Campylobacter concisus utilizes blood but not short chain fatty acids despite showing associations with Firmicutes taxa

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INTRODUCTION

Campylobacter species other than Campylobacter jejuni and Campylobacter coli have recently gained prominence as aetiological agents of a range of gastrointestinal malignancies (Kaakoush et al., 2015c). Often classified as emerging Campylobacter species, this subset of species includes Campylobacter concisus, which has been associated with gastroenteritis (Lastovica, 2009; Nielsen et al., 2013b), Barrett’s esophagus (Blackett et al., 2013; Kaakoush et al., 2015b), post-infectious irritable bowel syndrome (Nielsen et al., 2014) and inflammatory bowel diseases (IBD) (Kaakoush et al., 2014b; Castano-Rodriguez et al., 2015).

Abbreviations: AGS, adherent gastric epithelial; AICC, adherent and invasive C. concisus; AToCC, adherent and toxicogenic C. concisus; CD, Crohn’s disease; EEN, exclusive enteral nutrition; FBS, fetal bovine serum; HBA, horse blood agar; IBD, inflammatory bowel diseases; OE33, adherent esophageal epithelial; MOI, Multiplicity of infection; PBS, phosphate buffered saline; PCDAI, The Pediatric Crohn’s Disease Activity Index; ROS, reactive oxygen species; SSZ, sulfasalazine.

Four supplementary figures and one supplementary table are available with the online Supplementary Material.
Nonetheless, the involvement of *C. concisus* in disease has been somewhat controversial given that the bacterium is present in the oral cavity of many humans, and it can be isolated from faecal samples of healthy people (Kaakoush & Mitchell, 2012). However, this discrepancy may arise due to a number of factors. For example, the division of *C. concisus* strains into different pathotypes, including adherent and invasive *C. concisus* (AICC) and adherent and toxigenic *C. concisus* (AToCC), in addition to commensal strains (Kaakoush *et al.*, 2014b), may contribute to this inconsistency. Moreover, the composition of the host’s intestinal microbiota and the abundance of specific physiological factors they produce (e.g. hydrogen) may influence the translocation of *C. concisus* from the oral cavity to the colon (Kaakoush *et al.*, 2014a, c; 2015a). Further, defective host immune pathways (e.g. autophagy) or the increased presence of specific host factors (e.g. inflammatory cytokines) (Burgos-Portugal *et al.*, 2014; Man *et al.*, 2010) may also play a role in the bacterium establishing a niche in the human intestine.

In addition to the oral cavity being a potential source of *C. concisus*, the bacterium has been detected in broiler chicken faecal samples (Kaakoush *et al.*, 2014c), on chicken meat and minced beef (Lynch *et al.*, 2011) and in pets (Chaban *et al.*, 2010). Thus, contaminated meat products and pets may also play a role in the transmission of pathogenic strains of *C. concisus* to the human intestinal tract.

The association between *C. concisus* and IBD is of particular interest given the complex aetiology of these diseases. It is currently unclear what role the bacterium plays in the gastrointestinal tract of IBD patients, however, it has been found to be more prevalent and/or abundant in these patients (Kaakoush *et al.*, 2014a, b). Given that Crohn’s disease (CD) presents in any location across the gastrointestinal tract, in the current study, we examined the relationship between *C. concisus* and the upper gastrointestinal tract. Further, we investigated the presence of *C. concisus* in the lower gut of CD patients undergoing treatment, and the relationship between *C. concisus* and the lower gut microbiota of these patients.

**METHODS**

**Bacterial strains.** *Campylobacter concisus* strains UNSWCD, UNSW1, UNSW2, UNSW3, ATCC 51561, ATCC 51562, UNSWCS and BAA-1457 were used in this study. All strains were grown on Horse Blood Agar (HBA) plates [Blood Agar Base No. 2 supplemented with 6% defibrinated horse blood (Oxoid)], and incubated at 37°C under microaerobic conditions supplemented with hydrogen for 24 h.

**Bacterial growth studies.** Two base liquid media were employed for the growth studies, Bolton broth (Oxoid) and Mueller Hinton broth supplemented with a range of compounds including acetate (0.5–100 mM), butyrate (0.5–100 mM), lactate (0.5–100 mM) (Sigma-Aldrich), fetal bovine serum (FBS) (10%), and defibrinated horse blood (1% and 10%), Cultures (non-shaking or shaking at 150 rpm) were incubated at 37°C under microaerobic conditions supplemented with hydrogen for 24 h and 48 h. Bacterial growth was measured by observing the change in absorbance at 595 nm by and the plate count method. For absorbance measurements of cultures containing blood, 1 ml of bacterial culture was washed three times with PBS and resuspended in a final volume of 1 ml PBS. Measurements were then blanked against the respective bacteria-free media washed in a similar manner in order to ensure that the supplemented blood did not interfere with the absorbance measurement. For growth studies, two biological replicates, and three technical replicates within each biological replicate were performed.

**Adherence and gentamicin protection assays.** Two cell lines were used in this study, OE33 (adherent oesophageal epithelial) cell line and AGS (adherent gastric epithelial) cell line. Media for AGS cells consisted of Ham’s F-12K nutrient solution (Life Technologies) supplemented with 10% FBS (Bovogen) and 100 µg ml⁻¹ penicillin and streptomycin (Life Technologies). OE33 cell media consisted of RPMI-1640 medium (Life Technologies) supplemented with 10% FBS and 100 µg ml⁻¹ penicillin and streptomycin. Cells were grown in 25 cm² tissue culture flasks (In Vitro Technologies) at 37°C with 5% CO₂. Cells were harvested by trypsinization and either passaged at a concentration of 1×10⁶ cells ml⁻¹ into 25 cm² tissue culture flasks and maintained or seeded at a concentration of 5×10⁵ cells ml⁻¹ into 24-well plates and kept for 2 days at 37°C with 5% CO₂ in order to form a confluent monolayer for the adherence and invasion assays. Prior to seeding, the wells were coated with 0.338 mg ml⁻¹ collagen and incubated for 20 min at 37°C with 5% CO₂. Monolayers were infected with the bacteria at a multiplicity of infection (MOI) of 200. Adherence and gentamicin protection assays were performed as previously described (Kaakoush *et al.*, 2011). Results for Caco-2 cells were obtained from Kaakoush *et al.* (2011).

**Acid shock studies.** *C. concisus* strains grown on HBA plates were washed and resuspended in PBS, and then inoculated into media supplemented with 10% FBS and adjusted to a pH of 2, 3, 4, 5, 6 or 7, or left unadjusted (pH 7.4, control). Bacterial cells were incubated for 10 min or 30 min under microaerobic conditions at 37°C. Following incubation, bacterial concentrations were determined using standard plate count method. Three biological replicates were performed for all acid shock experiments.

**Extraction of bacterial metabolites and ¹H-NMR spectroscopy.** Bacteria grown on HBA plates were harvested and then washed three times using chilled phosphate buffered saline (PBS). Bacterial cells were resuspended in D₂O and lyed by freeze-thawing in liquid nitrogen in triplicate. Lysates were centrifuged at 14 650 g to pellet the debris. The supernatant was then filtered through a 10 kDa Microcon centrifugal filter (Millipore) and the filtrate was used for further analyses. Centrifugal filters were washed with D₂O prior to use. All ¹H spectra were acquired on a Bruker Avance III HD 600 spectrometer fitted with a TCI cryo-probe (Bruker; Billerica, MA, USA) and a refrigerated sample changer. Chenomx v8 software (Chenomx; Edmonton, Canada) was employed for the identification of metabolic compounds within the acquired NMR spectra.

**Patients with Crohn’s disease.** Five children attending the Sydney Children’s Hospital (SCH), Randwick, Inflammatory Bowel Disease Clinic were recruited prospectively at the time of diagnosis of CD. The diagnosis of CD was based upon standard histologic, endoscopic, and radiologic criteria, after performing upper gastrointestinal endoscopy, colonoscopy, and radiological assessment of the small bowel (IBD Working Group of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition, 2005). Following diagnosis, all five children were managed with exclusive enteral nutrition (EEN) following a standard protocol (Day *et al.*, 2006) to induce remission. The children were prescribed Osmolite (Abbott Laboratories; Sydney, NSW, Australia), a polymeric formula containing whole polypeptides and all nutritional requirements, to be taken as their sole nutritional...
Faecal sample collection and DNA extraction. Faecal samples from the five patients were collected at baseline (prior to endoscopy preparation, week 0) and then at 1, 2, 4, 6, 8, 12, 16 and 26 weeks following diagnosis. Owing to the lack of material, not all samples were available for analysis; however, all children had a minimum of a baseline, two time-points during EEN, 8- and 26-week samples. Samples were collected in sterile collection containers and immediately stored at ~20 °C, then transported frozen to the laboratory where they were stored at ~80 °C as described previously (Kaakoush et al., 2015d). DNA was extracted using the method of Griffiths et al. (2000). The concentration and quality of DNA was measured using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies; Wilmington, DE, USA).

Ethics. Informed consent was obtained from all children (or their parent/guardian for younger children) to be included in the study. This study was approved by the Research Ethics Committees of the University of New South Wales and the South Eastern Sydney and Illawarra Area Health Service Research Ethics Committee (ethics no. 03/163, 03/165, and 06/164).

Real-time PCR. Detection of C. concisus DNA was accomplished using real-time PCR on a Rotor Gene 6000 real-time PCR cycler (Qiagen). C. concisus DNA was amplified using the following primers: 5′-CTTGAGATATGCTATGGA-3′ (Concisus F) and 5′-CTCAT-TAGAGTGCTCAGCC-3′ (Concisus R), which had been previously optimized by Man et al. (2010). Cycling conditions were 40 cycles of 94 °C for 10 s, 65 °C for 10 s and 72 °C for 30 s. The equation for the C. concisus (r=0.966) PCR was log$_2$(C. concisus DNA concentration) = (C$_{\odot}$ value−14.675)/(−4.5726). The abundance of other microbial taxa in these samples was previously published by our group (Kaakoush et al., 2015d).

Genome-wide comparisons and phylogenetic analyses. Genes within the eight available C. concisus genomes with homology to bacteria from the Firmicutes phylum were defined using an iterative BLAST method. Briefly, C. concisus proteomes from NCBI were used as test proteins and were put through BLASTp (minimum 40 % identity and 90 % length hit) in sequential order against proteomes from the Firmicutes phylum. Identified hits were then manually verified through additional BLASTp searches. Alanine racemase, glutamate racemase and aspartate racemase sequences employed in this analysis were obtained from the NCBI website. All sequences for Campylobacteriaceae available at the time of the analysis were included. Phylogenetic trees were generated using the Mega5 tool (Tamura et al., 2011) by applying the neighbour-joining method with 1000 bootstrap replications.

RESULTS AND DISCUSSION

Selection of liquid media for the growth of C. concisus

Given that the growth of C. concisus in liquid media has not been properly investigated in the literature, we examined the ability of C. concisus to grow in liquid media supplemented with different nutrients to inform our downstream experiments. The results showed that at 24 h some strains were capable of growing (OD$_{595}$ ~0.45) in Mueller Hinton broth (e.g. strain ATCC 51561), while others appeared not to (e.g. strain UNSWCD) (Figs 1 and S2). However, by 48 h, poor levels of growth were observed for strain...
UNSWCD in Mueller Hinton broth (0.22±0.04). Nonetheless, *C. concisus* strains that were capable of growth in Mueller Hinton broth by 24 h still had weaker growth when compared to growth on HBA. Given this difference across strains and the weak growth in liquid media overall, we then examined other factors that may influence the growth of *C. concisus*.

We observed good growth for *C. concisus* UNSWCD in Bolton broth, a medium designed for pre-enrichment of *Campylobacter* species from food and clinical samples, and this did not change with the addition of serum (Fig. 1). Given that the bacterium grows at a very high rate on HBA, we also examined the effect of supplementation of the liquid media with horse blood on the growth of *C. concisus*. Blood supplementation improved the growth of all *C. concisus* strains considerably (Figs 1 and S2). Indeed, strain UNSWCD showed significant growth rates in Mueller Hinton broth following supplementation with either 1% or 10% horse blood (Fig. 1). These results suggest that while some strains are facultative haemophiles, capable of growing without the presence of blood (e.g. ATCC 51561), other strains are more reliant on blood in the medium for growth (e.g. UNSW1). Further, there appears to be a strain-specific difference in the requirement of blood in the medium, given that UNSW1, unlike UNSWCD, was incapable of growing in Bolton broth alone but could grow in media supplemented with horse blood (Fig. S2).

These results indicate that blood and not serum supplementation of liquid media is beneficial for *C. concisus* growth, and Bolton broth could serve as a simple alternative for growth of *C. concisus* in liquid media and for the enrichment of the bacterium prior to isolation from patient samples.

### Table 1. Levels of adherence to and invasion into gastrointestinal epithelial cells by *C. concisus* strains

Results for Caco-2 cells were obtained from Kaakoush et al. (2011).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Adherence (%)</th>
<th>Invasion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OE33 (Oesophageal)</td>
<td>2.4±0.5</td>
<td>0.037±0.003</td>
</tr>
<tr>
<td>BAA-1457</td>
<td>1.0±0.3</td>
<td>0.020±0.006</td>
</tr>
<tr>
<td>UNSWCS</td>
<td>0.93±0.17</td>
<td>0.061±0.007</td>
</tr>
<tr>
<td>AGS (Gastric)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UNSWCD</td>
<td>3.6±0.4</td>
<td>0.083±0.041</td>
</tr>
<tr>
<td>BAA-1457</td>
<td>0.25±0.02</td>
<td>0.0049±0.0013</td>
</tr>
<tr>
<td>UNSWCS</td>
<td>0.77±0.05</td>
<td>0.0017±0.0003</td>
</tr>
<tr>
<td>Caco-2 (Intestinal)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UNSWCD</td>
<td>4.5±0.8</td>
<td>0.47±0.04</td>
</tr>
<tr>
<td>BAA-1457</td>
<td>3.6±1.2</td>
<td>0</td>
</tr>
<tr>
<td>UNSWCS</td>
<td>4.6±1.5</td>
<td>0.00059±0.00015</td>
</tr>
</tbody>
</table>

### Ability of *C. concisus* to tolerate acidity and adhere to and invade oesophageal and gastric epithelial cells

Given that CD can present in several locations throughout the gastrointestinal tract including the oesophagus and stomach, and *C. concisus* would require passage through the stomach to reach the intestines, we first examined the ability of different strains of *C. concisus* to adhere to and invade gastrointestinal cells that were not of colonic origin, and the ability of the bacterium to tolerate acidic environments.

We found that all three strains tested could adhere to OE33 oesophageal cells, albeit at slightly lower levels than those...
observed for Caco-2 intestinal cells (Table 1). In contrast to intestinal cells where UNSWCD shows a hyper-invasive phenotype recently attributed to potential evasion of autophagy (Burgos-Portugal et al., 2014), all three strains were capable of invading oesophageal cells at relatively similar levels. This suggested that under certain conditions where C. concisus could colonize the oesophagus, it could potentially be involved in host tissue damage.

Early indications that C. concisus can tolerate acidity come from a study by von Rosenvinge et al. (2013) that found C. concisus to be highly active in gastric fluid. Further, Bik et al. (2006) have detected C. concisus in the human gastric mucosa. We found that C. concisus could tolerate acidic environments up to pH 4 with no effect on viability (Fig. 2). Some tolerance to pH 2 and 3 for a shorter time period of 10 min was observed. However, this corresponded to a highly significant reduction in viability (Fig. 2). Similar results were observed for other C. concisus strains (data not shown), as was for C. jejuni by Le et al. (2012). We then examined the adherence to and invasion of gastric AGS cells by C. concisus strains. Strain UNSWCD showed a higher ability to adhere to and invade gastric cells than the other strains, similar to the higher invasive potential observed for this strain in intestinal cells (Table 1). However, the difference was not as marked as that observed in intestinal cells (Table 1). These results would also suggest that under more alkaline conditions in the stomach, C. concisus may potentially colonize the gastric mucosa, adhere to and invade gastric cells or alternatively survive passage to the intestines.

Presence of C. concisus in the lower gut of CD patients

Given that C. concisus can invade human oesophageal and gastric cells, survive exposure to acidity, and adhere to and invade human intestinal cells, we then examined the relationship between C. concisus and the lower gut of CD patients. We recruited five CD patients sampled longitudinally at baseline, through 8 weeks of EEN therapy, and at 6 months from baseline (Figs 3 and S1).

Four out of five patients showed signs of colonization by C. concisus at some point during the 6-month period they were monitored, while one patient (CD4) was negative for the bacterium throughout this time period. Two out of five

![Fig. 3. Presence of C. concisus in paediatric patients with CD across a 26-week period. Figure was adapted from Kaakoush et al. (2015d) to include the positivity of C. concisus. Estimated C. concisus concentrations (fg/g faeces) were: CD1: Week 0, 18.5; Week 8, 248.0; Week 16, 45.9; Week 26, 4.7. CD2: Week 0, 36.5; Week 1, 63.0. CD3: Week 6, 128.8; Week 26, 0.3. CD5: Week 26, 1.1.](image)
patients (CD1 and CD2) were colonized by *C. concisus* at the time of diagnosis (baseline). *C. concisus* was eradicated upon treatment; however, in patient CD1, the bacterium returned upon termination of therapy, which also coincided with recurrence of disease. The patient remained positive for *C. concisus* while on sulfasalazine (SSZ) even though the patient showed signs of improvement. This could be due to the fact that SSZ is anti-inflammatory but its antibiotic component sulfapyridine does not have good activity against *Campylobacter* species (Andreasen et al., 1988). However, it is worth noting that the levels of *C. concisus* in patient CD1 decreased dramatically while on SSZ therapy (Fig. 3).

**Fig. 4.** Relative of abundance of *Faecalibacterium* (white) and *Lachnospiraceae incertae sedis* (black) in faecal samples of patients with CD. (+) and (-) refer to the presence of *C. concisus* within the sample. Y-axis scales were different across patients given the high variability in bacterial abundances.
Patient CD3 showed colonization by C. concisus upon ter-
mination of EEN therapy and slight aggravation of disease
(a PCDAI increase from 15 to 25). Patient CD3 was once
again positive for C. concisus at the 6-month time point dur-
ing 5-aminosalicylate (5-ASA) therapy, similar to the pat-
tern observed for patient CD1. The negative reading for
patient CD3 at the 8-week time point could not be readily
explained, given that the patient’s disease activity increased
between the 6 and 8-week time points. On the other hand,
the positive reading for patient CD3 at Week 26 despite
having a PCDAI of zero was attributed to very low levels of
the bacterium. It is interesting to note that patients with
severe disease (CD3 and CD4) either showed an abnormal
pattern of colonization or no colonization throughout the
6-month period.

Patient CD5 was negative for the bacterium across the time
period tested except for the 26-week time point which also
corresponded to recurrence of disease. The patient was
treated with metronidazole and 5-ASA. However, it is
unclear if this eradicated C. concisus given that there were
no further faecal samples beyond this time point.

While this is only a preliminary observational study, early
indications suggest that C. concisus is responsive to therapy
in a subset of CD patients, and this warrants further study
in larger cohorts.

The relationship of C. concisus with other
microbial taxa in the human gut

Previous studies have suggested that there is a correlation
between the abundances of C. concisus and microbial taxa
from the Firmicutes phylum in the gastrointestinal tract
(Kaakoush et al., 2014a, c). Thus, we examined the relative
abundance (species abundance relative to other species in a
defined community) of microbial taxa in the patient
samples for any potential qualitative association with C. concisus
positivity. The abundance of the microbial taxa in these
samples was previously analysed in Kaakoush et al. (2015d),
and relative abundances of taxa of interest are provided in
Table S1.

The presence of two microbial taxa within the Firmicutes
phylum (Faecalibacterium and Lachnospiraceae incertae
sedis) in relatively high abundance and C. concisus positivity
was observed concurrently in a number of patient samples
(Fig. 4). However, the relative abundance of these taxa
required for prediction of C. concisus positivity was patient-
specific. For example, patient CD1 was positive for *C. concisus* at baseline and this corresponded with a high combined relative abundance (78.6%) of *Faecalibacterium* and *Lachnospiraceae* incertae sedis. Upon treatment, levels of these taxa dropped and the patient tested negative for *C. concisus*. Following termination of therapy, the patient was positive for *C. concisus* and the total relative abundance of the two taxa had also increased to 56.5% (Fig. 4). Similar relationships were observed in patient CD2, CD3 and CD5 (Fig. 4). However, in the case of patient CD2, *C. concisus* positivity did not return at 16 weeks when *Faecalibacterium* levels were also high relative to the baseline of the patient. This could be explained by the fact that unlike other patients, patient CD2 received therapeutic levels of azithromycin (Fig. S1), which has strong activity against *Campylobacter* species.

In support of these findings, patient CD4, who tested negative for *C. concisus* throughout the 6-month period, also had relatively low levels of both *Faecalibacterium* and *Lachnospiraceae* incertae sedis, with combined levels of these taxa always remaining below 2.5% across this time period (Fig. 4). In comparison to *Faecalibacterium* and *Lachnospiraceae* incertae sedis, the relative abundance of common members of the human gut microbiota *Bacteroides* showed no potential association with *C. concisus* positivity (Fig. S3).

### Genetic similarity between *C. concisus* and Firmicutes

In an effort to further understand the relationship between *C. concisus* and Firmicutes, we examined the genomes of *C. concisus* for genomic similarity between the bacterium and microbial taxa within the Firmicutes phylum. We have previously reported that one gene (exotoxin 9) belonging to the extra-chromosomal restriction-modification system suggested to be associated with increased intracellular survival within host cells (Burgos-Portugal *et al.*, 2014; Kakkoush *et al.*, 2011), has very high homology (88–92%) to a replicative DNA helicase from *Lachnanaerobaculum saburreum* (belonging to the *Lachnospiraceae* family) (Deshpande *et al.*, 2013). Additional BLAST searches show that other genes within this restriction-modification system share high sequence similarity with *Streptococcus suis* (DNA helicase/Exotoxin 9, 84%) or *Mycoplasma californicum* (Tenericutes, restriction endonuclease, 86% and DNA-cytosine methyltransferase, 89%). Further, a recombinase on the same plasmid was found to share high similarity (90%) with *Streptococcus constellatus* subsp. *pharyngis*.

In addition to extra-chromosomal genes, several chromosomal genes encoding the amino acid racemases, aspartate racemase and glutamate racemase, were found to share high sequence similarity to Firmicutes taxa. While both of these

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**Fig. 6.** Metabolic profiles of *C. concisus* strains grown on horse blood agar obtained using $^1$H-NMR spectroscopy. Peaks corresponding to aspartate can only be observed in *C. concisus* ATCC 51562.
racemases were similar to racemases from other emerging Campylobacter species, similarity was relatively higher to Firmicutes than to other related taxa. For example, C. concisus aspartate racemase showed high homology to those from Mucispirillum, Cardiobacterium, Seleniumas, Anaerovibrio and Oscillibacter. Further, C. concisus glutamate racemase showed high homology to Seleniumas and Fusobacterium species. Interestingly, we did not observe this relationship with Firmicutes for the alanine racemase, which supports our argument given that aspartate and glutamate racemases originate from the same superfamily of proteins, which is distinct from alanine racemases.

Given that our BLAST searches showed homology to several other emerging Campylobacter species but not to the well-known C. jejuni and C. coli, we then examined if this relationship with Firmicutes was unique to racemases from emerging Campylobacter species. Phylogenetic analyses on aspartate racemases (Fig. 5a), glutamate racemases (Fig. 5b), and alanine racemases (Fig. 5c) from Campylobacterales species revealed that aspartate and glutamate racemases each form two distinct clusters (D1 and D2; E1 and E2), with C. jejuni and C. concisus belonging to differing clusters in both cases (Fig. 5a, b). This difference in origin was confirmed when additional sequences from a range of microbial taxa were included in the phylogenetic tree (Fig. S4). Interestingly, several emerging Campylobacter species had a copy of aspartate racemase from each of the two clusters (Fig. 5a). BLAST searches revealed that unlike C. concisus, C. jejuni aspartate racemase (D2), while also similar to aspartate racemases from Firmicutes taxa, showed high homology to those from Clostridium, Streptococcus and Fusobacterium species, while the C. jejuni glutamate racemase (E2) showed high homology to a range of glutamate racemases from Firmicutes and γ-Proteobacteria. In contrast, alanine racemases followed a more standard evolutionary path with Campylobacter, Arcobacter, enterohepatic Helicobacter and gastric Helicobacter species each clustering together (Fig. 5c).

Interestingly, one of the sequenced C. concisus strains, ATCC 51562, did not contain a homologue for aspartate racemase within its genome despite the fact that all other strains had aspartate racemases with 100 % homology. Analyses of the metabolic profiles of the eight strains had aspartate racemases with 100 % homology.

Analyses of the metabolic profiles of the eight strains grown under the same conditions revealed a potential functional consequence resulting from the lack of aspartate racemase in C. concisus ATCC 51562 (Fig. 6). Unlike the other seven strains, the metabolic profile of this strain showed signs of residual aspartate (Fig. 6). This build up is likely to be a result of accumulation of D-aspartate that could not be converted into L-aspartate to feed the aspartate metabolism pathway. Indeed, previous phylogeny of a range of C. concisus metabolic pathways did indicate that strain ATCC 51562 was genetically more distant from the other strains (Deshpande et al., 2013).

These results indicate that functional genetic elements with high homology between C. concisus and Firmicutes taxa are present.

**Effect of short chain fatty acids on growth of C. concisus**

Microbial taxa belonging to the Firmicutes phylum, particularly Ruminococcaceae and Lachnospiraceae, are known to produce short chain fatty acids (SCFAs) in the human gut (den Besten et al., 2013; Simpson & Campbell, 2015). Given the association between the presence of C. concisus and the relative abundance of microbial taxa within Firmicutes, and the genetic evidence suggesting an interaction between these bacteria, we examined the effect of SCFAs such as acetate and butyrate on the growth of C. concisus to determine if the production of these compounds attracted C. concisus to the colon.

Results from growth studies indicated that supplementation of the liquid medium with either acetate, butyrate or lactate did not result in an improvement of C. concisus growth (data not shown), indicating that the association between C. concisus and Faecalibacterium and Lachnospiraceae incertae sedis was not directly associated with SCFA production. In fact, acetate and butyrate were toxic to C. concisus at high concentrations (100 mM). Two possible explanations for this association include: (1) these organisms are obligate anaerobes and the association between the two could be a result of shared affinity to an environment that lacks reactive oxygen species (ROS); (2) the SCFA butyrate is believed to stimulate the production of mucus in colonic cells (Shimotoyodome et al., 2000), and the mucus layer appears to stimulate aggregation and biofilm formation by C. concisus on intestinal cells (Kaakoush et al., 2011).

**CONCLUSIONS**

Our results suggest that C. concisus has the potential to be pathogenic in the upper gastrointestinal tract. There appears to be a relationship between C. concisus and certain microbial taxa within the Firmicutes phylum, evidenced by both abundance levels and potential genetic exchange between the bacteria. Investigation into the possible role of SCFAs revealed that these were unlikely to play a role in the relationship between C. concisus and Firmicutes. Possible reasons for the relationship could be indirect attraction of C. concisus through the stimulation of mucin production by butyrate produced by Faecalibacterium or the shared susceptibility to ROS of these organisms. Importantly, Bolton broth was a good medium for C. concisus growth, and some strains of C. concisus appeared to be obligate haemophiles requiring blood, but not serum, for optimal growth. It remains to be seen what essential nutrients blood supplementation provides for the bacterium; however, this could explain their attraction to sites of damage in the oesophagus (Blackett et al., 2013; Kaakoush et al., 2015b), or sites of inflammation (Man et al., 2010). Our results collectively indicate that further investigation into the role of C. concisus in the gastrointestinal tract is warranted.
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REFERENCES


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