Colonisation and virulence characteristics of

Campylobacter concisus oral and clinical strains

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

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3rd March 2016
Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Signed Khaled Saleh A Allemailem

Date 03/03/2016
Dedication

In the name of Allah, the most gracious, the most merciful

My Mother and Father who have always supported me to reach my full potential

I am eternally grateful to you for your immense love and care.

My wife, kids, brothers and sisters who have provided emotional support

This work is a sign of my love to you.
Acknowledgements

I would like to express my sincere gratitude to vital contributions given by the following people within the entire period of my PhD candidature:

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I am forever indebted to my mother Ruqayya and my father Saleh for dedicating their life to care for me and my siblings. The words alone cannot express my gratitude. I would like to thank my wife Maha Alfraih for being my pillar of strength, even in the most stressful of times and for encouraging me during my studies. My son Waleed and my little princess Muhra always with their smiles, hugs and kisses, fuelled my motivation to accomplish this work. A big thank is expressed to all my siblings and friends, with their encouragement, reaching my goals seemed a lot easier.

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Preface

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Lastly, I owe everything to the Almighty God (Allah) without whose grace and guidance, none of this would be possible.
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Summary

*Campylobacter concisus* is a heterogenic bacterium which normally colonises the human oral cavity. It has been associated with oral cavity infections including gingivitis and periodontitis. It has also been isolated from other sites including enteric infections mainly in the elderly, immunocompromised patients, and children less than 24 months of age. To date there are little data about the mode of transmission, reservoir, and pathogenic potential of *C. concisus*. This bacterium is genotypically divergent with two or more genetic subgroups (genomospecies), however it is not known if a particular genomospecies is more associated with infections. This study investigated genetic diversity and colonisation of *C. concisus* amongst families with infants and newborn babies and other healthy individuals.

In this study, *C. concisus* strains were isolated from gum swabs of healthy volunteers of different age groups ranging from 1 week-50 years of age. The strains were collected from 7 different families and other unrelated healthy individuals. A total of 47 *C. concisus* oral strains, including 9 strains from infants before teething were isolated. The isolated strains were grouped into genomospecies (A and B) according to PCR amplification of the 23s rDNA. Interestingly, all infants were colonised with genomospecies B before teething except one infant whom was colonized with different *C. concisus* isolates from genomospecies A and B. Furthermore, three infants were followed up after teething and were all found to be colonised by genomospecies A isolates as well as genomospecies B. In general, 60% of *C. concisus* strains belonged to genomospecies A, while genomospecies B composed 40% of the strains. However, genomospecies B was found to be the dominant within children.
To investigate the phenotypic and genotypic relationship between *C. concisus* strains, SDS-PAGE was performed for each family on the outer membrane proteins (OMPs) of isolates from each member. Non-teething infants were also analysed separately. The dendrograms generated from SDS-PAGE profiles of the isolated *C. concisus* strains, showed that the OMPs were not identical even for the strains isolated from members of the same family. The only exception was the two strains isolated from twin infants within one of the families in this investigation.

This study also investigated motility as a virulence factor in *C. concisus* including the presence of the flagellin genes flaA, flaB and flaC and their expression and also the motility speed of selected *C. concisus* strains. Fifteen *C. concisus* clinical strains isolated from children suffering from gastroenteritis at the Royal Children’s Hospital, Melbourne, and two reference strains (ATCC 51561 and ATCC 51562) in addition to the oral strains from this study were tested. Using specific PCR primers, designed according to *C. concisus* 13826 published whole genome to amplify the full gene nucleotide sequence, flaA was amplified in some strains (26.5%) regardless of the genomospecies while flaB was detected in genomospecies B strains only (34.4%). In contrast, flaC was detected in all *C. concisus* clinical and oral isolates tested. New primer sets from shorter regions (<300 bp) of all these genes were designed from the conserved region in each gene to be used for gene expression. Conserved regions of flaA, flaB and flaC and their expression were thus studied for the first time in *C. concisus* grown in brain heart infusion broth (BHI). The expression of flagellin genes was investigated using RT-PCR compared to the reference strain *C. concisus* ATCC 51561. flaA was significantly up-regulated in only one *C. concisus* oral strain, while flaB was significantly up-regulated in two *C. concisus* strains including the strain with up-regulated flaA. However, only flaC gene expression was significantly up-regulated in all tested *C. concisus* strains.
Virulence characteristics related to motility, adhesion and invasion were investigated in selected *C. concisus* oral strains. The motility speed was measured using a modified method from Differential Dynamic Microscopy (DDM). Adhesion and invasion assays were also performed in an *in vitro* model using the human epithelial cell line INT-407. A *C. concisus* strain isolated from a healthy child (RMIT O17) was found to be significantly more invasive than the other tested strains. The motility speed of the tested strains varied, however *C. concisus* RMIT-O17 was found to be more motile than the other tested strains.

To conclude, Cape Town filtration protocol is suggested to be the best way to isolate *C. concisus* from clinical and oral samples. *C. concisus* genospecies B was found to first colonise the human oral cavity. Our results suggest that there are variations in the expression of flagellin genes within *C. concisus* strains and these genes are heterogeneous in different genospecies. The *flaC* primer set used in this study can be used as a species-specific primer set to detect new *C. concisus* isolates. Based on flagellin genes tested in this study, knock-out mutants of *flaA, flaB* and *flaC* genes would be necessary to investigate the role of these genes in motility.
Conference Proceedings


# Abbreviations

<table>
<thead>
<tr>
<th>Term</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>%</td>
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<tr>
<td>λ</td>
<td>lambda phage DNA</td>
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<tr>
<td>μM</td>
<td>micromole</td>
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<tr>
<td>mM</td>
<td>millimole</td>
</tr>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AFLP</td>
<td>amplified fragment length polymorphism analysis technique</td>
</tr>
<tr>
<td>AGRF</td>
<td>Australian Genome Research Facility</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>atp</td>
<td>ATP synthase F1 alpha subunit gene</td>
</tr>
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<td>BB</td>
<td>Brucella broth</td>
</tr>
<tr>
<td>BHI</td>
<td>brain heart infusion broth</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment sequence tool</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
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<td>C.</td>
<td><em>Campylobacter</em></td>
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<td>Columbia agar base</td>
</tr>
<tr>
<td>CB</td>
<td>Columbia broth</td>
</tr>
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<td>Description</td>
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<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CLO</td>
<td>Campylobacter like organisms</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>D</td>
<td>diffusion</td>
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<tr>
<td>DMEM</td>
<td>dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Deionised distilled water.</td>
</tr>
<tr>
<td>DDM</td>
<td>Differential Dynamic Microscopy</td>
</tr>
<tr>
<td>DGGE</td>
<td>Denaturing Gradient Gel Electrophoresis</td>
</tr>
<tr>
<td>DICF</td>
<td>Differential Intensity Correlation Functions</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DNase</td>
<td>deoxyribonuclease</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EICC</td>
<td>Enteric invasive <em>C. concisus</em></td>
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<td>exp</td>
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<td>FBS</td>
<td>foetal bovine serum</td>
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<td>flaA</td>
<td>Flagellin A gene</td>
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<tr>
<td>flaB</td>
<td>Flagellin B gene</td>
</tr>
<tr>
<td>flaC</td>
<td>Flagellin- like protein flaC gene</td>
</tr>
<tr>
<td>FPS</td>
<td>Frame per second</td>
</tr>
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<td>Description</td>
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</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GBS</td>
<td>Guillain-Barré syndrome</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>gyrB</td>
<td>gyrase subunit B gene</td>
</tr>
<tr>
<td>HBA</td>
<td>Columbia agar base with 5% horse blood agar</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'2-ethane sulfonic acid</td>
</tr>
<tr>
<td>HREC</td>
<td>Human Research Ethics Committee</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IBS</td>
<td>irritable bowel syndrome</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>one thousand daltons</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>Mb</td>
<td>Megabyte</td>
</tr>
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<td>milligram</td>
</tr>
<tr>
<td>MLST</td>
<td>multilocus sequence typing</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MOI</td>
<td>the multiplicity of infection</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MSD</td>
<td>mean squared displacement</td>
</tr>
<tr>
<td>ng</td>
<td>nanogram</td>
</tr>
<tr>
<td>N₂</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>NRMSE</td>
<td>normalize root mean squared error</td>
</tr>
<tr>
<td>NCTC</td>
<td>The National Collection of Type Cultures</td>
</tr>
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</table>
O  Oral (C. concisus strains)
OD  optical density
OMP  outer membrane protein
PAGE  polyacrylamide gel electrophoresis
PCR  polymerase chain reaction
PCR-DGGE  Polymerase chain reaction denaturing gradient gel electrophoresis
PFGE  Pulsed Field Gel Electrophoresis
pH  negative logarithm to the base ten of the concentration of hydrogen ions
q  scattering vector
qPCR  quantitative PCR
RAPD  randomly amplified polymorphic DNA
RCH  the Royal Children's Hospital, Melbourne, Australia
rDNA  ribosomal deoxyribonucleic acid
RT-PCR  Real time PCR
RXH  The Red Cross Children’s Hospital, Cape Town, South Africa
SD  standard deviation
SDS  sodium dodecyl sulfate
SEM  standard error of the mean
spp  species
t  time
TAE  Tris-acetate-EDTA

XX
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tm°C</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted Pair Group method with Mathematical Averages</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>WGS</td>
<td>whole-genome shotgun contigs</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>WCL</td>
<td>whole cell lysate</td>
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Chapter One - Introduction

Chapter One
Introduction

1.1 The genus *Campylobacter*

The first *Campylobacter* species to be isolated was *C. fetus*, from a sheep in 1906 and called *Vibrio fetus* by Smith and Taylor (Smith *et al.*, 1919), and was then renamed *Campylobacter fetus* in 1973 after it was shown as microaerophilic Vibrio (Veron *et al.*, 1973). The genus *Campylobacter* is now placed in the Epsilonproteobacteria (Logan *et al.*, 1994), and its family is Campylobacteraceae (Vandamme *et al.*, 1991a), which includes the genera *Campylobacter*, *Arcobacter* and *Sulfurospirillum*. The closest genera are *Helicobacter* and *Wolinella*, which are from Helicobacteraceae (Vandamme, 2000). Campylobacters were thought to be only veterinary pathogens until the 1970s, when they were shown to be isolated from stools of patients with acute enterocolitis (Skirrow, 1977). There are approximately 25 known species, and 11 subspecies in the *Campylobacter* genus (Murray *et al.*, 2009). Currently, the *Campylobacter* genus comprises several species and subspecies such as *C. curvus*, *C. fetus* subsp. *fetus*, *C. gracilis*, *C. helveticus*, *C. hyoilei*, *C. hyointestinalis* subsp. *hyointestinalis*, *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* (Gorkiewicz *et al.*, 2003). *C. coli* was first classified *C. hyoilei* and was isolated from lesions of porcine proliferative enteritis (On, 2001).

Campylobacters can be found at different sites including the intestinal tract and the oral cavity of humans and animals as normal flora or as pathogens (Garcia *et al.*, 1983, Vandamme, 2000). *C. pylori*, which is a human gastric pathogen was also included in the genus in 1984 (Marshall *et al.*, 1984), however this organism was later relocated in the genus *Helicobacter* according to the 5S and 16S RNA sequence data and DNA-DNA hybridization (Goodwin *et al.*, 1989). *Campylobacter upsaliensis* was added to the genus *Campylobacter*.  

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Wolinella curva was described in early 1980s and then added to the Campylobacter as C. curvus (Vandamme et al., 1991b). C. concisus was first described by Tanner et al. (1981), as small asaccharolytic, non-pigmenting gram-negative rods, and was first isolated from a person with gingivitis and periodontitis. C. mucosalis has been described to be most similar to C. sputorum and was named as C. sputorum subsp. mucosalis (Roop et al., 1985); sharing a highly similar phenotype and a common source (pig intestine) (Harrington et al., 1999). Hence, C. mucosalis is now distinguished from C. sputorum by negative arylsulfatase activity test. The study suggested that C. mucosalis is more strongly related to C. concisus compared to other Campylobacter spp (Vandamme et al., 1989).

Campylobacter spp. cells are normally motile, spiral-curved rods with a size of 0.5-0.8 µm by 0.2-5.0 µm and most members of Campylobacter genus are microaerophilic (Griffiths et al., 1990). They can grow at a wide range of temperature including 25°C, 37°C and 42°C. Under certain conditions, Campylobacter spp. may become round or coccoid and the shape is considered as viable but non-culturable (Rollins et al., 1986). Campylobacter ordinarily grow in a microaerophilic condition, however some species require anaerobic conditions or hydrogen in order to grow under microaerophilic conditions, such as C. concisus. Oxidase activity can be found in all Campylobacter species except C. gracilis (Wassenaar et al., 2006). The following table (Table 1.1) shows the valid species and subspecies in Campylobacter genus as of April 30, 2013, their common hosts and associated diseases in humans and animals.
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<table>
<thead>
<tr>
<th>Campylobacter spp.</th>
<th>Host species</th>
<th>Associated human diseases</th>
<th>Associated animal diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. avium</em></td>
<td>Poultry</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. canadensis</em></td>
<td>Whooping cranes</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>Pigs, poultry, ostriches, cattle, sheep</td>
<td>Gastroenteritis</td>
<td>Gastroenteritis, infectious hepatitis</td>
</tr>
<tr>
<td><em>C. concisus</em></td>
<td>Humans, domestic pets</td>
<td>Gastroenteritis, periodontitis</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. cuniculorum</em></td>
<td>Rabbits</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. curvus</em></td>
<td>Humans</td>
<td>Periodontitis, gastroenteritis</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. fetus subsp. fetus</em></td>
<td>Cattle, sheep, reptiles</td>
<td>Gastroenteritis, septicaemia</td>
<td>Spontaneous abortion</td>
</tr>
<tr>
<td><em>C. fetus subsp. venerealis</em></td>
<td>Cattle, sheep</td>
<td>Septicaemia</td>
<td>Infectious infertility</td>
</tr>
<tr>
<td><em>C. gracilis</em></td>
<td>Humans</td>
<td>Periodontitis</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. helveticus</em></td>
<td>Dogs, cats</td>
<td>Periodontitis</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td><em>C. hominis</em></td>
<td>Humans</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. hyointestinalis subsp. hyointestinalis</em></td>
<td>Cattle, deer, pigs, hamsters</td>
<td>Gastroenteritis</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td><em>C. hyointestinalis subsp. Lawsonii</em></td>
<td>Pigs</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. insulaenigrae</em></td>
<td>Seals, porpoises</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. jejuni subsp. doylei</em></td>
<td>Humans</td>
<td>Septicaemia, gastroenteritis</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. jejuni subsp. jejuni</em></td>
<td>Poultry, cattle, pigs, ostriches, wild birds</td>
<td>Gastroenteritis, Guillain-Barré syndrome</td>
<td>Spontaneous abortion, avian hepatitis</td>
</tr>
<tr>
<td><em>C. lanienae</em></td>
<td>Cattle</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. lari subsp. concheus</em></td>
<td>Shellfish</td>
<td>Gastroenteritis</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. lari subsp. lari</em></td>
<td>Wild birds, dogs, poultry, shellfish, horses</td>
<td>Gastroenteritis, septicaemia</td>
<td>Avian gastroenteritis</td>
</tr>
<tr>
<td><em>C. mucosalis</em></td>
<td>Pigs</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. peloridis</em></td>
<td>Shellfish</td>
<td>Gastroenteritis</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. rectus</em></td>
<td>Humans</td>
<td>Periodontitis</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. showae</em></td>
<td>Humans</td>
<td>Periodontitis</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. sputorum</em></td>
<td>Humans, cattle, pigs, sheep</td>
<td>Gastroenteritis, abscesses</td>
<td>Spontaneous abortion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td><em>C. subantarcticus</em></td>
<td>Birds in the subantarctic</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. upsaliensis</em></td>
<td>Dogs, cats</td>
<td>Gastroenteritis</td>
<td>Gastroenteritis</td>
</tr>
<tr>
<td><em>C. ureolyticus</em></td>
<td>Humans</td>
<td>Gastroenteritis, Crohn's disease</td>
<td>None as yet</td>
</tr>
<tr>
<td><em>C. volucris</em></td>
<td>Black-headed gulls</td>
<td>None as yet</td>
<td>None as yet</td>
</tr>
</tbody>
</table>

Table, modified from On (2013).
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1.1.1 Hydrogen-requiring Campylobacters

Some emerging species of *Campylobacter* including *C. concisus, C. showae, C. rectus,* and *C. curvus,* require hydrogen or formate to grow. These organisms can be found as normal flora in the human mouth, and are associated with gum diseases, although a causative role of these organisms has not been determined. These *Campylobacter* species have also been isolated from stool samples in patients with gastroenteritis, septicaemia, and other conditions (Lasticova *et al.*, 2002). Hydrogen-dependent strains are very difficult to recover using standard culture techniques that are routinely used in most laboratories (Lastovica, 2006), however, the number of the isolated strains can be increased using Cape Town protocol (Lasticova *et al.*, 2002).

*Campylobacter, Helicobacter* and *Arcobacter* strains require H$_2$-enriched microaerophilic conditions to grow, however some *Arcobacter* strains can also grow aerobically. Hence, the aerobic growth can be used to differentiate *Arcobacter* from *Campylobacter* and *Helicobacter* strains (Lastovica, 2006). The membrane filtration method can be used to isolate all known *Campylobacter, Arcobacter,* and *Helicobacter* species. This method was found to be a simple, efficient, and cost-effective isolation protocol. It is used onto media, without antibiotic and incubation at 37°C in an H$_2$-enriched microaerobic condition. This filtration method is the most appropriate method for isolating some hydrogen-dependent *Campylobacter* species like *C. concisus, C. rectus* and *C. curvus* (Lastovica, 2006).

A very recent study by Casanova *et al.* (2015) indicated that the number of *C. concisus* isolated from stool cultures was unusually increased and provoked a pseudo-outbreak. Two different microaerobic conditions were used, gas generator packs (5% O$_2$, 10% CO$_2$, and 85% N$_2$), or with evacuation and gas replacement of anaerobic jars (5% O$_2$, 8% CO$_2$, 15% H$_2$, and 72% N$_2$). In contrast to the microaerobic gas generator packs previously used, which cannot
produce hydrogen, the resulting atmosphere of the new system (evacuation and gas replacement of anaerobic jars) contains approximately 15% H₂ (Casanova et al., 2015). Some Campylobacters, like *C. concisus*, exhibit optimal growth with 6% (v/v) hydrogen (Fitzgerald, 2011). The growth of *C. concisus* with the previous methodology (gas pack) yielded weak or no growth. In contrast, *C. concisus* grew more optimally in H₂-enriched atmosphere. Changing the culture condition to an H₂-enriched atmosphere enhanced the *C. concisus* growth was deemed to be responsible for the increased isolation rate and the pseudo-outbreak (Casanova et al., 2015). Hydrogen-enriched atmosphere is currently used by our research group at RMIT University.

### 1.1.2 Isolation and Identification of *Campylobacter* spp.

Through the last few decades, *Campylobacter* spp. isolation methods have been improved. Isolation of thermophilic *Campylobacter* spp. from stool samples normally relies on some selective antibiotics and growth at 42ºC, to inhibit contaminating flora. Pre-enrichment procedures followed by culture on selective media are used for bacterial isolation, whether from food and environmental samples, or any other samples with low numbers of stressed bacteria. Most enrichment media of *Campylobacter* contain antimicrobials agents including cefoperazone, cycloheximide and vancomycin to inhibit growth of flora. *Campylobacter* species are found to be highly sensitive to peroxides, although they have superoxide dismutase and catalase. Some media are highly beneficial for *Campylobacter* species growth such as sheep or horse blood, and H₂-enriched atmosphere conditions. Sensitive *Campylobacter* spp. including *C. hyointestinalis, C. upsaliensis, C. mucosalis* or *C. jejuni* subsp. *doyle* may not be isolated with the use of selective antibiotics and a high incubation temperature. As some of these species grow slowly, incubation time is often extended (Wassenaar et al., 2006).
The Cape Town protocol, a method developed by Le Roux et al. (1998), was used in isolating Campylobacter spp. from blood and stool samples. A suspension from the stool sample is applied on a 0.6-µm filter placed on agar plates containing 10% horse blood. Blood samples can be applied directly to the plate without filtration. The plates are then incubated in an H₂-enriched atmosphere condition. The colonies of Campylobacter spp. are buff coloured or dirty yellow. C. helveticus spreads, forms thin flats films, becoming visible after 6 days. Gram staining confirms the cell morphology. The cells of C. mucosalis are quite short and stubby, while C. hyointestinalis cells are large and slightly curved. C. concisus, C. rectus, C. curvus and C. ureolyticus are straight or less curved (Wassenaar et al., 2006).

Several media can be used to grow Campylobacter spp., but Modified charcoal, cefoperazone, desoxycholate agar (mCCDA) is the recommended medium. The blood-containing media and charcoal-containing media are the two main groups of selective media. Preston agar, Skirrow agar, Butzler agar and Campy-cefex are examples of blood-containing media, while mCCDA, Karmali agar and CAT agar (cefoperazone, amphotericin and teicoplanin) are from charcoal-containing media (O.I.E., 2008). CAT agar medium is used to grow C. upsaliensis (Aspinall et al., 1993). The study by Piersimoni et al. (1995) demonstrated that CCDA medium along with the filtration is the best method for the optimal recovery of Campylobacter spp.

Biochemical tests can be used to identify and differentiate Campylobacter spp. These include catalase, urease, hippurate hydrolysis, nitrate reduction, indoxyle acetate hydrolysis, growth in bile esculin, growth in 1% glycine, and growth in 3.5% NaCl. C. sputorum can be distinguished by growing in 3.5% NaCl. C. fetus and C. sputorum are the only reported species for the growing with bile esculin, with C. sputorum able to grow and C. fetus unable to grow. C. jejuni can be easily differentiated from other Campylobacter spp. as it is the only Campylobacter spp. that can hydrolyse hippurate (Wassenaar et al., 2006). Campylobacter,
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*Helicobacter* and *Arcobacter* species can be differentiated from other bacteria (including both gram-negative & gram-positive), with these bacteria lacking L-Alanine amino-peptidase (L-ALA) found to be in most gram-negative bacteria (Hoosain *et al.*, 2009).

1.2 *Campylobacter* spp. in humans

*C. jejuni* is a well-known cause of human gastroenteritis. Similarly, *C. coli* is responsible for causing 1–25% of all *Campylobacter* diarrheal diseases. Emerging *Campylobacter* species like *C. concisus*, *C. upsaliensis* and *C. ureolyticus* have been found to be associated with a range of gastrointestinal diseases including periodontitis, gastroenteritis and Inflammatory Bowel Disease (IBD). Members of the *Campylobacter* genus can colonise several hosts including humans and domesticated pets, as well as both farm and wild animals. *Campylobacter* spp. can also be found in contaminated food, leading to high risk of transmission of infection to humans (Man, 2011).

1.2.1 *Campylobacter* spp. as colonisers

*C. jejuni* and *C. coli*, the most common *Campylobacter* spp. can colonise a wide range of sites in the human body and have been found in blood, cerebrospinal fluid, gall bladder, peritoneal fluid, urine and retroperitoneal abscesses (Blaser *et al.*, 1986). The intestinal tract of humans was shown to be colonised by a number of *Campylobacter* species including *C. concisus*, *C. curvus*, *C. gracilis*, *C. hominis*, *C. mucosalis*, *C. showae*, *C. upsaliensis* and *C. ureolyticus* (Inglis *et al.*, 2011).

*C. rectus* was found to colonise the human mouth, as detected in saliva and plaque samples healthy children (Umeda *et al.*, 2004) A number of *Campylobacter* spp., namely *C. concisus*, *C. sputorum bv. sputorum*, *C. curvus*, *C. rectus*, *C. gracilis*, and *C. showae*, have been isolated from the oral cavity of humans (Kamma *et al.*, 2000a, Kamma *et al.*, 1994). *C. fetus* can also be isolated from the intestinal tract of humans. It can also colonise the
intestinal tracts of a range of animals including sheep and turtles (Harvey et al., 1983). *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* were also isolated from the blood as well as abscess samples (Petersen et al., 2007).

1.2.2 *Campylobacter* spp. as pathogens

*C. jejuni* and *C. coli* are closely-related species, and both are a common cause of acute enteritis worldwide (Olson et al., 2008). The infection outcomes can vary, and for example, acute watery or bloody diarrhoea is reported in developed countries such as USA and UK. A high infectious dose is required to cause human diseases caused by these organisms (Black et al., 1993). *C. jejuni* and *C. coli* can use colonisation, attachment, invasion, and toxin production to initiate disease (Cróinín et al., 2012). *C. jejuni* infections can subsequently result in development of Guillian-Barre’ Syndrome (GBS). This syndrome presents as an acute neurological disease, due demyelination of peripheral nerves and can lead to ascending paralysis. Development of GBS was seen in approximately 0.05% of patients with *C. jejuni* infections in the USA (Nachamkin et al., 1998). Thirty-three *Campylobacter* outbreaks were previously reported in Australia in a period between January 2001 and December 2006, affecting 457 persons. Among these outbreaks, there were 8 foodborne outbreaks, 4 at aged care facilities and 3 at school camps (Unicomb et al., 2009). A campylobacteriosis outbreak was later reported with *C. jejuni* (Gardner et al., 2011). *C. jejuni* was also found to cause gastroenteritis and IBD (Newman et al., 1980). *C. coli* can also cause gastroenteritis and acute cholecystitis (Blaser et al., 1986).

The clinical manifestations of *C. jejuni* and *C. coli* infections include fever followed by diarrhoea. Bacteraemia and systemic infections can be found as intestinal illness specifically in immunocompromised patients, but in most cases, the infections are localised to the intestine. Diarrheic stools including watery and mildly bloody stools obtained from affected
people in developed countries were found to have leukocytes and erythrocytes. The disease is self-limiting and no antibiotic treatment is required. Diarrhoea caused by *Campylobacter* spp. may usually last up to one week, and the organism be excreted for several weeks (Melamed *et al.*, 1983, Perlman *et al.*, 1988).

Skirrow *et al.* (1993) reported that in 257 cases bacteraemia was caused by *Campylobacter* enteritis. The study found that the highest percentage was from *C. jejuni* or *C. coli* (89%), and 8.6% was from *C. fetus*, *C. lari*, and *C. upsaliensis*. *C. upsaliensis* is recognised as a human pathogen, causing bacteraemia and acute or chronic diarrhoea. *C. upsaliensis* has been isolated in high numbers from diarrhoea and blood cultures of paediatric patients in South Africa (Lastovica, 2006, Lastovica *et al.*, 2008, Lastovica *et al.*, 1989), has been haemolytic uremic syndrome (Carter *et al.*, 1996), and spontaneous abortion in humans (Gurgan *et al.*, 1994). The association between *C. rectus* and diseased subgingival sites was reported (Macuch *et al.*, 2000). *C. spotorum* has also been suggested to cause bacteraemia (Tee *et al.*, 1998).

To date, *C. fetus* is the most recognised *Campylobacter* spp. in animals. The *C. fetus* subsp. *fetus* main reservoir is the GIT of cattle and sheep. This subspecies can also be obtained from other animal faeces (Duncan *et al.*, 2014, Van Bergen *et al.*, 2005). *C. fetus* subsp. *fetus* is a rare cause of disease in humans and can cause bacteraemia, cellulitis, endocarditis, and meningoencephalitis, and abortion (Lastovica *et al.*, 2008). Human-to-human transmission of *C. fetus* is suggested to be more common in susceptible newborn babies (Wagenaar *et al.*, 2014).

*C. helveticus* and *C. hyointestinalis* have been reported to cause diarrhoea, while *C. upsaliensis*, *C. ureolyticus* and *C. mucosalis* are more strongly associated with gastroenteritis cases (Bullman *et al.*, 2012, Inglis *et al.*, 2011). Furthermore, *C. upsaliensis* was previously
found to cause campylobacteriosis with manifestations including fever, diarrhoea with or without blood, and vomiting (Goossens et al., 1990). A number of *Campylobacter* spp. are linked to chronic IBD and *C. hominis, C. showae, and C. ureolyticus* have been isolated from intestinal biopsies from of children with Crohn’s disease (CD) (Zhang et al., 2009). *C. concisus* was also isolated in a high detection rates in the patients with Crohn’s disease (CD) and ulcerative colitis (UC) (Hansen et al., 2011, Mahendran et al., 2011). A number of studies have investigated the association between *C. concisus* and oral diseases including periodontitis (Haffajee et al., 1984, Macuch et al., 2000), suggesting that *C. concisus* is an opportunistic pathogen.

1.2.3 *Campylobacter* spp. as a part of the human oral cavity microbiota

Several *Campylobacter* spp. colonise the human oral cavity including *C. concisus, C. gracilis, C. rectus, and C. showae*. *C. rectus* and *C. gracilis* are suggested to be associated with periodontitis (Abiko et al., 2010, Macuch et al., 2000). Macuch et al. (2000) isolated *C. rectus* in 90% of adult subjects with periodontitis, 20% of patients with gingivitis and 10% of healthy subjects (Macuch et al., 2000). *C. rectus* was also detected in high numbers in subgingival plaque or saliva samples of youths and adults groups suffering from advanced periodontal diseases, compared with healthy individuals or those who suffering from initial periodontitis (Lopez et al., 2011, Von Troil-Linden et al., 1995). The study by Umeda et al. (2004) also showed *C. rectus* detected in saliva and plaque samples in 95% of healthy children with permanent, mixed or deciduous teeth (Umeda et al., 2004). This suggested that *C. rectus* is able to colonise oral cavities in children at a young age, with no clinical symptoms (Man, 2011).
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*C. gracilis* was detected in high numbers, in permanent teeth of children and youth groups suffering from caries progression. It was also detected in permanent teeth of healthy controls. *C. showae* was also detected in low numbers in plaque from white-spot or dental lesions of children with caries, compared to healthy individuals (Aas *et al.*, 2008). However, Macuch *et al.* (2000) argued there was no association between *C. gracilis* or *C. curvus* and periodontal diseases (Macuch *et al.*, 2000). Based on these studies, it can be suggested that not all of oral *Campylobacter* spp. are pathogenic (Man, 2011).

1.3 *Campylobacter concisus*

*Campylobacter concisus* was first isolated in 1981 from a person suffering from gingivitis and periodontitis. *C. concisus* is an asaccharolytic, fastidious, slow-growing gram-negative rod shaped bacterium. It is motile with one or two polar flagella (Paster *et al.*, 1986, Tanner *et al.*, 1981), and requires microaerophilic conditions containing around 4% CO₂ and 7.5% H₂ to grow (Vandamme *et al.*, 1989). The isolation of *C. concisus* has mostly been from children with diarrhoea and immunocompromised patients, however *C. concisus* has also been isolated from healthy people (Schlenker *et al.*, 2009). Initially believed to be a human isolate of "*Vibrio succinogenes*", *C. concisus* was later renamed by Holdeman *et al.* (1984). *V. succinogenes* and related organisms that derive energy by reduction of fumarate or nitrate with formate or hydrogen were then reclassified as *Bacteroides gracilis, C. concisus*, or in a new genus, *Wolinella* (Holdeman *et al.*, 1984).

The first isolation of *C. concisus* in Australia was in the study by Russell (1995). The study was conducted on children with diarrhoea at the Royal Children’s Hospital (RCH), Melbourne, with an aim to isolate hydrogen-requiring campylobacters (*Campylobacter* like organisms; CLO). Among the 39 characterised CLO strains, 56% were later identified as *C. concisus* (Russell, 1995). *C. concisus* is divided into at least two different genomospecies that
are phenotypically not discriminable, but genetically different when analysed by DNA-DNA hybridisation (Vandamme et al., 1989) and AFLP analysis (On et al., 2000). Several studies have confirmed the heterogeneity of \textit{C. concisus}, and confirmed its classification into two major genomospecies (A and B), using 23S rDNA PCR (Istivan et al., 2004, Kalischuk et al., 2011). To date, only few reports are available regarding the source and route of transmission of \textit{C. concisus}.

1.3.1 The prevalence of \textit{C. concisus} as a normal flora

\textit{C. concisus} was suggested as one of the \textit{Campylobacter} spp. members to colonise the human oral cavity with higher numbers than initially predicted. \textit{C. concisus} was more frequently isolated from healthy oral sites than diseased sites, and was found in higher proportions from shallow than deep sites. Within diseased sites, \textit{C. concisus} was isolated more frequently from persons with initial periodontitis than other clinical groups, which led to describing it as an opportunistic pathogen (Macuch et al., 2000).

Using culture and PCR methods, \textit{C. concisus} prevalence in the saliva samples of healthy individuals and IBD patients was investigated and in samples from 18 patients with IBD (13 with CD and 5 with UC) and 59 healthy subjects who were not taking antibiotics at the time of sample collection. \textit{C. concisus} was detected in 97\% of saliva samples of healthy individual and in all saliva samples of IBD patients by PCR. However, \textit{C. concisus} was isolated by culturing from 75\% (44/59) of the healthy subjects; 85\% (11/13) of the CD patients and all (5/5) UC patients. The high prevalence of \textit{C. concisus} found in healthy individuals suggested that \textit{C. concisus} is a normal part of the human oral microflora. The study also suggested the possibility that some oral \textit{C. concisus} strains may colonise the lower part of GIT and are involved in some enteric diseases, including IBD (Zhang et al., 2010).
1.3.2 *C. concisus* in adults

*C. concisus* has firstly been described in gingival crevices of a person suffering from a mouth disease (Tanner *et al.*, 1981). A Belgian study also isolated *C. concisus* from enteritis cases in adults, and the isolation rate was 1.5% among the total number (Zhi *et al.*, 1996). *C. concisus* was also isolated from stool and blood samples of children and adults with and without diarrhoea (Vandamme *et al.*, 1989).

Mahendran *et al.* (2011) studied the prevalence *C. concisus* in CD adult patients from biopsies from different sites including ileal, cecal, colonic, and rectal sources. They found that the prevalence of *C. concisus* was significantly higher in colonic biopsies (53%; 8/15) as compared with controls (18%; 6/33). The study also identified *C. concisus* in UC patients with a significantly higher prevalence (69%; 9/13), compared with controls (36%; 12/33) (Mahendran *et al.*, 2011). The relationship between *C. concisus* and UC was further investigated in another study. It was also found that the prevalence of *C. concisus* was significantly higher in biopsies of patients with UC (33.3%; 23/69) as compared with controls (10.8%; 7/65) (Mukhopadhya *et al.*, 2011). A study by Nielsen *et al.* (2013) included 8302 patients (20-67 years); found 441 *C. concisus* isolates among 400 patients diarrhoeic stool samples. *C. concisus* was isolated from all age patients. The study found that 10% of *C. concisus* patients had co-infections dominated by *Salmonella enterica* and *Clostridium difficile*. It has also been found that three patients with *C. concisus* had co-infections with *Plesiomonas shigelloides*, *Yersinia enterocolitica*, or *Shigella sonnei*, respectively. Only 2 patients had *C. concisus* and *C. jejuni/coli* in their samples (Nielsen *et al.*, 2013). However, the pathogenic role of *C. concisus* is still unclear.
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1.3.3 *C. concisus* in children

A Belgian study by Lauwers *et al.* (1991) reported that *C. concisus* can be isolated from faecal samples of children and adults suffering from diarrhoea. *C. concisus* was isolated from the stools samples of 1.5% of adults and 2.4% of children. Interestingly, only 9% of *C. concisus*-positive children were >5 year old, while the majority (54%) of *C. concisus*-positive children were <1 year old (Lauwers *et al.*, 1991). Russell (1995) successfully isolated *C. concisus* from children with gastroenteritis who were admitted to the Royal Children’s Hospital (RCH), Melbourne, Australia. The children in this study had clinical manifestations including diarrhoea, vomiting, fever, and abdominal pain and were between 2 and 30 months old. The majority of *C. concisus* strains were isolated from children under 2 years old (18/19). The age distribution found in this study was similar to the global data of *C. jejuni* and *C. coli* infections. The study also suggested that there was a potential seasonal distribution for *C. concisus* infections, as the majority of *C. concisus* cases (85%) were identified from July to January (Russell, 1995).

In a similar study, Van Etterijck *et al.* (1996) compared *C. concisus* isolation rates in children with and without diarrhoea. Using DNA fingerprinting, *C. concisus* was isolated from 95% of children in the same care centre; however there was no significant difference in the isolation rates between patients with diarrhoea (13.2%) and controls (9%). This study concluded that there was no clear pathogenic role for *C. concisus* in enteritis (Van Etterijck *et al.*, 1996). However, a later study reported a significant difference with increased prevalence of *C. concisus* in faecal samples of children suffering from CD (65%), compared to healthy individuals (33%) and non-IBD controls (37%). Furthermore, all *Campylobacter* spp. prevalence was also found to be higher in children with CD (72%) than the healthy controls (30%) and non-IBD controls (30%) (Man *et al.*, 2010b).
A Scottish study of 100 recruited children, isolated *C. concisus* from 3 intestinal biopsies of children with IBD (2 CD and 1 UC), however no *C. concisus* was isolated from controls. Using the sequence analysis, there was no significant difference in the prevalence of *C. concisus* in IBD patients (38.6%, 17/44) compared with the controls (38.1%, 16/42) (Hansen et al., 2011). However, Zhang et al. (2009) previously found a significant occurrence of *C. concisus* and higher levels of antibodies against *C. concisus* in CD children than controls. The group investigated the possible role of *C. concisus* in newly diagnosed paediatric CD and children with newly diagnosed CD (51%), *C. concisus* DNA \((P < 0.0001)\) and *C. concisus* specific IgG antibody levels \((P < 0.001)\) were significantly higher, compared with controls (2%). Moreover, *C. concisus* strain UNSWCD was first isolated from an intestinal biopsy of a CD child (Zhang et al., 2009). Given these conflicting reports, it is not yet possible to draw a causative link between *C. concisus* and IBD, however it remains a possibility that some strains are more pathogenic than others.

In a study performed by Kamma et al. (2000b), *C. concisus* was detected in low numbers in the deciduous teeth of children, with higher numbers in the molars than incisors teeth. This group investigated the composition of subgingival microbiota in children with primary dentition aged 4-5 years (Kamma et al., 2000b). In the same year, the group published another study on children with mixed dentition aged 7-8 years and *C. concisus* was detected more frequently in permanent than deciduous teeth (Kamma et al., 2000a). Both studies more frequently isolated *C. concisus* from bleeding sites and suggesting an association of colonisation within these sites (Kamma et al., 2000a, Kamma et al., 2000b).

**1.3.4 *C. concisus* in other sources**

*C. concisus* has been reported to be isolated from unusual and systemic sites in rare cases including from a foot ulcer of a patient with diabetes mellitus (Johnson et al., 1987, Johnson
et al., 1985). It was also detected in synovial fluid (SF) samples from patients with reactive arthritis and other forms of post-infectious arthritis using RT-PCR (Cox et al., 2003). In addition, C. concisus was isolated from a 65-year-old male with a history of carcinoma of the right-sided maxillary sinus with intracranial growth using 16S rRNA sequencing analysis. The patient developed a brain abscess due to polymicrobial flora including C. concisus (De Vries et al., 2008). C. concisus has also been isolated from children’s blood samples and the isolation from such sites, which are normally considered to be sterile can justify their clinical significance, therefore further investigations are required (Lastovica et al., 2008, Lastovica et al., 2014).

1.3.5 C. concisus in animals

C. concisus was initially suggested to be adapted in human only (Engberg et al., 2005). However, Petersen et al. (2007) successfully isolated C. concisus from a cat’s saliva samples. Chaban et al. (2010) isolated C. concisus from 9.5% (6/65) of diarrheic dogs. Ninety seven percent of diarrheic dogs and 58% of healthy dogs had detectable levels of Campylobacter spp. including C. jejuni, C. coli, C. concisus and C. showae.

The first isolation of C. concisus from food was in chicken and beef by Lynch et al. (2011), who collected 550 meat samples from chicken, pork and minced beef in a 12 month period. C. concisus was then isolated in 10% of chicken and 3% of beef. The presence of C. concisus in poultry products in this study was considered to be significant (Lynch et al., 2011). Based on these studies, it was suggested that chickens, and not domestic pets, were more likely to be the reservoirs of C. concisus, with infection suggested to be via zoonotic transmission. Moreover, the presence of C. concisus in the human saliva supports a route of transmission from person to person (Kaakoush et al., 2012).
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1.4 Isolation and Identification of *C. concisus*

As *C. concisus* is a fastidious organism its isolation from clinical samples by culture techniques is not always successful. The Cape Town protocol was developed to isolate *Campylobacter* spp. from stool samples. It uses a membrane filter on an antibiotic-free agar plate. The plate is then incubated at 37°C in hydrogen-enriched microaerophilic conditions. This protocol was found to dramatically improve the number of *Campylobacter* and related genera *Arcobacter* and *Helicobacter* isolated from paediatric patients’ stool samples (Lastovica et al., 2002). This protocol was developed by Le Roux et al. (1998) at the Red Cross Children’s Hospital (RXH) in Cape Town, South Africa. When other conventional methods were used, *Campylobacter* spp. were only isolated from 7.1% of paediatric patients’ diarrheic samples. However, the percentage was dramatically increased to 21.8% after using the Cape Town protocol (Le Roux et al., 1998). This protocol has been modified (no filter is used) and shown to be effective for the isolation of *Campylobacter* and related genera from blood, pleural aspirates, and other clinical specimens, and for the isolation of *H. pylori* from gastric biopsy specimens (Lastovica, 2006).

The filtration method has been shown to be more effective for *C. concisus* strains as they normally grow slower than *C. jejuni* strains. Hydrogen-enriched environment is required for *C. concisus* to grow and it grows better at 37°C than at 42°C. Faecal samples or other samples including saliva can be cultured by preparing suspensions of a known dilution (1:2 to 1:10) in PBS (pH 7.4), or in brain heart infusion (BHI) broth. The plate should be incubated under hydrogen-enriched microaerophilic conditions (7% H₂, 7% CO₂ 7% O₂ and the balance of N₂). This method can be performed in two different ways; by using an anaerobic gas pack and an anaerobic jar without a catalyst, or by using the procedure of evacuation replacement, which evacuates the jar to -0.7 bar and re-gassing to the pressure of atmospheric with a gas mixture of 10% hydrogen, 10% carbon dioxide and a balance nitrogen. Three to five days
incubation period is the optimal duration before opening the jar. The colonies of *C. concisus* are small (1-2 mm), round, entire, greyish and semi-translucent. In studies in our laboratory, wet mounts of *C. concisus* are normally prepared in PBS or brain-heart infusion broth and *C. concisus* cells can be seen as very small, slightly curved rods with rapid darting motility under the microscope. *C. concisus* also forms long rods and may also be found in coccoid forms, like other *Campylobacter* spp. (Istivan et al., 2010).

### 1.4.1 Biochemical and conventional identification of *C. concisus*

Conventional phenotypic and biochemical tests can be used to identify *C. concisus* colonies (Table 1.2), including colony morphology, Gram stain, organism motility, oxidase, catalase hydrogen auxotrophy, H$_2$S production, indoxyl acetate hydrolysis, DNase production, susceptibility or resistance to specific antibiotics, hippurate hydrolysis, nitrate reduction and the ability to grow on MacConkey agar (Engberg *et al.*, 2005, Lastovica, 2009, On *et al.*, 1996). *C. concisus* can be differentiated from *C. mucosalis* by the test of sensitivity to cephalothin and nalidixic acid, the ability to grow at 25°C and 42°C, and colony colour (On, 1994). *C. concisus*, isolated in a pure culture from the stool sample of young male suffering from a chronic lymphatic leukaemia was initially misidentified as *C. mucosalis* because the diagnosis was based on the biochemical reactions and susceptibility tests only. It was then recognized as *C. concisus* using a 16S rRNA sequencing (Anderson *et al.*, 1996). Under the electron microscopy, *C. concisus* cells can be seen as curved rods, with a size of 0.5-1 x 2-6 μm and possessing a single polarised flagellum (Figure 1.1) (Zhang *et al.*, 2014). Figure 1.2 shows a gram-stained smear for a 5 day old *C. concisus* culture with short and long pale pink stained curved rods.
Table 1.2 Phenotypic characteristics of *C. concisus*.

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<td>Growth in air +CO₂</td>
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<td>Growth stimulated by formate and fumarate</td>
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<td>Growth stimulated by nitrate</td>
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<td>Benzyl viologen</td>
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<td>Benzidine</td>
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<td>Lysine and ornithine decarboxylase</td>
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<td>Indoxyl acetate hydrolysis</td>
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<td>Arylsulfatase</td>
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<td>Pyrazinamidase</td>
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*A, 95–100% positive; M, 60–93% positive; F, 14–50% positive; N, 0–11% positive; not available, “Trace quantities.

Table, modified from Kaakoush et al. (2012).
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Figure 1.1 Electron microscopy image of *C. concisus*. The bacterium is a slightly curved rod (0.5-1 x 2-6 μm) and has a polar flagellum. Magnification 24000x (Zhang *et al.*, 2014).

Figure 1.2 *Campylobacter concisus* gram-stained smear, showing both the normal short, and the long curved bacterial cells from the same colony. Magnification 1000x (Istivan *et al.*, 2010).
1.4.2 Molecular identification of \textit{C. concisus} strains

1.4.2.1 Grouping of \textit{C. concisus} by 23S rDNA

The molecular typing of \textit{C. concisus} strains using 23S rDNA sequences was initially performed by Bastyns \textit{et al.} (1995b). A PCR assay using 23S rDNA was developed to detect 306 bp in 12 \textit{C. concisus} strains as well as other closely related \textit{Campylobacter} spp. A combination of three primers, a forward primer MUC1 and two reverse primers CON1 and CON2 were used. The forward primer MUC1 was designed as a specific primer for both \textit{C. mucosalis} and \textit{C. concisus} (Bastyns \textit{et al.}, 1995a). The combination of the two reverse primers was used because some strains were undetectable with MUC1-CON1 only. This PCR assay was able to distinguish \textit{C. concisus} strains from other \textit{Campylobacter} spp. including \textit{C. mucosalis} (Bastyns \textit{et al.}, 1995b).

The 23S rDNA PCR was then modified by Istivan \textit{et al.} (2004) using combinations of either MUC1-CON1 or MUC1-CON2 and used to group 19 clinical \textit{C. concisus} isolates from RCH (Melbourne, Australia) as well as two reference strains (ATCC 51561 and ATCC 51562). The study classified these \textit{C. concisus} strains into two different genomospecies, A and B. Six \textit{C. concisus} strains (28.5\%) and the reference strain ATCC 51561 were belonging to genomospecies B, while the majority of the strains (71.5\%) and the reference strain ATCC 51562 were belonging to genomospecies A (Istivan \textit{et al.}, 2004). The sequences of these two reverse primers, CON1 and CON2 were found to be significantly different although both yield a similar-sized PCR fragment (306 bp), there is a major genomic difference within the 23s rDNA nucleotide sequences of two genomospecies where the reverse primers are binding between the sequences as indicated in Figure 1.3. The figure shows the alignment of the 23s rDNA nucleotide sequences between \textit{C. concisus} ATCC51561 representing genomospecies B and
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ATCC51562 representing genospecies A. The figure also indicates the locations where the forward and reverse primers can bind during PCR amplification.

The results of this study support the complex nature of *C. concisus* as they demonstrated two different molecular genotypes (which were named genospecies, A and B), amongst the Australian *C. concisus* isolates with the majority of these isolates belonging to genospecies A (Istivan *et al.*, 2004). Two subsequent studies in Denmark and Canada on faecal samples obtained from diarrhoeic and healthy individuals used the method of Istivan *et al.* (2004), and found that genospecies B isolates were the dominant (67%) while genospecies A isolates composed 33% of the total isolates (Engberg *et al.*, 2005, Kalischuk *et al.*, 2011). It is worth to mention that the distribution of *C. concisus* genospecies can be affected by geographical factors.

**Figure 1. 3 Alignment of 23S rDNA nucleotide sequence of *C. concisus* ATCC 51561 (genospecies B) and *C. concisus* ATCC 51562 (genospecies A), showing the forward primers, MUC1 and 2 reverse primers, CON1 (genospecies A) and CON2 (genospecies B). The forward primers are identical, while there is 9 bp difference in the reverse primers.**
1.4.2.2 Protein profiling of *C. concisus*

The SDS-PAGE protein profiles were performed by Tanner (1986) to differentiate between *C. concisus* and other small asaccharolytic, non-pigmenting gram-negative rods of the human oral cavity, as they were difficult to discriminate from each other. All tested species showed 73% intraspecies similarity using correlation coefficients’ clustering analysis and *C. concisus* showed different protein patterns (Tanner, 1986). In a following study, Vandamme *et al.* (1989) also used SDS-PAGE and found that *C. concisus* protein profiles were diverse (Vandamme *et al.*, 1989). Both studies were in an agreement between the grouping of electrophoresis after a numerical analysis of the protein profiles and the taxonomic groups.

The genetic diversity in the SDS-PAGE protein profiles between faecal and oral *C. concisus* isolates was also investigated by (Aabenhus *et al.*, 2002). Based on the protein patterns, 85% of *C. concisus* faecal strains were different from the *C. concisus* oral reference strain ATCC 33237. The study was then able to allocate the strains into two major groups. The first group included the *C. concisus* strains similar to the *C. concisus* oral type strain ATCC 33237, while the second group included the *C. concisus* strains whose protein profiles were different from that of the *C. concisus* type strain (Aabenhus *et al.*, 2002).

Istivan *et al.* (2004) also used SDS-PAGE to study the outer membrane protein profiles (OMPs) and whole cell lysates of 19 *C. concisus* clinical isolates. Five different protein profiles were found for *C. concisus* outer membrane proteins (OMP) extracts, compared with the *C. mucosalis* reference strain protein profile. At least 3 *C. concisus* protein profiles showed a significant similarity. An identical protein profile was observed with the majority of genomospecies B strains with an exception with the reference strain ATCC 51561 and RCH 14. Genomospecies A *C. concisus* strains were found to be more divergent in their SDS-PAGE protein profile (Istivan *et al.*, 2004).
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The protein profiles of total cell lysates of eight *C. concisus* strains, including UNSWCD and BAA-1457, were also investigated by Kaakoush *et al.* (2011a) using SDS-PAGE. The two strains, UNSWCD and BAA-1457, were previously found to be different in the size of their genome (1.8 Mb and 2.1 Mb, respectively) (Deshpande *et al.*, 2011). The protein profiles of the strains were analysed by Phoretix 1D software, clustering the strains based on their banding profiles. *C. concisus* strain BAA-1457 was found to be highly divergent from all other seven strains with an average similarity of 56.8%, suggesting that this strain is atypical in its nature (Kaakoush *et al.*, 2011a). Based on the studies presented above, *C. concisus* is shown to be a heterogeneous species.

1.4.2.3 Other molecular techniques for *C. concisus* identification

DNA–DNA hybridisation was first used to identify *C. concisus* isolates from diarrheic children by Vandamme *et al.* in 1989. The study confirmed that the faecal *C. concisus* strains exhibit between 42 and 50% of DNA–DNA hybridisation values with the oral strains concluding that *C. concisus* is a genetically diverse species (Vandamme *et al.*, 1989). Given this and according to the guidelines of taxonomy then, On *et al.* (2000) suggested that the diarrheal and oral isolates should be dissimilar, although they may not be phenotypically distinguished. Hence, *C. concisus* should be considered as a “complex species comprising at least two genomospecies” (On *et al.*, 2000)

The genetic diversity of *C. concisus* has also been investigated using Randomly Amplified Polymorphic DNA (RAPD), and showed that *C. concisus* strains isolated from 35 of 37 children with diarrhoea (94.6%) had distinct RAPD profiles. Forty-nine different types of PCR banding patterns were found, while the same DNA pattern was reported with two different children, and other children were observed to harbour unique strains (Van Etterijck *et al.*, 1996). Matsheka *et al.* (2006) also used RAPD analysis with the (GTG)$_5$
oligonucleotide as a primer to type 100 *C. concisus* isolates collected from 2 adults and 98 children with diarrhoea. This method indicated that 86% of the isolates were genotypically different. Twenty-five of these heterogeneous isolates were previously analysed by PFGE and were shown to have distinctive profiles (Matsheka *et al.*, 2002). The remaining strains (14%) could be classified to five distinct RAPD profiles (Matsheka *et al.*, 2006).

Matsheka *et al.* (2002) used Pulsed Field Gel Electrophoresis (PFGE) with the *Not* I restriction enzyme to assess the *C. concisus* genetic diversity of 53 strains isolated from faecal samples from children with gastroenteritis. Ninety-six percent of the *C. concisus* strains (51/53) were shown to have different *Not* I macro-restriction fragments, whereas the other 2 strains were resistant to the *Not* I digestion. Between 1 and 14 restriction fragments comprised the patterns, with type and reference strains of two well-defined genomospecies of oral and faecal isolates comprising 6 and 12 fragments, respectively (Matsheka *et al.*, 2002).

Soon after the previous study Petersen *et al.* (2007) developed a PCR-DGGE method, as a semi-nested PCR that allows a sensitive detection of Epsilobacteria, with separation of species using DGGE to detect and identify Epsilobacteria including *Campylobacter, Helicobacter* and *Arcobacter* species in saliva samples from humans, dogs and cats. This PCR-DGGE method was able to identify *C. concisus* in all human saliva samples (11/11) and in only one cat’s saliva sample (Petersen *et al.*, 2007).

The genotype of 62 *C. concisus* clinical isolates, 56 diarrheal strains, 4 oral strains, the oral type strains CCUG 13114, and the intestinal type strain CCUG 19995, were investigated using amplified length fragment polymorphism (AFLP) analysis method (Aabenhus *et al.*, 2005a). All tested strains were shown to have unique profiles; however, strains were allocated into four different clusters by phylogenetic tree which were suggested to be four distinct genomospecies, which could be linked to their pathogenic potential (Aabenhus *et al.*, 2005a).
Kalischuk et al. (2011) also used AFLP analysis to compare the genotypes of 23 different *C. concisus* isolates, from diarrheic and asymptomatic healthy individuals. Isolates were found to belong to one of two phylotypic clusters; with 34% similarity between each other. All AFLP cluster 1 isolates belonged to genomospecies A and included LMG7788 type strain. AFLP cluster 2 assigned 17 *C. concisus* isolates, a single isolates belonged to genomospecies A (5.9%), 12 isolates (70.6%) belonged to genomospecies B, 3 isolates were assigned to both genomospecies A and B (17.6%), and the last isolate did not fit into any of the previous groups. Although, these AFLP results contradicted what was previously shown in the study by Aabenhus et al. (2005a), the majority of those *C. concisus* isolates (90.3%) were classified into two main clusters (Kalischuk et al., 2011).

Kaakoush et al. (2014a) performed Real-time PCR to determine the levels of *C. concisus* DNA and exotoxin 9/Dnal (a putative virulence factor) in faecal samples of CD patients and healthy controls and to link these levels with the abundances of microbial genera identified in the same subjects. *C. concisus* DNA was detected in faecal samples of CD patients (83.3%) and healthy controls (76.7%). However, the level of *C. concisus* DNA was 145-fold more common in faecal samples from CD patients than healthy controls. The level of exotoxin 9/Dnal was also higher in CD patients, ~18-fold than in healthy controls. These findings suggested that CD patients had a higher abundance of *C. concisus*, and that their strains may be more virulent. The correlation between *C. concisus* levels and exotoxin 9 levels was only found in CD patients, which suggested that healthy controls may be colonised with non-virulent *C. concisus* strains (Kaakoush et al., 2014a).
1.5 *C. concisus* as a potential pathogen

1.5.1 The possible role of *C. concisus* in oral diseases

Several studies on *C. concisus* suggested the possible role of this bacterium in gingivitis and periodontitis. Moore *et al.* (1987) performed a study to compare the subgingival bacterial flora in children and adults with gingivitis, and in healthy controls and subjects with different levels of periodontitis. *C. concisus* was more frequently isolated from children and adults with gingivitis, compared with healthy controls or subjects with periodontitis (Moore *et al.*, 1987). Haffajee *et al.* (1984) also reported the association between the level of *C. concisus* with a gain in tooth attachment in a person with periodontitis, while Tyrrell *et al.* (2003) successfully isolated *C. concisus* from a patient with halitosis. The isolation rate of *C. concisus* in individuals suffering from initial periodontitis (68%) was significantly higher than in healthy individuals, those with gingivitis, or established periodontitis, 35%, 32%, 32%, respectively (Macuch *et al.*, 2000).

The immune response to *C. concisus* in mouth disease and controls has also been investigated. Ebersole *et al.* (1985) studied systemic antibody levels to several oral microorganisms involved in periodontal diseases including *C. concisus*. Level of antibody to *C. concisus* were found to be higher in subjects with periodontal diseases than healthy subjects, confirming what was seen in the previous study (Taubman *et al.*, 1992). *C. concisus* was also found to be significantly associated with the bleeding sites of the permanent teeth (Kamma *et al.*, 2000a, Kamma *et al.*, 2000b), supporting a previous study by the same group, indicating that *C. concisus* was associated with bleeding in young adults with periodontitis (Kamma *et al.*, 1994, Nakou *et al.*, 1998). The incidence of *C. concisus* was also significantly higher in smokers than non-smokers (Kamma *et al.*, 1997, Kamma *et al.*, 1999).
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Although there are several reports on the detection and isolation of *C. concisus* from people with periodontal disease, the evidence to support the role of *C. concisus* in oral disease is limited, as most of those reports only concentrated on isolates of this bacterium from the diseased oral sites, rather than from healthy controls. Therefore, it is still unclear whether *C. concisus* is just part of the normal flora, an opportunistic pathogen or an oral pathogen (Kaakoush *et al.*, 2012).

1.5.2 The possible role of *C. concisus* in gastroenteritis

The role of *C. concisus* in gastrointestinal diseases has been more widely investigated as compared to its role in oral diseases. The link between *C. concisus* and IBD in human has been reported. *C. concisus* has a significantly higher prevalence in intestinal biopsies and stool samples collected from IBD patients compared to controls (Mahendran *et al.*, 2011, Man *et al.*, 2010b, Mukhopadhy et al., 2011, Zhang *et al.*, 2009). *C. concisus* has been frequently detected in, or isolated from, diarrheal stool samples (Aabenhus *et al.*, 2002, Engberg *et al.*, 2000, Lastovica, 2006, Nielsen *et al.*, 2013).

*C. concisus* was also isolated from faecal samples of children with enteritis, some of them suffering from diarrhoea. The study suggested that this species can cause diarrhoea, specifically in immunosuppressed patients (Van Etterijck *et al.*, 1996). However, no significant difference was found between *C. concisus* isolation rates from diarrheal and non-diarrheal faecal samples (Engberg *et al.*, 2000, Van Etterijck *et al.*, 1996). *C. concisus* was detected in 9% of dog’s diarrheal stool samples and 0% in non-diarrheal samples using PCR. The role of *C. concisus* in diarrheal disease remains unclear (Chaban *et al.*, 2010).

Newly diagnosed CD children in Australia were reported to be significantly positive (82%) for *Campylobacter* as compared with 23% of controls by PCR. *C. concisus, C. showae, C. hominis, C. gracilis, C. rectus, C. ureolyticus*, and *C. jejuni* were detected in CD patients,
however the prevalence of *C. concisus* was significantly higher in patients with CD (51%) compared with controls (2%). This is the first report of association between *C. concisus* and paediatric CD (Zhang *et al.*, 2009).

A recent study by Hansen *et al.* (2013) did not successfully detect a significant difference in the prevalence of *C. concisus* and all *Campylobacter* spp. DNA in cohorts of children with UC and controls. The prevalence in the UC children was 30.8% (4/13) and 69.2%, respectively, while it was 38.1% (16/42) and 76.2%, respectively in the controls (Hansen *et al.*, 2013). These results suggest that there is no role of *C. concisus* in paediatric UC, however further studies are required to confirm these observations in larger groups of paediatric patients (Kaakoush *et al.*, 2014c).

Von Rosenvinge *et al.* (2013) studied the microbiota of human stomach fluid and *C. concisus* was found to be highly active (444% increase in transcriptomic activity) within the gastric fluid. In addition, the study stated that the presence of *H. pylori* was not associated with significant differences in *C. concisus* levels (Von Rosenvinge *et al.*, 2013). In a study to investigate the prevalence of *C. concisus* in Barrett’s esophagus patients, *C. concisus* was isolated from 57% of esophageal biopsies and but not from the controls (Macfarlane *et al.*, 2007). Further studies are required to determine whether this bacterium can play a role in the development of Barrett’s esophagus (Lastovica *et al.*, 2014). The pathogenic potential and virulence mechanisms employed by *C. concisus* are of significant interest in an association with IBD. Although *C. jejuni* is the most investigated species of all *Campylobacter* spp., it is predicted that *C. concisus* shares some similar mechanisms of *C. jejuni* pathogenesis (Kaakoush *et al.*, 2014c).
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1.5.3 C. concisus and Antimicrobial Susceptibility

Few studies have been performed to determine the antimicrobial susceptibility of C. concisus and other non-jejuni and -coli campylobacters. Twenty C. concisus isolates were tested for antimicrobial susceptibility using Etest to determine the minimum inhibitory concentrations (MICs) of six antimicrobial agents, though other methods were previously performed such as disk diffusion and broth microdilution. Due to the limited data on standardised susceptibility for Campylobacter spp., only two MICs were described, being MIC_{50} and MIC_{90}, as well as the percentages and ranges of resistance of the six antibiotics tested. Both MIC_{50} and MIC_{90} represent the MICs that completely inhibited the visible growth of 50% and 90% of the strains, respectively. The Clinical Laboratory Standards Institute (CLSI) does not recommend to include specific breakpoints for defining resistance in Campylobacter spp. Therefore, this study used CLSI criteria of Staphylococcus spp. for erythromycin and tetracycline on Campylobacter spp. Among the different Campylobacter spp. strains, 95% were found to be susceptible to erythromycin and ciprofloxacin (0.25 mg/L MICs). However, 80% were found to be resistant to nalidixic acid with 32 mg/L MICs. C. concisus and C. fetus isolates were susceptible to ampicillin, gentamicin and tetracycline with 5% of C. concisus been resistant to gentamicin, ciprofloxacin and erythromycin. As such, it was suggested that erythromycin be the recommended treatment for campylobacteriosis caused by C. concisus and C. fetus during severe intestinal infections (Vandenberg et al., 2006). A comparable study on C. concisus was performed in Denmark by Aabenhus et al. (2005b) using the E-test method and reported that among 109 tested C. concisus isolates, there were some with resistance to ampicillin (2%), tetracycline (3%), ciprofloxacin (5%) and erythromycin (7%) (Aabenhus et al., 2005b).
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In another study thirty-four *C. concisus* isolates were tested with eleven different antibiotics. All of them were found to be completely susceptible to all tested antibiotics, including ampicillin, tetracycline, gentamicin, ciprofloxacin, erythromycin and nalidixic acid (Engberg *et al.*, 2005). However, *C. concisus* was always reported to be completely resistant to nalidixic acid (Vandamme *et al.*, 1992). Vandenberg *et al.* (2006) reported that 80% of *C. concisus* isolates were found to be resistant to nalidixic acid, which was a similar to what was found by Van Etterijck *et al.* (1996) who found 51% of *C. concisus* isolates were resistant in Belgium. Another study stated that *C. concisus* was sensitive to three antimicrobial agents: amoxicillin, metronidazole and clindamycin, with E-test MICs of 0.500 mg/L, 1.500 mg/L, and 1.500 mg/L, respectively (De Vries *et al.*, 2008).

As infections with *Campylobacter* spp. are mostly self resolving, and only a few studies have been performed on *C. concisus* treatments. Erythromycin is currently the first choice for the initial treatment of intestinal campylobacteriosis caused by *C. concisus* and *C. fetus* (Skirrow, 1990, Vandenberg *et al.*, 2006). Rifaximin has been suggested to be effective with *Campylobacter* species without side effects (Guslandi, 2009). Rifaximin works to inhibit bacterial RNA and then protein synthesis by blocking the enzymatic activity of bacterial DNA-dependent RNA polymerase and is effective against both gram-negative and gram-positive bacteria (Scarpignato *et al.*, 2005).

### 1.6 Virulence factors in *Campylobacter* spp.

Several virulence factors have been studied in *Campylobacter* spp., especially in *C. jejuni*. Cytolethal distending toxin (CDT) is produced by some gram-negative bacteria including *Campylobacter* spp. and is considered to be a virulence factor (Smith *et al.*, 2006). The toxin can cause eukaryotic cells to arrest in the G2/M phase of cell cycle, which may lead to
apoptosis (Pickett *et al.*, 1996). It was demonstrated that CDT(+) *C. jejuni* strains were able to adhere and invade epithelial cells more efficiently than CDT(-) strains (Jain *et al.*, 2008).

Several other toxins have been identified in *Campylobacter* spp. It was found that *orf6* gene has a role in *C. jejuni* 81116 toxin production, and contains an RTX toxin-activating protein C signature. Orf6 protein may only be involved in *C. jejuni* cytotoxin activation rather than being an active toxin (Liu *et al.*, 2007). An RTX toxin was also found in *C. rectus*, *C. concisus* and *C. ureolyticus* (Burgos-Portugal *et al.*, 2012, Gillespie *et al.*, 1993, Kaakoush *et al.*, 2010). RTX proteins are pore-forming toxins synthesised by several Gram-negative bacteria. RTX-mediated cytotoxicity has two phases; which are a membrane insertion phase and adsorption onto the target cell surface (passive phase) (Lally *et al.*, 1999). Gatsos *et al.* (2012) examined CHO cell-active, Vero cell-inactive cytotoxin that may mediate the inflammatory diarrhoea due to *C. jejuni*, by one of six candidate proteins (Cj0114, Cj0289c, Cj0778, Cj0834c, Cj0998c and Cj1240c) that have potential cytotoxic activity (Gatsos *et al.*, 2012). The capsular polysaccharide (CPS) was found to play an important role to protect *C. jejuni* against host defence. Some studies suggest that a CPS-deficient *C. jejuni* mutant lacking the *kpsM* gene was unable to resist against serum killing (Bacon *et al.*, 2001, Maue *et al.*, 2013).

Biofilm formation is one of many survival mechanisms used by microorganisms to resist biological, environmental and physical stresses and also to persist and sustain in a several range of ecological niches. Biofilms may have an important role in infections caused by *Campylobacter* spp. (Kaakoush *et al.*, 2014b). Some researches on *C. jejuni* biofilm formation have focused on how *C. jejuni* can survive within a biofilm state. A study on *C. jejuni* survival within the biofilm suggested that this bacterium can persist for an extended period of time through localised biofilms formed in the water (Buswell *et al.*, 1998). Joshua
et al. (2006) reported that microaerobic bacteria in flocs (unattached aggregates) can survive for long periods, up to 24 days at room temperature, while planktonic bacteria can survive half of this period. Thus, the biofilm growth mode can provide protection against the environmental stresses (Joshua et al., 2006).

Other Campylobacter spp. have also been shown to form biofilms on glass, stainless steel and polystyrene including C. concisus, C. curvus, C. rectus, C. showae, C. mucosalis and C. gracillis. C. upsaliensis, C. sputorum, C. hyointestinalis and C. helveticus were found to form biofilms on stainless steel. The biofilm formation of eight microaerophilic Campylobacter spp. including C. jejuni, C. coli, C. lari, C. fetus, C. hyointestinalis, C. upsaliensis, C. sputorum and C. helveticus was tested. Of these, C. jejuni 81-176 was the only strain that produced a visible biofilm on several surfaces. Six Campylobacter spp. that can anaerobically grow including C. concisus, C. curvus, C. rectus, C. showae, C. mucosalis and C. gracillis also produced a visible biofilm on multiple surfaces. On the other hand, C. coli ATCC 33559 did not form biofilms on plastic, steel or glass (Gunther et al., 2009).

1.6.1 Motility as a virulence factor in Campylobacter spp.

C. jejuni has two different viscosity-dependent modes of motility into caecal mucosal scrapings from mice (Lee et al., 1986). C. jejuni was found to rely on its flagellum for propulsion at low viscosity, like any other flagellated bacterium. However, it is likely that the spiral morphology is important in concert with changes in flagellar conformation and/or rotation, when the viscosity is high (Ferrero et al., 1988). The swimming direction of C. jejuni is reversed in a back and forth pattern with a relatively short straight moving path, in high viscosity media (Shigematsu et al., 1998). Therefore, the two types of motility could play a role in the different regions of the mucus layer of gastrointestinal tract. Viscosity is thought to increase between the lumen and the epithelial surface. Darting motility may raise
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the number as well as the time of contacts between *C. jejuni* and the epithelium, thus increasing the adherence to or invasion of those cells (Szymanski *et al.*, 1995). Colonisation of the gut mucosa is based on the ability of the bacteria to attach to gut epithelium and the rapid motility, with the campylobacter spiral shape allowing the bacterium to penetrate the mucus layer of intestinal cells. The flagella are very important as virulence factors, as aflagellate bacteria cannot colonise cells *in vivo* (Aguero-Rosenfeld *et al.*, 1990).

The flagellum of *Campylobacter* spp. includes two homologous flagellins, FlaA and FlaB. The FlaA is very important for adherence, colonization of GIT, and invasion. *C. jejuni* seems to have fimbriae-like elements and cell surface proteins (PEB1, CadF), which help to function in attachment, colonisation and invasion (Dolg *et al.*, 1996). The invasive antigen (Cia) of *Campylobacter* spp. is also secreted through the flagellum and used to facilitate motility (Dasti *et al.*, 2010). FlaA was found to be important for flagellin and motility, however, FlaB requirement varies between species (Josenhans *et al.*, 1995, Suerbaum *et al.*, 1993). *C. jejuni* flagella can export the Cia and it is delivered to the host cells cytosol. *Campylobacter* is then able to attach to the epithelial cells (Larson *et al.*, 2008, Neal-Mckinney *et al.*, 2012). The importance of the flagellum in invasion of host cells has previously been reported and aflagellated *C. jejuni* mutants have a significantly reduced internalisation into host cells *in vitro* (Wassenaar *et al.*, 1999).

The flagellum of *C. jejuni* (Figure 1.3) involves three major parts including a basal body, a filament and a hook. FlaA, FlaB, which are the components of the flagellin filament, are O-linked glycosylated proteins. In *C. jejuni*, the flagella system is composed of the major flagellin (FlaA) and the minor flagellin (FlaB) (Logan *et al.*, 1987). Any mutation in the flaA gene may lead to a truncated flagellum, loss of motility and loss of the ability to colonise in human and animals (Wassenaar *et al.*, 1991), and an ability to invade the intestinal cells *in
vitro (Yao et al., 1994). C. jejuni with mutations in the flagellin gene flhB is affected in motility and flagellin expression. Therefore, this gene is important for C. jejuni flagellar biogenesis (Matz et al., 2002). Studies using knock-out mutants have shown that the stator elements, motA and motB were found to be responsible to obtain the energy for motility (Dean et al., 1984). Sigma28 is included in the transcription of a small subset of the major flagellin gene (flaA). Sigma54 is however, involved in the transcription of many genes encoding the flagellar rod, basal body, and the minor flagellin (flaB) (Jagannathan et al., 2001).

Some flagellin genes have been found to have important roles in the bacterial biofilm formation. For instance, Kalmokoff et al. (2006) showed that an aflagellate flhA mutant was found that it could not form the pellicle liquid culture interface and it also was no longer able to attach to a solid matrix and produce biofilm formation. The delay in pellicle formation was also found to be due to inactivation of some genes that known to affect the flagellar filament such as flaA, flaB, and flaG or that affect the adhesion like flaC (Kalmokoff et al., 2006). This was also supported by a study performed by Reeser et al. (2007), which indicated that C. jejuni flaA/B and luxS mutants were dramatically reduced in the ability to produce the biofilm formations compared to the wild-type. This suggested that flagella have a very important role in the optimal production of biofilm (Reeser et al., 2007). Using electron microscopy, the flagella were also found to behave as connectors between individual bacteria (Moe et al., 2010).

1.6.1.1 The structure of flagellum

The motility system of Campylobacter spp. includes the flagellum and the chemosensory system, which influences flagellar rotation in response to environmental conditions. Between 25 and 30 conserved proteins constitute the flagellum with additional factors required for
synthesis or secretion of these components. The chemosensory components are less conserved and can include several chemoreceptors and signalling proteins. Overall, 40 to 100 different proteins are essential to form a rotating, chemoresponsive flagellum (Lertsethtakarn et al., 2011). The description of flagellar structure and biosynthesis of *Campylobacter* and *Helicobacter* species are similarly described for well-studied systems of *Salmonella* species and *E. coli* (Chevance et al., 2008, Macnab, 2003, Minamino et al., 2008). Two main broad substructures divide the flagellum: the hook-basal body (HBB) complex and the extracellular filament. The HBB complex is also divided into three structures, these are located at the cytoplasm and inner membrane, the periplasmic rod and associated ring structures, and the surface-localized hook (Lertsethtakarn et al., 2011). FlaA and FlaB have been found to be included in the flagellum of *Campylobacter* and FlaA is more important for motility (Dasti et al., 2010). The hook of *Helicobacter* contains FlgE protein (O'toole et al., 1994).

The base of flagella contains the motor, the cytoplasmic C ring or switch complex, the flagellar type III secretion system (T3SS), and the MS ring. The MS ring is a homomultimer of FliF with a functional flagellar T3SS, composed of FlhA, FlhB, FliO, FliP, FliQ, and FliR within the central membrane patch of the ring (Figure 1.3). The flagellar T3SS also export some proteins that are part of flagellum beyond the inner membrane. The C ring functions as the switch of flagellum and helps in secretion. Together, FliG, FliM, and FliN or FliY form this complex, though Epsilonproteobacteria include both FliN and FliY (Lowenthal et al., 2009). FliG was found to be a responsible for the switch rotary component, turning the flagellum either clockwise or counter-clockwise (Sarkar et al., 2010). FliY may have 2 functions, sharing homology with FliN and has a partially redundant function in *H. pylori* (Lowenthal et al., 2009). The base motor components include MotA and MotB, which
work together to link proton flow and torque generation for flagellar rotation (Minamino et al., 2008).

The first rod secreted proteins are FliE, FlgB, and FlgC. These proteins polymerise on the periplasmic surface of the MS ring to form a hollow tube. Other secreted proteins like FlgF and FlgG rod proteins can also polymerise on the tip of the developing flagellar structure (Lertsethtakarn et al., 2011). The P ring in the peptidoglycan and the L ring in the outer membrane are formed by FlgI and FlgH. These rings can form around the rod, which likely then support these cell envelope components (Kamal et al., 2007, Ryan et al., 2005). In Salmonella species, FliK, is not only responsible to switch export from hook to filament proteins, but also works to controlling hook length (Minamino et al., 1999). FliK also works to measure the hook length. Simultaneous contact of FliK with FlgD through the N and C termini, which presents at the tip of the growing hook and also with FlhB at the flagellar base, indicating that an ideal length hook has formed (Erhardt et al., 2010).
Figure 1. 4 Major components of the flagellar structure *C. jejuni* and *H. pylori*. The components are coloured based on the respective genes classification in the transcriptional regulatory cascade for the organisms. The proteins encoded by genes whose transcription was not analysed or is still known to be outside the flagellar transcriptional hierarchy are coloured as grey (Lertsethtakarn *et al.*, 2011).
1.6.1.2 C. concisus motility genes

To date, there are limited data about C. concisus motility. Flagellin A has never been studied in C. concisus. There are some similarities between the flagellar systems of C. jejuni and C. concisus. The system of C. jejuni flagellar secretion has a significant role in its virulence (Kaakoush et al., 2010), and this may also be the case for C. concisus. Nuijten et al. (1990) have identified two flagellin genes, flaA and flaB of C. jejuni 8116 (Nuijten et al., 1990). The group also identified the presence of at least four distinct immunogenic regions of flagellin proteins of C. jejuni (Nuijten et al., 1991).

Kaakoush et al. (2010) used proteomics, mass spectrometry (MS) and comparative bioinformatics to predict putative virulence factors of C. concisus. Two motility secreted proteins have been identified: flagellin B encoded by ccc13826_2297 and the flagellin-like protein FlaC, encoded by ccc13826_2187 (Kaakoush et al., 2010). FlaB is included in the immunoreactive proteins detected in 10 C. concisus-positive CD patients. It is therefore one of the predominant antigens recognised by all patients’ sera, including FlaB, ATP synthase F1 alpha subunit, and OMP18. The sequence of C. concisus flaB has been compared with other members of Campylobacters, with 83% identity with C. curvus, 78% with C. rectus, 60% with C. lari and 57% with both H. pylori and C. jejuni (Kovach et al., 2011).

The flaC gene is conserved in several Campylobacter spp. including C. jejuni, C. coli, C. lari and C. upsaliensis. C. jejuni NCTC 11168 has a conserved flaC gene. A homologous sequence of flaC is also present in C. coli, C. lari and C. upsaliensis. C. coli strains tested also secrete a FlaC-like protein. The flaC gene appears to have evolved in Campylobacter spp., after the divergence of Helicobacter. The motility of flaC mutant has been compared with wild type, flaA and flgF mutant, and the motilities of a flaC and the wild type cells are similar, while a flgF mutant was non-motile (Song et al., 2004). A FlaC orthologue is not
present in *H. pylori* (Parkhill et al., 2000). *flaA* mutants were previously reported to have low motility (Guerry et al., 1992). The *flaC* gene does not appear to be essential for the formation of the flagellum or motility, and a *flaC* null mutant of *C. jejuni* TGH9011 maintained ability to adhere to HEp-2 cells, but was defective in cellular invasion capability (Song et al., 2004). *C. concisus* UNSWCD secreted flagellin-like protein FlaC, encoded by ccc13826_2187 (Kaakoush et al., 2010). Moreover, Holo-(acyl carrier protein) synthase, encoded by ccc13826_2069 interacts with FlaC, a flagellin-like protein. It was suggested that the presence of virulence factors in *C. concisus* secreted proteins, interact with the host cell more readily than membrane-bound proteins, providing supporting evidence to suggest that *C. concisus* is a gastrointestinal tract pathogen (Kaakoush et al., 2010).

### 1.6.1.3 Mechanism of *C. concisus* motility

The *C. concisus* flagellum is important for motility and can be used to mediate adhesion host cells. For instance, *C. concisus* uses its polar flagellum to adhere to host microvilli (Kaakoush et al., 2011b, Man et al., 2010a). Lavrencic et al. (2012) tested the motility of 8 *C. concisus* strains by growing them on solid agar and liquid media, all were found to be motile and five strains (UNSWCD, UNSW1, UNSW2, UNSW3 and UNSWCS) had a higher level of motility than the other three strains (BAA-1457, ATCC 51561 and ATCC 51562) (Lavrencic et al., 2012). Four of the five high motile strains (UNSWCD, UNSW1, UNSW2 and UNSW3) were previously isolated from chronic gastroenteritis patients, while one high and two lower motile strains (UNSWCS, BAA-1457 and ATCC 51562) were from acute gastroenteritis patients. One strain from a healthy individual (ATCC 51561) had a low motility level (Kaakoush et al., 2011b). These findings suggested that the motility level might have an effect on the outcome of disease. However, other pathogenicity factors may play a role in the virulence (Lavrencic et al., 2012).
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The motility of *C. concisus* was shown to increase when the bacterium was cultured in bacteriological media with a high viscosity (concentration of 20.0 viscosities and 74.7 centipoises). This may contribute to their ability to adapt toward the viscosities present in the outer and inner mucus layers of the colon (Lavrencic *et al.*, 2012). The following figure (Figure 1.4) shows some similarities and differences between the pathogenic potential of *C. jejuni* and *C. concisus*. To date, the known mechanisms of *C. concisus* pathogenesis include adherence, invasion, motility, biofilm formation intracellular survival and toxin production (Kaakoush *et al.*, 2014c).
Figure 1.5 Similarities and differences in the biology and pathogenic potentials between the established pathogen *C. jejuni* and *C. concisus*. RBC, red blood cell; T4SS, type IV secretion system; T6SS, type VI secretion system; TLRs, toll-like receptors; TLR5, toll-like receptor 5 (Kaakoush *et al.*, 2014c).
1.6.2 Adhesion and invasion as virulence factors in *Campylobacter* spp.

A number of adhesion and invasion related factors have been identified in *Campylobacter* spp. The plasmid-encoded *virB11* gene is a marker associated with the virulence of *Campylobacter* spp. (Bacon *et al.*, 2002). A mutation in one of the four genes in this plasmid (the plasmid – the *virB11* homologue genes) can lead to a 6-fold decrease in adherence as well as an 11-fold decrease in invasion compared with the wild-type strain. The *cj0588* gene was also found to encode a protein included in the adherence to the human epithelial Caco-2 cells (Salamażynska-Guz *et al.*, 2008). Motility is also needed for invasion of intestinal epithelial cells *in vitro* and for increasing adherence and invasion, under viscous conditions (Szymanski *et al.*, 1995). Studying using well-defined mutants indicated that *flaA* is not essential for adhesion; however it is necessary for invasion (Grant *et al.*, 1993). As discussed, motility and the spiral shape are considered important virulence factors *in vivo*.

Attachment to the epithelial surfaces is very important for the bacteria as it assists with invasion, translocation through intestinal barrier, and induction of damage to the host cells. Flagella play an important role during *Campylobacter* spp. attachment (Everest *et al.*, 2002). A number of *C. jejuni* adhesions were found to mediate attachment to the host cells. JlpA, CadF and FlpA are known to be receptors of host cells (Rubinchik *et al.*, 2012). Flagella proteins were determined to mediate adhesion in order to invade intestinal cells (Mcsweegan *et al.*, 1986). *C. jejuni* with *flaA* disruption (No flagellum, non-motile) had weaker in invasion and adhesion capability (0.5% and 1.5% of the level relative to the wild-type, respectively) (Yao *et al.*, 1994). Another study showed that the mutant strain of *C. jejuni* NCTC 11186 (−*flaA*, +*flaB*) showed a reduction in the level of invasion of INT-407 cells (Wassenaar *et al.*, 1991). Another study performed by Konkel *et al.* (2004) indicated that *C. jejuni* with mutations in *flgB* or *flgC*, *flgE2* or both *flaA* and *flaB* were unable to secrete CiA.

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proteins (invasion antigens) of *Campylobacter* (Konkel *et al.*, 2004). These finding suggest that *C. jejuni* flagella could mediate the adhesion protein secretion (Kaakoush *et al.*, 2014b). Several proteins were found to have important roles in *C. jejuni* attachment such as flagellin, JipA, CadF, FlpA, PEB1, PEB3, PEB4, CapA, CapB, Cj1349, Cj0091, TlyA and P95 (Rubinchik *et al.*, 2012). A study on a *C. jejuni* mutant with no PEB1 showed that this strain of *C. jejuni* had reduced capacity to attach to HeLa cells (50-100 times less) compared with the wild-type strain (Pei *et al.*, 1998). Several *Campylobacter* spp. were found to be capable of internalisation by host cells by a transcellular mechanism; meaning they can invade the epithelial cells and transcytose through intestinal barrier. Moreover, *Campylobacter* spp. were also found to be internalised by host cells by invasion of monolayers across a paracellular mechanism, meaning they can translocate through the intestinal barrier between two adjacent epithelial cells (Kaakoush *et al.*, 2011b, Man *et al.*, 2010a, Nielsen *et al.*, 2011).

Everest *et al.* (1992) used the Caco-2 cell line model to study the adhesion and invasion of intestinal epithelia by *C. jejuni* and *C. coli* clinical isolates. The tested strains were from two clinical groups of patients, suffering from either colitis or watery (non-inflammatory) diarrhoea. All *C. jejuni* and *C. coli* strains were able to adhere to Caco-2 cells. There was a correlation between the invasion of Caco-2 cells and the isolation of the strain from patients with “non-inflammatory” disease. It was suggested that these strains were isolated from the patients with colitis but the inflammatory cells in stool were missed or absent at the time of diagnosis. Isolation of non-invasive strain from non-inflammatory diarrhoeic patients could be a reflection of strain differences. Furthermore, the correlation between the invasion ability and colitis supports the role of invasion in the pathogenesis of *Campylobacter* spp. (Everest *et al.*, 1992).
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Both *in vivo* and *in vitro* studies show that *C. jejuni* is able to invade the intestinal cells and then damage the tissue in infected humans (Van Spreeuwel *et al.*, 1985). Additionally, *in vitro* studies showed that *C. jejuni* can also invade several intestinal cell lines such as non-polarised INT-407, HEp-2, MKN-28 or Caco-2 (Krause-Gruszczynska *et al.*, 2011, Novik *et al.*, 2010, Oelschlaeger *et al.*, 1993). The invasion ability of the *C. jejuni* 11168H wild-type and the non-motile *C. jejuni* 11168H *rpoN* mutant was analysed. The *C. jejuni* 11168H *rpoN* non-motile mutant was able to interact with and invade Caco-2 in significantly lower numbers of bacteria than the wild-type strain under both microaerobic and aerobic conditions. The study suggested that motility is very important for the interaction and invasion of *C. jejuni*, especially in early stages (Mills *et al.*, 2012).

It was shown that invasion antigen B (*ciaB*) gene has an important role in *C. jejuni* invasion in host cells and a null mutation in *ciaB* showed a non-invasive phenotype (Konkel *et al.*, 1999). Neal-Mckinney *et al.* (2012) reported that CiaC is also required for optimal invasion of host cells by *C. jejuni*, and induces cytoskeletal rearrangement of host cells (Neal-Mckinney *et al.*, 2012).

*C. coli* was also found to invade several human cell lines such as HeLa, T84, and Caco-2 cells, and also the monkey kidney epithelial cell line, Vero cells (Malagon *et al.*, 2010, Zheng *et al.*, 2006, Zheng *et al.*, 2008). *C. coli* was reported to be significantly more invasive at a lower temperature, although it is able to grow and move at 42ºC (Aroori *et al.*, 2013). Arce *et al.* (2010) found that *C. rectus* was able to invade human and murine trophoblastic cells. Moreover, the expression of pro-inflammatory cytokines IL-6 and TNF-α were significantly up-regulated. This in combination with the capability to invade trophoblastic cells, provides a link to the adverse pregnancy outcomes connected with human oral diseases including periodontitis (Arce *et al.*, 2010).
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*C. fetus* is able to invade and then exit from Caco-2 cells, and can translocate in an apical-to-basolateral and a basolateral-to-apical direction through the monolayer of Caco-2, within one day, with no any alteration in the permeability of the monolayer (Baker et al., 2010). On the other hand, *C. hominis*, *C. showae* and *C. ureolyticus* were found to attach to, but not invade Caco-2 intestinal cells (Man et al., 2010a). Based on these findings, it was suggested that not all campylobacters are able to invade. The nature of adhesion and invasion exhibited by some species implies that adhesion and invasion are used by *Campylobacter* spp. members to colonise and persist in the host GIT (Kaakoush et al., 2014b).

### 1.6.2.1 Adhesion and invasion of *C. concisus*

Kaakoush et al. (2010) found that CadF (*Campylobacter* adhesion to fibronectin) is secreted by *C. concisus* 13826, although the precise proteins involved in *C. concisus* invasion and attachment are still unclear (Kaakoush et al., 2010). CadF and other proteins like JIpA and FlpA have been proposed to be used by *C. jejuni* to attach the host cells (Rubinchik et al., 2012).

Both transcellular and paracellular invasion mechanism have been described for *C. concisus*. Bacterial cells penetrating the epithelial cell membrane usually induce a membrane ruffling effect on entry and were found to be included in the invasion by transcellular mechanisms. Electron microscopy studies have revealed that *C. concisus* prefers to attach and congregate at the intercellular junction of the Caco-2 cells or near these cells (Kaakoush et al., 2011b, Man et al., 2010a, Nielsen et al., 2011).

Man et al. (2010a) investigated the invasion of several *C. concisus* strains including *C. concisus* UNSWCD, UNSWCS, ATCC 51561 and ATCC 51562 strains. Using scanning electron microscopy, it was shown that the initial contact of *C. concisus* UNSWCD strain with the host cells was initiated by flagellum-microvilli interaction. The flagellum can attach
to the microvillus and wrap around it, allowing the bacterium to attach and anchor to the epithelial cell. Bacteria then invaded the host cells from the non-flagellated end. *C. concisus* was able to invade Caco-2 cells, create the membrane effect on the epithelial cells, and damage barrier functions using preferential attachment at the cell-cell junction. *C. concisus* UNSWCD (from CD patient) was the most invasive tested strain against Caco-2 cells (0.14% ± 0.04% SEM), and was 46 and 201 times higher than *C. concisus* UNSWCS and *C. concisus* ATCC 51562 levels, respectively. *C. concisus* ATCC 51561 did not invade Caco-2 cells (Man *et al.*, 2010a).

Kaakoush *et al.* (2011b) also studied the same *C. concisus* strains with 4 other *C. concisus* strains to examine the adherence to and invasion of the epithelial cell line Caco-2. *C. concisus* UNSWCD was the most efficient within the 3 CD strains, followed by UNSW3 and then UNSW2 (Kaakoush *et al.*, 2011b), supporting the earlier study by Man *et al.* (2010a). The invasion level of *C. concisus* UNSW1 (one of the new strains) was similar to the *C. concisus* UNSWCD level. No invasion was reported in two strains, *C. concisus* BAA-1457 (acute gastroenteritis) and *C. concisus* ATCC 51561 (healthy subject), whereas *C. concisus* ATCC 51562 and *C. concisus* UNSWCS (both from acute gastroenteritis sources) had no detectible invasion compared to chronic disease causing strains. No significant differences were seen between *C. concisus* UNSW1 (chronic gastroenteritis), *C. concisus* UNSW2 (CD), *C. concisus* UNSW3 (CD), *C. concisus* UNSWCS and *C. concisus* BAA-1457, compared to *C. concisus* UNSWCD (CD). However, both *C. concisus* ATCC 51561 (healthy individual) and *C. concisus* ATCC 51562 (acute gastroenteritis) were significantly different from these strains (Kaakoush *et al.*, 2011b).

Burgos-Portugal *et al.* (2014) investigated differential invasive phenotypes of *C. concisus* further with autophagy. The process was involved in clearing intracellular *C. concisus* from
within epithelial cells of the host. The group detected intracellular \textit{C. concisus} persisting within autophagic vesicles. Weakly invasive \textit{C. concisus} strains showed inhibition of autophagy, leading to dramatic accumulation within epithelial cells. This finding suggests that strains with low capacity for invasion appear to be killed quickly by autophagy once they invade host cells (Burgos-Portugal \textit{et al.}, 2014). These findings suggest the possibility that highly invasive strains may have some virulence factors inhibiting the autophagy pathway. This is supported by the finding that important genes within the autophagy pathway such as LAMP1, cathepsin D and cathepsin S were found to be down-regulated following infection with the highly invasive \textit{C. concisus} strain (Burgos-Portugal \textit{et al.}, 2014, Kaakoush \textit{et al.}, 2011b).

Based on the previous studies, the virulence mechanisms of \textit{C. concisus} were suggested to be classified into two important pathotypes. They are adherent and invasive \textit{C. concisus} (AICC), and adherent and toxinogenic \textit{C. concisus} (AToCC). These pathotypes are functionally and genetically dissimilar to non-pathogenic strains. For instance, they are characteristically different in the intracellular survival, paracellular survival and pathogenesis, compared with the commensal strains (Kaakoush \textit{et al.}, 2014c). The classification of the two pathotypes \textit{C. concisus} (AICC, AToCC), and the commensal \textit{C. concisus} strains is similar to the classification of \textit{E. coli} subtypes that are strongly associated with ileal CD (Darfeuille-Michaud \textit{et al.}, 2004, Darfeuille-Michaud \textit{et al.}, 1998). Based on functionality, AICC strains have ability to survive intracellularly, while the AToCC strains may produce a zonula occludens toxin (zot) to target the tight junctions of the host cells (Figure 1.5). The association between AICC and AToCC pathotypes, and IBD aetiology requires further investigation (Kaakoush \textit{et al.}, 2014c).
Figure 1. 6 AICC and AToCC pathogenic mechanisms; AICC attach to and invade the epithelial cells and induce immune responses by host epithelial cells. They can survive in the epithelial cells by subverting autophagy. The AToCC strains attach to and invade the epithelial cells. They can produce a Zot, that targets the tight junctions of the host cells, although they are efficiently eliminated from within the host cells by autophagy (Kaakoush et al., 2014c).
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1.6.3 Other putative virulence factors of *C. concisus*

The cytolethal-distending toxin (CDT) is the most characterised toxin in *C. jejuni*. It was shown to cause progressive cellular distension, and then death, in Chinese hamster ovary (CHO), Hep-2 and HeLa cells (Bang *et al.*, 2004, Johnson *et al.*, 1988). Engberg *et al.* (2005) investigated CDT in *C. concisus* and detected a CDT-like effect in all three healthy subjects’ *C. concisus* isolates and *C. concisus*-type strain, and in 90% (35/39) of *C. concisus* isolates from patients with diarrhoea.

A *C. concisus* haemolysin has been identified, and cell-associated haemolytic activity was observed in isolates from children with diarrhoea. Istivan *et al.* (2004) characterised the haemolysin and found it to be an outer-membrane phospholipase A (OMPLA), which induces a very strong cytolytic effect on Chinese hamster ovary (CHO) cells, and vacuolation of these cells (Istivan *et al.*, 2004). A follow-up study on these *C. concisus* strains demonstrated that a secreted hemolysin is present (Istivan *et al.*, 2008). A heat labile-secreted haemolytic activity was detected in *C. concisus* fresh cultures using a liquid haemolysin assay that tested cell-free supernatants (S) and retentates (R; 10kDa). The influence of iron on the haemolytic activity was also studied and the activity of haemolysin was reduced to 40% and to 25% of total blood haemolysis when 10 µM FeCl₂ and 20 µM FeCl₂ respectively, were contained in the medium. The study suggested that the iron-regulated hemolysins are virulence factors in *C. concisus* and may play a role in the diseases caused by this organism (Istivan *et al.*, 2008).

Zonula occudens toxins (Zot) are known tight junction toxins. Pathogens like *Vibrio cholerae* and *Neisseria meningitidis* use Zot to increase the permeability of the tissue (Fasano *et al.*, 1995). Some studies on Zot homologs in the host-related Campylobacterales suggested that this toxin is only present in *C. concisus* and may have a role in its pathogenesis. These
findings support the hypothesis that the expression of Zot in *C. concisus* contributes to attachment and invasion of host cells via a paracellular mechanism, by targeting the cell-tight junctions (Kaakoush *et al.*, 2010). Two genes encoding zonula occudens toxins (Zot) in *C. concisus* 13826 (CCC13826_2075 and CCC13826_2276) were reported. These Zot genes were found to be a unique to *C. concisus* and are not encoded in genomes of other *Campylobacter* species. The study also identified two hypothetical proteins encoded by ccc13826_0191 and ccc13826_1210 with 47% and 46% similarity to the *C. concisus* Zot, respectively (Kaakoush *et al.*, 2010).

The prevalence of the Zot gene was tested in 56 oral *C. concisus* isolates from IBD patients and healthy subjects. The Zot gene was found in 30% of the strains, of these; zot was present in 40% of healthy subject strains and 55% of active IBD patient strains. Some of active IBD patient’s oral cavities carried multiple Zot-positive *C. concisus* strains (Mahendran *et al.*, 2013). The sequences of Zot genes of *C. concisus* 13826 and *V. cholerae* 86015 were compared and the biological active domain of *V. cholerae* Zot protein (FCIGRL) was not found in *C. concisus* 13826 Zot (Kaakoush *et al.*, 2010). However, the *C. concisus* Zot was found to share conserved motifs not only with the *V. cholerae* Zot receptor binding domain but also with human zonulin receptor binding domain (Wang *et al.*, 2000), suggesting that Zot of *C. concisus* can increase the intestinal permeability by a similar mechanism to those in the human zonulin and *V. cholerae* Zot (Goldblum *et al.*, 2011). Zhang *et al.* (2014) also suggested that the action of Zot in oral *C. concisus* can trigger the onset or relapse of the IBD and that some oral *C. concisus* strains can acquire the Zot from a virus (prophage). It was also suggested that the *C. concisus* oral strain that colonises the intestinal tract may release Zot because of the prophage, which may lead to a prolonged primary epithelial wall defect that triggers the development of IBD (Zhang *et al.*, 2014), although no clear evidences were provided.
Kaakoush et al. (2010) also identified several secreted proteins and putative virulence factors of C. concisus using proteomics, MS and comparative bioinformatics methods. A total of eighty-six secreted proteins were successfully identified and divided into three groups. The first group was related to the physiology of bacterium and its survival (metabolic proteins), while the second one was involved in host-related functions (the virulence factors). The last group was those proteins associated with protection that against environmental stresses (oxidative stress response proteins) (Kaakoush et al., 2010). CjaA, CjaC and S-layer-RTX were also found to be secreted by C. concisus UNSWCD. CjaA and CjaC are known to be immunodominant in C. jejuni and they are possibly included in the surface-exposed proteins that are homologs of ABC-transport proteins. Several virulence and colonisation factors, which are present in other Campylobacter spp. were also found, such as Twitching motility protein, Fibronectin-binding protein CadF, Outer membrane protein 18, Invasin InvA, Hemolysin TlyA and Invasion protein CiaB (Kaakoush et al., 2010).

C. concisus has been observed to aggregate in huge numbers on the surfaces of intestinal epithelial cells. The aggregation appears to be induced by the presence of mucin (Kaakoush et al., 2011b, Man et al., 2010a). Lavrencic et al. (2012) found that some C. concisus strains isolated from GIT diseases patients, can produce biofilm as efficiently as healthy individual’s C. concisus isolates (Lavrencic et al., 2012). It was suggested that biofilm formation by C. concisus was a general strategy to colonise the natural environment. It is unclear if inhibition of biofilm formation reduces the ability of Campylobacter spp. including C. concisus to infect or colonise the host cells (Kaakoush et al., 2014b). Some C. concisus strains have been sequenced and analysed. The genetic basis of previously reported differences in invasion was then investigated (Deshpande et al., 2011, Deshpande et al., 2013). C. concisus UNSWCD, a
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strain isolated from CD patient was found to have a plasmid (30 kb) encoding several virulence-associated genes (Deshpande et al., 2011, Kaakoush et al., 2011b).

Although *C. concisus* is suggested to be associated with oral and GIT diseases, there is no causal link and only a few virulence factors been identified. In order to understand the role of *C. concisus* as a component of the oral flora and as a potential pathogen, we need to have a better understanding of its genetic diversity; the expression of putative virulence factors, and the possibility of the presence of pathogenic strains. To date, there is no previous study on the colonisation patterns of *C. concisus* in oral cavity of children before teething within a specific family unit with parents and siblings.

Therefore, the aims of this study are to:

- Investigate the carriage rate of *C. concisus* in healthy volunteers in different ages to determine the age of acquisition and any differences between genomospecies in relation to colonisation.
- Investigate if *C. concisus* strains isolated from the same family unit members are related.
- Investigate motility as a virulence factor in *C. concisus* in addition to other virulence characteristics.
- Investigate if *C. concisus* isolated from healthy individuals possess virulence characteristics.
Chapter Two
General Materials and Methods

This chapter describes general methods used in this study. Other specific methods are described in the relevant chapters.

2.1 General Procedures used prior to experiments

All glassware were cleaned using Pyroneg detergent (Diversey Pty Ltd, Melbourne, Australia), and soaked for several minutes. Glassware was washed thoroughly with tap water and rinsed with deionized water before use. The chemicals and reagents used in the experiments were of analytical laboratory grade. All solutions were prepared using deionised water and were obtained by filtration through a Millipore Milli-Q-water System (Merck, Australia). All PCR and RT-PCR reactions were prepared using molecular grade water (Qiagen, Australia). All sterilisation including media, reagents, glassware, and pipette tips was performed by autoclaving at standard conditions (121°C for 15 mins) unless specified. Micro pipettes were used to accurately measure and dispense small volumes of liquid. All solutions were dispensed using Finnpipette (Pathtech, Australia) for all volumes ranging from less than 0.5μl to 1000μl and these were calibrated regularly as per manufactures recommendations.
## 2.2 Chemicals and Equipment

### 2.2.1 General Materials

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Sodium Potassium Tartrate  
Merck, Australia

Sulphuric acid  
BDH Chemicals, Australia

TEMED  
Bio-Rad, Australia
(N,N,N’,N’-tetramethylethylenediamine)

Tetro cDNA Synthesis kit  
Bioline, Australia

Tissue culture flask (25 cm2, 75 cm2)  
VWR, Australia

Tris-HCl  
Merck, Australia

Triton-X-100  
Sigma-Aldrich Pty., Ltd, Australia

trypan blue  
Sigma-Aldrich Pty., Ltd, Australia

Trypsin – EDTA  
Life Technologies, Australia

Tryptone  
Cell BioSciences, Australia

Turbo DNA-free™ kit  
Life Technologies, Australia

Ultrapure water  
Bioline, Australia

UltraPure Tris-base  
Sigma-Aldrich Pty. Ltd., Australia

Wizard Genomic DNA Purification Kit  
Promega, Australia
### 2.2.2 General Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaerobic jar:</td>
<td></td>
</tr>
<tr>
<td>(i) 2.5 &amp; 3.5L jars</td>
<td>Oxoid, Australia</td>
</tr>
<tr>
<td>(ii) 9 &amp; 48 plates jars</td>
<td>Don Whitely, Australia</td>
</tr>
<tr>
<td>Balances:</td>
<td></td>
</tr>
<tr>
<td>(i) Analytical balance</td>
<td>Sartorius GMBH, Australia</td>
</tr>
<tr>
<td>(ii) Balance (0.1-500g)</td>
<td>U-Lab, Australia</td>
</tr>
<tr>
<td>Camera (With 18-200mm lens)</td>
<td>Canon, Australia</td>
</tr>
<tr>
<td>Cell counting chamber</td>
<td>Life Technologies, Australia</td>
</tr>
<tr>
<td>Cell Counter</td>
<td>Life Technologies. Australia</td>
</tr>
<tr>
<td>Cellulose Acetate Filter (0.65 µm)</td>
<td>Sartorius GMBH, Australia</td>
</tr>
<tr>
<td>Centrifuges:</td>
<td></td>
</tr>
<tr>
<td>(i) Microcentrifuge</td>
<td>ProSciTech, Australia</td>
</tr>
<tr>
<td>(ii) Bench top centrifuge</td>
<td>Eppendorf, Australia</td>
</tr>
<tr>
<td>(iii) High-Speed centrifuge</td>
<td>Thermofisher, Australia</td>
</tr>
<tr>
<td>(iv) Ultra-Speed centrifuge</td>
<td>Beckman, USA</td>
</tr>
<tr>
<td>Centrifuge tubes:</td>
<td></td>
</tr>
<tr>
<td>(i) 1.5ml Eppendorf centrifuge tube</td>
<td>VWR, Australia</td>
</tr>
<tr>
<td>(ii) 10ml centrifuge tube</td>
<td>VWR, Australia</td>
</tr>
<tr>
<td>(iii) 15ml centrifuge tube</td>
<td>VWR, Australia</td>
</tr>
<tr>
<td>(iv) 50ml centrifuge tube</td>
<td>VWR, Australia</td>
</tr>
</tbody>
</table>
Chapter Two – General Materials and Methods

Clone Manager Software  Scientific & Educational Software, USA
Cover slips  Mediglass, Australia
Cryoval  Mcfarlane Medical, Australia
Cuvette  Eppendorf, Australia.
DNA Thermocycler (for PCR)  G-Storm, England

Electrophoresis Power Supply:
(i) PowerPac Basic  Bio-Rad, Australia
(ii) EPS 3000xi  Bio-Rad, Australia

Electrophoresis Units:
(i) DNA
   (a) Mini Gel  Bio-Rad, Australia
   (b) Midi Gel  Bio-Rad, Australia
(ii) Protein
   (a) Mini Protein II gel system  Bio-Rad, Australia

Filters: Syringe Filters (0.22µm, 0.45µm)  Sarstedt, Germany
GELDOC system  Bio-Rad, USA

Incubators:
(i) Bellsouth 100 air incubator  Bellsouth, USA
(ii) Tissue culture (5% CO₂) incubator  Forma Scientific, USA

Loop; normal plastic & L-shape  Interpath, Australia

Microscopes:
(i) Light microscope  Olympus optical, Japan
Chapter Two – General Materials and Methods

(ii) Inverted Optical ix71 
Olympus optical, Japan

Needle (sterile 18G, 19G, 21G) 
Terumo Pty, Ltd., Australia

pH meter 
Metrohm, Swiss

Plate (24 well, 96 flat well) 
Greiner Bio-One, Australia

Pipette (10µl, 20µl, 200µl, 1000µl, 10ml) 
Thermofisher, Australia

Pipette (MultiChannel) 
BrandTech Scientific, USA

Phoretix 1D software 
Phoretix International, UK

Prism 6 Software 
GraphPad Software, Inc., USA

Rectangle Capillaries (For DDM test) 
Bioscientific Pty Ltd, Australia

Rotor-Gene Q (Real-Time PCR) 
Qiagen, Australia

Scanner (SDS-PAGE imaging) 
Canon, Australia

Shaker (with incubation service) 
Bioline Global, Australia

Slides 
LivingStone, Australia

Sonicator 
Branson Sonic Power Co., USA

Spectrophotometer (For OD$_{600}$) 
Eppendorf, Australia

SpectroStar Omega Reader (ELISA reader for Protein Concentration) 
BMG-LabTech, Australia

Syringe (1 ml, 5 ml, 10 ml, 20 ml, 50 ml) 
Livingstone International, Australia

Swab (Copan Eswab, For sample collection) 
COPAN, Italy

Tips (Sterile filtered 10µl, 20µl, 100µl, 200µl, 1000µl) 
VWR, Australia

Tips (10µl, 200µl, 1000µl) 
VWR, Australia

Vortex mixer (V ml) 
Ratek Instruments, Australia
Chapter Two – General Materials and Methods

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Bath</td>
<td>Ratek Instruments, Australia</td>
</tr>
<tr>
<td>Weight Trays</td>
<td>Mirella Research, Australia</td>
</tr>
</tbody>
</table>
2.2.3 General solutions and buffers

2.2.3.1 Ammonium acetate

The 10M solution was made up by dissolving ammonium acetate (NH₄ Ac) in dH₂O.

2.2.3.2 Ammonium persulfate (APS)

Freshly prepared by dissolving APS (10% w/v) solution in dH₂O, and used immediately.

2.2.3.3 Coomassie Brilliant Blue Stain

Prepared by mixing methanol (10% v/v), acetic acid (50% v/v) in dH₂O. Brilliant blue G (0.05% w/v) was then mixed to the mixture.

2.2.3.4 De-staining solution (for SDS-PAGE gels)

Prepared as ethanol (10% v/v) and acetic acid (10% v/v) in dH₂O.

2.2.3.5 EDTA

Prepared as 1mM, 100mM, and 0.5M by dissolving EDTA in dH₂O.

2.2.3.6 Ethidium bromide (EtBr)

Prepared as 6 mg EtBr (Sigma-Aldrich, Australia) in 2 L dH₂O.
2.2.3.7 Formaldehyde (2\%)

Prepared by diluting formaldehyde solution (40\%) (Merck, Australia) in dH2O.

2.2.3.8 Gel loading dye (10x)

It was prepared by dissolving 50 mM Tris-HCL (Merck, Australia) in sterilised dH2O and adjusted pH to 8.0. Ficoll 400 (10\% v/v), EDTA (10 mM), glycerol (50\% v/v), SDS (1\% w/v), and orange G dye (Merck, Australia) (0.5\% w/v) were then added to the solution. The solution was aliquoted into 1.5 ml tubes and stored at RT.

2.2.3.9 Lambda (\(\lambda\)) PstI DNA ladder

It was prepared by mixing 108\(\mu\)l \(\lambda\)DNA (50\(\mu\)g), 18\(\mu\)l PstI restriction enzyme (100U), 90\(\mu\)l 10x restriction enzyme Buffer H, 90 \(\mu\)l BSA (10mg/ml) and 900 \(\mu\)l dH2O. The mixture was incubated overnight at 37\(^\circ\)C. An 88\(\mu\)l of 10x gel loading dye (2.2.3.8) was then added to the mixture. The solution was aliquoted into 1.5 ml tubes prior to storing at -20\(^\circ\)C.

2.2.3.10 Lysozyme

It was prepared from ultra-pure grade lysozyme (Astral Scientific, Australia) by dissolving lysozyme powder in RNase-free water to make a final concentration as 1mg/ml and kept at -20\(^\circ\)C.

2.2.3.11 Phosphate buffered saline (PBS)

It was prepared by dissolving 1 PBS tablet (Astral Scientific, Australia) in 100ml dH2O to be 1x. The buffer was then sterilised by autoclaving.
2.2.3.12 Resolving gel buffer

It was prepared by dissolving Tris-HCl (1.5M) in dH$_2$O and pH was then adjusted to 8.8.

2.2.3.13 Running buffer (10x)

The SDS-PAGE running buffer (10x) was prepared by dissolving Tris-HCl (0.25M, pH 8.8), glycine (1.92M) and SDS (1% w/v) in dH$_2$O. The mixture was diluted to 1x before use.

2.2.3.14 Sample Buffer (for protein gels)

Sample buffer (5x) was prepared by mixing glycerol (10% v/v), bromophenol blue (0.02% w/v), β-mercaptoethanol (5% v/v) and Tris-HCl (0.125M, pH 6.8) in dH$_2$O.

2.2.3.15 Sarkosyl detergent (for outer membrane proteins)

Prepared by dissolving 1.88% Sarkosyl (N-laurylsarcosine) in 12.5mM Tris-HCl and pH was then adjusted to 7.4.

2.2.3.16 Stacking gel buffer

It was prepared by dissolving 0.5M Tris-HCl in dH$_2$O and pH was adjusted to 6.8.

2.2.3.17 50X TAE buffer (Tris-Acetate-EDTA)

It was prepared by mixing 2M Tris base (242.2g/L), 1M Acetic acid (60mL/L), 0.1M EDTA (37.2 g/L) and dH$_2$O up to 1 L. The buffer was diluted to 1x prior to use.

2.2.3.18 TE buffer

It was prepared by dissolving Tris-base (10 mM) and EDTA (1 mM) in RNAse-free water and the pH was adjusted to 8. The buffer was then filtrated using sterilised filter (0.22 μm).
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2.2.4 Enzymes stocks

2.2.4.1 Taq DNA polymerase

MyTaq DNA Polymerase was purchased from Bioline (Australia) as 5U/μl stock and was stored at -20ºC.

2.2.4.2 5xTaq Reaction Buffer

It was as 5x MyTaq Reaction Buffer (Bioline, Australia) and stored at -20ºC.

2.3 Microbiological Methods

2.3.1 Bacterial strains

Twenty *Campylobacter* spp. strains were used for this study from the RMIT University stock collections. Fifteen *C. concisus* clinical isolates (RCH strains) were isolated from faecal specimens of children with gastroenteritis at the Royal Children’s Hospital (RCH, Melbourne, Australia) in periods between 1995 and 2004 as explained by Istivan *et al.* (2004). All *C. concisus* clinical strains histories including 2 reference strains are indicated in Table 2.1. Some *Campylobacter* strains other than *C. concisus* strains were also used in this study and they are listed in Table 2.2. *C. concisus* oral strains were isolated from healthy people in different age groups (4 weeks – 50 years old) as will be fully explained in chapter 3.
Table 2.1 *Campylobacter concisus* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source and clinical history</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 51561</td>
<td>Faeces, 24Y, female</td>
<td>CCUG 20034, Sw, (Vandamme et al., 1989)</td>
</tr>
<tr>
<td>ATCC 51562</td>
<td>Faeces, 7M, male, D</td>
<td>CCUG 20700, UK, (Vandamme et al., 1989)</td>
</tr>
<tr>
<td>RCH 3</td>
<td>Faeces, 1Y, male, D &amp; V, 3 d</td>
<td>RCH, Au, (Istivan et al., 2004)</td>
</tr>
<tr>
<td>RCH 4</td>
<td>Faeces, 1Y, female, D &amp; V, 5 d</td>
<td></td>
</tr>
<tr>
<td>RCH 5</td>
<td>Faeces, 5M, female, D, 5 d</td>
<td></td>
</tr>
<tr>
<td>RCH 6</td>
<td>Faeces, 2Y, male, D, 3 d</td>
<td></td>
</tr>
<tr>
<td>RCH 7</td>
<td>Faeces, 16M, male, D &amp; V, 7 d</td>
<td></td>
</tr>
<tr>
<td>RCH 9</td>
<td>Faeces, 2Y, male, D &amp; fever, 7 d</td>
<td></td>
</tr>
<tr>
<td>RCH 11</td>
<td>Faeces, 2Y, male, D, 4 d</td>
<td></td>
</tr>
<tr>
<td>RCH 12</td>
<td>Faeces, 30M, male, D, 20 d</td>
<td></td>
</tr>
<tr>
<td>RCH 15</td>
<td>Faeces, 5M, female, D &amp; V, 9 d</td>
<td></td>
</tr>
<tr>
<td>RCH 19</td>
<td>Faeces, 2Y, female, D &amp; V, 5 d</td>
<td></td>
</tr>
<tr>
<td>RCH 20</td>
<td>Faeces, 10M, female, D &amp; V, 14 d</td>
<td></td>
</tr>
<tr>
<td>RCH 23</td>
<td>Faeces, &lt;15Y, G</td>
<td>RCH, Au, (Istivan, Personal communication)</td>
</tr>
<tr>
<td>RCH 24</td>
<td>Faeces, &lt;15Y, G</td>
<td></td>
</tr>
<tr>
<td>RCH 25</td>
<td>Faeces, &lt;15Y, G</td>
<td></td>
</tr>
<tr>
<td>RCH 26</td>
<td>Faeces, &lt;15Y, G</td>
<td></td>
</tr>
</tbody>
</table>

Y: years and M: months, W: weeks of age, H: Healthy, d: days of symptoms, D: diarrhoea, V: vomiting.


Table modified from Istivan et al. (2004).
Table 2.2 Other *Campylobacter* spp. strains used in the study

<table>
<thead>
<tr>
<th>Classification</th>
<th>Strain and origin</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. mucosalis</em></td>
<td>ATCC 43264, (Pig intestine)</td>
<td>USA, (Vandamme <em>et al.</em>, 1989)</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>81116, (Human gastroenteritis)</td>
<td>RMIT, Au, (Palmer <em>et al.</em>, 1983)</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>NCTC 11366, (Human faeces)</td>
<td>RCH, Au, (Istivan <em>et al.</em>, 2004)</td>
</tr>
</tbody>
</table>

**ATCC;** American Type Culture Collection, **RCH;** Royal Children Hospital, Melbourne. **NCTC;** National Collection of Type Cultures.
Country of isolation: **Au;** Australia, **USA;** United States of America.
Table modified from Istivan *et al.* (2004).
2.3.2 Bacteriological media

All used media were prepared according to manufacturer’s instructions. Standard conditions sterilization was performed at 121°C for 15 minutes. Media was then poured into sterile Petri dishes under germ-free conditions and left for 20 minutes in a laminar flow cabinet. Liquid media was ready to use after sterilising and cooling at RT. Media used in this study were as following:

2.3.2.1 Brain Heart Infusion Broth (BHI) medium

Brain heart infusion broth powder (Oxoid, Australia) as 3.7% (w/v) was dissolved in dH₂O and autoclaved at standard conditions. The media was aliquoted into 50 ml tubes prior to storing at 4-8°C.

2.3.2.2 Brucella semi-solid medium

Brucella broth powder (BD, Australia) as 1.4% (w/v) containing 0.4% agar base was dissolved in dH₂O and sterilised by autoclaving using standard conditions. It was cooled to approximately 50°C. Around 25 ml of the medium were then poured into sterile Petri dishes (90 mm). The plates were left at room temperature to solidify for 1-2 h prior to storage at 4-8°C.

2.3.2.3 Columbia agar medium

Columbia agar powder (Cell BioSciences, Australia) as 4.1% (w/v) was dissolved in dH₂O and autoclaved at standard conditions. It was then processed as described in 2.3.2.2.
2.3.2.4 Columbia broth medium

Columbia broth powder (Cell BioSciences, Australia) as 3.5% (w/v) was dissolved in dH₂O and autoclaved at standard conditions. The media was then aliquoted as in 2.3.2.1.

2.3.2.5 Horse Blood Agar (HBA) medium

Columbia agar powder (Cell BioSciences, Australia) as 4.1% (w/v) was dissolved in dH₂O and sterilised by autoclaving using standard conditions. It was cooled to approximately 50°C. The media was then supplemented with 5% (v/v) Horse blood (Oxoid, Australia) and mixed thoroughly. It was then processed as described in 2.3.2.2.

2.3.2.6 Tryptone/skim milk storage medium

Skim milk powder (Coles, Australia) as 10% (w/v), 1% (w/v) tryptone (Cell BioSciences, Australia) and 10 mM Tris-base were dissolved in dH₂O. The mixture pH was adjusted to 7.5 and each 10 ml was then aliquoted into a small glass container. The media was autoclaved at 109°C for 10 min prior to storing at 4-8°C.

2.3.3 Bacterial cultural conditions

All Campylobacter spp. strains used in this work were grown on HBA (2.3.2.5), unless otherwise specified. The plates were the incubated for 2-4 days at 37°C under microaerophilic conditions in a gas mixture consisting of 6% O₂, 8% CO₂, 6-8% H₂ and a balance of nitrogen in anaerobic jars (Oxoid, Australia and Don Whitely Scientific, Australia) without anaerobic catalyst. C. jejuni and C. coli strains were also grown in a jar with using CampyGen Sachet (GasPack) in the same atmosphere. For liquid cultures, all tested strains were grown in Columbia broth (Cell Biosciences, Australia) (2.3.2.4) or Brain-heart infusion (BHI) broth.
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(Oxoid, Australia) (2.3.2.1) media and incubated under the same conditions for 48 h. For semi-solid cultures, all tested strains were grown in Brucella broth with 0.4% agar (2.3.2.2) and incubated under the same conditions for 72h.

2.3.4 Bacterial strains storage

All *Campylobacter* spp. strains were stored at -80°C into tryptone/skim milk medium (2.3.2.6). The bacterial growth was harvested from the HBA plates (2-4 days of incubation) using a plastic L-shape loop and suspended in a cryovial containing ~1 ml tryptone skim milk. The bacterial suspension was stored at -80°C. For short term storage, strains were stored at 4-8°C on appropriate media plate.

2.4 DNA Molecular Techniques

2.4.1 Rapid boiling method to extract DNA templates for PCR

A 2-4 days old bacterial colonies (approximately $10^7$ CFU) was suspended in a 1.5ml microcentrifuge tube containing 100 µl of sterilised dH2O. The suspension was boiled using a block heater for 5 min and then cooled on ice for 10 min to disrupt the cells. Bacterial debris was removed by centrifugation at 12000xg for 5 min. The supernatant was then used as DNA template for PCR reactions purposes.

2.4.2 Extraction of genomic DNA

The 2-4 days old colonies from a pure bacterial culture were collected from a lawn culture on HBA plate and then suspended into 1 ml PBS. The suspension was centrifuged at 15000xg for 2 min at RT and collected the bacterial pellet. The pellet was then washed twice with 1 ml
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PBS and centrifuged under the same conditions after each wash. The bacterial pellet was used for the DNA extraction according to Wizard® Genomic DNA Purification Kit instruction (Promega, Australia). At the end, between 50 µl and 100µl of DNA rehydration solution was added to the tube and the solution was either incubated at 65°C for 1 h with periodically mixing the solution by gently tapping the tube or incubated overnight at 4°C. The genomic DNA was then stored at -20°C for long term storage.

2.4.4 Determining of DNA concentration

The concentration of the extracted genomic bacterial dsDNA was measured using Nanodrop 2000 (Thermofisher, Australia) at absorbance of 260 nm and 280 nm. Two µl were placed on the machine and the DNA concentration was measured as ng/µl.

2.4.5 Primer Design

PCR and RT-PCR primers were designed using the Clone Manager Professional suite software (Scientific & Educational Software, USA). Primers were designed to have 40-60% of GC content with a Tm°C in the range of 50-80°C. The software was used to confirm that the corresponding designed primers were correctly having the criteria. The software was also used to align genes sequences and compare similarities between them. The primers’ sequences were selected from the genome sequence of *C. concisus* strain 13826 which is available online unless otherwise mentioned. All designed primers were then sent to GeneWorks Company (SA, Australia) to be built up.

2.4.6 Polymerase Chain Reaction (PCR)

PCR was performed by a PCR Thermal cycler Machine (G-Storm, Australia) in sterile PCR tubes. PCR Master Kit was supplied by Bioline (Australia) and used to set up the reaction.
PCR was performed using the general protocol described in the instruction manual. The details of the PCR reaction and cycling conditions are stated below.

### 2.4.6.1 PCR amplification conditions

All PCR reactions were performed using the following reagents:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template (8ng/µl)</td>
<td>2.5µl</td>
</tr>
<tr>
<td>Primer 1 (100ng/µl)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Primer 2 (100ng/µl)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>10x reaction buffer</td>
<td>5µl</td>
</tr>
<tr>
<td>AmpliTaq DNA polymerase (5 units/µl)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>16µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25.0µl</td>
</tr>
</tbody>
</table>

A negative control containing all previous reagents except DNA template, which was replaced with an equal volume of sterile dH₂O, was used in all PCR amplifications to ensure the PCR mix purity.

All PCR amplifications were performed using a DNA Thermocycler under the following conditions:

<table>
<thead>
<tr>
<th>Number of cycles</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (denaturation)</td>
<td>95°C</td>
<td>2 min</td>
</tr>
<tr>
<td>30 (denaturation)</td>
<td>95°C</td>
<td>15 sec</td>
</tr>
<tr>
<td>30 (annealing)</td>
<td>58°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>30 (extension)</td>
<td>72°C</td>
<td>2 min</td>
</tr>
<tr>
<td>1 (extension)</td>
<td>72°C</td>
<td>7 min</td>
</tr>
</tbody>
</table>
2.5 Agarose gel electrophoresis

2.5.1 Agarose gel preparation

Agarose gel was prepared by dissolving DNA grade agarose powder (Bioline, Australia) in 1x TAE buffer (2.2.3.17) and boiled using a microwave until totally dissolved. The percentage of agarose used was 1-1.5% (w/v) in 1x TAE buffer. Midigels (100 ml) and minigels (50 ml) were used. The agarose was then allowed to cool with shaking to approximately 55°C. It was decanted into a casting tray and allowed to solidify for 25 min prior to use in electrophoresis.

2.5.2 Gel electrophoresis, staining, visualisation and photography of gels

PCR reactions including DNA templates were mixed with 10x loading dye (2.2.3.8) as 10:1 (v/v) prior to electrophoresis. An electric field was applied as 40–100V for 1–3 hours and the DNA fragments were separated according to the size. The size of the DNA was estimated by running the samples against the λDNA marker (2.2.3.9). After electrophoresis, the gel was stained by placing in EtBr bath (2.2.3.6) for approximately 10 min. The gel was then destained by washing under running tap water for 20 min before viewing. DNA fragments were visualized under UV illumination using the Gel Doc imaging system (Bio-Rad, Australia).

2.6 Protein analysis methods

2.6.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.6.1.1 Extraction of bacterial outer membrane proteins (OMP)

*C. concisus* strains were cultured on HBA under microaerophilic conditions for 2-4 days. Bacteria were then harvested from lawn culture using 1 ml of 10mM Tris-HCl (pH 7.4) per
agar plate (5-6 plates were used) and centrifuged at 2000xg for 5 min at RT. The bacterial pellet was resuspended in 5-10ml of 10mM Tris-HCl (pH 8) and was then sonicated on setting 6 for 6x30 sec bursts with cooling on ice between each sonication. The sonicated cells were centrifuged at 1000xg for 10 min at 4°C to remove unbroken cells. The supernatant was then collected and centrifuged at 12000xg for 60 min at 4°C to pellet the total membrane. The total membrane pellet was then resuspended with 8 volumes of sarkosyl detergent and incubated for 30 min at RT with mild shaking according to Filip et al. (1973) methods. The outer membrane proteins (OMP) were then collected using centrifugation at 12000xg for 30 min at RT. The OMP pellet was finally resuspended in 0.1ml sterile water and stored at -20°C.

2.6.1.2 Estimation of protein concentration

The OMP protein concentration was determined using a modification of Lowry method as described by Markwell et al. (1978). The preparation of Lowry assay reagents was as summarised in the following steps:

I. **Reagent A**: sodium carbonate (2% w/v), sodium hydroxide (0.4% w/v), sodium potassium tartrate (0.16% w/v) and SDS (1% w/v) were dissolved in dH2O.

II. **Reagent B**: Copper sulphate (4% w/v) was dissolved in dH2O.

III. **Reagent C**: was prepared by mixing of reagent A (50 ml) and reagent B (0.5 ml). This solution was prepared before use and only used fresh.

IV. **Reagent D**: Folin-ciocalteau reagent was dissolved in dH2O (1:1).

Several dilutions from standard albumin were performed to construct a standard curve. The dilutions were 0, 10, 20, 30, 40, 50, 70 and 100µl of protein standard (1mg/ml). The final volume of 200µl was completed by sterile dH2O. For OMPs, the volume of 200µl was made
by adding 10µl of protein to 190µl of dH₂O. The blank was made by adding 10µl of cell lysate buffer (without bacterial cells) to 190µl of dH₂O. Six hundred microlitres of reagent C was added to all prepared tubes (that include 200µl of blank, standard curve and OMP) and left at RT for 20 min. Sixty microlitres of reagent D was then added, mixed rapidly and left to stand at RT for 30 min. Two hundred microlitres of the mixture (for each tube) was pipetted into a 96-well plate and the samples concentrations were detected using SpectroStar Omega Reader (BMG-LabTech, Australia) and then compared to the protein standards. The standard curve was done using Microsoft Excel and the concentration was then calculated by the equation that obtained from the standard curve.

2.6.1.3 Protein sample preparation

Samples were prepared according to Laemmli (1970) methods with minor modifications. A total of 80mg/L protein concentration was used for each sample by diluting the OMP in sterile dH₂O. A 10 µl of diluted protein samples was then mixed with 5µl of 5x-SDS sample buffer solution (2.2.3.14). The mixtures were then boiled for 5 min and cooled prior to loading into individual wells of SDS-PAGE gels using a micro-syringe or micro tips (10µl).

2.6.1.4 Preparation of SDS-PAGE gels

Mini-PROTEAN Tetra Cell electrophoresis system was used to perform SDS-PAGE electrophoresis to analyse protein samples. A 12 % (w/v) acrylamide of the resolving gel and 4% (w/v) acrylamide of the stacking gel were prepared to make the protein gel. The composition of the resolving and stacking gels was as following:
Chapter Two – General Materials and Methods

**Resolving gel for SDS-PAGE (12%)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>2.44ml</td>
</tr>
<tr>
<td>1.5M Tris-HCl, pH 8.8</td>
<td>1.75ml</td>
</tr>
<tr>
<td>10% (w/v) SDS stock</td>
<td>100μl</td>
</tr>
<tr>
<td>Acrylamide (40% stock)</td>
<td>1.56ml</td>
</tr>
<tr>
<td>10% ammonium persulfate (fresh)</td>
<td>50μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>3μl</td>
</tr>
</tbody>
</table>

**Stacking Gel for SDS-PAGE (4%)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>1.58ml</td>
</tr>
<tr>
<td>0.5M Tris-HCl, pH 6.8</td>
<td>0.625ml</td>
</tr>
<tr>
<td>10% (w/v) SDS stock</td>
<td>25μl</td>
</tr>
<tr>
<td>Acrylamide (40% stock)</td>
<td>0.25ml</td>
</tr>
<tr>
<td>10% ammonium persulfate (fresh)</td>
<td>25μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.5μl</td>
</tr>
</tbody>
</table>

2.6.1.5 Running of SDS-polyacrylamide gels

The setting used to run the mini systems in electrophoresis power supply (Bio-Rad, Australia) was 60V for 30 min followed by 180V for 50 min. The electrophoresis was stopped when the dye front was about 5-10 mm from the bottom edge of the gel. A protein standard marker (Bio-Rad, Australia) was also run with each gel and the size range of the marker was 10-250 kD.

2.6.1.6 Staining, destaining for SDS-PAGE, photography of gels and storing

After the completion of electrophoresis, the gel was carefully removed from the apparatus and stained with coomassie blue stain (2.2.3.3) for 1-2h with mild shaking. The gel was then
removed and placed in destaining solution (10% ethanol and 10% acetic acid) overnight with mild shaking. Several changes of destaining solution were made. The gel was kept in 5% acetic acid at 4ºC for long storage. Image for each SDS-PAGE gel was taken either by Canon eos650 camera (Canon, Australia) or by a scanner (Canon, Australia).

2.7 Tissue Culture

The tissue culture work was performed in biological safety cabinet class II to decrease the contamination risks. All reusable containers used to store media and solution like glassware were cleaned by Pyroneg detergent, washed at least twice with dH2O and immersed in dH2O for overnight. Containers were then autoclaved and used to store tissue culture solutions. The sterile containers were re-autoclaved prior using in tissue culture.

2.7.1 Tissue Culture solutions and media

2.7.1.1 Antibiotic Stock Solutions

Antibiotic solutions were prepared by dissolving the antibiotics in sterilized MQ-water at the preferred concentration. These stock solutions were stored at -20ºC. Antibiotic stocks were:

2.7.1.1.1 Gentamycin

It was prepared as 100mg/ml stock solution in sterile MQ-water, and then sterilised using a 0.22µl filter and stored at –20ºC. It was then used in 400µg/ml final concentrations in DMEM/FBS in tissue culture experiment.
Chapter Two – General Materials and Methods

2.7.1.1.2 Penicillin-Streptomycin

Penicillin Streptomycin (100mg/ml) from Life Technologies (Australia) was used to prevent the cell culture from any contamination. It was stored at -20°C.

2.7.1.2 Dimethyl sulfoxide (DMSO)

A stock from Merck (Australia) and was filtered through 0.22 µm filter.

2.7.1.3 Dulbecco’s Modified Eagle’s medium (DMEM)

DMEM media (Life Technologies, Australia) was prepared by adding 10mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Life Technologies, Australia) to 500 mL DMEM (Life Technologies, Australia). The medium was well mixed by inverting the bottle several times. The DMEM medium was divided into 50mL sterile conical tubes and stored at 4°C. The medium was then supplemented by adding 5mL of filtrated heat-inactivated FBS (10% v/v) (Life Technologies, Australia) (2.7.1.4) to 45mL of DMEM in each tube (50ml DMEM/FBS).

2.7.1.4 Inactivated Fetal Bovine Serum (FBS)

The inactivation was performed to inhibit heat-labile complements in FBS (Life Technologies, Australia) prior to use in tissue culture media. Briefly, 500mL FBS bottle stored at -80°C was thawed at 4°C overnight and was then incubated in 37°C water bath until thawed completely. The bottle was inverted several times and poured into 50mL falcon tubes. They were incubated in 56°C water bath for 35 min and then allowed to cool down at RT for at least 30 min. The treated FBS tubes were stored at -20°C. The heat treated FBS was filtrated using a 0.22µm filter prior to use in tissue culture. Inactivated FBS was filtrated
using 0.22µl filter and stored at -20°C. It was used as 10% with DMEM (2.7.1.3) to prepare the complete medium (45mL DMEM + 5mL FBS).

2.7.1.5 Triton -X- lytic solution

A working solution of Triton X-100 detergent (0.3% v/v) was diluted in sterile PBS. The solution was sterilised by filtration using a 0.22µm filter and then stored at 4°C.
Chapter Three
Oral Colonisation of C. concisus in healthy adults and children

3.1 Introduction

C. concisus was first described by Tanner et al. (1981) as a gram-negative microaerophilic curved bacteria. It was isolated from a person with periodontitis and gingivitis (Tanner et al., 1981). While C. concisus has been detected in the saliva 100% of patients with IBD, it was however also detected in 97% of healthy subjects in the same study using a PCR method (Zhang et al., 2010), and in another study in healthy volunteers (100%) using PCR-DGGE methods (Petersen et al., 2007).

In studies aimed to investigate the role of C. concisus in the acquisition of oral microflora, the composition of the subgingival microbiota including C. concisus was investigated in children aged 4-5 years with primary dentition. C. concisus was isolated in greater numbers the molars (5%) than incisors (2.5%), while it was isolated in low numbers in the children with deciduous teeth (Kamma et al., 2000b). In a later study, C. concisus was isolated more often in permanent (10%) than deciduous (3.8%) teeth in children aged 7-8 who with mixed dentition (Kamma et al., 2000a). Furthermore, C. concisus was in both studies associated with bleeding sites from where it was isolated, suggesting that the mouth infected sites may be at greater risk of the outbreak of disease in the future (Kamma et al., 2000a, Kamma et al., 2000b). There are however, no published reports on isolation of C. concisus from the oral cavity of children before teething. Moreover, there are no previous reports on how toddlers can acquire C. concisus in early stages (e.g. from parents, siblings etc.).

The genetic diversity and relationship between C. concisus strains have been investigated. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) has been used to
detect the protein profile diversity of *C. concisus* isolates (Istivan *et al.*, 2004). Nineteen *C. concisus* clinical isolates were identified using amplification of the 23S rDNA gene by PCR. They were grouped into two groups, A and B (Istivan *et al.*, 2004). *C. concisus* strains have also been identified by amplifying the ORF3 region (Istivan *et al.*, 2004, Matsheka *et al.*, 2001). Analysis of DGGE profiles of bacterial samples from the dental plaque of cohorts children with and without gingivitis has revealed that 45% of healthy samples and 50% of gingivitis samples clustered together, suggesting that specific microbiota may be linked to health and disease (Gafan *et al.*, 2005). Given that *C. consicus* has been linked to gingivitis and gastrointestinal disease, it is possible that some strains are more pathogenic. In this study, *C. concisus* isolates were collected from family units, and the relationship between strains was investigated.

The aims of the experiments completed in this chapter were to study *C. concisus* isolates by:

- Isolating *C. concisus* from subjects in different age groups, including families with infants.

- Investigating the colonisation patterns of *C. concisus*, and whether a specific *C. concisus* genomospecies is dominant at different teething stages.

- Identifying the similarities within *C. concisus* strains isolated from members of the same family using genomic and phenotypic techniques.
Chapter Three - Oral Colonisation of *C. concisus* in healthy adults and children

### 3.2 Materials and Methods

#### 3.2.1 Collection of *C. concisus* strains and isolation methodology

Gum swabs were obtained from 28 healthy volunteers (0 month – 50 years old) including 16 adults, and 12 children, 8 of whom were infants before teething using Eswab (COPAN, Italia). The Eswab was taken from the surface of the gum (front and back) and stored in Eswab medium prior to processing. Eswab medium (50-100 µl) was poured on a 0.65µm filter (Sartorius stedim, Australia), named Cape Town protocol filtration method, on HBA medium and left for 15-45 min. The filter was then removed and the filtrate was streaked using sterile loop. The plates were incubated under microaerophilic conditions for 2-4 days as explained in 2.3.3. A single colony was isolated and then subcultured under the same conditions. Genomic DNA was extracted and a stock was kept at -80ºC for each bacterial isolate. The isolates were then used for all biochemical, molecular and protein analysis and other tests.

The *C. concisus* strains were isolated from gum swabs of healthy volunteers of different age groups ranging from 4 week - 50 years of age. The strains were collected from 7 different families and other healthy individuals. Through the families, the strains were isolated from parents and their children, including the infants prior to teething. Some *C. concisus* strains were also isolated from other healthy individuals not related to any family. Two *C. concisus* reference strains, ATCC 51561 and ATCC 51562 were used in all PCR tests included in this study (Table 2.1). *C. mucosalis* type strain ATCC 43264, *C. jejuni* 81116 and *C. coli* NCTC 11366 were also used in all PCR tests as negative controls (Table 2.2). The histories of the 2 *C. concisus* reference strains and other *Campylobacter* spp. used in this study are described in Tables 2.1 and 2.2.
3.2.2 Follow up for Newborn babies

The swabs were taken each week from week one, until the bacterium was isolated (approximately 4-9 times). Three newborn babies (from 2 different families) were also followed up after teething to detect if they were colonised by other C. concisus genomospecies. Swabs were taken when the children reached 2 years of age.

3.2.3. Confirmation of identification of C. concisus using biochemical tests and other conventional tests

A standard inoculum of $10^7$ cfu/ml was used to perform some phenotypic and biochemical tests to characterise C. concisus isolates only, described in Istivan (2005). C. concisus oral isolates were tested for growth conditions, oxidase and catalase production and Gram stain.

3.2.4 Molecular identification of C. concisus

3.2.4.1 PCR amplification of a 0.5-kb C. concisus species-specific fragment

The identification of C. concisus oral isolates was confirmed by the PCR amplification protocol described by Matsheka et al. (2001) using forward 5F cisus and reverse primer 6R cisus to amplify a 0.5 kb specific region in the genome of C. concisus (Table 3.1).

3.2.4.2 PCR amplification of 23S rDNA

The genomospecies of C. concisus oral strains isolated in this study was confirmed using the 23S rDNA PCR amplification protocol as described by Istivan et al. (2004), which was modified from the method used by Bastyns et al. (1995b). Two reverse primers (CON1 and
CON2) were used independently with forward primer (MUC1) to group the *C. concisus* isolates into 2 different genomospecies (A and B). *C. concisus* type strains ATCC 51562 (genomospecies A) and ATCC 51561 (genomospecies B) were used as controls. PCR amplification of the 23S rDNA in *C. concisus* oral isolates was performed as described in 2.4.6 using the primer sets described in Table 3.1.

### Table 3.1 DNA sequences of primers used in PCR amplifications for the molecular identification of *Campylobacter concisus* strains.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1</td>
<td>5'-ATGAGTAGCGATAATTGGG-3'</td>
<td>(Bastyns et al., 1995b)</td>
</tr>
<tr>
<td>CON1</td>
<td>5'-CAGTATCGGCAATTCGCT-3'</td>
<td>(Bastyns et al., 1995b)</td>
</tr>
<tr>
<td>CON2</td>
<td>5'-GACAGTATCAAGGATTTACG-3'</td>
<td>(Bastyns et al., 1995b)</td>
</tr>
<tr>
<td>5F cisus</td>
<td>5'-AGCAGCATCTATATACGTT-3'</td>
<td>(Matsheka et al., 2001)</td>
</tr>
<tr>
<td>6R cisus</td>
<td>5'-CCCGTTTGATAGCGATAG-3'</td>
<td>(Matsheka et al., 2001)</td>
</tr>
</tbody>
</table>

#### 3.2.5 SDS-PAGE analysis for protein profiles of *C. concisus* strains

The outer membrane proteins (OMP) of all *C. concisus* oral strains isolated in this chapter were extracted as described in 2.6.1.1 using the method of Filip *et al.* (1973). SDS-PAGE was completed as detailed in sections between 2.6.1.2 and 2.6.1.6 using the method of Laemmli (1970). An SDS-PAGE gel was performed for each of the seven families and a separate gel was also performed for genomospecies B strains isolated from all infants before teething.
3.2.6 Data analysis for clustering SDS-PAGE images

The SDS-PAGE profiles were analysed using Phoretix 1D software (TotalLab, Newcastle upon Tyne, UK). This software was used to determine the percentage of similarity of the protein profiles of *C. concisus* strains within each SDS-PAGE gel profiles and to cluster the lanes based on banding patterns using the Unweighted Pair Group method with Mathematical Averages (UPGMA) clustering. The images were firstly saved in TIFF format for analysis. A gel image for the *C. concisus* protein profiles for isolates from each family were analysed separately. Figure 3.1 shows the procedures followed to isolate and identify *C. concisus* after collecting the gum swabs from participants in this study.

3.2.7 Ethics statement

Ethical approval was granted by The RMIT University Human Research Ethics Committee HREC and the approval number was 57/13. A written consent form was obtained from adult participants and from the parents on behalf of their children. Informed assent was also obtained from older children who were able to understand the nature of the study.
Figure 3.1 Diagram showing workflow for processing *C. concisus* isolates.
Chapter Three - Oral Colonisation of *C. concisus* in healthy adults and children

### 3.3 Results

#### 3.3.1 *C. concisus* isolation and phenotypic analysis

A total of 47 *C. concisus* strains were isolated from all participants in this study including 26 strains from healthy adults, and 21 strains from healthy children, 9 of whom were from infants before teething. All *C. concisus* oral isolates were oxidase positive and catalase negative. *C. concisus* cells were Gram-negative with curved-rod shape and pale pink colour. The *C. concisus* RMIT-O17 strain which was isolated from a 5 year old healthy boy was observed to have a unique golden colour on HBA (data not shown). The normal colour of *C. concisus* colonies on HBA is greyish colour.

#### 3.3.2 *C. concisus* molecular identifications

**3.3.2.1 PCR amplification of *C. concisus* species-specific 0.5-kb fragment (**gyr** B gene)**

A 0.5 kb PCR product observed with all *C. concisus* strains except one strain (RMIT-O30) which was isolated from a 29 year old male in family 4 (Figure 3.2). This strain was further analysed in the following section (3.3.2.2). No PCR product was detected for other *Campylobacter* spp. strains including *C. mucosalis* ATCC 43264, *C. jejuni* 81116 and *C. coli* NCTC 11136 strains that were used as controls in this PCR experiment. All *C. concisus* species-specific 0.5-kb fragment PCR amplification (**gyr** B gene) results are shown in Table 3.2.
Figure 3.2 PCR amplification of the *C. concisus* specific-specific 0.5 kb fragment (*gyrB* gene) of oral isolates for Family 4. M, Lambda/ *PstI* marker. Lane 1, *C. concisus* ATCC 51561 (Positive control as genomospecies B grouping). Lane 2, *C. concisus* ATCC 51562 (Positive control as genomospecies A grouping). Lanes 3-8, *C. concisus* oral strains RMIT-O28 - RMIT-O33. Lane 9, *C. mucosalis* ATCC 43264. Lane 10, *C. jejuni* 81116. Lane 11, *C. coli* NCTC 11136.
Chapter Three - Oral Colonisation of *C. concisus* in healthy adults and children

### 3.3.2.2 *C. concisus* molecular typing by 23S rDNA PCR amplification

Figure 3.3 shows examples of PCR amplification of 23S rDNA using forward primer; MUC1 and reverse primer, CON1 to detect genomospecies A strains, and forward primer, MUC1 and reverse primer, CON2 to detect genomospecies B strains. A 306 bp PCR product was amplified in all *C. concisus* isolates. Overall, 60% of the isolates were from genomospecies A. Only 19 *C. concisus* oral isolates (40%) belonged to genomospecies B. Genomospecies B was dominant in children with 13 *C. concisus* isolates (13/21, 62%) from children belonging to genomospecies B. From *C. concisus* strains isolated from infants before teething, genomospecies B was also dominant with 89% (8/9), while only 1 *C. concisus* strain was isolated from genomospecies A. Among older children, only 5 of 12 strains were from genomospecies B (41.5%) and the other 7 strains belonged to genomospecies A. In adults, the majority of *C. concisus* strains isolated were from genomospecies A (20/26, 77%), while only 6 strains belonged to genomospecies B. Table 3.2 lists all 47 *C. concisus* oral strains isolated from healthy volunteers with their information.

The two *C. concisus* reference strains, ATCC 51561 from genomospecies B and ATCC 51562 from genomospecies A were used with all PCR tests for this grouping. There were no PCR products obtained with *C. mucosalis* ATCC 43264, *C. jejuni* 81116 or *C. coli* NCTC 11136. All *C. concisus* strains grouped in genomospecies A or B by 23S rDNA PCR amplification are shown in Table 3.2.
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Figure 3.3 PCR amplification of genomospecies A and B grouping for *C. concisus* oral isolates for family 4. M, Lambda/PstI marker. Lane 1, *C. concisus* ATCC 51561 (Positive control as genomospecies B grouping). Lane 2, *C. concisus* ATCC 51562 (Positive control as genomospecies A grouping). Lanes 3-8, *C. concisus* oral strains RMIT-O28 - RMIT-O33. Lane 9, *C. mucosalis* ATCC 43264. Lane 10, *C. jejuni* 81116. Lane 11, *C. coli* NCTC 11136. A, genomospecies A grouping using Con 1 reverse primer. B, genomospecies B grouping using Con 2 reverse primer.
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### 3.3.2.3 Further analysis of *C. concisus* RMIT-O30 strain

A colony with an expected identity of *C. concisus* was grown on HBA from a sample isolated from a 29 year old man in family 4 that was Gram-negative and positive for oxidase. However, PCR amplification for the species-specific 0.5-kb fragment (*gyr B* gene) was found to be negative (Figure 3.2). Further study using 23S rDNA PCR amplification showed it to belong to genomospecies A (Figure 3.3).

### 3.3.3 Follow up studies

Four *C. concisus* strains were isolated from 3 newborn babies from families 5, 6 and 7 when they were followed up from week 1 after birth. The *C. concisus* RMIT-O37 strain was isolated after 4 weeks from the newborn baby of family 5, while *C. concisus* RMIT-O40 and RMIT-O41 strains were isolated after 6 weeks from the newborn baby of family 6. Furthermore, this infant was the only infant colonised by both genomospecies (A and B). *C. concisus* RMIT-O45 strain was isolated after 9 weeks of follow up from the newborn baby of family 7.

Three infants who have genomospecies B strains, being infant of family 1 (RMIT-O19) and the twin infants of family 2 (RMIT-O22 and RMIT-O23), were also followed up after teething (when they became 2 years old) to detect whether they were colonised by other *C. concisus* genomospecies (genomospecies A). Interestingly, genomospecies A was successfully isolated from all of three children, as well as genomospecies B which had already been isolated. *C. concisus* RMIT-O46 strain was isolated from the follow up of family 1 volunteer, while *C. concisus* RMIT-O47 and RMIT-O48 strains were isolated from family 2 twins, respectively.
Table 3.2 Characteristics of \textit{C. concisus} oral strains isolated from healthy individual volunteers including 36 \textit{C. concisus} oral strains isolated from 7 different families.

<table>
<thead>
<tr>
<th>Family</th>
<th>Individual</th>
<th>Strain</th>
<th>Age &amp; Gender</th>
<th>Genomospecies typing (23S rDNA)</th>
<th>\textit{gyrB} gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family 1</td>
<td>1</td>
<td>RMIT-O4</td>
<td>31Y, M</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>RMIT-O18</td>
<td>25Y, F</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3 i</td>
<td>RMIT-O19</td>
<td>3M, F</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3 ii</td>
<td>RMIT-O46</td>
<td>2Y, F</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>RMIT-O20</td>
<td>4Y, M</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RMIT-O21</td>
<td></td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td>Family 2</td>
<td>1</td>
<td>RMIT-O2</td>
<td>39Y, M</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>RMIT-O8</td>
<td>3Y, F</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RMIT-O9</td>
<td></td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>RMIT-O10</td>
<td>26Y, F</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RMIT-O11</td>
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<td>B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>RMIT-O12</td>
<td>5Y, M</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RMIT-O13</td>
<td></td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5 i</td>
<td>RMIT-O22</td>
<td>2M, M</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5 ii</td>
<td>RMIT-O47</td>
<td>2Y, M</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6 i</td>
<td>RMIT-O23</td>
<td>2M, F</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6 ii</td>
<td>RMIT-O48</td>
<td>2Y, F</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td>Family 3</td>
<td>1</td>
<td>RMIT-O24</td>
<td>27Y, M</td>
<td>A</td>
<td>+</td>
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<td></td>
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<td></td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>RMIT-O26</td>
<td>23Y, F</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>RMIT-O27</td>
<td>2M, F</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td>Family 4</td>
<td>1</td>
<td>RMIT-O28</td>
<td>29Y, M</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RMIT-O29</td>
<td></td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RMIT-O30</td>
<td></td>
<td>A</td>
<td>-</td>
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<td>2M, M</td>
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Table 3.2, Characteristics of *C. concisus* oral strains, continued

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<td>3</td>
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<td>9W, F</td>
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<td></td>
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<td>5Y, M</td>
<td>A</td>
<td>+</td>
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</tbody>
</table>

Y; Years, M; Months, W; Weeks of age; A; Genomospecies A, B; Genomospecies B; Male, Female; i; First isolation before teething, ii; Second isolation after teething.
3.3.4 SDS-PAGE analysis of OMPs protein profiles of *C. concisus* isolates from different families

The *C. concisus* oral strains isolated from members of the same family were compared using SDS-PAGE to demonstrate protein profiles within their outer membrane proteins (OMPs). The same was performed for the 7 different families. Precision Plus Protein™ Kaleidoscope™ Standards (Bio-Rad, Australia) were also run with each gel to measure the molecular weight sizing on SDS-PAGE gels. There were variations in the outer membrane protein profiles (OMPs) for *C. concisus* oral isolates within each family. The variations found show that *C. concisus* strains have diverse OMP profiles. The *C. concisus* protein profiles were then clustered according to their banding patterns. Figures 3.4 and 3.5 show an example of SDS-PAGE of protein profiles for the *C. concisus* oral isolates from family 4 and gel for all genomospecies B strains (8 strains) isolated from the seven infants with no teething.

Furthermore, protein profiles for outer membrane proteins (OMPs) were carried out by SDS-PAGE and the analysis of similarity within each family was performed using Dendrogram clustering by Phoretix 1D software. The dendrogram analysis was performed separately for each family to detect the similarities through the members of each family (Figure 3.6 and Figure 3.7). The dendrogram analysis of protein profiles of all genomospecies B strains that were isolated from the seven infants with no teething is also shown separately in Figure 3.8. The outer membrane protein profiles (OMPs) for *C. concisus* oral isolates were not identical in all gels. The only exception was the twin infants in family 2 and they belonged to genomospecies B.
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**Figure 3.4** SDS-PAGE of OMP profiles of *C. concisus* oral isolates visualized by coomassie blue stain. M; SDS-PAGE Marker (Precision Plus Protein™ Kaleidoscope™ Standards from Bio-Rad, Australia). Lanes 1-6, family 4 *C. concisus* oral strains RMIT-O28 - RMIT-O33.

**Figure 3.5** SDS-PAGE of OMP profiles of *C. concisus* oral isolates visualized by coomassie blue stain. M; SDS-PAGE Marker (Precision Plus Protein™ Kaleidoscope™ Standards from Bio-Rad, Australia). Lane 1-8, all genomspecies B *C. concisus* strains isolated from healthy infants before teething, RMIT-O19, RMIT-O22 - RMIT-O23, RMIT-O27, RMIT-O33, RMIT-O37, RMIT-O41 and RMIT-O45, respectively.
In family 1, RMIT-O21 and RMIT-O19 had 83% similarity, although they both were from genomospecies B. Genomospecies A isolates, RMIT-O18 (mother) RMIT-O20 (4 years boy) had less similarity with 71%. Two major clusters were found, a cluster included genomospecies B strains (2 strains), while the other cluster was for genomospecies A strains (3 strains) (Figure 3.6 A). Like family 1, two clusters were also found in family 2, and each genomospecies strains were in separate cluster. The OMP protein profiles of the twins (RMIT-O22 and RMIT-O23) were 100% identical. The \textit{C. concisus} isolate RMIT-O12 was highly divergent from the other isolates in this family, as it had less than 40% similarity from all other isolates. The closest isolate to the father’s (RMIT-O2) was RMIT-O10 with 82.5% similarity (Figure 3.6 B).

In family 3, \textit{C. concisus} RMIT-O24 strain (the father’s genomospecies A) had a unique pattern 43% similarity with other strains. The isolate RMIT-O25 (the father’s genomospecies B) was 84% similar to RMIT-O27, the infant (Figure 3.6 C). The OMP profiles of family 4 were completely divergent and this family was the only one had 3 clusters within their OMP profiles. The highest similarity in this family was 75% between RMIT-O30 and RMIT-O31. There were 2 clades with 52% similarity (lowest similarity found), one containing RMIT-O28 and the other containing all other isolates (Figure 3.6 D).

All family 5, family 6 and family 7, \textit{C. concisus} strains had 2 major clusters (Figure 3.7). Each cluster within each family included only one genomospecies strain. In the family 5, the \textit{C. concisus} isolate from the infant, RMIT-O37 was the most identical isolate to the parents among the tested families, with 96% similarity to her mother. On the other hand, other \textit{C. concisus} isolate of mother, RMIT-O35 was a divergent from all other isolates within this family with 48% similarity (Figure 3.7 E). In family 6, the infant isolate, RMIT-O40 (genomospecies A) was found to be 74% similar with her father’s isolate, RMIT-O38.
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However, the other infant isolate, RMIT-O41 (genomospecies B) was different from all other isolates with only 34% similarity with them (Figure 3.7 F). In the last family tested (family 7), the highest similarity was found between RMIT-O42 and RMIT-O43 strains, both genomospecies A with 77%. The isolate from the infant female, RMIT-O45 was divergent from all other *C. concisus* isolates with only 39% similarity them (Figure 3.7 G).

These results show that all infants were only colonised by *C. concisus* genomospecies B with the exception of the infant from the sixth family who was colonised by 2 *C. concisus* strains, one from genomospecies A (RMIT-O40) and the other from genomospecies B (RMIT-O41). A separate analysis that included all the *C. concisus* strains from infants characterised as genomospecies B (8 isolates) was performed to investigate the similarities within these strains (Figure 3.8). As mentioned, in family 2, the OMP profiles of twin infants (RMIT-O22 & RMIT-O23) were the only exception with 100% identity. All OMP profiles of other infants were found to be divergent from each other. *C. concisus* RMIT-O19 had the lowest similarity with isolates from all other infants with only 18% (Figure 3.8).
Figure 3.6 Dendrogram clustering analysis for OMPs of C. concisus oral strains isolated from different families. A is family 1; RMIT-O4 and RMIT-O18 - RMIT-O21, B is family 2; RMIT-O2, RMIT-O8 - RMIT-O13 and RMIT-O22 - RMIT-O23, C is family 3; RMIT-O24 - RMIT-O27 and D is family 4; RMIT-O28 - RMIT-O33. A, genomospecies A; B, genomospecies B.
Figure 3.7 Dendrogram clustering analysis for OMPs of *C. concisus* oral strains isolated from different families. E is family 5; RMIT-O34 - RMIT-O37, F is family 6; RMIT-O38 - RMIT-O41, G is family 7; RMIT-O42 - RMIT-O45. A; genomospecies A. B; genomospecies B.
Figure 3.8 Dendrogram clustering analysis for OMPs of *C. concisus* oral strains isolated from infants (B). All *C. concisus* strains were genomospecies B isolated from healthy infants, RMIT-O19, RMIT-O22 - RMIT-O23, RMIT-O27, RMIT-O33, RMIT-O37, RMIT-O41 and RMIT-O45.
3.4 Discussion

To our knowledge, this is the first study that isolated *C. concisus* from gum swab samples of all participants using a culture method, and also from gum swab samples of infants before teething. In the present study, 47 *C. concisus* isolates were successfully cultured from 28 healthy volunteers (100%). *C. concisus* has previously been detected in 97% of saliva samples of healthy individuals and 100% of IBD patients; using nested PCR only. This is notably higher than the same study that used the culture method by filtration technique (Cape Town Protocol) achieved only from 75% of healthy individuals and 89% (16/18) from IBD patients (Zhang *et al.*, 2010). The high prevalence of *C. concisus* in the mouths of healthy individuals found in this study supports the idea that *C. concisus* is part of the human commensal microbiota, although it has also been isolated from gingivitis and periodontitis cases (Kamma *et al.*, 1999, Macuch *et al.*, 2000, Tanner *et al.*, 1981).

None of the volunteers in this study were smokers or had any symptomatic disease. A number of previous studies have associated the isolation of *C. concisus* with oral diseases (Macuch *et al.*, 2000, Moore *et al.*, 1987, Tanner *et al.*, 1981, Tanner *et al.*, 1987). The results of this study suggest that this is however not the case, as all participants were healthy. *C. concisus* can be isolated effectively using Cape Town Protocol (Lastovica, 2006), and this study shows that this protocol is a highly effective way to isolate *C. concisus* from gum swabs. This study also confirmed that this protocol can increase the number isolated *C. concisus*.

Two groups of children were included in the study, children with primary dentition (3 - 5 years) and infants before teething (4 weeks - 3 months old), which has not been performed previously. *C. concisus* was isolated in greater proportion in molar teeth than other type of teeth in children aged 4-5 years, and was also isolated in greater numbers in permanent than deciduous teeth in children aged 7-8 years (Kamma *et al.*, 2000a, Kamma *et al.*, 2000b),
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suggesting that *C. concisus* can be isolated from the mouth after teeth eruption. However, the results of this study showed that *C. concisus* can be found in the mouth even before teething, possibly because of contact with surrounding environment or with family members (parents or siblings). It is suggested that getting milk or food prepared by parents, kissing or coughing are a likely source of infection.

There is currently not enough evidence to support a causative role for *C. concisus* in oral diseases. Most of the previous studies isolated *C. concisus* from sites of oral disease without comparison to healthy controls. Therefore, it is not well known if *C. concisus* is an oral pathogen, an opportunistic bacterium, or just part of the normal flora in the mouth (Kaakoush *et al.*, 2012). The results of this study support the view that *C. concisus* is normal flora. However, there is not yet enough information on possible variations in virulence between strains.

In this study, a *C. concisus* 0.5 kb species-specific PCR fragment of gyr*B* gene was used to detect and confirm *C. concisus* isolation. This *C. concisus* species-specific PCR product was detected in all *C. concisus* oral isolates used in this study except RMIT-O30 *C. concisus* strain, genomospecies A (46/47, 98%). The colonies of RMIT-O30 were firstly predicted as *C. concisus* colonies on HBA; however its DNA could not be detected with *gyr* B gene PCR test. *C. concisus* RCH26 strain, isolated from faecal sample of patient suffering from diarrhoea, was previously found not to amplify this 0.5 kb *C. concisus* species-specific fragment (Istivan, Personal communication), suggesting that this gene is heterogeneous in different *C. concisus* isolates or genomospecies, or there is a mutation in the location of this gene. Therefore, the DNA was then tested for *C. concisus* grouping by 23S rDNA PCR amplification and was successfully identified as a *C. concisus* genomospecies A strain. The *gyrB* PCR method has been successfully used in a number of previously published studies.
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(Istivan *et al.*, 2004, Matsheka *et al.*, 2001); however the results of the current study suggest that it may be necessary to combine this approach with 23SrDNA analysis to avoid missing potential isolates.

In order to group oral *C. concisus* isolates into genomospecies A and B, we used the 23S rDNA PCR following the method of Istivan *et al.* (2004). In this study, 60% of oral isolates belonged to genomospecies A, while genomospecies B *C. concisus* oral isolates was only 40%. Overall, the majority of the isolates were genomospecies A, however genomospecies B was the dominant in *C. concisus* isolated from children. Among 21 *C. concisus* isolates from children, 13 isolates (62%) belonged to genomospecies B. It was also found that 89% (8/9) of *C. concisus* isolated from infants before teething belonged to genomospecies B. In a previous study performed in Australia on faecal samples from children with gastroenteritis, genomospecies A had also the higher percentage (71.5%) than genomospecies B (28.5%) (Istivan *et al.*, 2004). However, a following study in Denmark on faecal samples obtained from diarrhoeic and healthy individuals found that genomospecies B *C. concisus* isolates were the dominant (67%) comparing to genomospecies A isolates (33%) (Engberg *et al.*, 2005). The same percentages were also found in a later study in Canada with 67% in genomospecies B and 33% in genomospecies A (Kalischuk *et al.*, 2011). Taken together, these studies suggest that the abundance of *C. concisus* genomospecies varies between sources to and geographical locations. Thus, further studies are required to form an understanding of this relationship.

In this study, some interesting results were obtained. For example, each family has at least one member has more than one strain or genomospecies in the mouth. This finding is consistent with a previous study suggested that more than one *C. concisus* strain can be isolated from the oral cavity of one individual (Zhang *et al.*, 2010). Interestingly, all strains collected from infants before teething belonged to genomospecies B, except the newborn
Chapter Three - Oral Colonisation of \textit{C. concisus} in healthy adults and children

from family 6 who had both genomospecies (isolated after 6 weeks). Three newborn babies were followed up by taking samples weekly, from week 1 after birth to isolate this bacterium. \textit{C. concisus} was successfully isolated from the toddlers after few weeks (4, 6 and 9 weeks, respectively) from the first sample during the follow up process. Three children were followed up after teething to determine if the babies were colonised by other \textit{C. concisus} genomospecies. It was only possible to obtain samples from the infants of family 1 and family 2 after teething at this time, and both genomospecies A and B were successfully isolated at this time. The findings suggest that genomospecies B may colonise the human oral cavity before genomospecies A. It could be that the environment in the mouth is more suitable for the colonisation and survival of geneomospecies B due to an optimal pH or to the nature of nutrients relevant to the food consumed in that age.

This study used OMPs from \textit{C. concisus} strains to demonstrate protein profiles within their OMPs using SDS-PAGE. Previous studies on \textit{C. concisus} used the SDS-PAGE technique to investigate similarities between protein patterns of the tested \textit{C. concisus} strains as a surrogate for the phylogenetic relationship between isolates (Aabenhus \textit{et al.}, 2002, Istivan \textit{et al.}, 2004, Zhang \textit{et al.}, 2010). Unlike our study that used OMPs with SDS-PAGE, most previous studies used the whole cell proteins. Istivan \textit{et al.} (2004) was only study on \textit{C. concisus} that used SDS-PAGE to study outer membrane protein profiles (OMPs) along with whole cell lysates of 19 \textit{C. concisus} clinical strains isolated. Based on the protein profiles, \textit{C. concisus} strains were allocated into more than five groups (Istivan \textit{et al.}, 2004). Given the obvious variations between \textit{C. concisus} strains after SDS-PAGE, we used Phoretix 1D software to analyse the relationship of SDS-PAGE profiles within 7 families and genomospecies B strains of all infants before teething.
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Based on dendrogram analysis, we found that all families had two major clusters (Figures 3.6-8). Each cluster included one *C. concisus* genomospecies (A or B) strains. The only exception was family 4 that had 3 clusters, being 2 clusters included genomospecies B strains, while genomospecies A strains were included in only one cluster. According to this analysis, even within the same family, the protein profiles were divergent; the only exception was the twin infants in family 2, which were 100% identical. These findings suggest that *C. concisus* strains are highly heterogeneous, even within same genomospecies, family or even within the same person who colonised by more than one strain. Kaakoush *et al.* (2011a) previously used the same approach to analyse SDS-PAGE profiles of 8 *C. concisus* strains. The highest similarity percentage was 75%, between *C. concisus* UNSWCD and *C. concisus* UNSW2, while the lowest percentage was 57% for *C. concisus* BAA-1457 with all other seven *C. concisus* strains (Kaakoush *et al.*, 2011a), suggesting that *C. concisus* is a heterogeneous species.

In conclusion, to our knowledge, this is the first study to isolate *C. concisus* from gum swab samples of all participants by culture, and from samples of infants before teething. Moreover, it is the first study that has compared *C. concisus* oral strains within healthy families. *C. concisus* isolate RMIT-O17 has a unique golden colour on HBA plate. More than one *C. concisus* strain can be isolated from the same subject. The results of this study are in agreement with previous studies that *C. concisus* has only two genomospecies based on 23S rDNA PCR amplification method. More than half of the isolates were genomospecies A (60%), consistent with the previous Australian study. Each family had at least one member colonised with both genomospecies. Interestingly, all strains collected from infants before teething belonged to genomospecies B, except *C. concisus* isolate RMIT-O40 from family 6 which had both genomospecies. The results of the study also suggest that genomospecies B
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can colonise the human oral cavity before genomospecies A. From the dendrogram analysis, it can be concluded that even within the same family, OMP profiles were not identical, the only exception being the twin infants who did have identical profiles. Analysis of whole genome sequence from oral strains of the same family will be required to precisely determine the relationship between strains. Other potential methods like AFLP and RAPD can also be used to type *C. concisus* isolates.
Chapter Four

Detection of *C. concisus* flagellin genes in clinical and oral isolates

4.1 Introduction

It is well known that the flagella of *Campylobacter* spp. have an important role in their pathogenicity. *C. jejuni* mutants expressing either *flaA* or *flaB* differ in both motility and invasiveness *in vitro* and *flaA*-mutant strains were 50-fold less invasive than the wild type, but the expression of *flaB* is not essential for bacterial motility (Wassenaar *et al.*, 1994, Wassenaar *et al.*, 1993). A *C. jejuni* 81116 *flaA/flaB* double mutant and *flaA* (*flaB<sup>+</sup>*) mutant had significant differences in the invasive potential compared to the wild type (Konkel *et al.*, 2004). The current study is the first to investigate the *flaA* gene in a panel of *C. concisus* isolates. Given the importance of the flagellin genes for pathogenicity in related species, these genes are potential candidates for virulence factors of *C. concisus*.

*C. jejuni* was found to secrete FlaC protein and the invasion capability of a *C. jejuni* TGH 911 *flaC*-mutant for HEp-2 cells was significantly reduced compared to the wild-type (Song *et al.*, 2004). *flaC* is a highly conserved gene in *C. jejuni* NCTC 11168 and has 99% identity and 100% similarity to FlaC of *C. jejuni* TGH9011 (Parkhill *et al.*, 2000). A *C. concisus* UNSWCD strain was suggested to secrete flagellin B, which is encoded by ccc13826_2297 by bioinformatics study (Kaakoush *et al.*, 2010). Flagellin B (FlaB) was also found to be among the immune-reactive proteins that were detected in 10 *C. concisus*-positive CD patients. It was one of the predominant antigens that were recognised by patients’ sera (Kovach *et al.*, 2011). The sequence of *C. concisus* *flaB* has been previously compared with
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other *Campylobacter* members and found to have 83% identity with *C. curvus*, 78% with *C. rectus*, 60% with *C. lari* and 57% with both *C. jejuni* and *H. pylori* (Kovach et al., 2011). *C. concisus* UNSWCD was found to secrete flagellin-like protein FlaC, encoded by *C. concisus* gene ccc13826_2187 (Kaakoush et al., 2010). However, no molecular detection of *C. concisus* flagellin genes has previously been reported on a panel of *C. concisus* clinical or oral strains. Furthermore, the expressions of *C. concisus* flagellin genes studies have never been investigated.

The experiments presented in this chapter were conducted to fulfil the following aims:

- To detect the flagellin genes (*flaA*, *flaB* and *flaC*) in a panel of *C. concisus* clinical and oral isolates and to investigate the heterogeneity of the nucleotide sequences of these genes in strains from genomospecies A and genomospecies B.

- To investigate the expression of flagellin genes (*flaA*, *flaB* and *flaC*) in selected *C. concisus* strains grown in broth cultures (BHI).
Chapter Four - Detection of *C. concisus* flagellin genes in clinical and oral isolates

4.2 Methodology

4.2.1 Bacterial Strains

All *C. concisus* reference and clinical strains and other *Campylobacter* spp. strains used in this chapter were listed and described in Tables 2.1 and 2.2. The oral *C. concisus* strains used in this chapter were listed and described in Table 3.2.

4.2.2 PCR

4.2.2.1 *C. concisus* flagellin genes

Three motility genes were selected and tested in this work. They were:

- *flaA* (CCC13826_2298): encoding for flagellin A
- *flaB* (CCC13826_2297): encoding for flagellin B
- *flaC* (CCC13826_2187): encoding for flagellin like protein, FlaC

They were obtained from publically available sequences of *C. concisus* 13826 and other *C. concisus* strains available at [http://www.ncbi.nlm.nih.gov/genome/genomes/1439](http://www.ncbi.nlm.nih.gov/genome/genomes/1439).

4.2.2.2 Primer design and sequence alignment

All primers used in this work were designed as described in 2.4.5. The primer sets used are described in Table 4.1. Clone Manager was used to align and compare gene sequences.
Table 4.1 Primer sequences for identification of *flaA*, *flaB* and *flaC* genes in *C. concisus* strains.

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</thead>
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<td>5’-CTCCAGGACTTATGGCATTC-3’</td>
<td>This study, RMIT University</td>
</tr>
<tr>
<td><em>flaA</em>-R</td>
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</tr>
<tr>
<td><em>flaB</em>-F</td>
<td>5’-AACGCAGTTAGCAACAACG-3’</td>
<td></td>
</tr>
<tr>
<td><em>flaB</em>-R</td>
<td>5’-CGTCCCTGATTTGTGATTC-3’</td>
<td></td>
</tr>
<tr>
<td><em>flaC</em>-F</td>
<td>5’-CTCGTAGTGTCGTAGCTTTAG-3’</td>
<td></td>
</tr>
<tr>
<td><em>flaC</em>-R</td>
<td>5’-CAAACCAGGCTTCAGGAAAC-3’</td>
<td></td>
</tr>
</tbody>
</table>

4.2.2.3 PCR and Agarose gel electrophoresis performance

PCR was performed using the PCR Master Kit (Bioline, Australia) as recommended by the manufacturer. The details of the PCR reaction and cycle conditions are stated in 2.4.6. Gel electrophoresis was also used to investigate the purity and integrity of RNA. Agarose gels preparation was described in 2.5.1. The gel staining, de-staining, and visualising are fully described in 2.5.2.

4.2.3 RT-PCR

All RT-PCR experiments were performed in a biological safety cabinet class II to avoid environmental contaminants. Moreover, all reusable items such as glass bottles were treated with diethylpyrocarbonate (DEPC) prior to autoclaving in order to eliminate any chance of contamination with nucleases. RNAse-free water (Qiagen, Australia) was used to prepare all solutions in RNA experiments. These solutions were then stored in DEPC washed containers or in plastic sterilised RNAse-free containers. Sterile, RNAse-free disposable plasticware was used for RNA techniques. Pipettes were sterilised prior to use and all used tips were sterile and RNAse-free with filters. Formaldehyde 2% (2.2.3.7) was used for decontamination.
purposes. All glassware and plasticware were treated before each experiment using UV for approximately 20 min to cross-link DNA and prevent contamination.

4.2.3.1 C. concisus strains and growth conditions

*C. concisus* strains used for gene expression experiments were RMIT-O2 and RMIT-O17 as representatives for genomospecies A, whereas RMIT-O16, RMIT-O22 and RMIT-O23 were representatives for genomospecies B. *C. concisus* ATCC 51561 was also used as a reference strain and it was from genomospecies B. *C. concisus* strains were grown in BHI broth under microaerophilic conditions (2.3.3) with shaking for 48 h.

4.2.3.2 RNA extraction and cDNA generation

RNA extraction was performed using ISOLATE II RNA Mini Kit (Bioline, Australia) following the manufacturer instructions after collecting RNA from bacteria grown in BHI broth by adjusting the OD<sub>600</sub> reading to be 0.095 – 0.105 (~10<sup>9</sup> CFU/mL). At the end, RNA was eluted by placing the column in elution tube (clean 1.5mL) and then adding 80µL of RNase free water directly onto the membrane and incubated at RT for 60 sec. The column was centrifuged at 6000xg for 60 sec and the isolated RNA was cleaned by DNase treatment and then used to generate cDNA.

Extracted RNA was treated to remove any genomic DNA residual contamination. Treatment was performed using Turbo DNA-free<sup>TM</sup> kit (Life Technologies, Australia) following the manufacture instructions with minor modifications. Briefly, 5µL of 10X turbo DNase buffer and 1µL of Turbo DNase were gently mixed with 50µL of extracted RNA in a PCR tube. The mixture was incubated at 37°C for 1 h to degrade any contaminant DNA within the extracted RNA. The reaction was then inactivated by adding 5µL of DNase inactivation and incubated at RT for 5 min with frequent mixing. The mixture was centrifuged at 10000xg for 2 min, and
the supernatant containing clean RNA was then collected. The cleaned RNA concentration was estimated and either used for cDNA synthesis or stored at –80°C.

The Tetro cDNA Synthesis Kit (Bioline, Australia) was used to generate cDNA from the cleaned RNA template following the manufacture instructions. At the end, the reaction was then terminated by incubation at 85°C for 5 min followed by chilling in ice. The cDNA was stored at -20°C for long-term storage.

4.2.3.3 Flagellin genes’ primers

The expression of the three flagellin genes (flaA, flaB, and flaC) was investigated. New primer sets from shorter genomic regions (<300 bp) were designed to find a conserved area in each gene and to be used for gene expression with RT-PCR. Some of RT-PCR primers included degenerate bases such as B, D, R, Y, W and M, which represent GCT, AGT, AG, CT, AT and AC, respectively. The primer sets used in this work are described in Table 4.2.

ATP synthase F1 alpha subunit gene (atpA) was used as a house-keeping gene (Mr. Elshagmani, Personal communication). Figures 4.1, 4.2 and 4.3 show flaA, flaB and flaC sequences obtained from C. concisus 13826 with primer sets used for standard PCR and RT-PCR.
Table 4.2 Primers used in the RT-PCR for gene expression assay of motility virulence genes of C. concisus strains. Blue colours indicate degenerating bases.

<table>
<thead>
<tr>
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<th>Sequence</th>
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<td>5’-CTAAA-BCCATCATCAACTAC-3’</td>
<td>This study, RMIT University, Melbourne, Australia</td>
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<td><em>flaA-R</em></td>
<td>5’-CACGGCCCTTCTTGGTATGAC-3’</td>
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<tr>
<td><em>flaB-F</em></td>
<td>5’-CAACACAGCTGCAGATGAC-3’</td>
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<tr>
<td><em>flaB-F</em></td>
<td>5’-GTCAAGCTCCTCCATTAGAC-3’</td>
<td></td>
</tr>
<tr>
<td><em>flaC-F</em></td>
<td>5’-GADDCCRATCTCYGAGCGAAGTGG-3’</td>
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</tr>
<tr>
<td><em>flaC-R</em></td>
<td>5’-MGGCGAGCTWTCAGYAAAG-3’</td>
<td></td>
</tr>
<tr>
<td><em>atpA-F</em></td>
<td>5’-TGCGCCTATGGACTACACAA-3’</td>
<td>Mr. Elshagmani, Personal communication</td>
</tr>
<tr>
<td><em>atpA-R</em></td>
<td>5’-TCAAAAGATCCAGCGCTAGT-3’</td>
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* atpA was used as a House-Keeping gene.

4.2.3.4 RT-PCR solutions

RT-PCR for the gene expression assays was performed using the SensiFAST™ SYBR No-ROX kit (Bioline, Australia). The total mixture of 12 µl reaction consisted of 6µL of the master mix, 5 pmol of each primer set (0.5µL from each) and 5µL from cDNA template (0.4µg).

4.2.3.5 RT-PCR cycle conditions

Thermal cycling was performed with a standard protocol according to the manufacturer's instructions (Bioline, Australia) with some modifications as follows:

<table>
<thead>
<tr>
<th>Number of cycles</th>
<th>Temperature</th>
<th>Duration</th>
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</thead>
<tbody>
<tr>
<td>1 (denaturation)</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>40 (denaturation)</td>
<td>95°C</td>
<td>15 sec</td>
</tr>
<tr>
<td>40 (annealing)</td>
<td>55°C</td>
<td>15 sec</td>
</tr>
<tr>
<td>40 (extension)</td>
<td>72°C</td>
<td>1 min</td>
</tr>
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</table>
Chapter Four - Detection of *C. concisus* flagellin genes in clinical and oral isolates

The melting curve analysis was also performed to confirm the specificity of PCR using the standard protocol (Rotor-Gene Q software, Qiagen Rotor-Gene RT-PCR machine)

### 4.2.3.6 Standard Curve and data analysis

A standard curve was generated by performing with serial dilutions from the genomic DNA. Three dilutions (descending by 30 folds) were generated as 0.2, 0.00667 and 0.00002 µg/µL, respectively. When using 5 µL RT-PCR reactions; 1, 0.0335 and 0.0001 µg/5µL were used as final concentrations for each reaction. Relative gene expression was calculated using the method described by Pfaffl (2001) with the *atpA* gene. Prism6 software (GraphPad Software, Inc., USA) was used for statistical analysis to calculate the mean and standard deviation. It was also used to generate the graphs in RT-PCR experiment of all flagellin genes. A change of more than 2 fold relative to control was considered a real change in expression and further statistical analysis using unpaired T-test was performed, a value of \( p < 0.05 \) was considered statistically significant.
Figure 4.1 Nucleotide sequence of flaA gene of *C. concisus* 13826. The blue colour font is for the standard PCR primer set and the green coloured font is for the RT-PCR primer set, while the red colour is for the nucleotide replaced by degenerate base in the forward RT-PCR primer flaA-F.
Figure 4.2 Nucleotide sequence of *flaB* gene of *C. concisus* 13826. The blue colour font is for the standard PCR primer set and the green coloured font is for the RT-PCR primer set.
Chapter Four - Detection of *C. concisus* flagellin genes in clinical and oral isolates

**Figure 4.3** Nucleotide sequence of *flaC* gene of *C. concisus* 13826. The blue colour font is for the standard PCR primer set and the green coloured font is for the RT-PCR primer set, while the red colour is for the nucleotide replaced by degenerate base in the forward RT-PCR primer *flaC*-F and *flaC*-R.
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4.3 Results

4.3.1 Detection of the presence of flagellin genes (flaA, flaB and flaC)

The full sequence of flaA gene was only detected in some strains, regardless of genospecies, with a PCR product of 0.55 kb. The flaA gene was detected in a total of 17 C. concisus strains (17/64, 26.5%); including 5 clinical strains (RCH) and 12 oral strains (Table 4.3). No PCR product was detected for the two C. concisus reference strains (ATCC 51561 & ATCC 51562) or for the other Campylobacter spp. (C. jejuni, C. mucosalis and C. coli). C. concisus RCH6 was used as the positive control as no PCR product was detected with any of the two C. concisus reference strains. Figure 4.4 shows an example of flaA PCR results for some selected strains.

On the other hand, the full sequence of flaB gene was only detected in C. concisus clinical and oral isolates belonging to genospecies B, with a PCR product of approximately 1.5 kb. The flaB gene product was detected in a total of 22 C. concisus strains (22/64), being 2 clinical strains (RCH strains), and 19 C. concisus oral strains (Table 4.3). The flaB gene was also detected in the reference strain C. concisus ATCC 51561, which was used as the PCR positive control but it was not detected in the reference strain C. concisus ATCC 51562 (genospecies A) or in strains from other Campylobacter spp. (Table 4.3). Figure 4.5 (A) shows an example of flaB PCR results for some selected strains.

The flaC gene was detected in all tested C. concisus clinical and oral strains (100%). The size of the PCR product for this gene was approximately 0.75 kb in all C. concisus clinical and oral isolates, as well as in both C. concisus reference strains ATCC 51561 and ATCC 51562. flaC was not detected with any other Campylobacter spp (Table 4.3). Figure 4.5 (B) shows
Chapter Four - Detection of *C. concisus* flagellin genes in clinical and oral isolates

an example of *flaC* PCR results in some selected *C. concisus* strains. The results for all flagellin genes are summarised in Table 4.3.

![PCR amplification of *C. concisus* flaA gene products.](image)

**Figure 4.4** PCR amplification of *C. concisus* flaA gene products. M, Lambda/ Pst I marker. Lane 1, *C. concisus* ATCC 51561. Lane 2, *C. concisus* ATCC 51562. Lane 3, *C. concisus* RCH6. Lanes 4-9, *C. concisus* oral strains RMIT-O28 - RMIT-O33. Lane 10, *C. mucosalis* ATCC 43264. Lane, *C. jejuni* 81116. Lane 12, *C. coli* NCTC 11136.
Table 4.3 Summary of flagellin genes (flaA, flaB and flaC) results of all tested *C. concisus* strains and other *Campylobacter* spp.

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<thead>
<tr>
<th>Strains</th>
<th>Genomospecies</th>
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<th>flaB</th>
<th>flaC</th>
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### Table 4.3 Summary of flagellin genes (flaA, flaB and flaC) results of all tested *C. concisus* strains and other *Campylobacter* spp., continued.

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Table 4.3 Summary of flagellin genes (flaA, flaB and flaC) results of all tested *C. concisus* strains and other *Campylobacter* spp., continued.

<table>
<thead>
<tr>
<th>strains</th>
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Blue colour, *C. concisus* clinical isolates obtained from RMIT University stocks. Black colour, *C. concisus* oral isolates isolated in this study from healthy people as indicated in chapter 3. Red colour, other *Campylobacter* spp. isolates obtained from RMIT University stocks.

ATCC; American Type Culture Collection, RCH; Royal Children Hospital, O; Oral, NCTC; National Collection of Type Cultures, A; Genomospecies A, B; Genomospecies B.
4.3.2 Sequence alignment of *C. concisus* fla*A* and fla*B* genes

As indicated in the standard PCR results (4.3.1), the *fla*A gene was only detected in some *C. concisus* strains (17/64) and *fla*B gene was detected with all genomospecies B *C. concisus* strains (19/64). Therefore, two fragments of the *fla*A (516 bp) and *fla*B (501 bp) genes were aligned with sequences of *C. concisus* ATCC 51562 and UNSW1 to identify conserved regions. Primer sets were designed in the conserved regions and used for RT-PCR. The percentage similarity for *fla*A gene with *C. concisus* strains ATCC 51562 and UNSW1 were 66% and 63%, respectively. However, the percentage similarity for *fla*B was higher, being 99% and 96%, respectively. Figures 4.6 and 4.7 indicate the conserved regions and primer locations within *fla*A and *fla*B genes. The next part (4.3.3) shows the amplification results of *C. concisus* flagellin genes used in RT-PCR, which targeted shorter regions.
Chapter Four - Detection of *C. concisus* flagellin genes in clinical and oral isolates

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</tr>
<tr>
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<tr>
<td>3: UNSW1 flaA</td>
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| flaA | 1: gctaaaagcctactcaactcaactcaactatcattttgcccttttagatattttaagaagtctac |
| 51562 flaA | 1: gctaaaaccctactcaactcaactcaactatcattttgcccttttagatattttaagaagtctac |
| UNSW1 flaA | 1: atatagcataatactcatcaactcaactcaactatcattttgcccttttagatattttaagaagtctac |

| flaA | 57: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| 51562 flaA | 57: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| UNSW1 flaA | 61: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |

| flaA | 121: atatagcataatactcatcaactcaactcaactcaactatcattttgcccttttagatattttaagaagtctac |
| 51562 flaA | 121: atatagcataatactcatcaactcaactcaactcaactatcattttgcccttttagatattttaagaagtctac |
| UNSW1 flaA | 121: atatagcataatactcatcaactcaactcaactcaactatcattttgcccttttagatattttaagaagtctac |

| flaA | 177: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| 51562 flaA | 177: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| UNSW1 flaA | 181: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |

| flaA | 236: ccaagaagcgccgtttttattggtgtatccgactgagagttgagatatttgcctctca |
| 51562 flaA | 236: ccaagaagcgccgtttttattggtgtatccgactgagagttgagatatttgcctctca |
| UNSW1 flaA | 240: ccaagaagcgccgtttttattggtgtatccgactgagagttgagatatttgcctctca |

| flaA | 296: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| 51562 flaA | 296: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| UNSW1 flaA | 300: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |

| flaA | 356: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| 51562 flaA | 356: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| UNSW1 flaA | 360: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |

| flaA | 416: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| 51562 flaA | 416: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| UNSW1 flaA | 420: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |

| flaA | 476: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| 51562 flaA | 476: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |
| UNSW1 flaA | 480: ttatcagatcataccaccaagccccacatatatctatattggtgtctgagttgctagccagtccaggttgcac |

Figure 4. 6 Alignment of flaA gene nucleotide sequence of *C. concisus* 13826 with ATCC 51562 & UNSW1, to obtain a conserved area within these strains. The first 516bp was used for the alignment. The conserved area used to construct the primer set was approx. 0.24 kb.
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Summary of Percent Matches:

<table>
<thead>
<tr>
<th>Ref</th>
<th>flaB 501</th>
<th>1 to 501 (501 bps)</th>
<th>2: UNSW1</th>
<th>1 to 501 (501 bps)</th>
<th>3: 51562</th>
<th>1 to 500 (500 bps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>atgagttttcgttataacacaaagctaaacacacagcgaatggctgtgtagc</td>
<td>1</td>
<td>atgagttttcgttataacacaaagctaaacacacagcgaatggctgtgtagc</td>
<td>61</td>
<td>aacaacagtgcacactaatatacagttctatctataggtctgtgtttagaatccaaaca</td>
<td>121</td>
</tr>
<tr>
<td>2</td>
<td>UNSW1</td>
<td>191</td>
<td>ttagttcgcagctttgtaataacagctatattctatattgaatgccgacaa</td>
<td>181</td>
<td>ctattgttaaaatctcaaacagaactaaagactaatctcaatattcagct</td>
<td>121</td>
</tr>
<tr>
<td>3</td>
<td>51562</td>
<td>301</td>
<td>caagacgccaaaccataactcaaacagccgaagctgtgatatagttctcttaaatg</td>
<td>301</td>
<td>caagacgccaaaccataactcaaacagccgaagctgtgatatagttctcttaaatg</td>
<td>301</td>
</tr>
<tr>
<td>4</td>
<td>flaB 501</td>
<td>481</td>
<td>atgttgtcggactacaatcctgac</td>
<td>481</td>
<td>atgttgtcggactacaatcctgac</td>
<td>481</td>
</tr>
</tbody>
</table>

Figure 4.7 Alignment of *flaB* gene nucleotide sequence of *C. concisus* 13826 with ATCC 51562 & UNSW1, to obtain a conserved area within these strains. The first 501bp was used for the alignment. The conserved area used to construct the primer set was approximately 0.26kb.
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### 4.3.3 Amplification of *C. concisus* flagellin genes used in RT-PCR

The *flaA* was not detected in *C. concisus* ATCC 51561 reference strains, RMIT-O2 and RMIT-O17. However, it was detected in RMIT-O16, RMIT-O22 and RMIT-O23 (Table 4.3). To investigate *flaA* gene expression by RT-PCR, a new primer set was designed in the conserved area (approx. 0.24kb). This primer set was tested by standard PCR prior to performing the RT-PCR. The *flaA* gene was detected in all selected strains including the strains which failed to produce a *flaA* PCR product by standard PCR, including ATCC 51561, RMIT-O2 and RMIT-O17 (Figure 4.8 A).

On the other hand, the whole sequence of the *flaB* gene was detected in *C. concisus* genospecies B strains only by standard PCR. This gene was not detected in RMIT-O2 and RMIT-O17, being from genospecies A. However, it was detected in *C. concisus* ATCC 51561, RMIT-O16, RMIT-O22 and RMIT-O23 (Table 4.3). To test *flaB* gene expression by RT-PCR, a new primer set was designed from a small conserved area of this gene (approx. 0.26kb). This primer set was tested by standard PCR with the selected *C. concisus* strains before performing the RT-PCR. It was detected in all selected strains including RMIT-O2 and RMIT-O17 (Figure 4.8 B).

The *flaC* gene was the only gene detected by standard PCR, in all *C. concisus* strains (Table 4.3). To test *flaC* gene expression by RT-PCR, primer set was designed from a small area of this gene (approx. 0.27kb) and then tested by PCR with all selected *C. concisus* strains before performing the RT-PCR. As previously described, the *flaC* gene was detected in all selected strains (Fig. 4.8 C).
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Figure 4. 8 RT-PCR amplification of 3 flagellin genes products obtained from cDNA synthesised from extracted RNA from BHI broth for 6 *C. concisus* strains used in RT-PCR for gene expression. M, Lambda/ Pst I marker. Lanes 1, *C. concisus* ATCC 51561 reference strain. Lane 2, *C. concisus* RMIT-O2. Lane 3, RMIT-O16. Lane 4, RMIT-O17. Lane 5, RMIT-O22. Lane 6, RMIT-O23. Lane 7, negative control. A, *flaA* gene results (0.24kb). B, *flaB* gene results (0.26kb). C, *flaC* gene results (0.27kb). All tested *C. concisus* strains were detected when using a short targeted area.
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4.3.4 Expression of flagellin genes

4.3.4.1 Expression of *flaA* gene

The *flaA* gene only was significantly up-regulated in *C. concisus* RMIT-O23 oral strain (3.063 fold) relative to the reference strain *C. concisus* ATCC 51561 (Figure 4.9). *flaA* expression was not significantly different from the reference in *C. concisus* RMIT-O22 (-0.390, *p*=0.1416). The expression of this gene was significantly down-regulated within the other three *C. concisus* strains (RMIT-O2, RMIT-O17 and RMIT-O16). The most significantly down-regulation of this gene was in RMIT-O2 with -14.400 fold. Figure 4.9 shows the results of *flaA* gene expression relative to the expression levels of the gene in the reference strain *C. concisus* ATCC 51561.

4.3.4.2 Expression of *flaB* gene

The *flaB* gene was significantly up-regulated in 2 oral strains, RMIT-O22 and RMIT-O23 relative to *C. concisus* ATCC 51561 (5.910 and 24.447 respectively). In the other *C. concisus* tested strains *flaB* was down-regulated compared to the reference. *C. concisus* RMIT-O2 was the most significantly down-regulated strain in the expression of the *flaB* gene (-75.00, *p*<0.001), followed by *C. concisus* RMIT-O17 (-11.400, *p*<0.05), both belonging to genomospecies A. *C. concisus* RMIT-O16 was the only strain among genomospecies B that down-regulated the expression the *flaB* gene (-3.167, *p*<0.05). Figure 4.10 shows the results of *flaB* gene expression relative to the reference strain *C. concisus* ATCC 51561.

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### 4.3.4.3 Expression of *flaC* gene

The *flaC* gene was significantly up-regulated by all tested oral strains relative to *C. concisus* ATCC 51561 reference strain. Remarkably, *C. concisus* RMIT-O17 had the highest levels of *flaC* gene expression (9.267, *p*<0.001). This gene was also significantly up-regulated by *C. concisus* RMIT-O22 and RMIT-O23 strains, being 6.990 and 6.627, respectively. The lowest *flaC* expression value was found in *C. concisus* RMIT-O2 (2.217). Although, RMIT-O16 showed greater than 2-fold higher expression (3.110), this was not statistically different unpaired T-test analysis (*p*=0.0601). Figure 4.11 shows the full results of *flaC* gene relative to the reference strain *C. concisus* ATCC 51561.
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Figure 4. 9 RT-PCR of *flaA* gene expression in 6 *C. concisus* strains, ATCC 51561, RMIT-O2, RMIT-O16, RMIT-O17, RMIT-O22 and RMIT-O23. Expression was normalised to *atpA*, and expressed as relative to the *C. concisus* ATCC 51561 reference strain lines indicate 2-fold changes. The expression of the *flaB* gene was significantly up-regulated by only *C. concisus* RMIT-O23 strain compared to the reference strain ATCC 51561. * p<0.05 and **p<0.01.
Figure 4. 10 RT-PCR of *flaB* gene expression in 6 *C. concisus* strains, ATCC 51561, RMIT-O2, RMIT-O16, RMIT-O17, RMIT-O22 and RMIT-O23. Expression was normalised to *atpA*, and expressed as relative to the *C. concisus* ATCC 51561 reference strain. Lines indicate 2-fold changes. The expression of the *flaB* gene was significantly up-regulated by only *C. concisus* RMIT-O22 & RMIT-O23 strains compared to the reference strain ATCC 51561. * p<0.05, **p<0.01 and *** p<0.001
Figure 4. 11 RT-PCR of *flaC* gene expression in 6 *C. concisus* strains, ATCC 51561, RMIT-O2, RMIT-O16, RMIT-O17, RMIT-O22 and RMIT-O23. Expression was normalised to *atpA*, and expressed as relative to the *C. concisus* ATCC 51561 reference strain lines indicate 2-fold changes. The expression of the *flaC* gene was significantly up-regulated by all tested *C. concisus* strains compared to the reference strain ATCC 51561. * p<0.05 and **p<0.01.
4.4 Discussion

The flagellum is known to be important for bacterial motility. The rapid motility and curved shape of *Campylobacter* spp. allows it to swim through the mucous layer of intestinal cells (Aguero-Rosenfeld *et al.*, 1990). In the case of *C. jejuni*, the flagella help the bacterium to penetrate the intestinal mucus and then adhere to, and invade the host intestinal epithelial cells (Szymanski *et al.*, 1995). The *Campylobacter* spp. flagellum has two homologous flagellins, FlaA and FlaB (Dasti *et al.*, 2010).

*C. concisus* is a motile bacterium and it uses one polar flagellum for the motility (Mahendran *et al.*, 2011, Man *et al.*, 2010a). To date, there has been no thorough investigation on the flagellin genes *flaA*, *flaB* or *flaC* in *C. concisus*. Due to the importance of the flagellin genes in *Campylobacter* species and their role in pathogenicity, this study investigated the presence of three flagellin genes; *flaA*, *flaB* and *flaC*, in a total of 64 *C. concisus* clinical and oral strains. It has also studied the gene expression of these genes in selected *C. concisus* strains. A previous bioinformatics study performed on a number of putative virulence factors associated with disease outcomes in *C. concisus* UNSWCD suggested that this bacterium is a pathogen in GIT. These factors included the flagellin-like protein FlaC, encoded by ccc13826_2187 and the flagellin B, encoded by ccc13826_2297 (Kaakoush *et al.*, 2010), however, the flagellin A gene (*flaA*) of *C. concisus* has never been studied.

In this study, using primers designed from the publically available sequence of *C. concisus* 13826, the whole *flaA* gene was detected in just 26.5% of *C. concisus* clinical and oral isolates regardless of genomospecies. The *flaA* gene was previously detected in 83% *C. jejuni* culture positive samples (Waegel *et al.*, 1996). It was also found in all tested *C. jejuni* isolates.
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(74) and *C. coli* isolates (11) isolated from different sources, including children, pigs, dogs and chickens (Krutkiewicz *et al.*, 2010). *flaA* was detected in 100% of *C. jejuni* tested isolates (65) isolated from 2 farms, retail carcasses, and a processing facility (Hanning *et al.*, 2010). It was also detected in all tested *Campylobacter* spp. including 42 *C. jejuni* isolates and 23 *C. coli* isolates (Zhang *et al.*, 2007).

On the other hand, in this study, the whole *flaB* gene was only detected in all genomospecies B *C. concisus* clinical and oral strains, which was 34.4% (22/64) of strains in the study (Table 4.3). A previous study found *flaB* in all tested *C. jejuni* (Muller *et al.*, 2006), being very similar to the *flaB* size fragment used in our study, 1.5kb. It was also detected in 75% of *Campylobacter* spp. isolated from children (Krutkiewicz *et al.*, 2010). Since the whole *flaA* was detected in only 17 of 64 *C. concisus* strains used in this study and the whole *flaB* was detected in only 22 of these strains, these genes may have been absent, or have a variable nucleotide sequences. Shorter regions might be targeted by designing new primers within the conserved regions for the detection of these genes. Therefore sequence variation between strains, rather than absence of the gene may have accounted for this result.

Unlike *flaA* and *flaB* genes, the *flaC* gene was detected in 64/64 (100%) of *C. concisus* tested strains. This gene was also found in 93% of *C. jejuni* isolates, from 2 farms, retail carcasses, and a processing facility (Hanning *et al.*, 2010). As *flaC* was detected in all tested *C. concisus* strains in this study, this indicates that this gene is a conserved in this bacterium. Therefore, the *flaC* primer set designed in this study can be used as a species-specific primer set to detect *C. concisus* isolates by standard PCR rather than the previously used cisus primer set (Matsheka *et al.*, 2001). As described in chapter 3 (3.3.4), the target of the cisus primer set may not be detected in all tested *C. concisus* isolates.
flaA, flaB and flaC genes were detected in all six tested C. concisus strains using primers designed from conserved regions. The failure to amplify flaA and flaB genes using primers from larger regions suggests that these genes are heterogeneous in different genomospecies. It was therefore of interest to determine whether the flagellin genes were differentially expressed in different isolates. Only few studies have previously investigated the expression of flagellin genes Campylobacter species (flaA, flaB and flaC). Here, the expression of these genes in selected C. concisus strains grown in BHI for 2 days under microaerophilic conditions was assessed.

In this study, differences in flaA and flaB expression relative to the reference strain were observed under standard conditions. Only one C. concisus strain, (RMIT-O23) from genomospecies B has a significant up-regulated level of the flaA gene expression, while no statically significant difference was observed with RMIT-O22 from genomospecies B as well. This gene was down-regulated with all other C. concisus tested strains. flaA was previously found to be expressed at higher levels than flaB in C. coli VC167 strain (Guerry et al., 1990). An association between the expression of flaA gene and motility in C. jejuni was reported (Wassenaar et al., 1991). In in vitro growth conditions, the flaA gene was the only flagellin gene expressed by C. jejuni 81116 (Nuijten et al., 1990). The strains RMIT-O22 and RMIT-O23, both from genomospecies B exhibited up-regulation in the flaB gene expression. These two strains RMIT-O22 and RMIT-O23 were isolated from healthy twin infants. Generally, the flaB gene was down-regulated in genomospecies A C. concisus strains (RMIT-O2 and RMIT-O17) (Fig. 4.10). These findings suggest that flaA and flaB expression is variable between C. concisus strains under this condition.

Unlike flaA and flaB, we found that flaC was the only gene that was up-regulated in all C. concisus strains tested relative to the reference strain in this study, with the highest significantly up-regulation in RMIT-O17, from genomospecies A. There is little published
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data on the flaC expression in *Campylobacter* spp. The flaC mutation of *C. jejuni* 81–176 strain had less invasiveness to INT407 cells than wild-type strain (Goon et al., 2006). Two criteria were applied in this study to analyse gene expression results. Firstly, to reduce the risk of technical errors, only changes greater than 2-fold were considered. Secondly, fold changes relative to the reference strain were tested using unpaired T-test. Although, RMIT-O16 showed higher than 2-fold (3.110), it did not show any significant result when using unpaired T-test analysis \( p=0.0601 \). It may be due to that its error bar was high.

In conclusion, although sequence variation appears to be significant, the flaA gene was detected in selected *C. concisus* strains regardless of genomospecies, while flaB gene was amplified in all genomospecies B strains only. Unlike flaA and flaB, the flaC gene was found in all tested strains. These findings support the previous observations that *C. concisus* is a heterogeneous species, and suggests that there are variations in the flagellin genes within *C. concisus* strains and these genes are heterogeneous in different genomospecies. Given that the flaC gene was detected in all tested *C. concisus* strains but not in other tested *Campylobacter* spp., the primer set designed to detect flaC in this study can be used as a species specific primer set to detect new *C. concisus* isolates. After using primer sets from the conserved regions of flaA, flaB and flaC those genes were amplified in all selected strains.

Under the condition tested, the flaA gene was significantly expressed by only one *C. concisus* strain, while lower expression was detected in all other tested strains compared to the reference strain, which was a pathogenic gut isolate. Only *C. concisus* RMIT-O22 and RMIT-O23 strains had significantly higher expression of the flaB gene, whereas the other strains had significantly lower expression under the test conditions. In general, flaC was stronger expressed in the oral isolates tested compared to the reference strain. *C. concisus* RMIT-O17 strain is the most significant up-regulated strain that expressed the flaC gene, which supports
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that this oral strain has unique features and further investigations are required to characterise other virulence factors in this strain. It is suggested that the environmental factors, the presence of O₂ in the oral cavity and CO₂ in the gut could affect the expression of these genes. Further studies are also required to evaluate genetic variations and virulence of clinical isolates from the two genomospecies to assess the role of these genes in pathogenesis of *C. concisus*. As the flagella of *Campylobacter* spp. were found to have an important role in their pathogenicity, proteomic studies on the *flaA, flaB* and *flaC* genes in *C. concisus* are essential to investigate if there are any differences in the proteins’ characteristics between genomospecies A and B. A knock-out mutant in one or more of the *flaA, flaB* and *flaC* genes is also necessary to investigate the role of these genes in pathogenicity of *C. concisus*. 
Chapter Five
Motility and invasion capability as virulence factors in *C. concisus*

5.1 Introduction

A number of virulence factors have been investigated and detected in *Campylobacter* spp., these include factors associated with adherence, invasion, motility, and toxins (Ali *et al*., 2012). Both adherence (mediated by the flagellum), and invasion are predicted to be critical for pathogenesis in *C. concisus*. There have been few studies to investigate the differences between strains isolated from healthy patients and from patients with disease, or those isolates from the oral cavity compared to the intestine. In *in vitro* studies, the polar flagellum of *C. concisus* was observed to facilitate attachment to the Caco-2 and HT-29 human intestinal cell lines. In these studies, *C. concisus* strain UNSWCD was reported to possess an increased ability to invade Caco-2 cells as compared with strains isolated from patients with acute gastroenteritis or healthy controls such as *C. concisus* ATCC 51561 (Man *et al*., 2010a). Furthermore, *C. concisus* strains isolated from healthy individuals were able to attach to, but were unable to invade Caco-2 cells, unlike strains isolated from patients with intestinal diseases (Kaakoush *et al*., 2011b). Enteric invasive *C. concisus* (EICC) was detected in oral isolates from 50% of IBD patients but was not detected in the healthy controls (saliva samples). Oral isolates of IBD patients also had high invasion indexes (Ismail *et al*., 2012).

Motility is an important virulence factor for *Campylobacter* spp., and there is little data on the relative motility in different strains of *C. concisus*. The flagellum of *C. concisus* is very important for motility for both movement through mucous (Lavrencic *et al*., 2012) and to
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mediate adhesion onto the host cells. *C. concisus* uses the polar flagellum to adhere to host microvilli (Kaakoush *et al.*, 2011b, Man *et al.*, 2010a). Although differences in motility between strains have been reported by other authors (Lavrencic *et al.*, 2012), there are to date no reports on a direct link between motility and disease outcome.

Wilson *et al.* (2011) developed the Differential dynamic microscopy (DDM) technique for fast, high-throughput characterization of the dynamics of active particles. The DDM results were highly accurate compared to conventional tracking methods for motility characterisation (Wilson *et al.*, 2011). This technique was applied for the first time to measure the swimming speed and motile cell fractions in *E. coli* suspensions and used to capture essential aspects of the dynamics of a mixed population of both non-motile and motile *E. coli* (Wilson *et al.*, 2011). In this study, the swarming motility assay and DDM were used to evaluate the relationship between motility and invasion in *C. concisus* strains isolated from gum swabs of healthy individuals.

The aims of the experiments carried out in this chapter were to:

I. Assess the interaction between *C. concisus* strains from both genomospecies in an *in vitro* model using INT407 intestinal epithelial cells using adhesion and invasion assays.

II. Investigate motility ratios of different *C. concisus* strains (oral and intestinal)

III. Investigate the motility and invasion as potential virulence factors in *C. concisus* strains.
5.2 Methodology

5.2.1 Bacterial strains isolation and growth conditions

*C. concisus* strains used in this experiment were RMIT-O2, RMIT-O16, RMIT-O17, RMIT-O22 and RMIT-O23. *C. concisus* ATCC 51561 was used as a reference strain (2.3.1). Isolation methods for oral strains are explained in section 3.2.1. All *C. concisus* strains were grown and maintained on HBA for 48-72 h at 37°C under microaerophilic conditions as described in 2.3.3. BHI broth or BB semi-sold agar were also used in specific assays.

5.2.2 *In vitro* intestinal epithelial cell model

5.2.2.1 Culture of intestinal epithelial cells

Tissue culture media composition and preparations are described in 2.7.1. Human embryonic intestinal cell line (INT407), obtained from RMIT tissue culture stock collection was used for the *in vitro* analysis. The preparation of cell lines was as follows; the cells were seeded in large flasks (75 cm²) and allowed to reach pre-confluent monolayer over 48-72 hours. DMEM medium (Thermofisher, Australia) was used for maintenance of INT407 cells. Cells were subcultured every 2 days by trypsinising. To maintain and harvest the cells, after reaching the target confluence, the cells were washed 2 times using PBS. Two millilitres of trypsin were then added and the flask was incubated for 10min. The cells were collected and transferred to 10ml tube that includes 3-5ml DMEM. The tube was then centrifuged at 1000xg for 3min. The supernatant was discarded and repeated this step. After discarding the supernatant, 2ml of DMEM/FBS were added to the pellet. The mixture was used for subculturing, cells storage or *in vitro* assays. The cells were counted using the Countess®
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Automated Cell Counter (Life Technologies. Australia) and the viability was determined using trypan blue stain.

### 5.2.2.4 Adhesion/Invasion Assays

The capability of *C. concisus* strains to adhere and invade INT407 *in vitro*, was determined according to the method described by Elsinghorst (1994) and modified by Chang (2002). INT407 cells (viability of ≥95%) were diluted in fresh DMEM/FBS to reach $2 \times 10^5$/mL of concentration and then 0.5mL seeded per well into a 24 well plate (i.e. $10^5$ cells/well). The 24 well plates containing INT407 were incubated for 10-16 h to allow the cells to attach. On the day of the procedure, the old medium was discarded and the cells were washed 3 times in PBS. Fresh media (400µL of DMEM/FBS) was added to each well after washing.

Several bacterial colonies were collected from HBA culture (48-72 h) and suspended into Columbia broth. The suspension was then measured and adjusted to be 0.1 OD$_{600}$ reading using spectrophotometer (Eppendorf, Australia). Serial dilutions, being $10^5$, $10^6$ and $10^7$ were performed from the original OD$_{600}$ inoculum of each strain and plated onto HBA and then incubated under microaerophilic conditions to count the live cells and to determine the CFU.

#### 5.2.2.4.1 Infection of INT407 cultures and adhesion assay

A 100µL (~$10^7$ CFU) of bacterial suspension was inoculated into each well containing INT407 to perform multiplicity of infection (MIO) of 10 – 100 (bacterial cells/infected cell). The plates were centrifuged 5 min at 200xg to enhance bacterial contacts with intestinal cells. The inoculated plates were then incubated at 37°C under microaerophilic conditions for 6 h to allow the bacterial strains to adhere and invade the cell line.
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Wells were washed 3 times with PBS after 6 h incubation to remove non-adherent bacterial cells. The intestinal cells were lysed by adding 200 µL of 0.3% triton-X-100 /well, to release the attached and intracellular bacterial cells, and then diluted by adding 800 µL PBS. The plates were incubated in 5% CO₂ at 37°C for 15 min. The bacterial cell number measured as colony forming units (CFU). A 100µL from several dilutions including 10¹, 10³ and 10⁵ was then inoculated onto HBA. The viable bacterial cells adhering onto the intestinal cells were calculated using the following formula:

\[
\text{Adhesion\%} = \frac{\text{No. of recovered bacterial cells}}{\text{No. of total bacterial cells initially used/\text{ml}}} \times 100
\]

### 5.2.2.4.2 Invasion Assay

After 6 hours incubation as above, wells were washed 3 times with PBS. One mL of DMEM/FBS containing of 400µg gentamycin was added to each well and incubated in 5% CO₂ at 37°C for 60 min to kill non invaded bacterial cells as gentamycin is acting in extracellular to kill the bacterial cells. The cells were then washed 3 times by PBS after the treatment to remove the gentamycin. The intestinal cells were lysed by adding 200 µL of 0.3% triton-X-100 and diluted by adding 800 µL PBS to release the invaded bacterial cell from the intestinal cell. The plate was incubated in 5% CO₂ at 37°C for 15 min. The dilutions 10¹, 10² and 10³ were made. An inoculum of 100µL from each of these dilutions was cultured onto HBA. Viable bacterial cells invaded into intestinal cells line was calculated by the following formula:

\[
\text{invasion\%} = \frac{\text{No. of recovered bacterial cells/\text{ml}}}{\text{No. of total bacterial cells initially used/\text{ml}}} \times 100
\]
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The invasion/adhesion ratio was also calculated according to the following formula:

\[
\text{Invasion/Adhesion Ratio} = \frac{\text{No. of invaded bacterial cells}}{\text{No. of adhered bacterial cells}} \times 100
\]

5.2.4 Data analysis

All experiments were performed with three independent experiments and the results reported as the average value ± Standard Error of the Mean (SEM). Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc., USA) using unpaired T-test to calculate \( P \) value and to also generate graphs in both experiments (adhesion & invasion). A value of \( p<0.05 \) was considered statistically significant.

5.2.5 Measurement of bacterial motility by Differential Dynamic Microscopy (DDM)

5.2.5.1 Bacterial strains and culture conditions

\textit{C. concisus} strains ATCC 51561, RMIT-O2, RMIT-O16, RMIT-O17, RMIT-O22 and RMIT-O23 were grown in Brain-heart infusion (BHI) broth medium in a microaerophilic environment with shaking for 48 h as described in 2.3.3. The concentration of suspension was adjusted to be \(~0.3\) (OD\(_{600}\)).

5.2.5.2 Sample preparation and video capture

The video capture for DDM analysis was performed using a modification of a method described by Wilson \textit{et al.} (2011). Briefly, 150\( \mu l \) of a 48 h bacterial suspension (\(~0.3\) OD\(_{600}\)) grown in BHI broth were loaded into a capillary tube (Bioscientific Pty Ltd, Australia) and
placed on an Inverted Optical ix71 microscope (Olympus, Japan). Videos were recorded at 1024x1024 frame rate using a high speed camera (EoSens MC1362 camera) from MIKROTRON (Germany). Two different videos were recorded for analysis. The first video was 30 sec with 60x magnification and 100 FPS (Frame per second) with bright field illumination to observe the presence of motile cells. The second video was 60 sec with 10x magnification and 100 FPS on phase contrast for DDM analysis.

5.2.5.3 DDM data analysis

The video file was processed using a custom written program in LabVIEW. The processing stage calculated the Differential Intensity Correlation Functions (DICF) from each input video. For a 1024x1024 video, 511 DICF were obtained and the DICFs were fitted using MATLAB software (Mathworks, USA) with a double exponential model. In a previous study that used DDM to characterise motility in \textit{E. coli} (Wilson \textit{et al.}, 2011), the DICF values were found to scale when replotted against $q \times t$ ($q$ is scattering vector and $t$ is the time). If data scales to $qt$, the detected motions are isotropic ballistic motion. Therefore, in that case it was possible to fit a model which describes ballistic motion and calculate the speed of the bacteria. For \textit{Campylobacter} bacteria, the calculated DICF scale in accordance to $q^2t$, which implies that the motility is diffusive in nature, rather than ballistic as is the case for \textit{E. coli}; therefore a model must be fitted which describes diffusion motion $exp(-D_1q^2t)$, where $exp$ indicates an exponential and $D_1$ is the diffusion coefficient for the fast motility process. The best fit required a double exponential ($exp$) due to the background Brownian motion that all cells experience. Therefore a double exponential fit was fitted and retrieved two fitting parameters $D_1$ and $D_2$. The fitted data was checked for goodness of fit using the normalize root mean squared error (NRMSE) function from MATLAB software. Data was only
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accepted if the NRMSE was above 0.95. These results were averaged, and a standard deviation calculated.

5.2.6 Motility swarming assay on semi-solid media

Motility swarming assay on semi-solid media (Takata *et al.*, 1992) was performed using a modification of a method described by Adler *et al.* (2014). Briefly, brucella broth (BB) containing 0.4% agar (BBA) was used. The swarming ability of 6 *C. concisus* strains (ATCC 51561, RMIT-O2, RMIT-O16, RMIT-O17, RMIT-O22 and RMIT-O23) was investigated at 37°C on semi-solid agar medium plates (BB). Three day old cultures of 6 *C. concisus* strains were adjusted to $10^8$ CFU/ml ($\text{OD}_{600} = 0.1$) and 3.8 µl dropped on semi-solid medium plates. The diameters of the swarming halos (zones of motility around the inoculation points) were then measured after 72 h incubation period at 37°C. The data were generated from three independent experiments and the mean was taken.
5.3 Results

5.3.1 In vitro

5.3.1.1 Adhesion assay

All the *C. concisus* oral strains tested were able to adhere to INT407 cells. The adhesion assay statistical analysis was in comparison with the reference strain *C. concisus* ATCC 51561. *C. concisus* RMIT-O2 and RMIT-O17, had a lower proportion of adherence compared to *C. concisus* strains RMIT-O22 and RMIT-O23 with 1.028, *P*= 0.0004 and 0.933, *P*= 0.0004, respectively. *C. concisus* RMIT-O16 adherence proportion was similar to RMIT-O2 and RMIT-O17 results, being 0.943, with a *P* value of 0.0011. The highest adherent proportions in the oral isolates were detected in *C. concisus* RMIT-O22 and RMIT-O23, being 2.070, *P*= 0.0027 and 2.110, *P*= 0.0021, respectively. The proportion of adherent bacteria for all the isolates was significantly lower than *C. concisus* ATCC 51561 (Figure 5.1).

5.3.1.2 In vitro invasion assay

All tested *C. concisus* strains had the ability to invade INT407 (Figure 5.2). *C. concisus* RMIT-O17 strain was found to be more invasive than the reference strain ATCC 51561 and other tested strains. It had a significantly higher invasion proportion (0.087, *P*=0.0030) than the reference strain ATCC 51561(0.0337). All other oral strains were significantly lower in invasion proportion than the reference strain, with *C. concisus* RMIT-O16 producing the lower invasion proportion (0.001, *P*=0.0050), followed by *C. concisus* RMIT-O2 strain (0.007, *P*=0.0100). The invasion proportions of *C. concisus* RMIT-O22 and RMIT-O23 were 0.007, *P*=0.0106 and 0.009, *P*=0.0129, respectively.
Figure 5.1 Proportion of adherent bacteria from five *C. concisus* oral isolates and the reference strain ATCC 51561. The oral strains were; RMIT-O2 and RMIT-O17 from genomospecies A. RMIT-O16, RMIT-O22 and RMIT-O23 were from genomospecies B. The data shown represent the mean number of adherent bacteria from 3 independent experiments ± SEM. *p*<0.05, **p*<0.01, ***p*<0.001
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Figure 5.2 Proportion of invasive bacteria from five *C. concisus* oral isolates and the reference strain ATCC 51561. The oral strains were; RMIT-O2 and RMIT-O17 from genomospecies A. RMIT-O16, RMIT-O22 and RMIT-O23 were from genomospecies B. The data shown represent the mean number of invasive bacteria from 3 independent experiments ± SEM. *p* < 0.05, **p** < 0.01.
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### 5.3.1.3 Invasion/Adhesion ratio

The results of the adhesion and invasion assays above showed that, *C. concisus* strains were more likely to adhere rather than to invade INT407. Except RMIT-O17, all tested strains including the reference strain ATCC 51561 had a value less than 1.00 in their invasion index which was calculated as:

\[
\text{Invasion/Adhesion Ratio} = \frac{\text{No. of invaded bacterial cells\%}}{\text{No. of adhered bacterial cells\%}} \times 100
\]

No statistically significant difference was found in the invasion index of *C. concisus* RMIT-O2 strain (0.659, \(P=0.5003\)), whereas *C. concisus* RMIT-O16 strain had a significantly lower invasion index (0.162, \(P=0.0053\)). The invasion indexes of *C. concisus* RMIT-O22 and RMIT-O23 were also significantly lower than the reference strain ATCC 51561, 0.363, \(P=0.0131\) and 0.410, \(P=0.0209\), respectively (Figure 5.3). Bacterial strains with invasion indexes >1 were considered to be enteric invasive strains (EICC) (Larson *et al.*, 2008). Therefore, only one enteric invasive *C. concisus* strain (EICC) was detected from oral strains and it was RMIT-O17, with 9.313, \(P < 0.0001\).
Figure 5.3 Invasion index (invasion/adhesion ratio) of five *C. concisus* oral strains compared to *C. concisus* ATCC 51561 reference strain. The oral strains RMIT-O2 and RMIT-O17 from genomospecies A and RMIT-O16, RMIT-O22 and RMIT-O23 were from genomospecies B. The data shown represent the mean number of invasive/adherent ratio of bacteria from 3 independent experiments ± SEM. *p*<0.05, **p*<0.01, ****p*<0.0001.
5.3.2 Differential Dynamic Microscopy (DDM)

5.3.2.1 Calculation of the Differential Intensity Correlation Functions (DICF)

The table below shows the average and standard deviations for the D1 and D2 values obtained. D1 is the active diffusion of the motile cells, and D2 is the Brownian diffusion coefficient. All D1 values of *C. concisus* oral tested strains were found to be significantly higher than the reference strain. In the reference strain *C. concisus* ATCC 51561 and the oral strain *C. concisus* RMIT-O16, the D1 and D2 are close together, indicating that the motility is not much faster than normal Brownian motion. However, the D1 value for RMIT-O16 was significantly higher than the reference strain (*P*=0.0067). All other oral *C. concisus* strains were also significantly higher than the reference strain and RMIT-O16, and had the same *P* values, <0.0001. However, *C. concisus* RMIT-O17 was found to be the most motile strain compare to all tested strains, 11 times faster than the background Brownian motion, followed by *C. concisus* RMIT-O2. *C. concisus* RMIT-O22 and *C. concisus* RMIT-O23 strains have motilities which are well above the Brownian motion background, but lower than *C. concisus* RMIT-O17 and *C. concisus* RMIT-O2. The diffusion value for the motile component (D1) was converted to a mean squared displacement (MSD) to determine the motility speed of each *C. concisus* strain (Figure 5.4).
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Table 5.1 Differential Intensity Correlation Functions (DICF) of six *C. concisus* strains. D1 (Motility, $\mu m^2/s$) and D2 (Brownian motion, $\mu m^2/s$). **$p<0.01$, **** $p<0.0001$.

<table>
<thead>
<tr>
<th><em>C. concisus</em> strains</th>
<th>D1 ± SD</th>
<th>D2 ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 51561</td>
<td>0.46 ± 0.35</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>RMIT-O2</td>
<td>2.12 ± 1.83****</td>
<td>0.3 ± 0.07</td>
</tr>
<tr>
<td>RMIT-O17</td>
<td>4.19 ± 1.61****</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>RMIT-O16</td>
<td>0.51 ± 0.23**</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>RMIT-O22</td>
<td>1.12 ± 0.43****</td>
<td>0.4 ± 0.79</td>
</tr>
<tr>
<td>RMIT-O23</td>
<td>1.6 ± 0.22****</td>
<td>0.18 ± 0.07</td>
</tr>
</tbody>
</table>

Figure 5.4 The mean squared displacement (MSD) as a representation of velocity for six *C. concisus* strains, ATCC 51561 reference strain, RMIT-O2, RMIT-O17, RMIT-O16, RMIT-O22, RMIT-O23. The fitted data was the checked for goodness of fit using the normalise root mea n squared error (NRMSE) function. MSD unit was $\mu m^2$ and the time (t) unit was second (s). Strain RMIT-017 had the highest calculated MSD.
5.3.3 Motility swarming assay on semi-solid media

The six *C. concisus* tested strains produced different size swarming zones around the inoculation points as shown in Figure 5.5. *C. concisus* ATCC 51561 reference strain and *C. concisus* RMIT-O16 were found to have the smallest swarming zones (≥0.65 cm) while *C. concisus* RMIT-O23 and *C. concisus* RMIT-O22 exhibited bigger swarming halos (~0.70 cm). *C. concisus* RMIT-O2 exhibited a swarming zone of (0.75 cm). Interestingly, *C. concisus* RMIT-O17 produced the biggest swarming halo (~1.00 cm) compared to *C. concisus* ATCC 51561 reference strain and the other oral strains in this test. Figure (5.5) shows the swarming zone of 3 days old cultures of 6 *C. concisus* tested strains on BB semi-solid agar.
Figure 5. 5 Swarming zone of 3 days old cultures of six *C. concisus* tested strains on brucella semi-solid agar (0.4% agar). 1; ATCC 51561. 2; RMIT-O2. 3; RMIT-O16. 4; RMIT-O17. 5; RMIT-O22. 6; RMIT-O23. RMIT-OO17 strain (Genomospecies A) isolated from young healthy boy had the biggest swarming zone compared to ATCC 51561 reference strain and other tested strains. ATCC 51561 and RMIT-O16 had the smallest swarming zones. The zone was measured as centimetre (cm).
Motility and invasiveness are considered to be important virulence factors in *Campylobacter* spp. A number of oral and enteric *C. concisus* strains have been reported to have adhesion and invasion abilities, and to stimulate epithelial apoptosis in intestinal epithelial cells *in vitro*. Adhesion to the host epithelial cells was found to be an important stage in the pathogenesis of several bacteria (Pizarro-Cerda *et al.*, 2006). The highest reported invasion level of *C. concisus* strains in an *in vitro* in Caco-2 cells was reported to be at minimum of six hours (Ismail *et al.*, 2012, Kalischuk *et al.*, 2011, Man *et al.*, 2010a, Nielsen *et al.*, 2011). We here assessed the interaction between five *C. concisus* oral isolates from both genomospecies (A and B) with enteric cells in an *in vitro* model of INT407 cell line using adhesion and invasion assays and compared them with *C. concisus* ATCC 51561 reference strain.

The motility of *E. coli* was previously measured by the DDM technique and the diffusivity of non-motile cells was found to be enhanced in proportion to the concentration of motile cells (Wilson *et al.*, 2011). The swarming assay has recently been used to determine the swarming ability of *C. jejuni* wild-types and mutant isolates (Adler *et al.*, 2014). In this study, DDM was applied to determine the swimming speed of 6 *C. concisus*. Motility was also assessed using a standard swarming motility assay. Furthermore, we investigated the relationship between the motility and the invasion in the same tested strains.

In this study, adhesion and invasion assays were performed to determine the capability of the oral isolates to adhere to and invade INT407 cell compared to the *C. concisus* ATCC 51561 reference strain. The INT407 cell line model has previously been used to assess invasion and adhesion for other *Campylobacter* spp. (Konkel *et al.*, 2004, Neal-Mckinney *et al.*, 2012).
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The proportion of *C. concisus* ATCC 51561 reference strain that were adherent INT407 cell was 4.41% (Figure 5.1). This is higher than what reported by Kaakoush *et al.* (2011b), 0.11%, for the same strain in Caco-2 cells. The adhesion result obtained for the ATCC 51561 reference strain in this study was however similar to that of 5 *C. concisus* strains isolated from intestinal biopsies and faeces of patients in Caco-2 cells (Kaakoush *et al.*, 2011b). Furthermore, all of oral isolates were found to be significantly less adherent than the reference strain. *C. concisus* RMIT-O23 was found to be the most adhesive oral strain (2.110, \(P=0.0021\)), followed by RMIT-O22 (2.070, \(P=0.0027\)) and both were from genomospecies B. Genomospecies A strains, RMIT-O2 and RMIT-O17 were found to be lowest adherent tested strains, being 1.028, \(P=0.0004\) and 0.933, \(P=0.0004\), respectively. Our findings suggest that the tested strains from genomospecies B could be more adherent than genomospecies A strains. This finding indicates that healthy individuals have the possibility of being colonised with oral *C. concisus* strains that have the ability to adhere to the intestinal epithelial cells.

Interestingly, although RMIT-O17 was weakly adherent in this assay, it was found to be the only oral strain that had significantly higher proportion of invasion (0.087, \(P=0.0030\)) than the reference strain (0.0337) (Figure 5.2). *C. concisus* ATCC 51561 reference strain was able to invade the INT407 cell line in this study, but did not invade the Caco-2 cell line in previously reported studies (Kaakoush *et al.*, 2011b, Man *et al.*, 2010a). The invasion index was calculated as a measure of enteric invasive ability. *C. concisus* strains that have invasion indexes >1 are considered to be enteric invasive strains (EICC) and as previously reported that *C. jejuni* strains that had invasion indexes of >1 showed clinical symptoms in infected piglets with similar to human symptoms, including bloody diarrhoea (Larson *et al.*, 2008). The oral isolate RMIT-O17 (9.313, \(P<0.0001\)) was the only strain to have a significantly higher invasion index than the reference strain (0.752) (Figure 5.3). These findings indicate
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that the invasion is conditions-dependant, and further that different cell lines can greatly affect the outcome. No oral EICC isolated from a healthy control has previously been reported. EICC oral strains were previously investigated in oral isolates from 50% of IBD patients and none of the control isolates (Ismail *et al*., 2012). Our study supports previous studies that indicated that *C. concisus* strain may be an enteric invasive pathogen (Ismail *et al*., 2012, Nielsen *et al*., 2011). Our findings also suggest that healthy individuals can carry invasive *C. concisus* strains in their oral cavity. In the future, it is suggested to use other types of cell line like Caco-2 and Hep-2 cells to study the adhesion and invasion of these *C. concisus* strains.

DDM has not been previously applied on any bacteria other than *E. coli*. In this work, DDM was used to measure the swimming speed of 6 *C. concisus* strains and also to differentiate between motile and non-motile cells within each strain. The motility of tested strains varied from strain to another and also from genomospecies to another, as differentiation between the motility speed of genomospecies A and genomospecies B strains was noted. All oral *C. concisus* tested strains were found to be significantly more motile than the reference strain, ATCC 51561. *C. concisus* RMIT-O17 had the highest motility speed (D1=4.19 \( \mu \text{m}^2/\text{s} \pm 1.61 \)) compared to the reference strain and other tested oral strain, followed by RMIT-O2 (D1=2.12 \( \mu \text{m}^2/\text{s} \pm 1.83 \)), and both only belong to genomospecies A. RMIT-O16 was also significantly higher than the reference strain but had the lowest significant result, \( P=0.0067 \), although both *C. concisus* ATCC 51561 reference strain and RMIT-O16 were found to have the lowest motility speed (D1 for both 0.46±0.35 and 0.51±0.23, respectively). Our findings suggest that genomospecies A strains could be more motile than strains from genomospecies B.
Moreover, variations in the swarming zone were detected between *C. concisus* tested strains. *C. concisus* RMIT-O17 had the largest swarming zone followed by RMIT-O2, both are from genomospecies A. The reference strain ATCC 51561 and RMIT-O16 had the smallest zones; both belong to genomospecies B (Figure 5.5). These findings indicate that isolates from different genomospecies may have different growth characteristics, including growth swarming zone. The swarming ability of a number of *C. jejuni* strains have recently been studied on BB and MH semi-solid media. No significant differences were found between the wild types in either media. However, a *C. jejuni* NCTC 11168 luxS-mutant had reduced swarming ability 42% of wild-type on BB (Adler et al., 2014). Differences in swarming zones were previously found between different strains or the same strains growing in BB and MH, or growing in different temperatures (37°C and 42°C) (He et al., 2008). Therefore, type of media, temperature, bacterial genomospecies and the source of strains affect the swarming and growth characteristics and therefore interpretation of the comparison between different studies is limited. In this study, genomospecies A strains exhibited larger swarming zones than genomospecies B strains. Taken all results together; the data from the motility and invasion assays also suggests that genomospecies A may be more virulent than genomospecies B. It is noteworthy however that although *C. concisus* RMIT-O17 strain had the fastest motility, and was the most invasive strain, it was isolated from healthy young boy with no intestinal symptoms.

In summary, the *C. concisus* RMIT-O17 strain isolated from a healthy young boy is unique and has some different virulence characteristics. It was found to be significantly more invasive than the reference strain and other tested strains, and was observed to be more motile than other tested strains and had the largest swarming zone, as reported in DDM and swarming assay, respectively. This strain also produced a unique golden colour on HBA.
(3.3.1) and had the highest levels of flaC gene expression (Figure 4.11). As reported, healthy individuals can carry invasive C. concisus strains in their oral cavity. Furthermore, genospecies A strains was faster than genospecies B strains in motility tests. These results may suggest that genospecies A are more potentially virulent than genospecies B.

In conclusion, this is the first time that INT407 cell lines were used in adhesion and invasion assays with C. concisus oral isolates. This is also the first report of isolation of an enteric invasive C. concisus strain (EICC) from the oral cavity of a healthy individual (RMIT-O17). The motility of isolates as measured using the DDM method also correlated with adhesion and invasion capacity of the isolates, consistent with a role for these characteristics as virulence factors. Further studies are however required to confirm the relationship between in vitro measures of virulence and in vivo virulence.
C. concisus is a fastidious hydrogen-requiring bacterium that was first described by Tanner et al. (1981) from a periodontitis lesion. It constitutes a part of the normal flora of the human oral cavity; however several studies suggested the possible role of this bacterium in gum diseases (Macuch et al., 2000, Moore et al., 1987, Tanner et al., 1981). C. concisus has been isolated from non-oral sources since 1989, and from faecal samples of children and adults suffering from diarrhoea (Lauwers et al., 1991). The first isolation and identification of C. concisus in Australia was performed by Russell (1995) on gastroenteritis cases from children at RCH in Melbourne (Russell, 1995).

The aim of this study was to investigate the colonisation and virulence characteristics of C. concisus. In this study, a total of 47 C. concisus oral strains were successfully isolated from healthy volunteers among 7 families and other non-related healthy individuals, including 12 children (8 being infants before teething). Of the 47 C. concisus oral strains, 26 strains were isolated from healthy adults, and 21 strains were from healthy children, 9 of which were from infants before teething. C. concisus oral strains were confirmed by biochemical and molecular identification methods. The identity of the isolated strains was confirmed by the 0.5 kb C. concisus species-specific PCR product for gyr B gene following the method described by Matsheka et al. (2001). The species-specific PCR product was not detected for one C. concisus oral strain (RMIT-O30). However, this strain was confirmed to be C. concisus when further investigated by PCR amplification of the 23S rDNA (Bastyns et al., 1995b). All C. concisus isolated strains were then grouped into two major genomospecies (A
and B), following the method described by Istivan et al. (2004) as a modification of the 23S rDNA amplification method previously used by Bastyns et al. (1995b).

In general, genomospecies A was dominant (60%, 28/47); however, genomospecies B was more dominant in infants (89%, 8/9). In older children aged between 2 and 5 years, 5 of the 12 isolates belonged to genomospecies B, and the other 7 strains belonged to genomospecies A. A previous Australian study on faecal samples from gastroenteritis cases in children found that genomospecies A were in the majority (71.5%), with genomospecies B only accounting for 28.5% (Istivan et al., 2004). Other studies have reported a predominance of genomospecies B (Engberg et al., 2005, Kalischuk et al., 2011) and it is suggested that the prevalence of *C. concisus* relevant to genomospecies to be studied to determine if there are any biological basis for these differences.

To our knowledge, this is the first report of the isolation of *C. concisus* from the gum swabs of healthy infants before teething. It is also the first study to isolate *C. concisus* from all participants using the culture method. All strains collected from infants before teething exclusively belonged to genomospecies B, except the infant of family 6, who had both genomospecies. Among 2 families, three children were followed up after teething and genomospecies A strain was successfully isolated. The findings suggest that genomospecies B may colonise the human oral cavity before genomospecies A. Each family has at least one member with more than one strain or genomospecies in the mouth, which means more than one *C. concisus* strain can be isolated from the same volunteer, consistent with the previous study (Zhang et al., 2010).
Chapter Six - General Discussion

Gram-negative bacterial outer membrane proteins (OMPs) are the interface between the pathogen and its host, and may play a role in pathogenesis (Istivan, 2005). No previous study attempted to analyse the relationship between the OMPs of isolates of members of the same family. In this study, OMPs were extracted from 36 *C. concisus* strains isolated from 7 different families. Analysis of SDS-PAGE profiles showed differences between *C. concisus* oral strains within each family. These results supported the ideas of the heterogeneity and complex nature of *C. concisus* as a species. The heterogeneity of *C. concisus* OMP profiles was also reported by Istivan *et al.* (2004), and six groups were detected based on SDS-PAGE profiles for these 21 *C. concisus* isolates (Istivan, 2005). Likewise, Aabenhus *et al.*, (2002b) were able to identify and differentiate between *C. concisus* strains isolated from immunocompromised patients with diarrhoea, grouping them into two groups based on the whole cell lysates (WCL) protein profile patterns, with 85% of *C. concisus* isolates being from the same group.

In this study, Phoretix 1D software was used to assess the relationship between protein profiles of *C. concisus* strains. All families were found to have 2 clusters, with exception of family 4, which had 3 clusters. Each cluster included one *C. concisus* genmospecies (A or B) strains. Family 4 had 2 different clusters for genmospecies B strains, while the third cluster included genmospecies A strains. The similarity percentage range was 18-100%. In family 2, the OMP profiles of twin infants (RMIT-O22 and RMIT-O23) were 100% identical. However, *C. concisus* RMIT-O19 had the lowest similarity percentage with the strains from all other infants with only 18%. Based on this analysis, the protein profiles were different even within the same family. These findings are in agreement with previous reports (Kaakoush *et al.*, 2011a) and show that *C. concisus* strains are heterogeneous and have a
complex nature, even within the same family or individual multiple strains or genomospecies may be present.

Flagellin genes are essential for motility and virulence in *Campylobacter* spp. To date, no thorough molecular studies on *C. concisus* flagellin genes have been conducted. In this study, the presence of three genes related to bacterial motility (flaA, flaB and flaC) was studied in selected *C. concisus* strains using PCR, in addition the expression of these genes in liquid environment (BHI broth) was performed using RT-PCR. The whole flaA gene region was detected in just 26.5% of *C. concisus* clinical and oral isolates, regardless of genomospecies. On the other hand, the whole region of flaB gene was detected in all genomospecies B *C. concisus* clinical and oral strains (22/64). Therefore, the flaB primer set used in this study can be used to confirm the identity of genomospecies B strains. The flaC gene was the only gene detected in all tested *C. concisus* strains. Few studies on these genes have previously been conducted on *C. jejuni* and *C. coli* isolates to detect their presence, and also to determine their expressions. flaA was detected in 83% of *C. jejuni* culture positive samples (Waegel *et al.*, 1996), and in 100% of *Campylobacter* spp., being 42 *C. jejuni* isolates and 23 *C. coli* isolates (Zhang *et al.*, 2007). flaB was found in all tested *C. jejuni* (11 strains), and was also detected in 75% of *Campylobacter* spp. isolated from children (Krutkiewicz *et al.*, 2010, Muller *et al.*, 2006). Hanning *et al.* (2010) found the flaC gene in 93% of *C. jejuni* isolates. The flaC primer set used in this study is suggested to be used as species-specific primer to detect *C. concisus* rather than using the 0.5 kb *C. concisus* species-specific fragment (gyrB) (Istivan *et al.*, 2004, Matsheka *et al.*, 2001), which was not amplified in strain RMIT-O30 isolated in this study and in RCH26 from a previous study by Istivan (Personal communication).

Since the whole flaA was detected in only 17 *C. concisus* strains, and the whole flaB was detected only in genomospecies B strains, two fragments of the flaA (516 bp) and flaB (501
bp) genes were aligned with sequences of *C. concisus* ATCC 51562 and UNSW1 to identify conserved regions. Given that sequence variation between strains (rather than absence of the gene) may have accounted for this result, new primer sets were designed within conserved regions of the genes and used to investigate expression of these genes. The conserved regions of *flaA*, *flaB* and *flaC* were detected in all selected strains when targeting a smaller fragment (<300 bp). PCR products from the conserved regions of *flaA* and *flaB* genes were detected in *C. concisus* strains that were not previously amplified by the previous PCR method which targeted the full gene, suggesting that these genes are heterogeneous in different genomospecies.

No studies have been published on the expression of the flagellin genes of *C. concisus*. In this study, we used RT-PCR to determine the motility of gene expression for selected *C. concisus* strains, being the same strains tested in *in vitro* and in motility experiments. The *flaA* gene was significantly up-regulated in only one *C. concisus* strain from genomospecies B (RMIT-O23). No significant difference was found with RMIT-O22, while this gene was down-regulated in all other *C. concisus* tested strains. The results from this study show that *flaA* was expressed in higher proportions in genomospecies B strains than in genomospecies A strains. On the other hand, both *C. concisus* RMIT-O22 and RMIT-O23 strains possessed significantly up-regulated *flaB* expression, while other tested strains were significantly down-regulated. The *flaA* was previously found to be expressed at higher levels than *flaB* in *C. coli* VC167 strain (Guerry *et al.*, 1990). Generally, *flaA* and *flaB* were expressed by *C. concisus* genomospecies B more than genomospecies A strains. We found that *flaC* was the only gene up-regulated by all *C. concisus* strains tested, with a higher level of expression detected in the RMIT-O17 strains belonging to genomospecies A. The expression of *flaC* was previously found to be up-regulated in the *C. jejuni* biofilms (Guerry, 2007). This study is the first to investigate the presence and expression of the flagellin genes in *C. concisus* in relation to the
Chapter Six - General Discussion

genomospecies. Further studies on the presence and expression other putative motility-related virulence genes known to be related to *C. concisus* are required.

Motility and invasiveness are considered to be important virulence factors. The highest reported invasion level of *C. concisus* strains *in vitro* was noticed at a minimum of six hours. *C. concisus* strains isolated from healthy individuals were able to attach, but were unable to invade, unlike the strains isolated from CD patients (Man *et al.*, 2010a, Nielsen *et al.*, 2011). Other virulence characteristics in *C. concisus*, including motility, adhesion and invasion, were also investigated. Adhesion and invasion assays were performed using INT-407 epithelial cell line. In general, all tested *C. concisus* strains were able to adhere to INT407. However, all oral strains were significantly lower in adherence proportion than the reference strain ATCC 51561 which was originally isolated from faeces of a healthy individual. Notably, while *C. concisus* ATCC 51561 was poorly adherent to Caco-2 cells (Kaakoush *et al.*, 2011b), it did adhere better to INT407 cells in this study. *C. concisus* RMIT-O23 and RMIT-O22 were the most adhesive oral strains (2.110 and 2.070 respectively), and both were from genomospecies B.

All of oral strains tested were significantly less invasive than the reference strain ATCC 51561, with the exception of *C. concisus* RMIT-O17 strain. *C. concisus* ATCC 51561 was found to be invasive to INT407 in this study, but it was not considered as enteric invasive strain. This strain did not previously invade Caco-2 cells (Man *et al.*, 2010a). The *C. concisus* RMIT-O17 strain (genomospecies A) was the only enteric invasive *C. concisus* strain (EICC) amongst the other isolated oral strains in this study. The EICC term has previously been used to describe the relationship between *C. concisus* oral strains and enteric epithelial cells (Ismail *et al.*, 2012). RMIT-O17 had an invasion index >1 (9.313), which fits the criteria of Larson *et al.* (2008). All other tested strains, including the reference strain ATCC 51561,
have invasion indexes <1. This is the first report to find EICC from healthy individuals, as EICC oral strains were previously reported in 50% (4/8) of \textit{C. concisus} oral strains isolated from IBD patients only, and none of the healthy controls’ isolates using Caco-2 cells (Ismail \textit{et al.}, 2012). Therefore, healthy individuals may carry enteric invasive \textit{C. concisus} strains (EICC) in their oral cavity. Comparison between this study and other published reports suggests that the cell line used can significantly impact the results. It is suggested to use other types of cell lines like Caco-2 and Hep-2 cells to investigate the adhesion and invasion capabilities of these \textit{C. concisus} strains and compare the results with current findings.

Motility was also measured using a modified method from Differential Dynamic Microscopy (DDM). This method has not been applied to bacteria other than \textit{E. coli}. DDM was used to measure the swimming speed and motile cell fraction of \textit{E. coli} (Wilson \textit{et. al.}, 2011). In this study, DDM was employed on selected \textit{C. concisus} strains grown in BHI broth. The D1 (motile cells) and D2 (Brownian motion) for \textit{C. concisus} ATCC 51561 and RMIT-O16, are close together, indicating that the motility is not much faster than normal Brownian motion. However, D1 values of RMIT-O16 and all other oral \textit{C. concisus} strains were significantly higher than the reference strain and considered to be motile. \textit{C. concisus} RMIT-O17 was the most motile strain compared to the reference strain and the other tested strains (11 times faster than the background Brownian motion), followed by \textit{C. concisus} RMIT-O2. Both strains are from genomospecies A. This finding suggests that genomospecies A could be more motile than genomospecies B.

Motility swarming on brucella semi-solid medium was performed. After 3 days of incubation, a halo zone of motility was observed around the inoculation point using motility swarming assay on BB semi-solid medium. Interestingly, \textit{C. concisus} RMIT-O17 has the biggest swarming zone (~1.00 cm) compared to the other tested strains, followed by RMIT-O2.
(−0.75 cm), both belonged to genomospecies A (Figure 5.5). This finding is consistent with
the DDM findings that genomospecies A is faster than genomospecies B. In previous studies,
C. jejuni mutant strains had smaller swarming halos than wild type strains (Adler et al.,
2014), and variations were also noted within different C. jejuni strains growing in different
media (BB and MH), or growing in different temperatures (37°C and 42°C) (He et al., 2008),
suggesting that motility is affected by a number of environmental factors, which is probably
ture for C. concisus. The finding of the current study suggests that genomospecies A strains
were more motile than genomospecies B and may therefore be more capable of swimming
through intestinal mucus and reaching the epithelium.

To summarise, this is the first study to isolate C. concisus from gum swab samples of all
participants by culture method. It is also the first study to isolate this bacterium from samples
of infants before teething. Genomospecies A (60%) was represented more than B (40%);
however, genomospecies B was dominant in children. We found that the oral cavity is firstly
colonised by genomospecies B strains. Healthy individuals can carry enteric invasive C.
concisus strains in their oral cavities, raising questions about what might trigger these strains
to cause infection. Analysis of the DDM method indicated that the motility speed of C.
concisus strains was different and genomospecies A was more motile than genomospecies B.
C. concisus RMIT-O17 is a unique strain, in that it was isolated from a healthy child. It had
unusual colony morphology, and a higher expression level of flaC gene, and was found to
have greater motility speed than others. This strain was also found to be significantly more
invasive than the other tested strains. It was also the only enteric invasive C. concisus strain
(EICC).

Further studies are required to isolate C. concisus from healthy infants before teething to
confirm which genomospecies firstly colonise their mouths. Future studies should be
expected to include more families to differentiate between *C. concisus* strains within the same family. It is recommended to study people and families from different backgrounds to confirm which molecular group is dominant. Another technique like MALDI-TOF MS can be developed and used to identify *C. concisus*. MALDI-TOF MS has been only applied to identify *C. concisus* from stool samples by Casanova et al. (2015), although it does not always identify two different species present in the same sample (Bessede et al., 2011).

It is suggested that additional whole genome sequencing for oral strains is required to facilitate the detection and investigation of *C. concisus* potential virulence genes. Knock-out mutants of *flaA*, *flaB* and *flaC* genes will be required to investigate the role of these genes in *C. concisus* motility. Moreover, *C. concisus* strains isolated from healthy volunteers should also be screened for the presence of other possible virulence genes related to motility to determine the role of these virulence genes. If possible, another study could be conducted to follow up on the same participants when they become ill to isolate *C. concisus* from their stool or biopsies, and compare these isolates with the original isolates from their mouths. *In vitro* and *in vivo* virulence related assays may also be used to determine if the gut isolates are more pathogenic. The expression of motility genes (*flaA*, *flaB* and *flaC*) should also be studied in different conditions to investigate which factors influence expression, and therefore motility. The DDM is a reliable method to compare the motility of *Campylobacter* spp. and can provide insight into more virulent strains. Although some individuals can carry enteric invasive *C. concisus* strains in their oral cavities, these strains cannot become pathogens because the hosts are healthy and presumably have effective immune control of these potential pathogens. However, it is likely that these enteric invasive *C. concisus* strains could be significant pathogens in immune suppressed persons.
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