

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

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

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11 **Abstract**

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

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## 32 Introduction

33 The gut microbiota is a highly diverse collection of trillions of microbes comprised of  
34 hundreds of species (Gill *et al.*, 2006). In the densely colonised large intestine, the  
35 microbiota can reach numbers of up to  $10^{12}$  cfu/g colon contents (Gueimonde & Collado,  
36 2012). Such vast numbers of symbionts can have a considerable impact on the health of the  
37 host. The gut microbiota has evolved with humans to a complex inter-dependent state,  
38 where their genome in addition to our own generates a profound ability to metabolise the  
39 diverse array of substrates in the human diet (Xu *et al.*, 2007). Predominant phyla in the  
40 human gut are Bacteroidetes and Firmicutes, making up over 90% of all resident colonic  
41 bacteria with the two other subdominant phyla being Actinobacteria and Proteobacteria  
42 (Eckburg *et al.*, 2005, Ley *et al.*, 2006). There have been numerous studies conducted  
43 recently on the use of purified and processed foods or food additives to modify bacterial  
44 composition. It is clear that diet has an effect on microbiota and this in turn affects health; as  
45 many as a third of all diseases, including cardiovascular disorders such as coronary heart  
46 disease and hypertension, type 2 diabetes, functional bowel problems and cancer, are  
47 lifestyle related and their risk may be mitigated through dietary means (Tuomilehto *et al.*,  
48 2001, Johnson *et al.*, 2006, Shahidi, 2009). Prebiotic supplementation is commonly used to  
49 treat gastrointestinal dysfunction. These are a class of non-digestible food ingredients such  
50 as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and xylo-  
51 oligosaccharides (XOS) (Gibson, 2004) that confer a health benefit to the host through  
52 selectively modulating bacterial composition (Gibson *et al.*, 2010). Prebiotic molecules are  
53 often sourced from plants, where specific oligosaccharides are isolated and concentrated in  
54 order to be used as a supplement to a regular diet. An alternative to prebiotic  
55 supplementation is the use of whole fruits and vegetables as health promoting foods, which  
56 are easier to implement into a dietary routine (Lipsky *et al.*, 2012). In addition to the health  
57 benefits of whole foods, they make for a more marketable product. An absence of  
58 processing maintains the natural structure of nutrients which are potentially more  
59 bioavailable in whole foods (van der Sluis *et al.*, 2002, Chandrasekara *et al.*, 2012).

60 The most commonly sold kiwifruit are from the species *Actinidia deliciosa* (typically green  
61 fleshed e.g. 'Hayward') and *A. chinensis* (typically yellow fleshed e.g. 'Gold3'). Kiwifruit are  
62 rich in vitamin C, potassium, folate, and phytochemicals (Ferguson & Ferguson, 2003). The  
63 principal carbohydrate found in kiwifruit is starch, with non-starch polysaccharides (NSP)  
64 such as pectic polysaccharides, hemicelluloses and celluloses amounting to 2–3% of total  
65 kiwifruit constituents (Dawson & Melton, 1991, Seager & Haslemore, 1993, Ferguson &  
66 Ferguson, 2003, Carnachan *et al.*, 2012). NSP are essentially resistant to digestion by  
67 enzymes encountered in the human stomach and small intestine. Therefore, they reach the

68 colon largely intact where pectic polysaccharides and, to a lesser extent, hemicelluloses and  
69 celluloses are fermented by the gut microbiota (Cummings & Englyst, 1987). Prebiotic  
70 effects, namely beneficial changes to the composition of the existing microbiota and colonic  
71 metabolites, may subsequently be observed. Several studies have examined kiwifruit fibre  
72 digestion *in vitro*, finding a chemically unaltered structure with only minor modifications to  
73 galacturonic acid residues and molecular weight profiles in the soluble fibre fraction (Dawson  
74 & Melton, 1991, Carnachan *et al.*, 2012). In a recent study, upper gastrointestinal tract  
75 digestion had little effect on either green or gold kiwifruit in an *in vivo* porcine model, with the  
76 dietary fibre fraction being completely undigested at the terminal ileum (Henare *et al.*, 2012).

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

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## 99 **Materials and Methods**

### 100 *Simulated Gastrointestinal Digestion (SGD)*

101 Two kiwifruit substrates were used in the batch culture models: Green kiwifruit (*Actinidia*  
102 *deliciosa*) 'Hayward' and Gold kiwifruit (*Actinidia chinensis*) 'Hort16A'. As a negative control,  
103 no exogenous substrate was added. Green and gold kiwifruit were peeled, chopped and  
104 mashed finely. The samples were subjected to the simulated gastric digestion procedure as  
105 detailed by Mills *et al.* with minor modifications (Mills *et al.*, 2008). Briefly, 60 g of sample  
106 was weighed and added to 150 mL of autoclaved distilled water in a stomacher bag where it  
107 was homogenised (Stomacher 400, Seward, West Sussex, UK) for 5 min at normal speed  
108 (460 paddle beats / min). After addition of 0.001 mol/L salivary  $\alpha$ -amylase the solution was  
109 incubated for 30 min on a shaker at 37 °C. The pH was adjusted to 2.0 using 6 M HCl.  
110 Pepsin solution was added to the mixture which was incubated at 37 °C gently shaking for 2  
111 h. The pH was adjusted to 7.0 following addition of a pancreatin/bile mixture (P8096/B8631  
112 Sigma, Poole, Dorset, UK) and the solution was incubated at 37 °C for 3 h. Samples were  
113 then transferred to a 500 Da dialysis membrane (Spectra/Por, Spectrum Laboratories Inc.,  
114 UK) to remove most di- and mono-saccharides. This was dialysed for 15 h against a 10 mM  
115 NaCl solution at 4 °C. The dialysis fluid was replenished and the samples dialysed for a  
116 further 2 h. Samples were then frozen at -80 °C and freeze-dried.

### 117 *pH controlled anaerobic faecal batch cultures*

118 Batch culture systems allow the study of microbial fermentation in a simulated colonic  
119 environment. The apparatus was set up the day before the experiment and sterilised by  
120 autoclaving. The basal culture medium used for the batch cultures contained (per L): 2 g  
121 peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K<sub>2</sub>HPO<sub>4</sub>, 0.04 g KH<sub>2</sub>PO<sub>4</sub>, 0.01 g MgSO<sub>4</sub>·7H<sub>2</sub>O,  
122 0.01 g CaCl<sub>2</sub>·6H<sub>2</sub>O, 2 g NaHCO<sub>3</sub>, 2 mL Tween 80, 0.05 g haemin (dissolved in a few drops  
123 of NaOH), 10 µl vitamin K, 0.5 g cysteine HCl, 4 mL resazurin solution (0.025 g/100 mL) and  
124 0.5 g No.3 bile salts. The solution was made up to 1 L with distilled water and sterilised by  
125 autoclaving. All chemicals were obtained from Sigma (Poole, Dorset, UK). One hundred and  
126 thirty-five millilitres of freshly autoclaved medium was aseptically poured into 280 mL  
127 capacity water-jacketed batch culture vessels. The medium was continually mixed using a  
128 magnetic stirrer and maintained at 37 °C with a circulating waterbath. Oxygen free N<sub>2</sub> gas  
129 was bubbled through the media overnight to establish an anoxic environment. Excess gas  
130 was vented outside through a 0.22 µm filter.

131 On the morning of the experiment, calibrated pH electrodes were inserted into each vessel.  
132 A freshly voided stool sample was obtained from a healthy volunteer who had not taken any

133 supplemental probiotics, prebiotics or antibiotics for 6 months prior. The stool was diluted  
134 1:10 in sterile PBS, stomached for 2 min and 15 mL was added to the vessels, yielding a  
135 total volume in each vessel of 150 mL. Then 1.5 g (1% w/v) of each kiwifruit substrate was  
136 added to the vessels (excluding the negative control). Approximately 5.5 mL of sample was  
137 taken from each vessel immediately upon addition of substrate representing the 0 h time  
138 point. Samples were then taken at 5, 10, 24 and 48 h time points. Each sample was placed  
139 on ice, dispensed into aliquots and stored appropriately. The batch culture systems were  
140 monitored throughout the 48 h run, with any adjustments of stirrer speed, N<sub>2</sub> flow rates or  
141 temperature carried out as required. This initial batch culture experiment was repeated twice  
142 with different faecal donors giving a total of three biological replicates.

#### 143 *Nuclear Magnetic Resonance (NMR) Spectroscopy*

144 One millilitre of fermenta was taken and centrifuged at 16,200 g for 10 min; then the  
145 supernatant was decanted and frozen at -80 °C until analysis. Samples were then defrosted,  
146 vortexed and 400 µL transferred into a sterile eppendorf. Two hundred microlitres of  
147 phosphate buffer (containing 1 mM of the internal standard TSP (3-(trimethylsilyl)-[2,2,3,3-  
148 d<sub>4</sub>]-propionic acid sodium salt), the bacteriostatic sodium azide in 100% D<sub>2</sub>O) was added to  
149 the samples which were then vortexed and centrifuged at 10,000 g for 10 min. The  
150 supernatant (550 µL) was then transferred to a 5 mm glass NMR tube. All samples (and a  
151 batch culture medium only control) were run on a Bruker Avance III 700 MHz NMR  
152 spectrometer. Initial spectral processing was conducted using Bruker's Topspin software.  
153 Spectra were baseline corrected to remove systemic offsets, phased to yield accurate peak  
154 integration and peak shape and the TSP (internal chemical shift standard) adjusted to 0  
155 ppm. Further data processing was carried out using MATLAB 7.8.0 R2013b. The spectral  
156 regions containing the resonances from residual water and polyethylene glycol were  
157 removed to minimise the effects of baseline distortions. Principal Component Analysis (PCA)  
158 was performed on the metabolic profiles in Matlab using scripts provided by Korrigan  
159 Sciences Ltd., United Kingdom. This unsupervised approach was used to determine inherent  
160 changes between batch culture samples of different substrates or at different time-points.

#### 161 *Gas Chromatography*

162 Concentrations of acetate, butyrate, formate, heptanoate, hexanoate, isobutyrate,  
163 isovalerate, lactate, propionate, succinate and valerate were quantified by gas  
164 chromatography equipped with a flame ionization detector (GC-FID) following a modified  
165 method of Richardson *et al.* (Richardson *et al.*, 1989). In brief, 1.5 mL of sample was  
166 centrifuged at 16,200 g for 10 min. The supernatant was diluted in 0.01 M phosphate  
167 buffered saline with 2-ethylbutyric acid (5 mM) as an internal standard. The sample was then

168 centrifuged at 3000 *g* for 5 min (4 °C). The clarified supernatant was acidified with  
169 concentrated hydrochloric acid and diethyl ether added, and following vortexing, was  
170 centrifuged at 10,000 *g* for 5 min (4 °C). The upper diethyl ether phase was collected and  
171 derivatised with N-tert-butyltrimethylsilyl-N-methyltrifluoroacetamide with 1% tert-  
172 butyldimethylchlorosilane (MTBSTFA + TBDMSCI, 99:1; Sigma-Aldrich) by heating to 80 °C  
173 for 20 min. To allow complete derivatisation, the samples were left for 48 h at room  
174 temperature before analysis. Standards containing 2-ethylbutyric acid (5 mM) as an internal  
175 standard were prepared alongside the samples. Analysis was performed on a Shimadzu gas  
176 chromatograph system (GC-17A, Kyoto, Japan) equipped with a flame ionization detector  
177 and fitted with a HP-1 column (10 m × 0.53 mm ID × 2.65 μm) (Agilent Technologies, Santa  
178 Clara, CA, USA). The carrier gas was helium with a total flow rate of 37 mL/min and  
179 pressure of 7 kPa. The temperature profile began at 70 °C, increasing to 80 °C at 10 °C/min,  
180 with a final increase to 255 °C at 20 °C/min, holding for 5 min. The pressure program was  
181 set to 7 kPa, increasing to 15 kPa at 0.8 kPa /min, holding for 4 min. Injector and detector  
182 temperatures were set at 260 °C. Samples were injected (1 μL) with a splitless injection. The  
183 instrument was controlled and chromatograms acquired using GC Solution Chromatography  
184 Data System software, Version 2.3 (Shimadzu). The acquired GC data were used to plot  
185 standard curves and calculate compound response factors in relation to the internal  
186 standard, enabling a sample result of μmol SCFA/mL fermentation supernatant to be  
187 calculated.

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#### 189 *DNA Extraction, 16S rRNA gene sequencing and bioinformatics*

190 A 1.5mL sample aliquot was centrifuged at 16,200 *g* for 10 min. The pellet was resuspended  
191 in 500 μL sterile 50% glycerol in PBS (v/v) and frozen at -20 °C until analysis. This sample  
192 was centrifuged at 16,200 *g* for 5 min and the pellet washed in 1 mL of PBS. The sample  
193 was centrifuged again at 16,200 *g* for 5 min and the pellet resuspended in 500 μL of TES  
194 buffer pH 8.0 (Trizma HCl 0.254 g, Trizma base 0.048 g, NaCl 0.116 g, EDTA 0.068 g,  
195 sterile distilled water 400 mL). The samples were placed on ice and 8 μL lysozyme (10  
196 mg/mL) and 2 μL mutanolysin (1 mg/mL) were added. The cell suspension was vortexed  
197 and incubated at 37 °C for 30 min. The samples were removed and placed on ice where 10  
198 μL proteinase K (20 mg/mL) and 10 μL RNase (10 mg/mL) were added. The cell suspension  
199 was vortexed and incubated at 65 °C for 1 h. Then 100 μL of 10% SDS was added and the  
200 tubes were gently mixed by inversion. The samples were then incubated for a further 15 min  
201 at 65 °C. The samples were cooled on ice for 30 min and then 620 μL  
202 phenol/chloroform/water mix was added to the samples. The tubes were gently mixed by  
203 inversion for 2 min and then centrifuged at 4,100 *g* for 10 min. The upper (aqueous) layer



204 was transferred to a clean eppendorf tube and 1 mL of ice-cold ethanol was added. The  
205 samples were left on ice for 30 min or stored overnight at -20 °C. The samples were  
206 centrifuged for 5 min at 16,200 g, the supernatant was carefully removed and the pellet  
207 allowed to air dry for 2–3 h or overnight. The pellet was resuspended in 50 µL sterile H<sub>2</sub>O,  
208 mixed well and DNA purity and concentration was assessed by running 2 µL on the  
209 NanoDrop ND-100 spectrophotometer.

210 DNA was used as a template to amplify variable regions V2-V3 of the 16S rRNA gene  
211 (position 336-535 in the *Escherichia coli* rRNA gene) using primers HDA-1  
212 (cgatcgcctccctcgcgccatcagACTCCTACGGGAGGCAGCAGT) and HDA-2  
213 (ctatcgccttgccagcccgctcagNNNNNNNNNGTATTACCGCGGCTGCTGGCAC) (Rosendale  
214 *et al.*, 2012) where the sequences of the forward and reverse primers are shown in lower  
215 case, the four base library “key” sequence is underlined, the letter N denotes the 10 base  
216 barcode sequence and the remaining capital letters the template-specific HDA primers  
217 (Tannock *et al.*, 2000). Twenty-five microlitres of HotStarTaq master mix (Qiagen,  
218 Melbourne, Australia) was mixed with 1 µL template DNA and 100 nM of each primer (total  
219 reaction volume 50µL). PCR conditions were as follows: Initial denaturation 95 °C for 15 min  
220 then thirty cycles of 1 min 95 °C denaturation, 45 sec 65 °C annealing, 1 min 72 °C  
221 extension. The PCR products were gel purified using the QIAquick PCR purification kit,  
222 (QIAGEN, Melbourne, Australia), quantified using the Qubit 2.0 fluorometer (Life  
223 Technologies), pooled in equimolar quantities, and submitted for sequencing on the Roche  
224 454 GS FLX Titanium platform (Macrogen Inc., Korea).

225 QIIME software V1.8.0 was used to analyse the 454 sequencing data (Caporaso *et al.*,  
226 2010). Reads were clustered into operational taxonomic units (OTUs) based on a 97%  
227 identity threshold value. Alignment of the sequences was carried out using PyNAST  
228 (Caporaso *et al.*, 2010) with reference to the Greengenes core reference database (version  
229 13\_8) (DeSantis *et al.*, 2006). Taxonomic assignment was made using the RDP Naive  
230 Bayesian classifier (Wang *et al.*, 2007). Chimeric sequences were removed from the reads  
231 using the ChimeraSlayer algorithm (Haas *et al.*, 2011, Claesson *et al.*, 2012). Alpha  
232 rarefaction was calculated using the Phylogenetic Diversity (PD) whole tree, Chao1,  
233 Observed Species and Shannon diversity metrics. Beta diversity was determined using  
234 UniFrac distances as input and EMPeror to visualise relationships in three dimensions  
235 (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.

## 248 Results

### 249 16S rRNA gene sequencing

250 The V2-V3 hypervariable region of the 16S rRNA gene sequencing results yielded 253,852  
251 reads that passed the quality filters at an average of  $5903 \pm 1703$  reads per sample. Reads  
252 were clustered into non-chimeric OTUs based on a 97% identity threshold value. Inspection  
253 of the relative abundance data at phylum and genus levels (Figure 1) indicated that the  
254 substrates had distinct effects on the microbial ecology within the *in vitro* fermentations.  
255 Bacterial profiles at time 0 for both substrates and control were very similar (Figure S1) and  
256 form a baseline from which changes over time caused by fermentable substrate can be  
257 measured. Several bacterial groups increased in abundance in all three vessels over the  
258 course of the experiment including *Enterobacteriaceae*, *Sutterella* spp., *Veillonella* spp.,  
259 *Collinsella* spp., and *Citrobacter* spp.. Genera which decreased in abundance, irrespective of  
260 vessel, over time were *Faecalibacterium* spp., *Blautia* spp., *Prevotella* spp. and *Lachnospira*  
261 spp.. *Bifidobacterium* spp. increased when the vessel was supplemented with green kiwifruit  
262 digesta (Figure 1B). This enrichment was observable despite the *P*-value falling short of  
263 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  
264 most likely due to the variation in response to the treatment between donors, which is quite  
265 common and is also demonstrated in the beta diversity biplots (Figure 2). For example,  
266 donor 1 had a significantly higher occurrence of bifidobacterial OTU *denovo1575* than  
267 donors 2 and 3 ( $P < 0.001$ ). *Bacteroides* spp. multiplied in the green and gold kiwifruit  
268 vessels after 5 to 10 h and then dropped back by 48 h, whereas *Bacteroides* spp. in the  
269 negative vessel remained relatively constant (Figure 1C). Although barely detectable in the  
270 kiwifruit vessels, *Oscillospira* spp. became prominent in the negative vessel, reaching almost  
271 5% of total abundance. At species-level, it was found that *Faecalibacterium prausnitzii*  
272 accounted for the majority of the *Faecalibacterium* species which dropped from about 13%  
273 of total abundance at time 0 to less than 2% by 24 h in all 3 vessels. Conversely, *Collinsella*  
274 *aerofaciens* increased in all three vessels and was the predominant *Collinsella* species.

275 Alpha diversity analysis was calculated by time, fermentation and substrate using four  
276 rarefaction metrics (PD whole tree, Chao1, Observed species and Shannon diversity) at  
277 2800 reads rarefaction depth. No significant associations were evident when plotted by time  
278 or fermentation, but a clear trend towards higher diversity in the negative control and lower  
279 diversity in the gold kiwifruit vessel was observed (see Figure S2). For the PD whole tree  
280 metric, gold kiwifruit had significantly lower species richness than the negative control ( $P =$   
281  $0.003$ ) at an average of  $15.84 \pm 1.59$  (gold kiwifruit) vs  $17.56 \pm 0.93$  (negative) as assessed  
282 by the nonparametric two-sample t-test (Monte Carlo permutation).

283 When the diversity of the bacterial populations between vessels was examined (beta  
284 diversity), it was evident that separation was greatest when plotted by fermentations on a  
285 principal coordinates analysis (PCoA) plot (Figure 2); this demonstrated how samples were  
286 grouped by donor. In the PCoA plots an ordination method was used to plot variance against  
287 orthogonal axes. When the PCoA was overlaid with the ten family level bacterial groups  
288 most responsible for causing variance using the biplot function, it showed that  
289 *Coriobacteriaceae*, *Clostridiales*, *Ruminococcaceae* and *Lachnospiraceae* were the core  
290 taxa changing in the vessels. When plotted by time (Figure 2), it was clear that as time  
291 progressed the points moved from the upper right space of each of the three fermentations  
292 towards the bottom left area of the plot. This direction of migration indicates an increase in  
293 the *Bacteroidaceae*, *Alcaligenaceae*, *Veillonellaceae* and *Enterobacteriaceae*. When  
294 comparing samples as a function of substrate, there was a tendency for the negative control  
295 to cluster close to the time 0 point (data not shown).

#### 296 *NMR Spectroscopy*

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

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316 *Gas Chromatography*

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

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## 342 Discussion

343 This study demonstrated that non-digestible components from green and gold kiwifruit can  
344 be utilised as fermentable substrates, effecting change to both bacterial composition and  
345 metabolism. The microbial profiles at time 0 for all three vessels were relatively similar but  
346 after 5–10 h *Enterobacteriaceae* and *Collinsella* spp. begin to markedly increase while  
347 *Faecalibacterium* spp. and *Blautia* spp. decreased, irrespective of the presence or absence  
348 of substrate. As these changes were also observed in the negative control, it is more likely a  
349 function of the gut model system, rather than a substrate response. There were un-  
350 fermented substrates present within the faecal inoculum and the medium to a small extent,  
351 which may have contributed to the initial changes in fermentation seen in the model. We  
352 note that early proliferation of *Enterobacteriaceae* was also observed by a group running the  
353 simulator of the intestinal microbial ecosystem (SHIME) models to measure the impact of  
354 polyphenols on the human gut microbiota (Kemperman *et al.*, 2013). Despite these  
355 background changes, the green and gold kiwifruit substrates exerted an additional powerful  
356 influence. *Bifidobacterium* spp. increased in abundance after 24 h of fermentation following  
357 exposure to green kiwifruit polysaccharides. *Bacteroides* spp. increased in relative  
358 abundance in response to the kiwifruit substrates prior to reverting to control levels. The  
359 genus *Bacteroides* include species that have a diverse array of substrate utilisation  
360 machinery encoded on polysaccharide utilisation loci (PUL) (Martens *et al.*, 2008).  
361 Potentially, the easily fermentable substrate was metabolised in the first few hours, after  
362 which the *Bacteroides* were able to engage PULs to utilise the more recalcitrant substrates,  
363 thereby increasing their relative abundance. These increases in *Bifidobacterium* spp. and  
364 *Bacteroides* spp. are consistent with previously published green and gold kiwifruit  
365 fermentation results (Parkar *et al.*, 2012).

366

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

378 However, despite the variability between donors, there were still consistent trends in the  
379 modulation of the microbiota.

380 Acetate was the most prevalent SCFA produced in all three fermentations. Acetate is  
381 absorbed into the bloodstream and used by peripheral tissue and muscle (Wong *et al.*, 2006)  
382 and is a major metabolite of bifidobacteria (Wolfe, 2005). Propionate, butyrate and ethanol  
383 were also seen to increase over time. Organic acids are quantitatively the most abundant  
384 end-products of microbial fermentation in the human colon and their production lowers pH  
385 and directly inhibits the growth of pathogens (Cummings & Englyst, 1987, Blaut, 2002). The  
386 accumulation of propionate and butyrate over time was expected as these cannot be used  
387 by cross-feeding bacteria (Louis *et al.*, 2007). Although acetate can be metabolised, it tends  
388 to accrue over time, the generation of this oxidised molecule being an energy favourable  
389 reaction (Macfarlane & Macfarlane, 2003). Given the comparable rates of butyrate  
390 production in the kiwifruit vessels compared with the negative control, it can be concluded  
391 that kiwifruit polysaccharides are not particularly butyrogenic. Green and gold kiwifruit  
392 substrates stimulated an increase in succinate and lactate production in the first several  
393 hours post inoculation before dropping back by the 24 h and 48 h time points. These  
394 intermediates can serve as substrate for other bacteria and may have been further  
395 converted to acetate or propionate (Louis *et al.*, 2007). Like succinate and lactate, formate  
396 also behaved in this intermediate manner. This could be explained by the onward  
397 conversion of formate to methane which can be performed by methanogens such as  
398 *Methanobrevibacter smithii* or to acetate by acetogens such as *Blautia hydrogenotrophica*  
399 (Flint *et al.*, 2012).

400 Protein only accounts for about 1% of the edible portion of green kiwifruit and a considerable  
401 proportion of this would be unable to make it to the colon without being digested or absorbed  
402 in the small intestine (Ferguson & Ferguson, 2003). However, small amounts of protein are  
403 included in the medium, plus low levels of protein may have been introduced along with the  
404 faecal inoculum and turnover of microbes could also add to amino acids available as  
405 substrates. The BCFAs, isovalerate and isobutyrate are products of the fermentation of  
406 branched chain amino acids such as leucine, isoleucine and valine which are potentially  
407 available in the fermentation medium (Macfarlane & Macfarlane, 2003). The negative control  
408 produced more BCFA than the kiwifruit vessels: this could indicate that microbiota were  
409 scavenging any available substrate as they would in the carbohydrate-deficient distal colon.  
410 Given that protein fermentation is an undesirable phenomenon which can lead to detrimental  
411 health effects, the lower level of BCFA production associated with kiwifruit fermentations is a  
412 positive outcome (Mortensen *et al.*, 1992, Nyangale *et al.*, 2012). It is not clear exactly which  
413 components of kiwifruit NSP were responsible for the changes observed in this study.

414 Certain differences exist between green and gold kiwifruit constituents; for example, gold  
415 kiwifruit is higher in hemicellulosic polysaccharides and lower in pectic polysachharides than  
416 green kiwifruit (Sauvageau *et al.*, 2010). The greater amount of hemicellulose in gold kiwifruit  
417 may be responsible for stimulating the *Enterobacteriaceae*, resulting in a lower abundance of  
418 bifidobacteria. More complex investigations are warranted that take into account the different  
419 microbial niches encountered in the large bowel and the fermentation of other sources of  
420 carbohydrates such as host mucin (Macfarlane *et al.*, 2005)..

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

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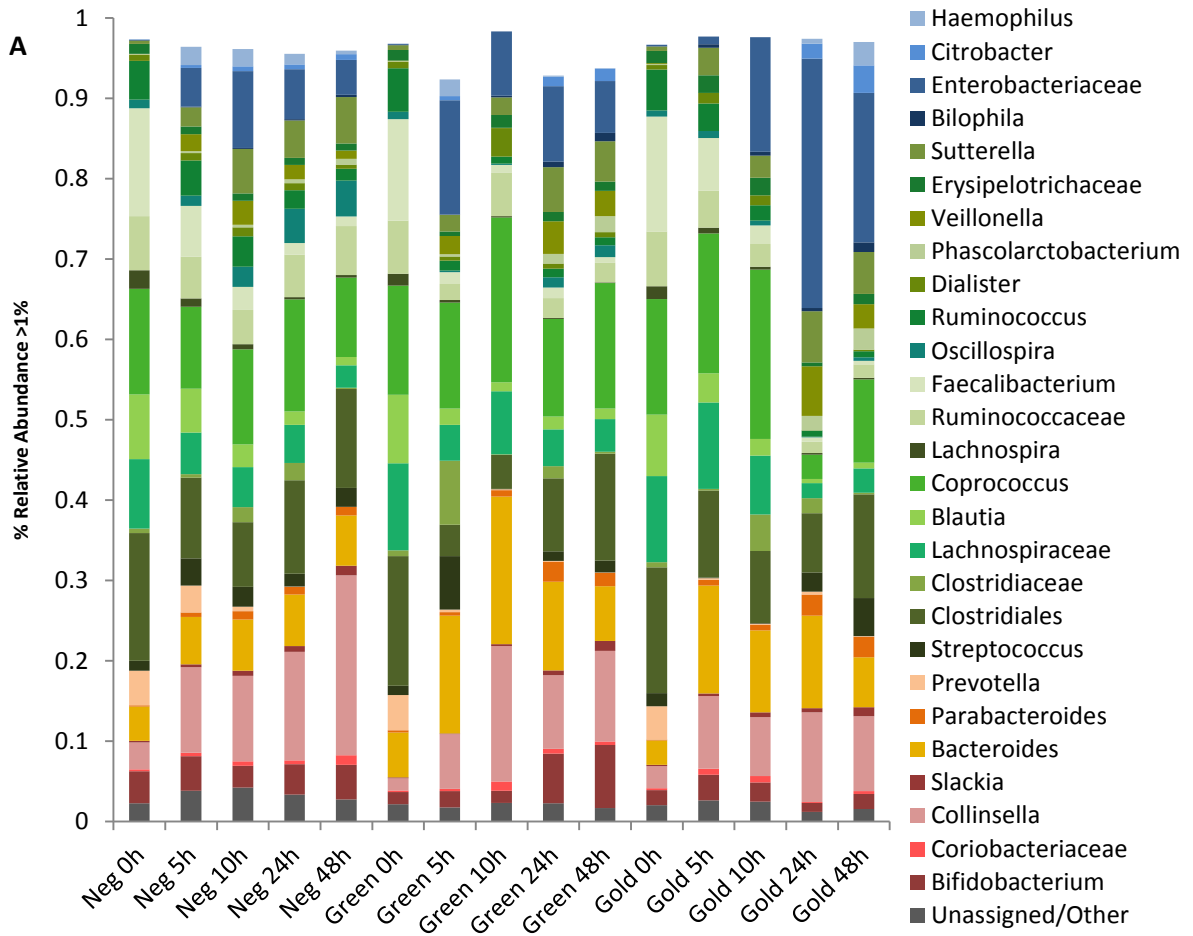
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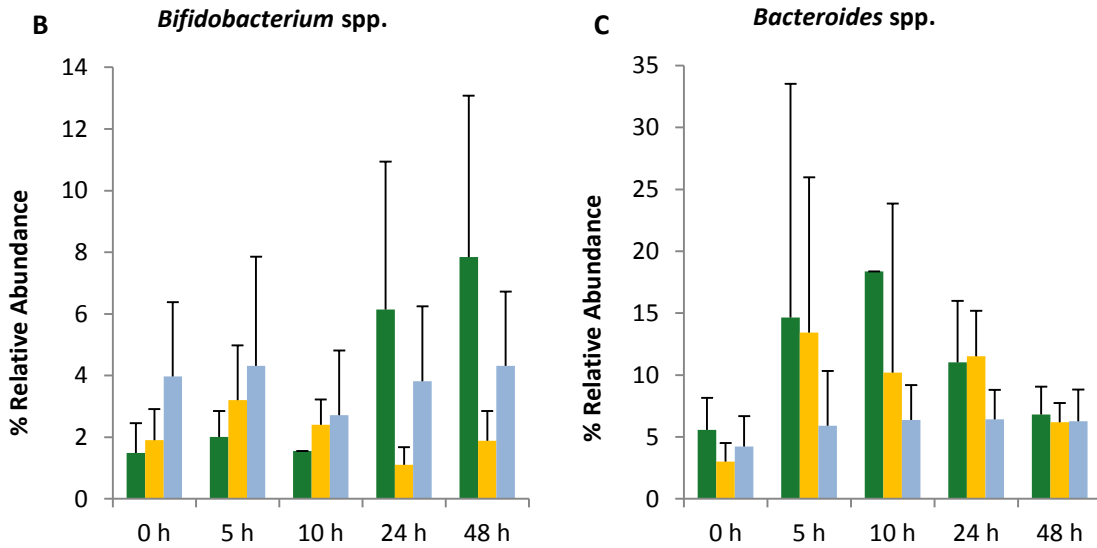
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575 **Figures:**



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581 **Figure 1 – Microbial composition of samples at all time points as determined by 16S rRNA**  
582 **gene sequencing.** (A) Mean (n=3). The four most abundant phyla are depicted as four colours –  
583 *Actinobacteria* (red), *Bacteroidetes* (yellow), *Firmicutes* (green) and *Proteobacteria* (blue) with family  
584 and genus level taxonomy portrayed as differing shades of those colours within each phylum. Only  
585 genera of greater than 1% total read composition were included in this graph. (B) Relative abundance  
586 of *Bifidobacterium* spp. and (C) *Bacteroides* spp.. Green, gold and blue bars represent green kiwifruit,  
587 gold kiwifruit and the negative control respectively. Error bars are the SEM of the three fermentations.  
588 SEM are displayed as error bars ( $n = 3$ ).

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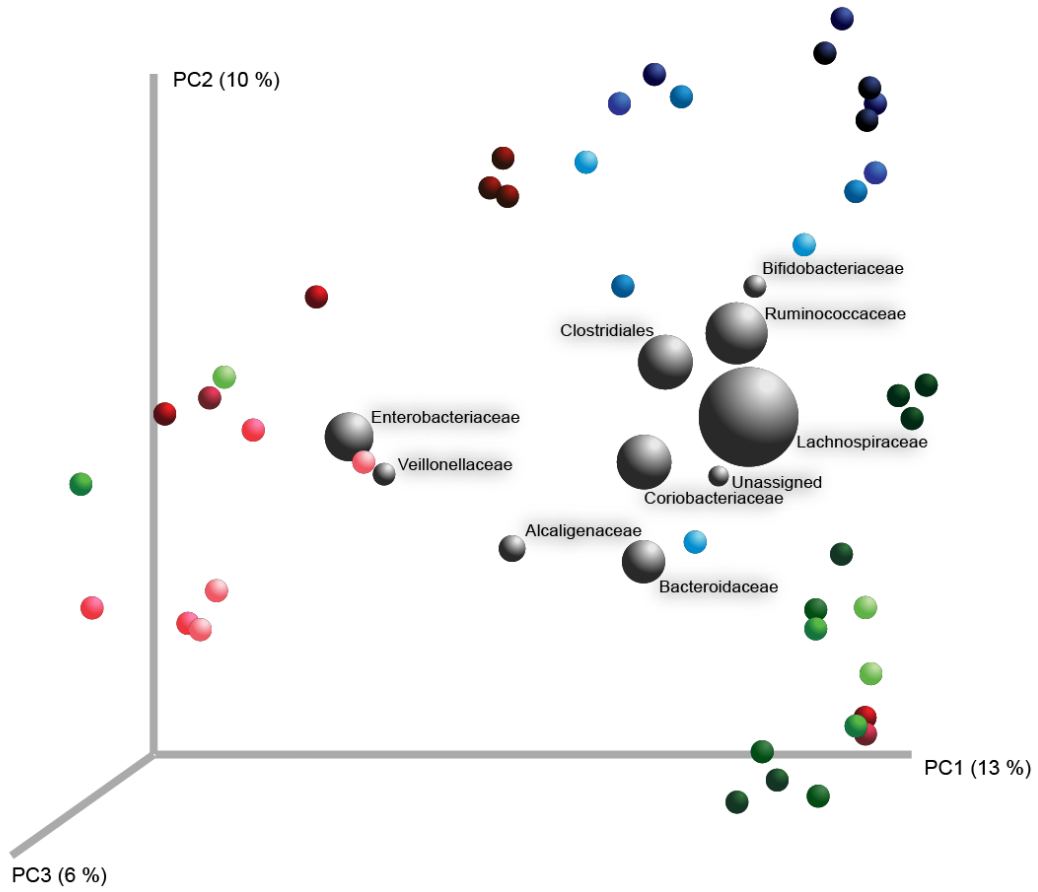
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620 **Figure 2 – Jackknifed beta diversity PCoA biplots showing unweighted UniFrac distances.**  
621 Plotted by faecal donor (fermentation), where donor 1, 2 and 3 are shown with blue, green and red  
622 spheres, respectively. Within the three colours, 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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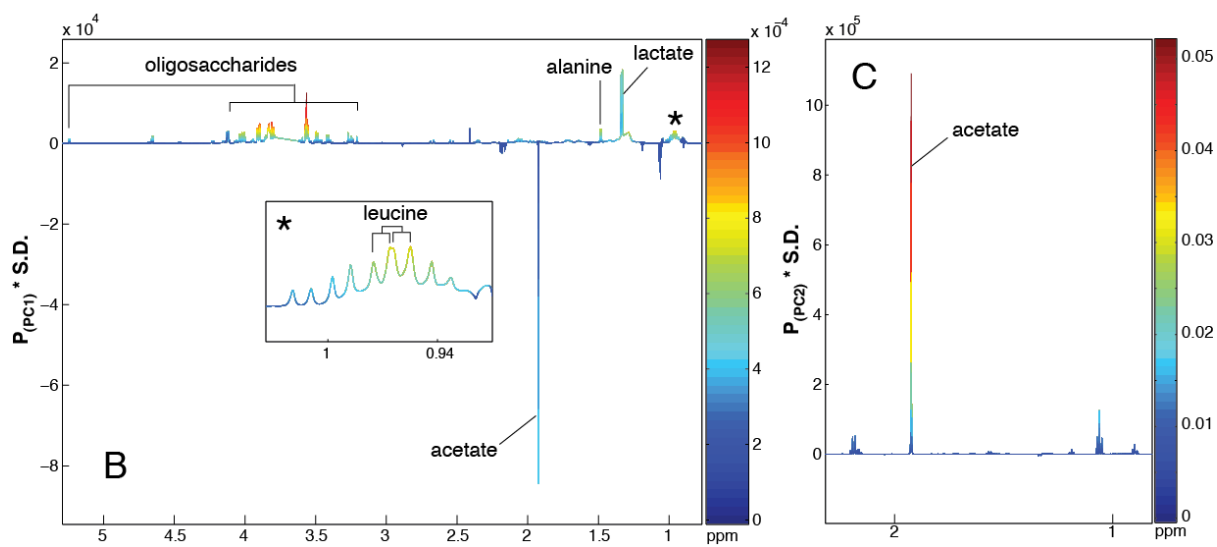
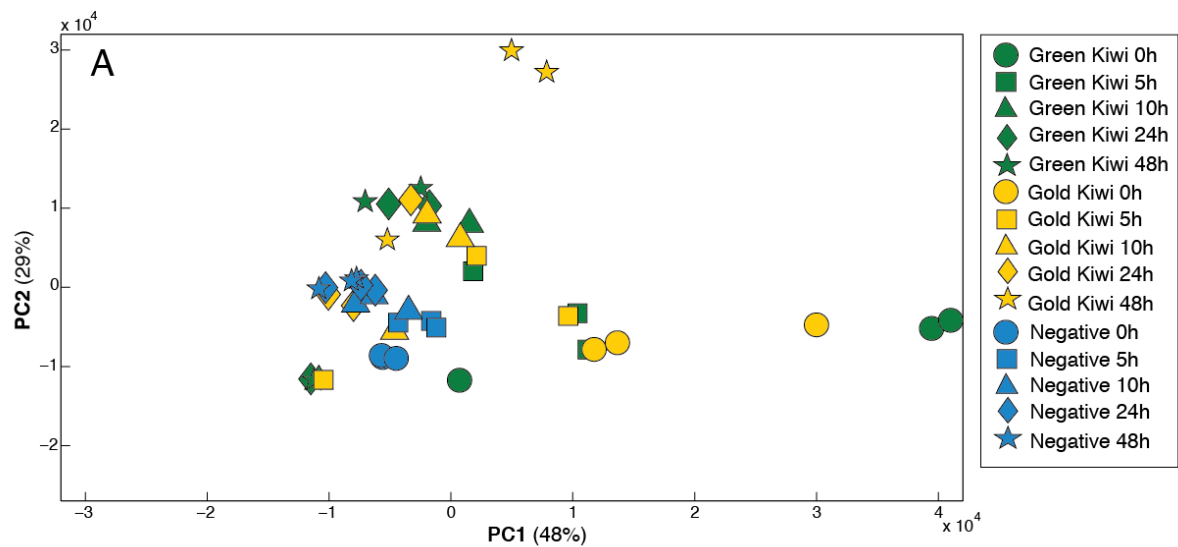
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646 **Figure 3:** Principal Component Analysis (PCA) model of the metabolic profiles of all batch culture  
647 supernatants. (A) Scores plot for PC1 vs PC2 (% variance explained in parenthesis). Product of PC  
648 loadings with standard deviation of the entire data set is plotted and coloured by the square of the PC  
649 for (B) PC1 and (C) PC2.

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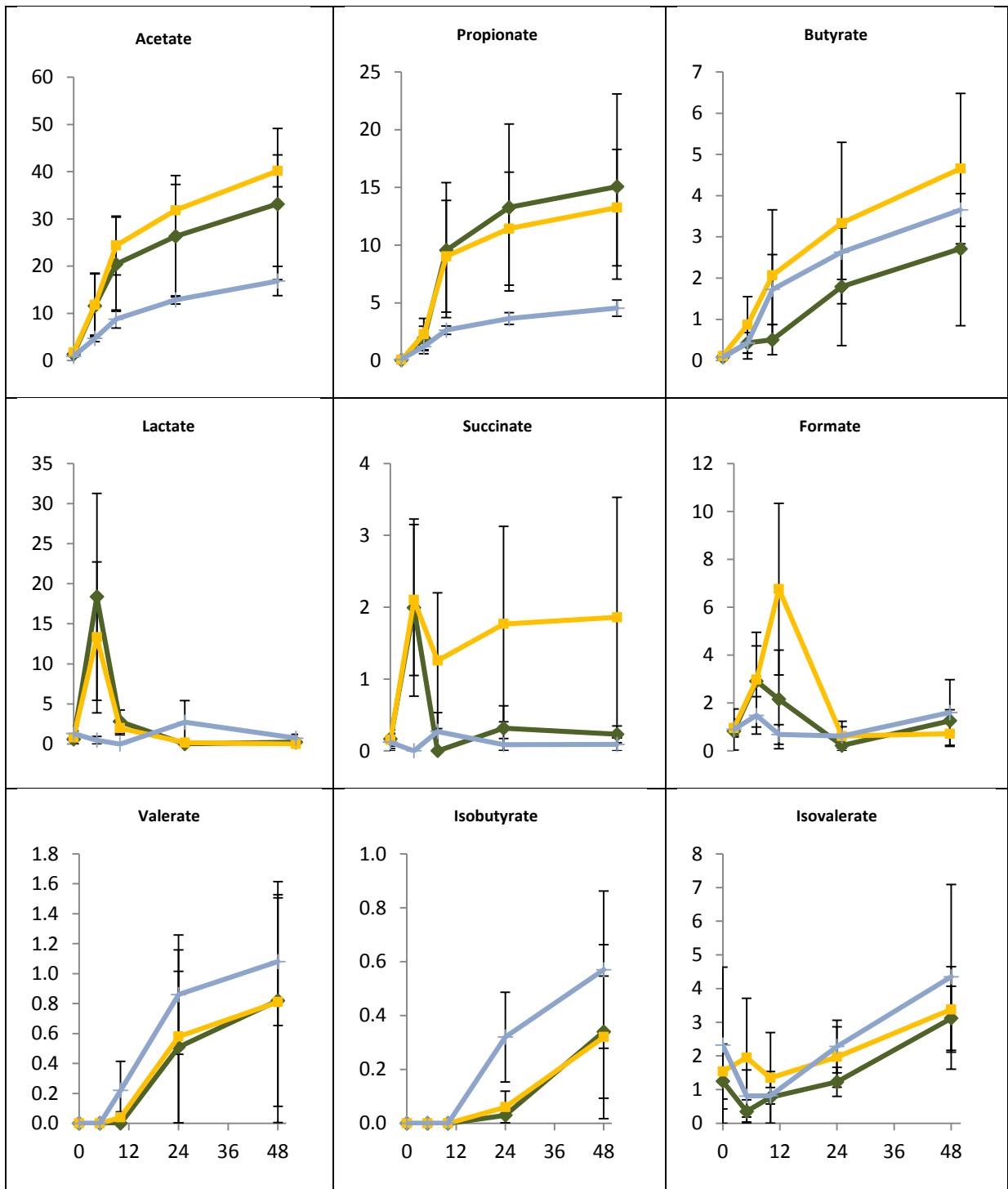
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677 **Figure 4 – Short-chain fatty acid and organic acid production as determined by GC-FID**  
678 **analysis.** The units of the vertical axes are concentration ( $\mu\text{mol/mL}$  fermenta) and the horizontal axes  
679 are time (hours). Green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

681