Ruminococcaceae
Lachnospira
Coprococcus
Blautia
Lachnospiraceae
Clostridiaceae
Clostridiales
Streptococcus
Prevotella
Parabacteroides
Bacteroides
Slackia
Collinsella
Coriobacteriaceae
Bifidobacterium
Unassigned/Other

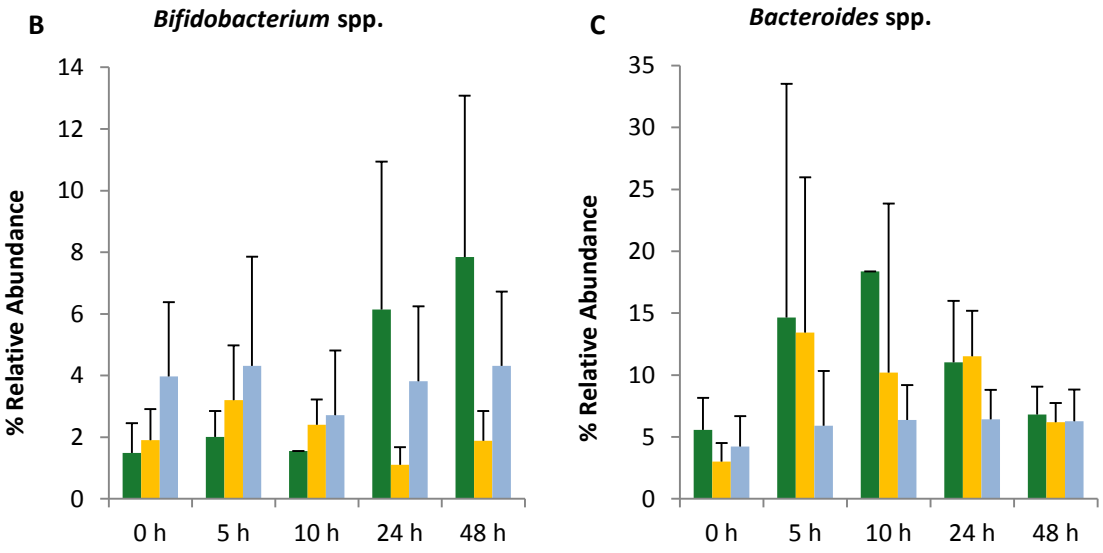
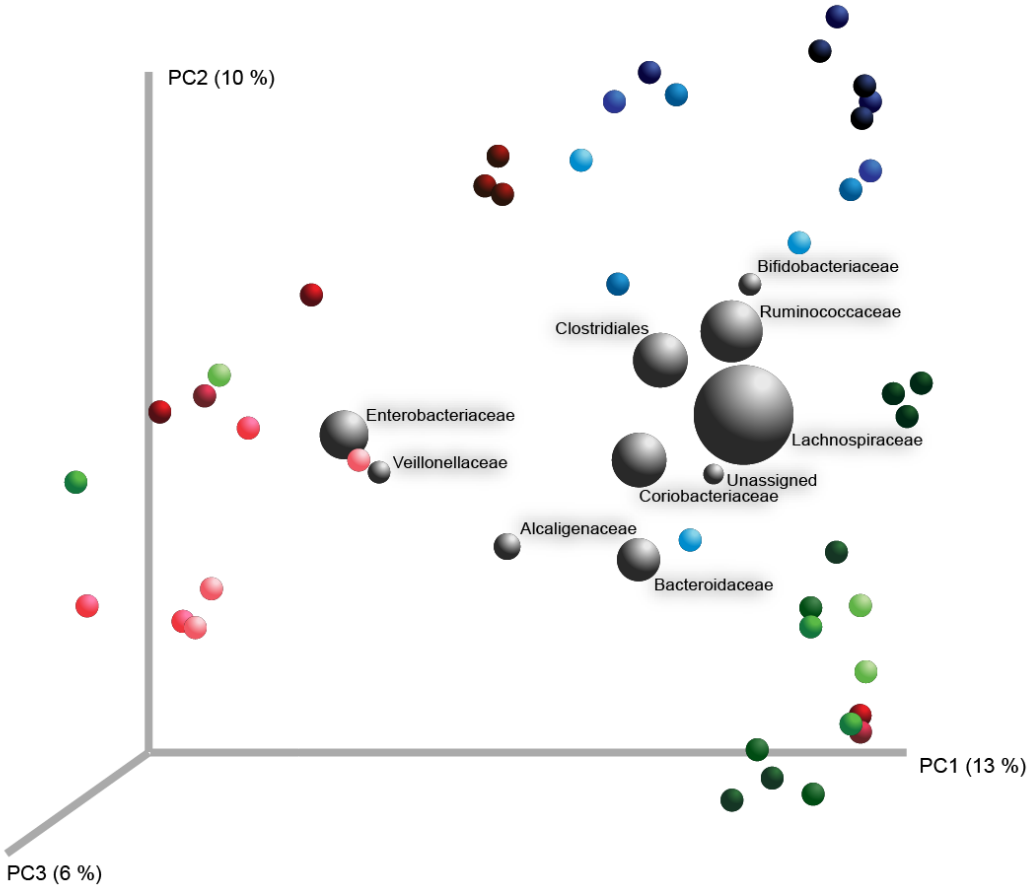

B
Bifidobacterium spp.
% Relative Abundance
14
12
10
8
6
4
2
0
0 h
5 h
10 h
24 h
48 h
C
Bacteroides spp.
% Relative Abundance
35
30
25
20
15
10
5
0
0 h
5 h
10 h
24 h
48 h

Figure 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 negative control respectively. Error bars are the SEM of the three fermentations. SEM are displayed as error bars (*n* = 3).


PC2 (10 %)
PC1 (13 %)
PC3 (6 %)
Bifidobacteriaceae
Ruminococcaceae
Clostridiales
Enterobacteriaceae
Veillonellaceae
Lachnospiraceae
Unassigned
Coriobacteriaceae
Alcaligenaceae
Bacteroidaceae

620 **Figure 2 – Jackknifed beta diversity PCoA biplots showing unweighted UniFrac distances.**
621 Plotted by faecal donor (fermentation), where donor 1, 2 and 3 are shown with blue, green and red
622 spheres, respectively. Within the three colours, the fermentations are plotted by time, with darker
623 hues moving to lighter hues from 0 h, 5 h, 10 h, 24 h, and 48 h. Vector loadings (or weighting) by
624 bacteria at the family level are shown with grey spheres, the size of which corresponds to their
625 contribution to differentiation in the plot.

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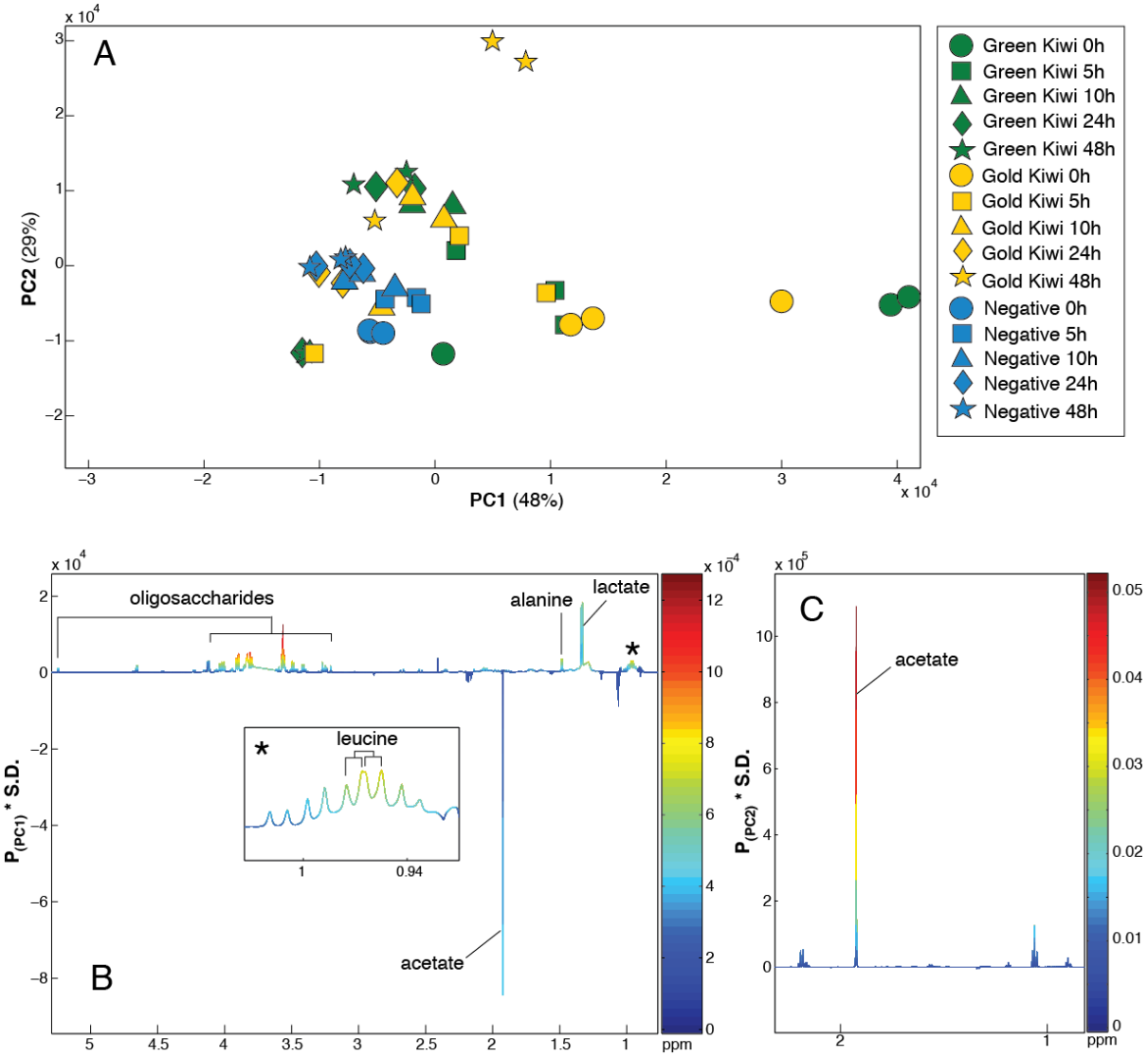

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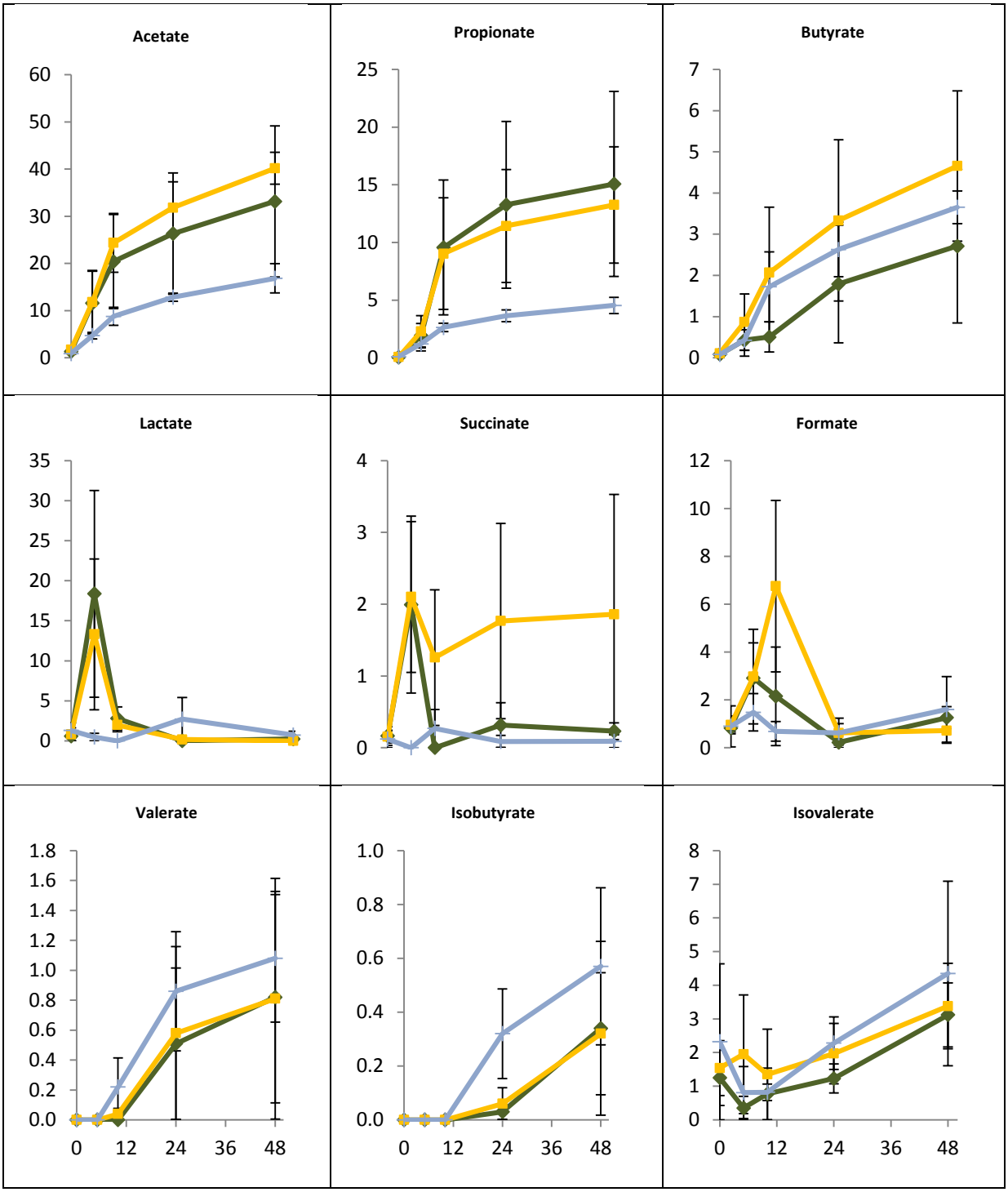
Figure 3: Principal Component Analysis (PCA) model of the metabolic profiles of all batch culture supernatants. (A) Scores plot for PC1 vs PC2 (% variance explained in parenthesis). Product of PC loadings with standard deviation of the entire data set is plotted and coloured by the square of the PC for (B) PC1 and (C) PC2.


Acetate
60
50
40
30
20
10
0
Propionate
25
20
15
10
5
0
Butyrate
7
6
5
4
3
2
1
0
Lactate
35
30
25
20
15
10
5
0
Succinate
4
3
2
1
0
Formate
12
10
8
6
4
2
0
Valerate
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
12
24
36
48
Isobutyrate
1.0
0.8
0.6
0.4
0.2
0.0
0
12
24
36
48
Isovalerate
8
7
6
5
4
3
2
1
0
0
12
24
36
48

677 **Figure 4 – Short-chain fatty acid and organic acid production as determined by GC-FID**
678 **analysis.** The units of the vertical axes are concentration (µmol/mL fermenta) and the horizontal axes
679 are time (hours). Green kiwifruit (green line), gold kiwifruit (gold line), negative control (blue line). SEM
680 are displayed as error bars (*n* = 3).

681