Diets supplemented with chickpea or its main oligosaccharide component raffinose modify faecal microbial composition in healthy adults

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Abstract

The effects of diets supplemented with either chickpea or its main oligosaccharide raffinose on the composition of the faecal microbial community were examined in 12 healthy adults (18-65 years) in a randomised crossover intervention study. Subjects consumed their usual diet supplemented with soups and desserts that were unfortified, or fortified with either 200 g/d of canned chickpeas or 5 g/d of raffinose for 3 week periods. Changes in faecal bacterial populations of subjects were examined using 16S rRNA-based terminal restriction fragment length polymorphisms (T-RFLP) and clone libraries generated from the diet pools. Classification of the clone libraries and T-RFLP analysis revealed that Faecalibacterium prausnitzii, reported to be an efficient butyrate producer and a highly metabolically active bacterium in the human intestinal microbiota, was more abundant in the raffinose diet and the chickpea diet compared to the control diet. However, no significant difference was observed in the faecal total short chain fatty acid concentration or in the levels of the components (butyrate, acetate and propionate) with the chickpea diet or the raffinose diet compared to the control diet. Bifidobacterium species were detected by T-RFLP in all three diet groups and quantitative real-time PCR (qPCR) analysis showed a marginal increase in 16S rRNA gene copies of Bifidobacterium with the raffinose diet compared to control (P>0.05). The number of individuals showing TRFs for the Clostridium histolyticum - Clostridium lituseburense groups, which include pathogenic bacteria species and putrefactive bacteria, were lower in the chickpea diet compared to the other two treatments. Diet appeared to affect colonisation by a high ammonia-producing bacterial isolate which was detected in 83%, 92% and 42% of individuals in the control, raffinose and chickpea groups, respectively. Our results indicate that chickpea and raffinose have the potential to modulate the intestinal microbial composition to promote intestinal health in humans.

Keywords: prebiotic, chickpea, microbiota, 16S rRNA, phylogenetic analysis, clone library, T-RFLP

1. Introduction

The composition of the human intestinal microbiota has an important role in health and disease (Hart et al., 2002; Hooper and Gordon, 2001). Dietary fibre consumption is considered to have a major impact on compositional diversity and metabolic activity of the microbiota in the human gastrointestinal tract. Changes in the type and quantity of non-digestible carbohydrates in the human diet influence both the metabolic products formed in the lower regions of the gastrointestinal tract and bacterial populations detected in faeces. Of particular interest to gut health and well-being are dietary oligosaccharides claimed to be ‘prebiotics’ and shown to have the ability to be...
fermented selectively by *Bifidobacterium* and *Lactobacillus* species. Human colonic bacteria ferment resistant starch and non-starch polysaccharides to produce short chain fatty acids, mainly acetate, propionate and butyrate, as well as gases. Butyrate is the primary energy source for colonocytes and appears to lower the risk of malignant transformation in the colon (McGarr *et al*., 2005). Protein fermentation results in the formation of ammonia, phenols, indols and secondary amines, some of which at higher concentrations have been proposed to act as co-carcinogens (McGarr *et al*., 2005).

Chickpea, contains significant levels of oligosaccharides, specifically ciceritol and the galactosides, raffinose and stachyose (Sanchez-Mata, 1998). Levels of oligosaccharides vary with species, cultivar and growing conditions. *Bifidobacterium* strains utilise raffinose as a fermentable carbon source *in vitro* (Pompei *et al*., 2006). It is not known whether raffinose and other oligosaccharides contained in pulses may preferentially promote the growth of *Bifidobacteria* and other health-promoting bacterial species *in vivo* and thereby function as prebiotics. Furthermore, the impact of other components of chickpea on microbiota, such as the non-starch polysaccharides, resistant starch and resistant protein in chickpea has not been investigated. The prebiotic potential of chickpea oligosaccharides alone or as components of chickpea has yet to be explored. The objective of this research was to determine the effects of chickpea or raffinose consumption on faecal microbiota.

Our understanding of the complex microbial communities found in the intestine has been greatly enhanced by the development of molecular ecological techniques based on the 16S rRNA gene (Suu et al., 1999). The most established 16S rRNA-based technique relies on amplifying, cloning and sequencing 16S rRNA genes from bacterial DNA. Phylogenetic analysis of libraries of cloned 16S rRNA genes has been applied to investigate the diversity of human intestinal microbiota and has revealed sequence information for both known and novel bacterial species (Hold *et al*., 2002; Suau *et al*., 1999). We used a combination of 16S rRNA-based molecular techniques to follow changes in faecal microbiota of subjects fed either a control diet, or the control diet supplemented with raffinose or chickpea. Terminal restriction fragment length polymorphism (T-RFLP) analysis and qPCR were used to track changes in the microbiota in individual subjects, while 16S rRNA clone libraries were generated for pooled samples of each of the three diets. Predominant terminal restriction fragments (TRFs) in the faecal communities were further identified by comparison with *in silico* digested TRFs using the clone sequences.

2. Subjects and methods

Subjects

Twelve healthy adults (18-65 years) consisting of employees and students were recruited through poster advertisements placed in various locations at the University of Saskatchewan (Saskatoon, Canada). Exclusion criteria included antibiotic use in the month prior to the study and current intake of probiotics.

Study design and experimental diets

Study participants were randomly assigned to three groups to receive over a 9-week period three weeks of a control diet, three weeks of the control diet plus 5 g/d raffinose, a dose expected to be bifidogenic (Bouhnik *et al*., 2006), and three weeks of the control diet plus 200 g/d canned chickpea, and intake expected to contain 3-5 g of oligosaccharides, in random order. Study participants consumed their usual diet with the additional supplements added to soups and desserts. All diets had similar macronutrient content. Control and raffinose interventions were double-blinded. However, blinding chickpea treatment was difficult and subjects may have been able to determine when they were assigned to this intervention. Ethics approval was obtained through the Biomedical Ethics Research Board at the University of Saskatchewan.

Faecal samples and DNA extractions

Participants collected faecal samples on three days in the third week of each three-week study period, and the samples were kept frozen until they were transferred to the -20 °C freezer in the research laboratory. Frozen faecal samples were thawed in a 50 °C water bath and homogenised before a sub-sample for DNA extraction was taken. Genomic DNA was extracted from 0.35 g of faeces using the protocol described by Dumonceaux et al. (2006). An equal volume of extracted genomic DNA was pooled for each of the three collection days within subjects, giving one sample per subject per period (n=36). The DNA concentration of each sample was determined using PicoGreen (molecular probes) and the DNA concentrations were adjusted to 2-5 ng/μl with double-distilled water.

PCR amplification, cloning and sequencing

Genomic DNA extracts representing each diet group were pooled on a volume basis and used as template in PCR reactions at a 1:10 dilution. The universal primers F1 (5’-GAG TTT GAT CCT GGC TCA G-3’) and R2 (5’-GWA TTA CCG CGG CKG CTG-3’) (Dorsch and Stackebrandt, 2006) were used to amplify the region corresponding to nucleotides 11-536 of the *Escherichia coli* 16S rRNA gene. PCR amplifications were performed in 50 μl consisting of...
5 µl of a 10× PCR reaction buffer, 2 mM of MgCl₂, 0.2 mM each of a dNTP mixture, 0.4 µM of each primer and 1 U of Taq DNA polymerase (UBI, Calgary, Canada) using an Eppendorf Mastercycler EP (Hamburg, Germany). The amplification program was 3 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension of 10 min at 72 °C. The resulting PCR products from each template pool were purified using a Qiagen gel extraction kit (Alameda, CA, USA) and ligated into cloning vector pGEM T Easy (Promega, Madison, WI, USA). Ligation reactions were used to transform E. coli JM109 competent cells and recombinants were identified by plating on Luria broth agar containing ampicillin, X-gal and IPTG. White colonies containing plasmids with inserts (576 per library) were picked into 96-well plates and sequenced with the T7 sequencing primer (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Canada). For processing of clone sequences, the vector sequence was initially trimmed from each read and sequences were clustered by similarity to identify multiple occurrences of the same sequence using the Staden Package (Staden et al., 2000) and Blastclust (Altschul et al., 1990).

Clone library analysis

Unique sequences and their frequency of occurrence in each library were recorded and consensus sequences were classified using the sequence analysis tool provided by the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu). The tool uses the RDP naïve Bayesian classifier, version 2.0 (Wang et al., 2007) to provide rapid classification of library sequences into the bacterial taxonomy. For phylogenetic analysis, the sequences of all three libraries were subjected to NAST multiple alignment tool (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi; De Santis et al., 2006) and the aligned sequences and closest cultured relatives were imported into ARB (Ludwig et al., 2004). A phylogenetic tree was constructed using the maximum-parsimony algorithm and the neighbour-joining method. The branches with species belonging to similar groups (genus level) were subsequently collapsed.

T-RFLP analysis

T-RFLP analysis of DNA extracted from each subject during each treatment period (n=36) was performed using 8F (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 926R (5’-CCG TCA ATT CCT TTR AGT TT-3’). The 8F primer was labelled at the 5' end with FAM (carboxyfluorescein-N-hydroxysuccinimide ester-dimethyl sulfoxide). The PCR conditions were the same as those used for amplification of 16S rRNA gene sequences. The fluorescently-labelled PCR products from triplicate PCR reactions were pooled and purified on QIAquick PCR purification kit columns (Qiagen, USA) and eluted in a final volume of 30 µl of double-distilled water. The DNA concentrations of the purified PCR products were determined using the PicoGreen quantification (molecular probes). Restriction enzyme digestions of the purified DNA (100 ng) were carried out in 20 µl reactions with Msp1, HaeIII or HhaI and incubated for 5 h at 37 °C.

Aliquots (2 µl) of restricted DNA were mixed with 9 µl of deionised formamide and 0.5 µl of 600 LIZ internal size standard (Applied Biosystems, Foster City, CA, USA). Each sample was then denatured at 95 °C for 2 min and chilled on ice prior to analysis. Lengths of the fluorescently-labelled terminal restriction fragments (TRFs) were analysed by the ABI PRISM 310 genetic analyser (Applied Biosystems, USA). The T-RFLP profiles were analysed using GeneMapper software (version 3.7) (Applied Biosystems, USA) and the size, in base pairs, of TRFs was estimated by reference to the internal standard using the Local Southern method. Sample data consisted of size (base pairs) and peak area for each terminal restriction fragment. To standardise the DNA loaded on the capillary, the sum of all TRF peak areas in the pattern was used to normalise the peak detection threshold in each sample. Therefore, the relative abundance of each TRF was calculated as the peak area of the respective TRF divided by the total peak area of all TRFs detected within a fragment length range between 50 and 600 bp. To remove background, TRFs whose relative areas were less than 1.0% of the total area were excluded from the analysis. TRF relative peak area was transformed by arc(sin)√ x, and profiles for each sample imported into Bionumerics software (version 5.1, Applied Maths, Austin, TX, USA) and subject to UPGMA cluster analysis using the dice similarity coefficient with area sensitivity. In silico digests of cloned sequences in each library were then performed for the three enzymes, Msp1, HaeIII and HhaI to generate predicted TRFs and their putative bacterial representation. Predicted TRFs were compared with the TRFs observed by individual profiles. Analysis of bacterial diversity was performed using the Shannon-Weiner index (Shannon and Weaver, 1949) based on relative peak areas of Msp1 TRFLP profiles of individual subjects.

Real time qPCR with group specific primers

The abundance of selected taxonomic groups was determined using real-time quantitative PCR and primer sets identified in Table 1. Quantitative PCR was performed with the iCycler real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) in a 25 µl final reaction mix containing 12.5 µl Platinum SYBRGreen qPCR Supermix UDG (Invitrogen, Carlsbad, CA, USA), 0.8 µM of each primer, calibration standards or 2 µl of template DNA. The amplification conditions were 95 °C for 2 min followed by 40 cycles of 30 s at 95 °C, 30 s at the appropriate primer annealing temperature (Table 1) and 1 min at 72 °C. Following amplification, a dissociation step was included to analyse the melting profile of the amplified
product to test for product specificity. The standards for the qPCR assay were made using a gel-purified PCR product obtained by PCR amplification of genomic DNA from the test samples using the relevant qPCR primers. Purified PCR product was quantified using Picogreen (molecular probes) and converted to copy number based on the calculated molecular weight of the PCR amplicon. A 10-fold dilution series of standards, ranging from 5 pg/µl to 0.0005 pg/µl, was used to construct the standard curve.

### Volatile fatty acid and lactic acid analyses

Faeces from each subject, period and diet were analysed for volatile fatty acid (VFA) content. Previously frozen faecal samples were thawed and 2 g samples were taken. Each faecal sample was diluted in 8 ml of distilled water, acidified with 8 M HCl (100 μl), mixed and centrifuged at 4,200 rpm for 15 min at 4 °C. One ml of the clear supernatant was then separated into a fresh tube and 50 μl of the internal standard (trimethyl acetic acid) and 450 μl of acetonitrile were added. The solutions were mixed well and centrifuged at 14,000 rpm for 10 min in a Beckman Coulter Microfuge (Brea, CA, USA). Approximately 0.5 ml supernatant was filtered using a 0.45 μm filter with PVDF membrane (Whatman Puradisc, Whatman, Clifton, NJ, USA) into an Agilent GC vial with a glass insert. The VFA concentrations were determined using an Agilent 6890 gas chromatograph with FID detector (Agilent, Santa Clara, CA, USA). A standard solution containing 10 mM each of acetate, propionate, butyrate, valerate, caproic, heptanoic, iso-butyrate, iso-valerate and iso-caproic acid was injected and a standard curve was determined.

Lactic acid enantiomers (D and L) in the faeces were separated and quantified by high performance liquid chromatography using a Waters 715 Ultra WISP autosampler, a Waters 600 controller and a Waters 486 Tunable Absorbance Ultraviolet detector (Waters, Milford, MA, USA) as previously described (Omole et al., 1999; Ewaschuk et al., 2004) and modified for human samples. Data collection and integration was performed using the Waters Millenium Chromatography Manager version 4 (Waters, USA).

### Statistical analysis

Statistical analysis of dietary effects on bacterial count, volatile fatty acid and lactic acid concentrations and Shannon diversity indices was conducted by analysis of variance using the general linear models procedure where diet (control, raffinose, chickpea), experimental period and the interaction were considered as sources of variation (SPSS for Windows, Chicago, IL, USA). No significant experimental period or interactions were observed for any parameter, hence these sources of variation were subsequently removed from the model. Diet effects were considered significant at $P<0.05$.

### 3. Results

Twelve participants (7 male; 5 female) with a mean age of 25.6 ± 8.7 completed the study. The participants completed, on a daily basis, a subject diary to indicate if they consumed the soup and dessert which contained the diet supplements, whether they experienced any gastrointestinal symptoms, and to record stool frequency. These data will be comprehensively described in a separate publication.

### DNA sequencing and phylogenetic analysis

In order to provide an initial assessment of the composition of the microbiota of the subjects in the study and to create a database of 16S rRNA sequences for generating *in silico* TRFs for assisting in identification of experimental TRFs, libraries of PCR amplified partial 16S rRNA sequences were generated from pooled DNA templates from each of the three diet groups. A total of 459, 415 and 440 cloned sequences were examined for the control, raffinose

<table>
<thead>
<tr>
<th>Target bacterial group</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
<th>Annealing temp (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium cocoides - Eubacterium rectale group</em></td>
<td>Cocc-F AAATGACGGTACCTGACTAA&lt;br&gt;Cocc-R CTTTGAGTTTCATTCTTGCAA</td>
<td>440</td>
<td>50</td>
<td>Matsuki et al., 2002</td>
</tr>
<tr>
<td><em>Clostridium leptum subgroup</em></td>
<td>Clept-F GCACAAGCAGTGGAGT&lt;br&gt;Clept-R CTTCCCTCGGTTTTGCTAA</td>
<td>239</td>
<td>50</td>
<td>Matsuki et al., 2004</td>
</tr>
<tr>
<td><em>Bifidobacterium</em></td>
<td>Bifid-F CTCTGGAAACGGGTGGG&lt;br&gt;Bifid-R GGTGTCTCTCCGATATCTACA</td>
<td>550</td>
<td>55</td>
<td>Matsuki et al., 2002</td>
</tr>
<tr>
<td><strong>Bacteroidetes</strong></td>
<td>Bact-F GGCAGCAGCAGGCGACGGG&lt;br&gt;Bact-R GRTCCTCTCTCAGAACC</td>
<td>212</td>
<td>60</td>
<td>Nakanishi et al., 2006</td>
</tr>
<tr>
<td><strong>Lactobacillii</strong></td>
<td>Lacto-F GCACGAGTGGAAATCCTCCA&lt;br&gt;Lacto-R GCATTYCACCGCTACACAG</td>
<td>345</td>
<td>55</td>
<td>Walter et al., 2001</td>
</tr>
</tbody>
</table>
and chickpea libraries, respectively. Based on sequence assignments using the RDP Classifier tool and phylogenetic clustering based on the Greengenes database (Figure 1) the largest proportion of clones in each of the three libraries corresponded to the Clostridiales clade. Within this broad taxon, sequences corresponding to *Clostridium* cluster IV were most abundant, accounting for 47%, 51% and 40% of the sequences in the control, raffinose and chickpea libraries. Within cluster IV, sequences classified as *Faecalibacterium* accounted for 17%, 33% and 24% of the libraries while the next largest cluster IV group was affiliated with *Subdoligranulum* (29%, 11% and 10% of sequences). *Clostridium* cluster XIVa sequences were also abundant, accounting for 40%, 41% and 35% of cloned sequences in the

![Phylogenetic Tree](image)

**Figure 1.** The phylogenetic tree showing the relationship of 16S rDNA sequences in faecal samples of subjects. The frequency of sequences in each library generated from the diet pools is given in the order of control: raffinose: chickpea for each bacterial group. The phylogenetic tree was constructed using the maximum-parsimony algorithm and the neighbour-joining method using ARB (Ludwig et al., 2004).
three libraries. Sequences classified as Bacteroidetes were rarely detected in the libraries, accounting for less than 3% of sequences. Within the Actinobacteria, *Bifidobacterium* were detected only in the raffinose and chickpea libraries while Coriobacteria were slightly more abundant and were detected in all three libraries.

The taxonomic composition of the three libraries was similar with no major differences observed between the diet pools. This observation was confirmed using Unifrac (Lozupone et al., 2006) where environment clustering, Unifrac significance calculation and a lineage specific analysis failed to consistently identify any statistically significant differences in the taxonomic profile between the three libraries.

**T-RFLP analysis of bacterial population present in individual subjects**

The faecal microbiota of the subjects who consumed the raffinose, chickpea, and control diets were followed by T-RFLP analysis of amplified 16S rRNA genes using restriction enzymes *MspI, HaeIII* and *HhaI*. In *silico* digests using our library sequence data and TRF assignments of human colonic microbiota analysed by PAD-HCM (Matsumoto et al., 2005) provided predicted TRF lengths and assignment of bacterial families or species. A comparison of observed and predicted TRFs as well as the taxonomic assignments is presented in Table 2.

The T-RFLP profile of each subject was then individually characterised for each period to test whether there was a change in microbial community profiles with the introduction of the diets. The DNA extracted from two samples from the chickpea diet group could not be PCR-amplified while one PCR-amplified product from the control group failed to digest with the *MspI* enzyme. Using UPGMA, dendograms of TRF profiles did not demonstrate consistent clustering according to diet or treatment period (data not shown). However, considering the bacterial species or phylogenetic group assignments (Table 2), it was determined that the *MspI* TRFs provided the best discrimination of species groups, thus quantitative comparisons of TRF peaks among diets were made using the *MspI* enzyme digests. Predominant *MspI* TRFs of 55, 217 bp, 281-285 bp, and 480 bp were observed in nearly all individuals irrespective of diet. The 217 bp and 281-285 bp TRFs corresponded to *Clostridium* subcluster XIVa and the *Faecalibacterium prausnitzii* and *Subdoligranulum* forks of cluster IV, whereas TRFs at 55 bp and 480 bp could not be assigned to a specific bacterial group. For *MspI* TRFs assigned to *Clostridium* clusters XIVa, I/II, *Lactobacillus* and *Bifidobacterium*, the percentages of individuals positive for a specific TRF in each treatment group are presented in Figure 2. The data

<table>
<thead>
<tr>
<th>TRF Lengths (bp)</th>
<th>Bacterial species</th>
<th>Taxonomic cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>217</td>
<td><em>Faecalibacterium prausnitzii</em></td>
<td>XIVa</td>
</tr>
<tr>
<td>281-285</td>
<td><em>Subdoligranulum</em> spp.</td>
<td>IV</td>
</tr>
<tr>
<td>55</td>
<td><em>Bacteroides</em> spp.</td>
<td></td>
</tr>
<tr>
<td>221</td>
<td><em>C. histolyticum</em> and relatives</td>
<td>II</td>
</tr>
<tr>
<td>287</td>
<td><em>L. casei</em>/<em>L. bifermentans</em> gp</td>
<td>XI</td>
</tr>
<tr>
<td>322</td>
<td><em>H. pseudofaecalis</em></td>
<td>unassigned</td>
</tr>
</tbody>
</table>

1 Base pair lengths (bp) for TRFs generated by *MspI*, *HaeIII* and *HhaI* digests are indicated. TRFs predicted by *in silico* digests of clone sequence data and observed following T-RFLP analysis are shown in bold. TRFs predicted from clone sequences data but not detected in T-RFLP analysis are given in regular font. TRFs detected only by T-RFLP analysis are in italics.

2 Bacterial species assignment was based on greater than or equal to 97% sequence identity between library sequences producing *in silico* digested TRFs and reference strains.
showed a decrease in the number of positive individuals, *MspI* TRFs 516-523 (*Clostridium* clusters I/II) and 192-194 (*Clostridium* cluster XI) with the chickpea diet (7.24±1.3) compared to the control diet (10.8±3.4).

**Shannon diversity index**

The total bacterial species diversity of each subject based on the *MspI* TRFs was analysed by the Shannon-Weiner diversity index (Shannon and Weaver, 1949) and the mean diversities of the treatment groups for each of the three-week periods are presented in Figure 3. No significant differences were observed between the chickpea and raffinose diets and the control diet in any of the periods. The mean number of TRFs based on *MspI* digests was 18.1±1.94 for individuals on the control diet, 17.9±2.0 for individuals on the raffinose diet and 18.4±2.4 for individuals on the chickpea diet.

**Real-time PCR quantification using group specific bacterial primers**

Real-time PCR analysis was performed to quantify the *Clostridium* cluster IV and I species group, *Lactobacilli*, *Bacteroidetes* and *Bifidobacterium* species in faeces of the subjects from the three diet groups. Group specific bacterial primers were selected to approximate those taxa which were identified as affected by diet based on *MspI* TRFLP.

**Volatile fatty acids and lactic acid**

The concentrations of VFAs and D- and L-lactic acid in faeces are reported in Table 4. Neither the total VFA nor the individual VFAs or lactic acid showed any significant differences among the three diets.

**4. Discussion**

Evidence that bacterial populations in the large intestine respond to changes in diet, particularly the type and quantity of dietary carbohydrate, is increasing. In this study, we used a combination of 16S rRNA-based molecular profiling methods in order to obtain complimentary data to investigate the composition of faecal microbiota in the subjects who consumed diets supplemented with chickpea or raffinose, as well as their normal diet. A detailed picture was obtained of the faecal microbial community members by sequencing 16S rRNA clone libraries generated from each diet pool. Phylogenetic relationships of the libraries generated from the chickpea, raffinose and control libraries revealed that *Clostridium* cluster XIVa and cluster IV species, members of the *Firmicutes*, were the most dominant members of the faecal microbiota of the subjects. In agreement, an investigation of the faecal microbial composition in healthy human adult volunteers...
Table 3. Mean 16S rRNA gene copies in faeces (copies/g ± standard error) collected from subjects consuming the control, raffinose or chickpea diets as determined by real-time quantitative PCR.

<table>
<thead>
<tr>
<th>Bacterial group</th>
<th>Control</th>
<th>Raffinose</th>
<th>Chickpea</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium cluster IV</td>
<td>8.65 ± 0.34</td>
<td>9.34 ± 0.14</td>
<td>8.80 ± 0.32</td>
<td>ns</td>
</tr>
<tr>
<td>Clostridium cluster I</td>
<td>5.27 ± 0.13</td>
<td>4.91 ± 0.23</td>
<td>5.51 ± 0.04</td>
<td>ns</td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>6.05 ± 0.34</td>
<td>6.45 ± 0.27</td>
<td>5.60 ± 0.34</td>
<td>ns</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>7.24 ± 0.49</td>
<td>8.08 ± 0.28</td>
<td>7.40 ± 0.69</td>
<td>ns</td>
</tr>
<tr>
<td>Bifidobacterium spp.</td>
<td>9.48 ± 0.65</td>
<td>9.85 ± 0.55</td>
<td>8.82 ± 0.55</td>
<td>ns</td>
</tr>
</tbody>
</table>

1 ns = not significant (P>0.05).

Table 4. Mean (± SD) volatile fatty acid (µmol/g or mmol/kg) and lactic acid (mmol/l) concentration in faeces collected from subjects consuming the control, raffinose or chickpea diets.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control diet (µmol/g)</th>
<th>Raffinose diet (µmol/g)</th>
<th>Chickpea diet (µmol/g)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic</td>
<td>48.02 ± 2.66</td>
<td>43.51 ± 3.99</td>
<td>43.61 ± 9.20</td>
<td>0.25</td>
</tr>
<tr>
<td>Propionic</td>
<td>13.98 ± 2.94</td>
<td>12.27 ± 3.07</td>
<td>11.48 ± 3.08</td>
<td>0.09</td>
</tr>
<tr>
<td>Butyric</td>
<td>13.31 ± 4.44</td>
<td>11.94 ± 5.43</td>
<td>12.58 ± 4.69</td>
<td>0.70</td>
</tr>
<tr>
<td>Valeric</td>
<td>1.39 ± 0.62</td>
<td>1.310 ± 0.85</td>
<td>1.190 ± 0.63</td>
<td>0.67</td>
</tr>
<tr>
<td>Caproic</td>
<td>0.41 ± 0.53</td>
<td>0.47 ± 0.43</td>
<td>0.484 ± 0.43</td>
<td>0.93</td>
</tr>
<tr>
<td>Heptanoic</td>
<td>0.39 ± 0.21</td>
<td>0.44 ± 0.33</td>
<td>0.428 ± 0.21</td>
<td>0.88</td>
</tr>
<tr>
<td>i-valeric</td>
<td>1.95 ± 0.79</td>
<td>1.92 ± 0.86</td>
<td>1.850 ± 0.89</td>
<td>0.95</td>
</tr>
<tr>
<td>i-butyric</td>
<td>1.40 ± 0.57</td>
<td>1.40 ± 0.74</td>
<td>1.312 ± 0.60</td>
<td>0.80</td>
</tr>
<tr>
<td>i-caproic</td>
<td>0.07 ± 0.06</td>
<td>0.11 ± 0.11</td>
<td>0.29 ± 0.19</td>
<td>n/a</td>
</tr>
<tr>
<td>Total VFA</td>
<td>80.91 ± 4.30</td>
<td>70.71 ± 6.15</td>
<td>73.06 ± 4.85</td>
<td>0.34</td>
</tr>
<tr>
<td>D-lactic acid</td>
<td>43.8 ± 20.0</td>
<td>42.7 ± 14.7</td>
<td>44.9 ± 12.2</td>
<td>0.23</td>
</tr>
<tr>
<td>L-lactic acid</td>
<td>35.3 ± 19.0</td>
<td>32.1 ± 18.8</td>
<td>37.6 ± 19.0</td>
<td>0.12</td>
</tr>
</tbody>
</table>

1 n/a = significance not tested; P>0.05 = not significant.

from five locations in Europe, it was shown that Clostridium cluster XIVA subgroup and Clostridium cluster IV subgroup were the predominant bacteria, accounting for 28% and 25%, respectively, of the total bacteria (Blaut, 2006). In contrast to the study by Blaut (2006) and others (Eckburg et al., 2005; Suau et al., 1999), sequences identified as members of the Bacteroidetes phylum were rarely identified (<3% of sequences) in the present study. Similarly low relative abundance of Bacteroidetes has been reported in obese individuals (Ley et al., 2006) and Bacteroidetes were also recovered in very low quantity from faeces of healthy subjects consuming diets high in resistant starch and non-starch polysaccharides (Abell et al., 2008). Sequence abundance within clones libraries is probably dependent on a number of factors including PCR primer bias, nevertheless, qPCR enumeration of Bacteroidetes and Clostridium Cluster IV bacteria in the present study indicate that the relative abundance of Bacteroidetes is 10-fold (1 log) less than Clostridium Cluster IV bacteria in approximate agreement with library representation at 3 and 25%, respectively.

In the present study, of the sequences placed within the Clostridiaceae family, a high proportion clustered with the F. prausnitzii and Subdoligranulum species. F. prausnitzii is reported as one of the most abundant species of Clostridium cluster IV in the large intestine of healthy adults, accounting for about 4-7% of the total clone library (Hayashi et al., 2002). Furthermore, members of Clostridium cluster IV, such as F. prausnitzii (Suau et al., 2001), and Roseburia species of cluster XIVA, have been reported to contribute significantly to butyrate production in the human intestine (Barcinella et al., 2000). In the present experiment, the raffinose library had the highest frequency of clones of F. prausnitzii, compared to the chickpea library and the control. However, since the libraries were created from sample pools they provide a good picture of the overall taxonomic composition of the microbiota but are not well suited for quantitative analysis beyond gross changes and rankings. Thus, since no indication of increased abundance of F. prausnitzii was supported by T-RFLP conducted on individual samples, we did not pursue this finding using qPCR.
We used T-RFLP to monitor the faecal communities of individual subjects, and the bacterial groups that corresponded to the predominant TRFs were identified by comparing the fingerprint patterns of bacterial communities to those of 16S rRNA gene clones showing >97% sequence identity to the reference strains. The T-RFLP method is sensitive and reproducible and is currently one of the most powerful tools for rapidly comparing temporal changes between microbial communities (Dicksved et al., 2007). In the present study, the majority of TRFs predicted by the 16S rRNA clone sequences were detected by T-RFLP analysis, indicating amplification of a common pool of the microbiota by both techniques. Importantly, in silico restriction digestion of the 16S rRNA library sequence data provided the opportunity to predict the specific bacterial taxa represented in each TRF. Unfortunately, no clustering of T-RFLP patterns was observable using UPGMA based on diet or period which was likely a result of large inter-individual differences that obscured any patterns.

We therefore elected to focus on MspI T-RFLP profiles which we were most successful at assigning to specific bacterial groups. As shown by MspI T-RFLP and clone library analysis, a marginal increase in putatively beneficial Bifidobacterium spp. counts was observed in the raffinose diet compared to both the chickpea and the control diets. In addition, MspI TRF profiles indicated the number of individuals positive for Clostridium clusters I/II and XI (Clostridium histolyticum - Clostridium lituseburense group), including pathogenic and putrefactive bacteria, were low in the chickpea diet compared to the control. Protein fermentation in the human distal colon is known to produce toxic metabolites such as ammonia, amines and phenolic compounds (Hughes and Rowland, 2003). Moreover, high concentrations of ammonia in the colon have been linked to increased DNA synthesis and neoplastic proliferation (Tuohy et al., 2006). Our hypothesis was that including chickpea in the diet would enhance fermentable substrate availability in the distal gut compared to the control diet, promoting protein nitrogen utilisation for microbial biomass and reducing the elaboration of nitrogen-containing toxins. We screened the faecal microbiota of the subjects for the presence of specific bacterial taxa previously characterised as high ammonia-producing (Cotta et al., 2003). Standard PCR amplification of the DNA samples from the faeces of the twelve subjects (data not shown) showed the presence of a high ammonia-producing strain, in 83%, 92% and 42% of individuals in the control, raffinose and chickpea groups, respectively. While we do not consider a 9% increase in the presence of this ammonia-producing isolate in individuals fed raffinose significant, a 50% reduction in the number of positive individuals fed chickpea supplemented diets is noteworthy. It is likely that this reduction in the chickpea diet is associated with any of the other major oligosaccharides found in whole chickpeas.

The total VFA concentrations in faeces observed in the study and the proportion of the main components (acetate:propionate:butyrate) were comparable to published literature in controlled human studies (McGarr et al., 2005). No significant differences in faecal VFA content were observed in the present study although we were surprised to see a numerical decline associated with oligosaccharide fortification in the raffinose and chickpea diets. However, a significant change in VFA concentrations was not observed in a majority of human experiments testing the prebiotic potential of oligosaccharides and diets containing inulin (Gibson and Roberfroid, 1995). The amount of VFA excreted in faecal matter depends mainly on the absorption, amount of cross-feeding of organic acids and transit time (Topping and Clifton, 2001) and may therefore not be an accurate assessment of VFA production rates. Similarly, since no significant differences were observed among concentrations of the different VFA measured, correlations with diet-associated changes in relative abundance of major taxonomic groups could not be made.

In conclusion, T-RFLP analysis demonstrated that both the chickpea and the raffinose diets modulated the gut microbiota of subjects with potentially beneficial effects associated with an increase in Bifidobacterium spp. and a decrease in Clostridium clusters I/II and XI including pathogenic and putrefactive bacteria. Despite the reported biases in amplifying Bifidobacterium spp. by 16S rRNA-based molecular methods (Hayashi et al., 2002), both clone libraries and T-RFLP showed the presence of this genus while real-time enumeration revealed a marginal increase in Bifidobacterium counts with the raffinose diet. Discordance among the three profiling approaches used in the study (sequence library, T-RFLP, qPCR) was observed but not entirely unexpected as each approach does not target identical bacterial groups (i.e. the group of bacteria represented in a TRF are different from those quantified by group specific qPCR primers). As shown by the Shannon diversity index, the study also further confirmed the notion that colonic microbial populations are particularly stable and resilient to change in healthy adults (Zoetendal et al., 1998), suggesting that short-term dietary intervention may have a limited capacity for modifying colonic microbiota in the longer term. Studying the effects of longer term dietary interventions in order to be able to manipulate the responses to dietary supplements for enhanced human health outcomes is also necessary.

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References


