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The Effects of Bile on *Campylobacter concisus* Growth and Protein Expression

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Abstract

Campylobacter concisus has been considered as a possible triggering agent of Inflammatory Bowel Disease (IBD). This aim of this project was to determine the impact of bile on the growth and survival of *C. concisus* strains isolated from patients with IBD and controls. Furthermore, the effects of bile on *C. concisus* protein secretion were examined and cloning of a *C. concisus* gene that is related to bile exposure was attempted.

C. concisus strains (42 strains) previously isolated from saliva samples of patients with IBD and healthy individuals were examined. *C. concisus* strains were cultured on horse blood agar plates containing 2% ox bile (HBA-B). The inhibition of bile on *C. concisus* growth was further quantified by the examination of the colony forming unit (CFU). *C. concisus* protein secretion in response to bile exposure was examined using mass spectrometry analysis. Gene cloning was performed following standard procedures.

Of the 42 *C. concisus* strains examined, 45.2% strains (19/42) were resistant, 38.1% strains (16/42) were tolerant and 16.7% (7/42) were sensitive to 2% bile. The rates of resistant, tolerant and sensitive to bile of *C. concisus* strains isolated from patients with CD, UC and healthy controls were not statistically significant ($P > 0.05$). The degree of concentration dependent bile sensitivity varies between strains of *C. concisus* strains. The presence of 2% bile greatly inhibited the growth of *C. concisus* strains; the percentage CFU of bile resistant strains on HBA-B and HBA plates were 0.01% - 6.34%. Furthermore, bile impacted *C. concisus* growth in a time and concentration dependent manner.

A hypothetic protein CCC13826_1330 was detected in the supernatant of a *C. concisus* strain cultured in medium containing 2% bile, but not in the bile-free medium. Cloning of CCC13826_1330 gene attempted. However, it was not successful.

In summary, this study found that *C. concisus* is a bacterium that has low resistance to bile. Difference in bile resistance between individual strains was observed, which is unlikely a factor that contributes to the high prevalence of *C. concisus* in the intestinal tract of patients with IBD.

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Abbreviations

GBS - Guillain-Barres Syndrome

PCR - Polymerase Chain Reaction

IBD - Inflammatory Bowel Disease

UC - Ulcerative Colitis

CD - Crohn's Disease

BSEP - Bile Salt Export Pump

ASBT - Apical Sodium-Dependent Bile acid Transporter

GIT - Gastrointestinal Tract

CDT - Cytolethal Distending-like Toxin

RND - Resistance-Nodulation-Division

MDR - Multidrug Resistance

LPS - Lipopolysaccharides

BSH - Bile Salt Hydrolase

HBA - Horse Blood Agar

HBA-B - Horse Blood Agar with Bile

HiB - Heart Infusion Broth

OD$_{600}$ - Optical Density (600nanometers)

R - Resistant

T - Tolerant

S - Sensitive

CFU - Colony Forming Units

NCBI - National Centre of Biotechnology Information

NA - Nutrient Agar

NB - Nutrient Broth

MCS - Multiple Cloning Site

Chapter 1: Introduction

1.1 The *Campylobacter* genus

Campyolobacter belong to the family *Campylobacteraceae*, a diverse group of pathogenic, commensal and environmental Gram-negative bacteria, currently comprising 3 genera: Campylobacter (31 taxa, Arcobacter (17 taxa), and Sulfurospirillum (7 taxa) (79). These bacteria are found in surface water, ground water, and food animals such as cattle, pigs and sheep, and their products such as milk. Campylobacter and Arcobacter colonize the mucosal surfaces of the intestinal tracts, urogenital tracts and oral cavities of humans and a broad range of animal and bird hosts. The *Campylobacter* genus contains 31 species with morphology presenting as curved, spiral rod shaped cells ranging between 1.5 and 3.5µm in length and 0.2 and 0.4µm in width. They are also Gram negative, nonsporing, flagellated and appear to move in a spiralling corkscrew-like motion when examined under a light microscope (71).

Differing species of *Campylobacter* can be found in the gastrointestinal tracts of humans and animals forming long term commensal relationships with occasional pathogenic results (49, 71, 84).

1.2 *Campylobacter jejuni, Campylobacter coli* and *Campylobacter fetus* are established human enteric pathogens

Of all the known species of *Campylobacter*, two species are established human pathogens. These include *Campylobacter jejuni, Campylobacter coli* and *Campylobacter fetus*, the first of these being considered the most common cause of bacterial gastroenteritis in industrialized nations. *C. jejuni* has been isolated from a variety of animals but typically infects humans via consumption of contaminated poultry products (60, 83, 84). In chickens, *C. jejuni* is considered a commensal organism. However, in humans it can result in intestinal inflammation and diarrhoea (60, 84).

C. coli is primarily found in pigs as opposed to chickens, although strains of *C. coli* have been isolated from chickens and cattle. *C. fetus* is a known pathogen associated with abortions in cattle and sheep (34).

In humans, *C. coli* has been reported to cause fewer cases of gastroenteritis when compared to *C. jejuni.* For example, a study by Gillespie *et al*. examined 3,764 cases of *Campylobacter* based enteritis in England as well as Wales and found 93% of cases to be associated with *C. jejuni*, 7% with *C. coli* and other *Campylobacter* species accounting for less than one percent of these cases (15).

In a different study conducted by Rajan *et al*., *C. fetus* was isolated from 45 of 305 stool samples (14.8%) from a stratified randomized sample of the population (379 families) of a village in rural India. The isolation rate was found to be highest in pre-school children under the age of 5 (37%) (66).

In addition to gastroenteritis, *C. jejuni* has been associated with other human disorders. Guillain-Barre syndrome (GBS) is an acute polyneuropathy able to result in defects in both motor and sensory function in humans; the condition arises from immune recognition of peripheral nerve cells. Due to approximately 67% of GBS patients reporting infections one to four weeks before onset, GBS is considered a post-infectious disease, where preceding infections generate an immune response cross-reacting with peripheral nerve antigens (27).

A wide range of pathogens have been reported in GBS patients (27, 38). *C. jejuni*, in particular, has demonstrated a strong association with GBS in a series of studies (27). An example includes a study conducted by Jacobs et. al investigated the preceding infections and antibody titres of 154 GBS patients compared to the same number of controls; *C. jejuni* infections were found in 49 GBS patients compared to 18 controls in the same time period (27).

C. jejuni has been associated with the production of anti-ganglioside antibodies with cross-reactivity for ganglioside GM1 (56). In the same study by Jacobs *et al*., GM1 antibodies were found in 31 of 154 GBS patients, 20 of them following *C. jejuni* infections compared to 10 in controls (27). It is suggested that these antibodies are related to axonal degradation of peripheral nerves in GBS (16).

1.3 Emerging pathogenic species of *Campylobacter*

In addition to *C. jejuni, C. coli* and *C. fetus,* other species of *Campylobacter* have been isolated from the stools of patients with gastroenteritis, septicemia, gingivitis and other conditions. These include *Campylobacter concisus, Campylobacter showae, Campylobacter rectus* and *Campylobacter curvus* and *Campylobacter lari* (34, 44).

Studies has indicated that emerging *Campylobacter* species have pathogenic potential. *Campylobacter upsaliensis,* for exmaple, is able to cause acute and chronic diarrhoea in healthy as well as immunocompromised humans. *C. upsaliensis* has also been frequently isolated from stool samples of dogs (64% of 98 dogs) (34). *Campylobacter hyointestinalis*, more commonly known for causing enteritis in pigs, has also been isolated from human stools and thought to be associated with diarrhoea in humans (34).

The exact pathogenic role of these species has not been well studied as isolation requires more specific growth conditions than what is typically available in laboratories. A hydrogen enriched environment as well as a lower incubation temperature (37ºC compared to 42ºC for *C. jejuni*) are required for effective laboratory growth (35).

1.4 *C. concisus*

C. concisus was first isolated by Tanner et. al in 1981 from oral lesions of patients with periodontal disease (73). It was later found by Zhang *et al*. that *C. concisus* is commonly found in the oral cavities of humans. In this study, *C. concisus* was isolated from 97% of saliva samples collected from healthy individuals and confirmed by PCR in 100% of the samples. Some individuals are even colonized by multiple strains of *C. concisus* (85).

Figure 1.1: Electron Microscopic image of an enteric *C. concisus* isolate. (25).

1.5 *C. concisus* and human diseases

1.51 Gingivitis

Despite being a common oral bacterium, *C. concisus* has been linked with a number of human diseases. Gingivitis is a chronic inflammatory disease responsible for connective tissue and bone degradation resulting in tooth loss (59). In an attempt to examine whether *C. concisus* is associated with periodontal disease, Machuch *et al*. compared the isolation rates of *C. concisus* from gingival plaque collected from the shallow periodontal pockets of patients with gingivitis and healthy individuals (44). This study isolated 6 *Campylobacter* species *(Campylobacter gracilis [37.5%], Campylobacter sputorum [6.5%], C. concisus [19.7%], C. showae [1.3%], C. curvus [3.2%] and C. rectus [9.8%])* (44) from these patients. *C. concisus,* in particular, was found in 70% of patients with initial periodontitis compared to 40% in healthy individuals. The association between *C. concisus* and ginigivitis is currently not conclusive; further studies examining a larger number of patients and controls are required for clarification.

1.52 Gastroenteritis

A population-based study by Nielsen *et al*. was conducted in North Jutland, Denmark between January 2009 and December 2010 looking at 29,424 human diarrhoeal stool samples found a high prevalence of *C. concisus* in these samples. Out of 1,532 patients, 400 were positive for *C. concisus* and a total of 441 isolates were found in stool samples of said patients. This figure was comparable to the isolates for *C. jejuni*, where 541 isolates were found in 489 patients (53). The study also found a trend between age groups; the age groups with the highest rates of *C. concisus* infections were between 0-4 years of age (90 cases per 100,000 population per year) and over 65 years of age (66 cases per 100,000 population per year) (53) while *C. jejuni* and *C. coli* had higher incidence rates in people between 15-24 years of age (67 cases per 100,000 population per year). This information suggests that those with underdeveloped or weakened immune systems (infantile and elderly) are more susceptible to *C. concisus* infections. This information is further supported by other researchers; Engberg *et al*. found 52 isolates of *C. concisus* from 39 clinical gastroenteritis cases (11). In this study, *C. concisus* was isolated more frequently from extreme age groups (i.e. 0-9 years of age and 60+ years of age (11)), a result similar to the previously mentioned investigation.

Aabenhus *et al*. conducted a study on severely ill and immunocompromised patients with gastroenteritis (healthy individuals were not included); pathogens were isolated from 11,550 stool samples. Of these, 224 were identified as *Campylobacter*. Further examination included 110 out of the 224 (49%) *Campylobacter* stool sample isolates testing positive for *C. concisus* (1). The study found no observable trend between age groups and *C. concisus* infection (as opposed to the previously mentioned studies) (1).

Another study by Nielsen *et al*. documented the short and long term clinical manifestations of *C. concisus* infections. Symptoms such as nausea, vomiting, headache, dizziness, abdominal pain and muscle aches were relatively similar (within 10% of each other) but large differences were found in fever and chill incidences; 23.9% of 139 *C. concisus* patients presented with fever compared to 69.7% of 187 *C. jejuni/C. coli* patients and chills were reported at 34.1% and 71.0% respectively. Another difference was observed in the duration of infection; the majority of *C. jejuni/coli* infections subsided after 14 days (68%) with 32% of infections lasting longer than 14 days. *C. concisus*, on the other hand, was mostly involved with cases lasting longer than 14 days (79.9%) and only 20.1% of infections subsiding before 14 days. This information indicates that there may be differences in virulence between *C. concisus* and *C. jejuni/coli* that lead to differences in their infection duration and severity (54).

1.53 *C. concisus* and the Henle-Koch Postulate

The Henle-Koch Postulate, loosely translated by Rivers in 1937, states the following (12):

1. The parasite occurs in every case of the disease in question and under circumstances which can account for the pathogenical changes and clinical course of the disease.

2. It occurs in no other disease as a fortuitous and non-pathogenic parasite.

3. After being fully isolated from the body and repeatedly grown in pure culture, it can induce the disease anew.

In addressing the first statement, *C. concisus* is not able to fulfil the Henle-Koch postulate. While *C. concisus* has a high prevalence in patients with gastroenteritis (1, 11, 53, 54), it is not the only causative agent behind *Campylobacter* based gastroenteritis, as other species of *Campylobacter* have demonstrated strong associations as well (e.g. *C. jejuni*). In addition, *C. concisus* has also been isolated from the stools of healthy individuals without pathogenic onset. Finally, because the pathogenic role of *C. concisus* in human disease is not fully known, it cannot be said that pathogenical changes in *C. concisus* accounts for the clinical course of the disease. However, with additional research, there remains the possibility that *C. concisus* would be able to fulfil the statement in time.

The second statement also cannot be fulfilled as *C. concisus* has also shown association with other diseases (e.g. Gingivitis) (59). It is also able to exist in humans in a non-pathogenic state (85).

Finally, due to limited research, *C. concisus* has not yet been found to fulfil the third statement as gastroenteritis has not yet been replicated in animal studies through the introduction of *C. concisus* (although it has been done for other species e.g. *C. jejuni*) (41). Again, with increased research in the area, *C. concisus* may one day meet the third statement of the Henle Koch postulate.

1.6 Inflammatory Bowel Disease (IBD)

IBD is both Ulcerative Colitis (UC) and Crohn's Disease (49), chronic inflammatory disorders of the intestines. UC is characterised by inflammation confined to the mucosa and superficial submucosa of the colon while CD displays a more elaborate inflammatory response; initial development of CD in the mucosa of the intestines resembles that of UC except CD persists in deeper layers of intestinal walls. CD is also able to occur in multiple areas of the intestines while UC is typically confined to the colon (61).

Incidences of IBD are reported all over the world, with the majority of cases being focused in Northern Europe, the United Kingdom and North America (42). However, recent reports indicate that IBD is increasing in incidence in other areas including the rest of Europe, Asia, Africa and South America (42).

In North America, 2.2 - 14.3 cases were observed per 100,000 persons for UC and 3.1 - 14.6 cases per 100,000 for CD between 1980-1996. In Europe, 1.5 - 20.3 cases per 100,000 persons were reported for UC while 0.7 - 9.8 cases per 100,000 relate to CD in the same time frame. In other areas of the world, incidences are increasing in number where they were previously considered to be more rare; South America and Asia, for example, observed a rise in UC (Between 1987 and 2000 in Asia and 1981 - 1994 in Africa) (42). Studies suggest that

'Westernized countries' or countries becoming more 'Westernized' observe higher incidences of IBD; this evidence suggests that a number of environmental factors could contribute to the onset of UC and CD.

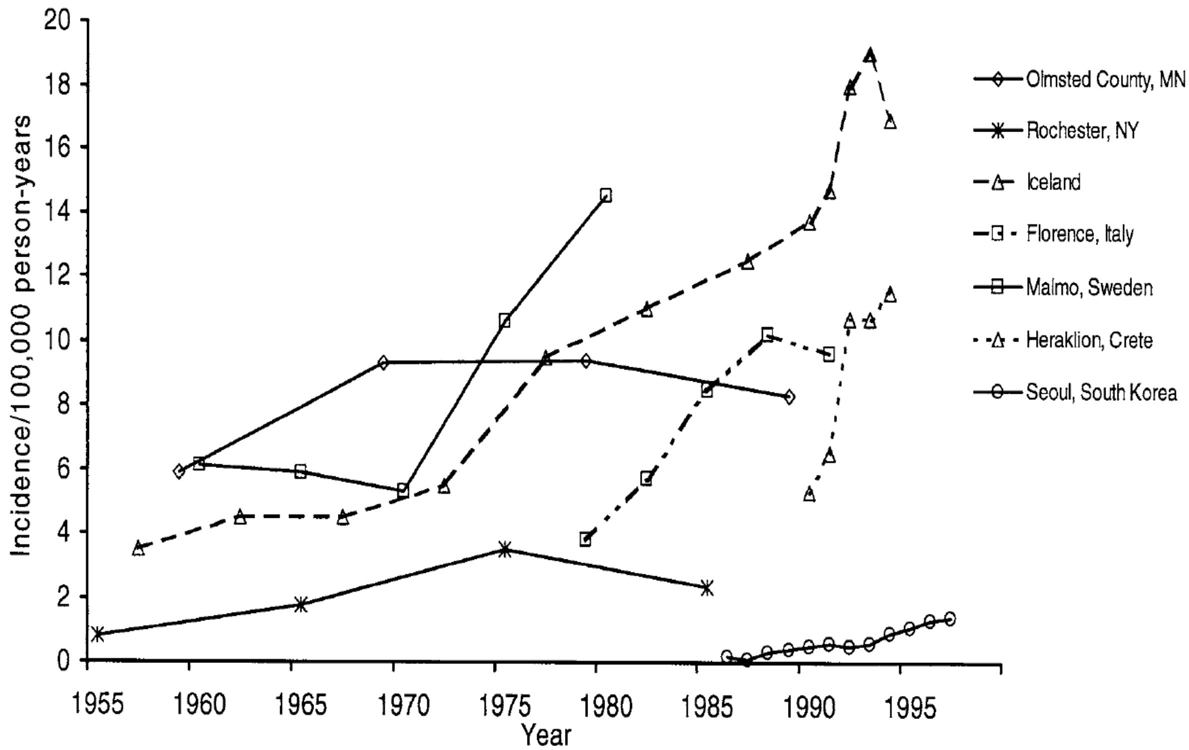

Figure 1.2: Trends in prevalence of UC per 100,000 persons over the years in selected geographical regions (Minnesota, New York, Iceland, Italy, Sweden, Greece and South Korea) (42).

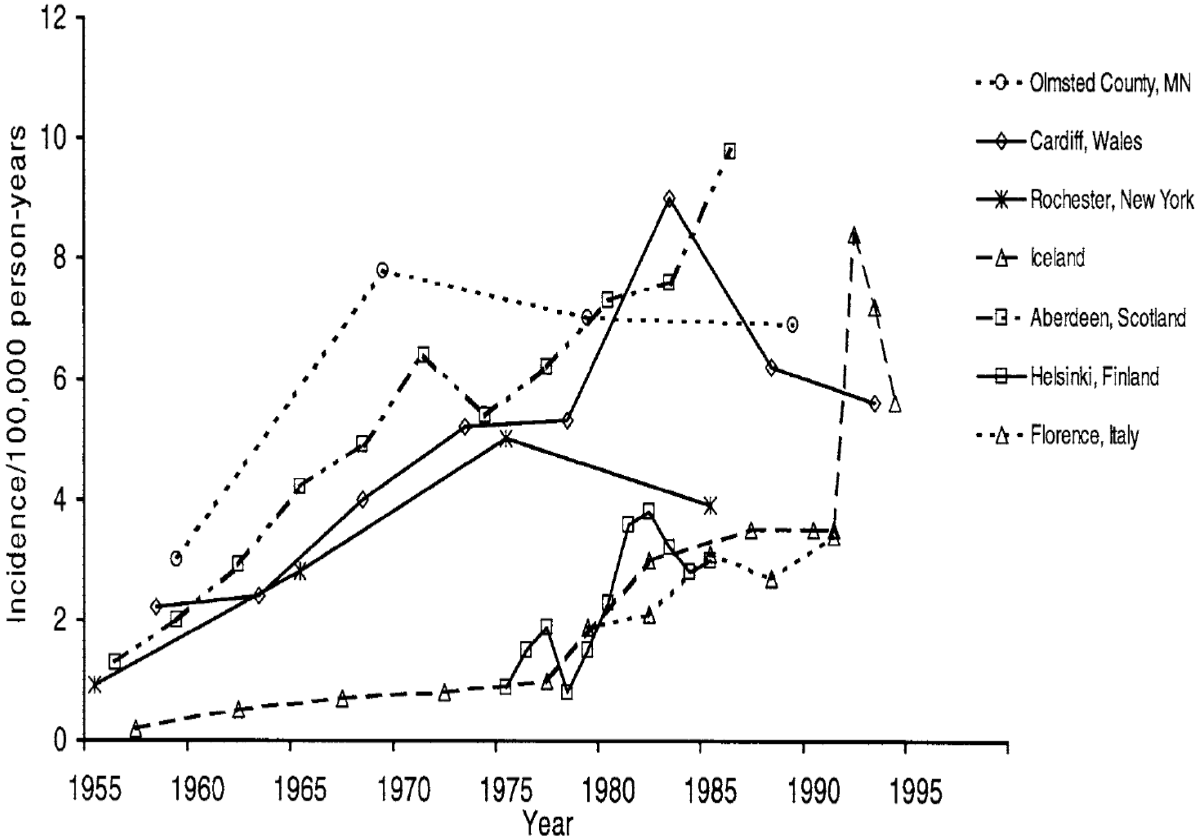

Figure 1.3: Trends in prevalence of CD per 100,000 persons over the years in selected geographical regions (Minnesota, Wales, New York, Iceland, Scotland, United Kingdom, Finland and Italy) (42).

The etiology of IBD is still not fully known but there is a current prevailing hypothesis. It has been established that there is a genetic element behind the onset of IBD; Orholm *et al*. illustrated this by conducting a study on medical records of 662 IBD patients from Copenhagen, Denmark. Initial impressions were that first-degree family members of a patient had a ten-fold risk of developing the same disease as the patient (UC or CD) as well as a lesser but present risk of developing the other condition encompassed by IBD (58). Following this pattern, second-degree relatives and more distantly related relatives of IBD patients had a lesser risk of developing the same disease (58). Orholm et. al also conducted a study on Danish twins in which 58.3% of twins were concordant in CD development as well as 18.2% in UC, while dizygotic twins were 0% and 4.5% respectively (57).

Another current hypothesis regarding the onset of IBD relates to microbiota; it is thought that alterations to the microbial community of the intestines can result in adverse immune responses in genetically susceptible individuals. Works by Sartor *et al*., for example, determined that the culprit could be a member of commensal bacteria; the introduction of bacterial cell wall fragments in rats resulted in inflammation of the colon (69). This information suggests a possible microbial agent behind the onset of IBD.

There are a number of potential microbial agents for the onset of IBD; research so far suggests that the inflammatory response is largely due to incorrect recognition of commensal microbes by the immune system. A combination of genetic and environmental factors can be responsible for this event. The environmental factors have a large amount of variation in this case; for example, bacterial dysbiosis is commonly observed in IBD. However, studies to date have shown that intestinal inflammation does not occur without intestinal microbiota (24, 69, 74).

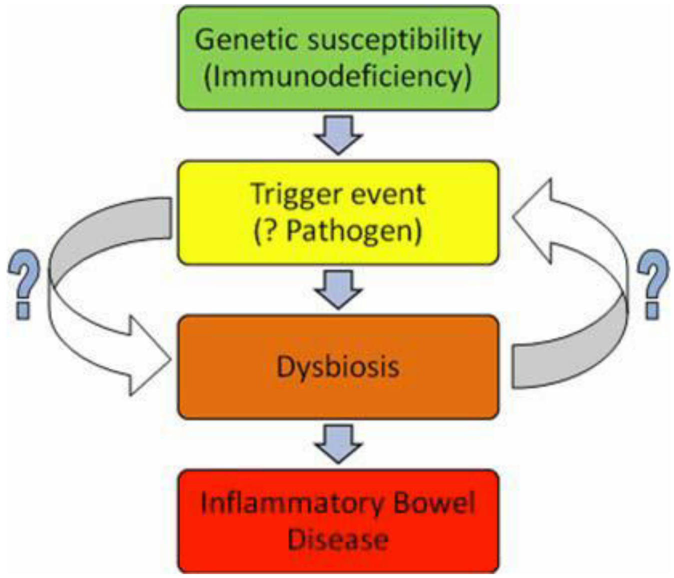

Figure 1.4: Potential sequence of events in the microbial hypothesis of the development of IBD (19).

It is unclear whether or not the dysbiosis is a cause or result of IBD but a heavy proliferation of Gram negative anaerobes is commonly observed in IBD cases, particularly CD (72). Works by Leach *et al*. found that even diet can play a role in IBD pathogenesis; children suffering from CD underwent exclusive enteral nutritional therapy in an attempt to alter the composition of bacterial flora. This was successful in reducing levels of Gram negative anaerobes, particularly *Bacteroides* species associated with increased levels of inflammation (36).

1.7 *C. concisus* and IBD

C. concisus has been found to be associated with IBD in a number of studies; studies by Zhang *et al*. found a high prevalence of *C. concisus* in children diagnosed with CD; about 51% of patient intestinal biopsies were found to be positive for *C. concisus* compared to healthy control samples (2%), indicating a possible relationship between *C. concisus* and IBD (47, 88). Mukhopadhya *et al.* found that *C. concisus* was also found more frequently in UC patients when compared to healthy control samples; 73.9% of UC patient biopsies were found to contain high levels of intestinal *C. concisus* when compared to 32.3% of controls (50). Finally, Mahendran *et al*. detected *C. concisus* in 68% of patients with IBD when compared to healthy individuals (18%) (46). *C. concisus* having a high prevalence in patients with IBD suggests a connection but the role of this bacterium in the onset and development of IBD is still not fully known.

Studies by Istivan *et al.* revealed an ability for *C. concisus* to produce a cytolethal distending-like toxin (CDT) among other virulence factors allowing the bacteria to effectively invade host intestinal cells (10). Aabenhus *et al.* determined the presence and activity of haemolytic phospholipases as well as finding that different strains of *C. concisus* possess different abilities to colonize a mouse gastrointestinal tract (GIT), suggesting that if *C. concisus* were to play a significant role in IBD development, perhaps this only occurs in certain individuals due to varying virulence between *C. concisus* strains (2, 26).

Works by Mahendran *et al.* also identified preferential infection sites for *C. concisus*; it was found that there was a much higher prevalence of *C. concisus* in the descending colon and rectum when compared to the caecum and ileum. These sites are exclusive targets of UC related inflammation and possible targets of CD related inflammation. Controls (healthy patients), however, had a low rate of *C. concisus* prevalence in all biopsy sites (46).

1.8 Colonization of oral *C. concisus* in the intestinal tract

Recent work by Ismail *et al*. revealed that strains that have successfully colonized human intestines can originate from the oral cavities of the same patient through the identification of identical protein bands between an oral and intestinal *C. concisus* strain in the same host (25). This suggests that these oral strains have travelled down the alimentary tract to the large intestines without destruction by host defences. An individual is likely to possess multiple strains of *C. concisus* in the oral cavity, so it is likely that some strains would be able to successfully colonize the colon. Similarities in previous studies were also found between an intestinal *C. concisus* strain and an oral strain of another patient. This suggests that infectious *C. concisus* strains may originate from the oral cavities of other humans (25).

In order for oral *C. concisus* strains to colonize the intestinal tract, the bacterium would have to survive exposure to multiple host defences and natural antimicrobial agents in the GIT such as gastric acid in the stomach and bile in the small intestine.

1.9 Bile - A natural microbial detergent

Bile fluid contains mostly cholesterol, bile acids (bile salts), bilirubin, lipids and proteins (e.g. IgA antibodies) (52). It also contains water, potassium, sodium and other trace elements such as copper (3, 22, 52).

Bile acids are produced in the liver at a rate of 0.2-0.6 g/d and is secreted by canalicular bile salt export pumps (BSEP) before being stored in the gallbladder (8). After eating, the gallbladder contracts and empties bile acids into the intestines in response to lipid content in ingested food (40, 52) as bile has a direct role in lipid emulsification and digestion. Bile is almost completely reabsorbed before it reaches the large intestine for storage and re-release (52), a process facilitated by both passive diffusion (ionic and non-ionic diffusion (31)) and through the activity of apical sodium-dependent bile acid transporters (ASBT) found in the membranes of ileum brush borders (8).

This cycle repeats multiple times throughout the day, eventually creating a large pool of bile. This process is dependent on fecal excretion of bile being less than or equal to synthesis rates (31).

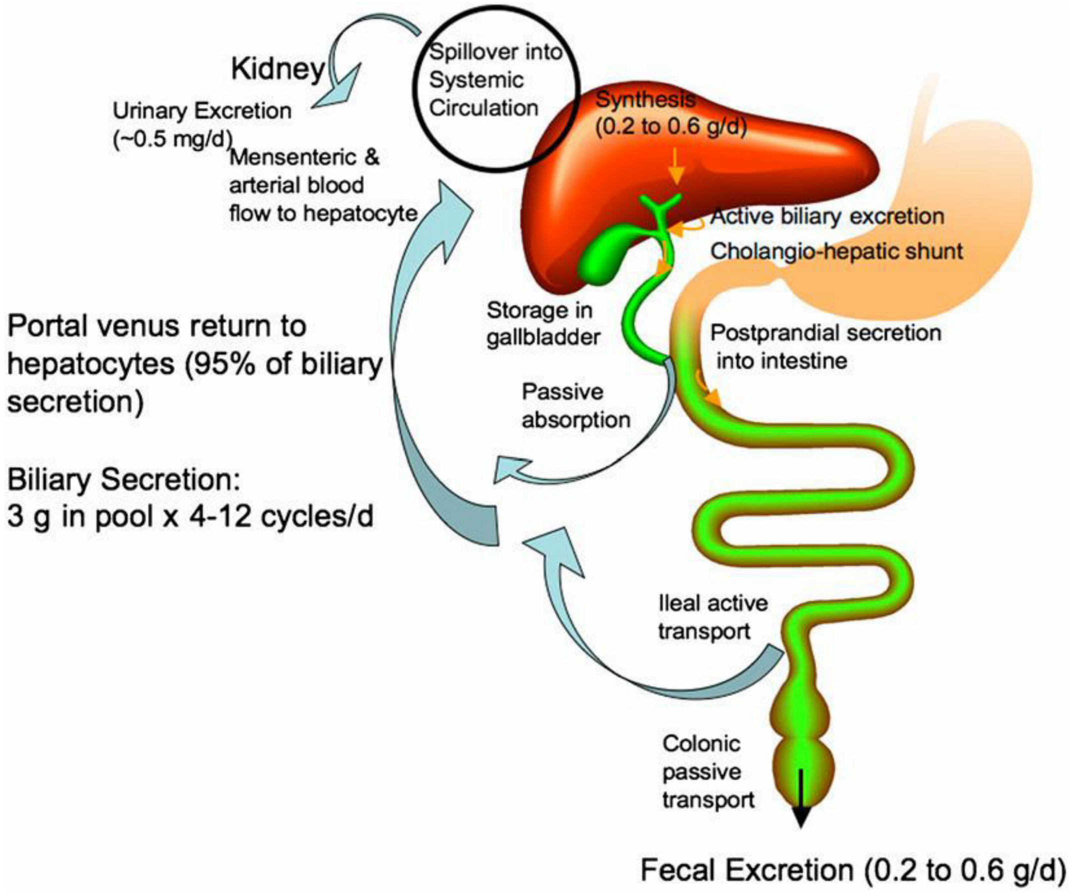

Figure 1.5: Diagrammatic illustration of the bile cycle. (8)

Bile acids are produced as primary bile salts (taurine and glycine conjugated cholic and chenodeoxycholic acids) which undergo deconjugation to yield secondary and tertiary bile salts (e.g. cholic acid and chenodeoxycholic acid). Non-conjugated bile acids (cholic, deoxycholic and lithocholic acids) are most effective against Gram negative bacteria; the amphipathic molecules destroy the lipid bilayer of bacterial cell membranes resulting in death of the cells (18). It can be assumed that bacteria persisting in the intestines possess a certain degree of bile resistance; their cell walls and membranes can interfere with the rate of bile acid penetration but is unable to stop it outright, presenting a need for an active method of bile acid removal (75).

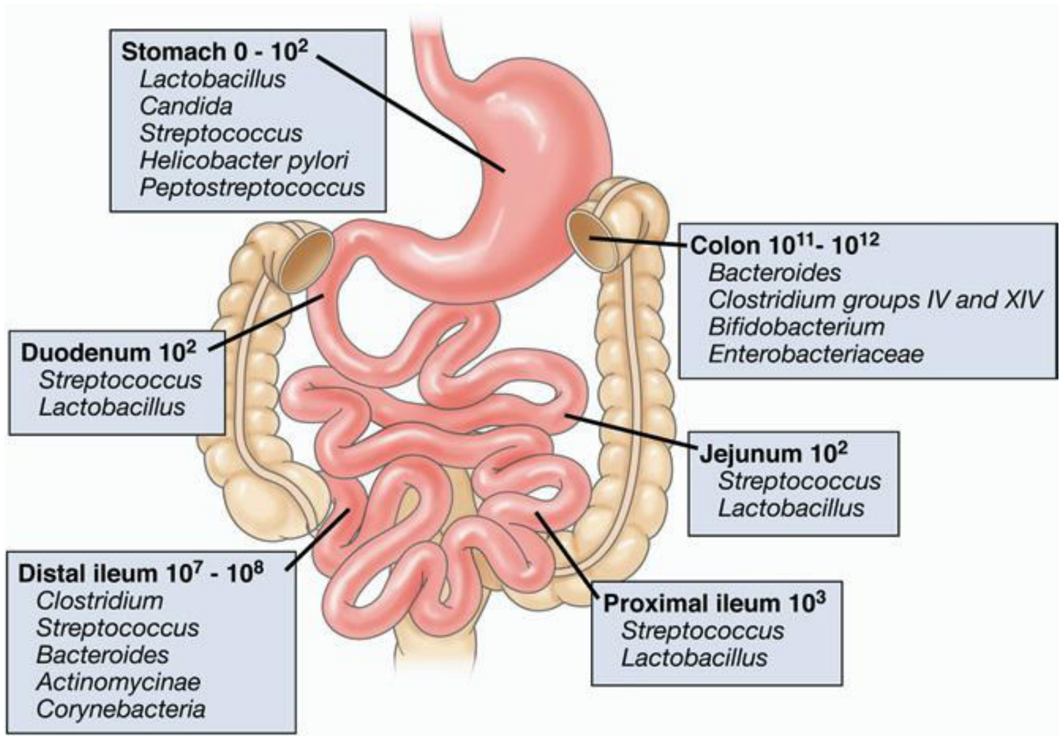

Figure 1.6: Illustration of general bacterial concentrations and variety in the gut (70).

Bacteria with strong abilities to resist bile would be able to survive or even thrive in the duodenum, jejunum and proximal ileum. For pathogens of the GIT, bile tolerance can facilitate colonization (3, 4, 9, 17, 40, 41, 62, 64, 75, 78)

1.9.1 Bile resistance in *Salmonella typhimurium*

S. typhimurium typically targets the small intestines, areas where bile concentrations are particularly high (37, 52). As a result, the species would require a high level of bile resistance; *S. typhimurium* is able to tolerate bile concentrations up to 18%, a level much higher than those typically found in the human ileum (62). This resistance is dependent upon the presence of PhoP and PhoQ; two components of a genetic regulatory system necessary for infection in mammalian hosts (i.e. mice, humans) by facilitating persistence within macrophages (13). Interestingly, through an unknown mechanism, this system also allows for high levels of bile resistance in *S. typhimurium (62)*.

S. typhimurium can develop a chronic asymptomatic infection of the gall bladder. This usually occurs in 1-3% of patients (63). The PhoP/PhoQ regulatory system essentially activates or deactivates various proteins and virulence factors in response to the host environment; activation of this system from bile exposure can increase the resistance of the bacteria to bile concentrations over 60% (78). Mutants of this regulatory system, in addition, are sensitive to significantly lower levels of bile, dying in concentrations as little as 2% (78).

1.9.2 Bile resistance in *Bacteroides fragilis*

B. fragilis thrives in human large intestinal conditions and is a prominent member of commensal microorganisms with potential to act as an opportunistic pathogen. This is due to certain factors including being able to utilize a wider range of dietary polysaccharides, the ability to tolerate high concentrations of bile (up to 5%) and high genetic variability in surface antigens for immune evasion (64).

The toleration of bile salts is important in order for *B. fragilis* to effectively colonize the intestines; this is facilitated by efflux. *B. fragilis* shares the Resistance-Nodulation-Division (RND) family efflux pump with other Gram negative intestinal bacteria (e.g. *S. typhimurium).* The pump has the function of actively transporting bile salts out of cells, among others (64).

1.9.3 Bile resistance in *Listeria monocytogenes*

L. monocytogenes is a Gram positive bacteria and does not share the same methods of bile resistance as Gram negative enteroinvasive bacteria. Begley *et al*. identified three genes thought to be involved with bile resistance through the use of deletion mutants (3). Only one of the genes was associated with bile hydrolysis; *bsh* encodes hydrolaze enzymes and is regulated by the two genes PrfA and *sigB*. *sigB* appears to have a greater effect on *bsh* expression, as mutants of said gene were found to be highly sensitive to bile salts (3). In addition, murine infection studies revealed that *sigB* expression is increased in anaerobic conditions; such expression can allow certain strains of *L. monocytogenes* to tolerate bovine bile concentrations of up to 30% (3). PrfA also has some effect on BSH production as a positive regulator of *bsh* (9).

L. monocytogenes has been isolated from the human gall bladder, the site where synthesized bile is concentrated and stored (6). It is therefore clear that even though the exact mechanism of bile resistance is not well understood, *L. monocytogenes* certainly exhibits a high capacity for bile tolerance (3, 6, 21). While *L. monocytogenes* typically persists through invasive activity in intestinal cells, its ability to survive in the lumen of the gall bladder indicates an extracellular mechanism of survival and reproduction (20, 21). Works by Hardy *et al*. supported this information by successfully growing *L. monocytogenes* in the gall bladders of mice (20).

1.9.4 The acrAB efflux pump

Efflux of bile salts from cells is facilitated by an acrAB efflux pump, a system shared by various Gram negative intestinal pathogens (e.g. *S. typhimurium, Escherichia coli [commensal and pathogenic] and B. fragilis)*. Lacroix *et al*. identified similarities in the system between *S. typhimurium* and *E. coli,* the transcription of which is under the control of a repressor. In the presence of deoxycholate bile, MarR (transcriptional repressor) is bound and rendered unable to interact with the *mar* operon, allowing increased transcription and resultant production of acrAB (33).

In terms of bile resistance, upregulation of the acrAB efflux pump allows for the active removal of deconjugated bile salts from the cell itself and therefore plays a crucial role. Active removal is achieved through two gene clusters; *acrAB* and *emrAB.* emrAB pumps out lipophilic compounds and acrAB pumps out dyes and detergents. While acrAB is much more critical in bile resistance, works by Thanassi *et al*. found that mutants of both *acrAB* and *emrAB* were more sensitive to bile than mutants of *acrAB*, suggesting that emrAB plays a minor role in bile resistance (75).

1.9.5 Bile resistance in *C. jejuni.*

C, jejuni possesses the Multidrug Resistance (MDR) efflux pump, a surface protein designed to actively remove various threats to cellular survival (e.g. bile acids, antibiotics). This system is common to Gram negative bacteria and is important for survival in host intestines (41). The encoding sequence for *Campylobacter's* MDR system is a tri-gene operon under the control of a transcriptional repressor; these are known as CmeABC and CmeR respectively (40, 41). This system allows *C. jejuni* to survive in bile concentrations as high as 5% (14).

The CmeABC operon contains three genes; *cmeA, cmeB* and *cmeC*. These genes encode periplasmic proteins, inner membrane drug transporters and an outer membrane protein respectively and work in conjunction for the removal of harmful substances from bacterial cell cytoplasm (41). To evaluate the importance of these genes, Lin *et al.* introduced *C. jejuni* mutants to bile and observed their responses. *cmeB* mutants impaired production of CmeB and CmeC, increasing the mutant's susceptibility to certain bile acids. Mutations in *cmeC* inhibited expression of CmeC but not CmeA, while interference with *cmeC* disabled the pump entirely (41).

The same mutants were also incubated in chicken duodenal extract as well as live chickens but were unable to grow. The wild type strains, on the other hand, grew successfully; this further demonstrates the importance of the MDR efflux pump in the successful colonization of intestines by *C. jejuni* (41).

1.10 Bacterial response to bile exposure

It is presumable that any bacteria able to survive in the GIT would have at least some degree of bile resistance; but investigative works show that these bacteria also respond in other ways to bile exposure. The presence of bile can be an indication to the bacteria that it is in an intestinal environment; it is plausible that various virulence factors or proteins could be upregulated when bile is encountered. As little work has been conducted on *C. concisus* with regards to exposure to bile and less on protein expression in the presence of bile, studies on other intestinal pathogens will be observed.

1.10.1 Protein expression by *S.typhimurium* in response to bile

The presence of bile upregulates *S. typhimurium's* own bile resistance mechanism; as previously mentioned, deconjugated bile acids bind MarR (which typically represses transcription by binding to *marRAB* operon) resulting in the upregulation of *mar* and increased production of the acrAB efflux pump. This process does not only facilitate resistance of *S. typhimurium* to bile but also other antimicrobials (e.g. antibiotics). (33).

The PhoP/PhoQ regulatory system is crucial in *S. typhimurium's* response to environmental conditions via regulation of virulence factors (48). PhoQ is a membrane bound kinase which activates PhoP via phosphorylation after interacting with a particular environmental cue (e.g. bile acids). PhoP then induces expression in a number of targeted genes depending on the desired response (48, 78).

In the case of bile resistance, PhoP/PhoQ activates genes associated with the modification of surface lipopolysaccharides (LPS) to facilitate survival against cationic bile acids. Works by Velkinburgh et. al tested specific bile acids against this two-component regulatory system and found it to be able to act specifically towards deoxycholate and glycochenodeoxycholate but not glycocholate or taurocholate; the former group being most detrimental to the survival of *S. typhirium* in the intestines (78).

Non PhoP/PhoQ regulatory systems that respond to bile are also in place. For instance, high concentrations of bile have been shown to deactivate certain virulence factors in a manner not mediated by PhoP/PhoQ. If the lumen of the gall bladder is infected, *S. typhimurium* do not produce a type III secretion system typically expressed for intestinal infection (78).

1.10.2 Protein expression by *E. coli* in response to bile

The acrAB efflux pump has been described to mainly remove bile acids from the bacterial cell, but it also protects *E. coli* from a variety of stress factors including dyes, solvents, antibiotics (both lipophilic and ampiphilic) as well as chemical therapeutic agents (55). However, if the pump is over-expressed it can become lethal to the cell through the production of toxic metabolites necessary to facilitate removal of harmful agents from the cell (43). The expression of the *acrAB* gene therefore requires a regulation mechanism; in the case of *E. coli,* there are multiple (67).

Nakamura *et al*. found that mutations in the *acrR* locus can cause the host cell to become highly susceptible to agents that acrAB would typically protect against (51), suggesting that *acrR* is a transcriptional repressor of acrAB (43). The *mar* locus contains a tri-gene operon; *marA, marB* and *marR. marR* is directly controlled by *marA* for effective control of acrAB production (67) and mutations in *marR* can lead to excessive levels of antimicrobial resistance due to uncontrolled activation of *marRAB* (43).

At the highest level, repressor protein Rob exhibits much greater control over the expression of *marRAB* than *marA* does. It is a much larger protein that typically exists in high concentrations in cells. It also has a distinct domain that allows for binding by low molecular weight effectors such as deconjugated bile salts . Binding of Rob by bile acids greatly increases expression of acrAB (32, 67).

True to the infectious nature of *E. coli*, expression of acrAB can also be affected by quorum sensing; Wei *et al*. as well as Rahmati *et al*. found that SdiA, a homologue of the LuxR quorum sensing regulator, is able to regulate a wide range of genes (including activation of acrAB) to facilitate adaptation to environmental stresses (65, 82).

1.10.3 Protein expression by *B. fragilis* in response to bile

B. fragilis is a Gram negative anaeorobic bacterial species which is part of the normal human intestinal flora, predominantly colonizing the large intestines (7, 68). Pumbwe *et al*. showed that exposure of *B. fragilis* to bile generated a number of responses likely related to bacterial virulence. Firstly, exposure to bile contributed to the formation of biofilms while the growth rate remained unaffected. Other responses included an upregulation in the production of adhesive fimbrae allowing effective adhesion to HT-29 cells and biofilm formation as well as cellular vesicle and efflux pump genes (64).

1.10.4 Protein expression by *L. monocytogenes* in response to bile

As mentioned, bile salt hydrolysis is facilitated by BSH (Bile Salt Hydrolase) enzymes which is under transcriptional control in *L. monocytogenes. SigB,* an important transcriptional regulator, promotes bacterial resistance factors (including *bsh)* in response to stresses as well as being able to promote transcription of *prfA,* a gene encoding an array of vital virulence factors (9, 30).

1.10.5 Protein expression by *C. jejuni* in response to bile

In *C. jejuni,* the previously mentioned transcriptional repressor of the *cmeABC* gene, CmeR, becomes bound by bile salts while *C. jejuni* is in an intestinal environment. This binding prevents the repression of *cmeABC* gene expression (without affecting the transcription rate of *cmeR*) resulting in a heavy increase in CmeABC production. Both conjugated and non-conjugated bile salts increase the expression of *cmeABC*, which in turn increases the resistance of *C. jejuni* to antimicrobials (e.g. antibiotics) (40). The degree of this interaction is also dependent on bile concentration as well as the duration of exposure (40).

With regards to proteins expressed by *C. concisus* when exposed to bile, the topic remains largely uninvestigated. This project aims to provide some insight with regards to IBD related strains of *C. concisus'* resistance to bile.

1.11 A preferential colonization site for *C. concisus*

In humans, bile is almost completely (95%) reabsorbed before it exits the ileum (52). This makes the colon the ideal site for infection for enteric pathogens without strong mechanisms of bile resistance provided they survive bile exposure in the ileum long enough to establish infection.

In a study by Mahendran *et al*., patients with IBD had intestinal biopsies taken at various sites and were compared to controls to identify possible preferential infection sites for *C. concisus;* the results found that a higher percentage of *C. concisus* were found in the descending colon (43% compared to 18% in controls) and the rectum (26% compared to 9% in controls) (46). Coincidentally, these are the areas with low concentrations of bile. Currently, it is unclear whether there is any difference in bile resistance among *C. concisus* strains isolated from patients with IBD and controls and whether such a difference contributes to the higher intestinal prevalence of *C. concisus* found in patients with IBD.

1.12 Project aims

The aim of this investigation is to examine the growth and survival of various *C. concisus* strains in the presence of bile to determine whether or not *C. concisus* strains isolated from patients with IBD are more resistant to bile than strains from healthy individuals and to identify unique proteins expressed during bile exposure.

1.13 Project hypothesis

- *C. concisus* strains isolated from patients with IBD have a greater resistance to bile compared to those isolated from healthy patients .

- Exposure to bile increases expression of virulence proteins in *C. concisus.*

Chapter 2: The impact of bile on *Campylobacter concisus* growth

2.1 Introduction

C. concisus is a Gram negative bacterium that is ubiquitously present in the human oral cavity (85). Previous studies found a significantly higher presence of *C. concisus* in the intestinal tracts of patients with Inflammatory Bowel Disease (IBD) compared to controls (50). However, what contributed to the higher prevalence of IBD remains unknown. Previous studies found that some oral strains of *C. concisus* are able to colonize the intestines in human hosts with potential inflammatory results (49, 71, 84).

As *C. concisus* travels through from the oral cavity to the large intestines, it is exposed to numerous host defences including gastric acids and intestinal bile (5, 22). Each of these factors can hinder bacterial growth and survivability. *C. concisus* strains that have a higher ability to resist the adverse effects of the natural defences in the stomach (gastric acid) and small intestine (bile) would therefore have a greater opportunity to establish intestinal colonization.

The abilities of *C. concisus* strains isolated from patients with IBD and controls to resist bile have not been previously investigated. In this chapter, the effects of bile on the growth and survival of *C. concisus* strains isolated from patients with IBD and controls were investigated. The studies included in this chapter were to examine the first hypothesis of this thesis; that *C. concisus* strains isolated from patients with IBD have a greater resistance to bile compared to those isolated from healthy patients.

2.2 Materials and methods

2.2.1 *C. concisus* strains used in this study

A total of 42 strains of *C. concisus* previously isolated in the lab of Li Zhang were used in this study. These strains were isolated from patients with UC or CD as well as healthy individuals (45, 86, 87).

Table 1.1: Isolation source and date of *C. concisus* strains

Strain	Diagnosis
P10CD-O-S2	CD
P10CD-O-S1	CD
P1CD-O2	CD
P1CDO-S2	CD
P2CD-O-S2	CD
P2CDO-S1	CD
P2CD-O6	CD
P6CDO-S1	CD
P11CD-S1	CD
P11CDO-S2	CD
P18CDO-S1	CD
P4CD-S1	CD
P4CDO-S3	CD
P4CDO-S4	CD
P12CDO-S2	CD
P1CDB-S1	CD
P7UC-S1	UC
P8UCO-S1	UC
P14UCO-S2	UC
P14UC-S1	UC
P16UCO-S2	UC
P16UC-S1	UC
P16UCO-S3	UC
P15UC-S1	UC
P15UC-S2	UC
P3UCLW-S1	UC

P3UCLW-S2	UC
P3UC-S1	UC
H11O-S1	H
H11O-S2	H
H6O-S1	H
H6O1-S1	H
H10O-S1	H
H12O-S1	H
H13O-S2	H
H17O-S2	H
H14O-S1	H
H15O1-S1	H
H9O-S2	H
H8O-S1	H
H8O-S3	H
H1O1-S1	H

Isolated strains were stored at -20°C for use in various investigations in accordance with health and safety requirements of the University of New South Wales.

2.2.2 Culturing *C. concisus*

In preparation for tests, bacteria were aseptically streaked via platinum loop on Horse Blood Agar (27) plates containing 20g of Oxoid Blood Agar Base (CM0271) and 30ml SR0050 defibrinated horse blood (6%) in distilled water (total volume of 500ml). Plates were incubated under hydrogen enriched anaerobic conditions (created using an Oxoid *Campylobacter* anaerobic gas generating kit [BR0038B]) at 37ºC for 48 hours.

In tests involving bile exposure using HBA plates, HBA plates containing bile (HBA-B) plates were prepared, which includes the same makeup as HBA but with the addition of 10g (2%) ox bile extract. Incubation conditions were the same as HBA plates.

Liquid media incubation of *C. concisus* consists of a 3.7% Heart Infusion Broth (HiB) solution incubated under previously mentioned conditions.

2.2.3 Growth of *C. concisus* strains in HBA plates containing 2% bile

All 42 strains of *C. concisus* were used in this investigation. After culturing, confirmation of bacterial morphology was achieved through a light microscope. Each strain was then collected in 1ml PBS solution via hockey stick and centrifuged at 9,000rcf and 15°C using the Eppendorf Centrifuge 5415 R. Supernatant PBS was then removed and bacterial pellets were washed and resuspended in 1ml PBS.

Optical Density (OD_{600}) was measured at 600nm through the SpectraMax 340 molecular device system run by SoftMaxPro40 software. OD_{600} was read at a wavelength of 595 after 5 seconds of automixing. Samples were then diluted with PBS until an OD_{600} of 0.1 was reached. Each strain (10µl) was then streaked on two HBA plates as well as two 2% HBA-B plates and incubated at 37°C under hydrogen enriched anaerobic conditions for 48 hours.

Each plate was then observed under a dissection microscope for the presence of colonies. If colonies were observed the strain was recorded as R (Resistant). If colonies were not present on the 2% HBA-B plates, a platinum loop was used to aseptically collect bacteria from the inoculum site and re-streaked onto HBA plates. The plates were then incubated for another 48 hours under the same conditions. Finally, resultant HBA plates were observed for *C. concisus* colonies; the presence of colonies marks the corresponding strain as T (Tolerant). If no colonies were observed, corresponding strains were marked as S (Sensitive).

The procedures for this study are outlined in figure 2.1.

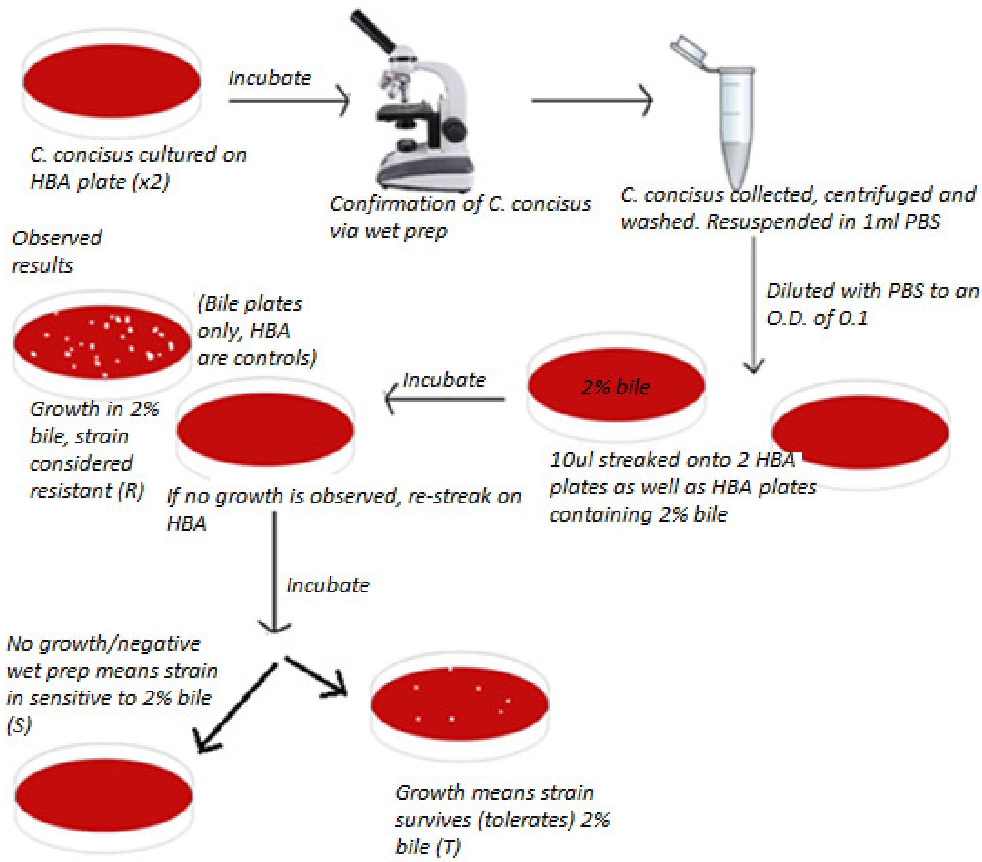

Figure 2.1: Diagrammatic illustration of the procedures used in examining the growth of *C. concisus* strains in HBA plates containing 2% bile (HBA-B). The details of the method were described in 2.2.3

2.2.4 Quantitative measurement of the impact of 2% bile on the growth of *C. concisus*

Twenty-five *C. concisus* strains were randomly selected and cultured on duplicate HBA plates before being incubated at 37°C under hydrogen enriched anaerobic conditions for 48 hours. Bacteria were then collected in 1ml PBS solution via hockey stick and centrifuged at 9,000rcf and 15°C before washing and re-suspending bacterial pellets in 1ml PBS. 20µl of each strain were cultured on two HBA and 2% HBA-B plates. Plates were then incubated under the same conditions for 48 hours.

Resultant bacteria were collected in 1ml PBS via hockey stick and were centrifuged at 9,000rcf in 15°C before being washed and resuspended in 1ml PBS. The solutions were then serially diluted to a concentration of 10^{-6} and 10^{-4} for cultures collected from HBA and HBA-B plates respectively. Finally, each dilution was blotted onto HBA plates (plates were dried for 20 minutes in a fume cupboard prior to blotting) in 5µl aliquots and three blots per dilution. Plates were then incubated under the same conditions previously used before CFU/ml was established via colony count using a clicker and microscope as well as the following formula:

$$\%CFU = \frac{\textit{average CFU of 2\% HBAB plates}}{\textit{average CFU of HBA plates}} \times 100$$

The procedures for this study are outlined in figure 2.2.

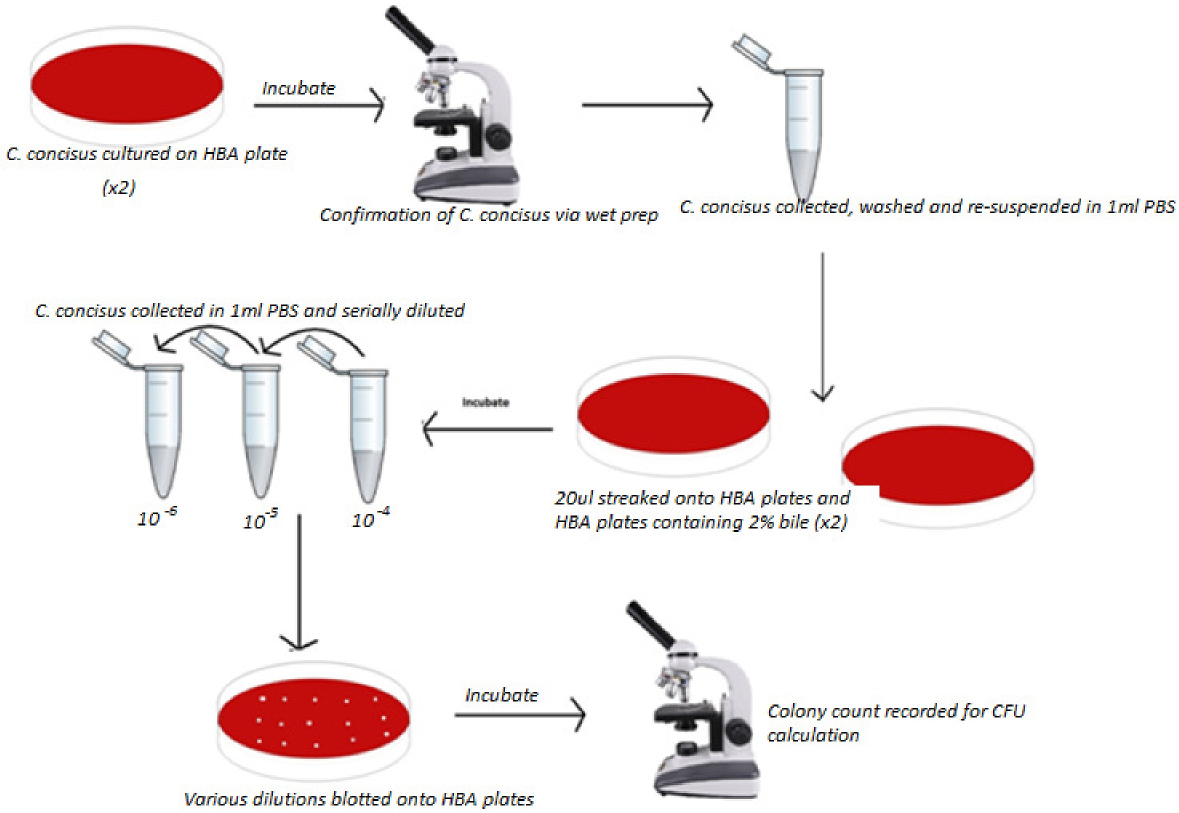

Figure 2.2: Diagrammatic illustration of the procedures used in quantitative measurement of *C. concisus* growth in HBA-B plates. The detailed methods were described in 2.2.4

2.2.5 The impact of two and six hours of bile exposure on the growth of bile sensitive strains of *C. concisus*

Ten sensitive strains from the test outlined in 2.2.4 were randomly selected and cultured on two HBA plates per strain before being incubated at 37°C under hydrogen enriched anaerobic conditions for 48 hours. Bacteria were then collected in 1ml of PBS via hockey stick and centrifuged at 9,000rcf and 15°C before washing in PBS and reading OD_{600}. The bacteria were then diluted with PBS until an OD_{600} of 0.01 was reached. The solution was then centrifuged at 9,000rcf for 2 minutes, the supernatant discarded and the bacterial pellet was resuspended in 900µl 3.7% HiB (CM1032). Next, 100µl of 20% bile solution was added to achieve 2% bile in total. The total solution was placed in an incubator at 37°C and 10µl was collected at regular time intervals and serially diluted in 900µl PBS (to 10^{-5} at 0 hours, 10^{-3} at 2 hours and no dilution at 6 hours) before blotting 5ml aliquots onto duplicate HBA plates. All plates were then incubated at 37°C under anaerobic conditions for 48 hours. Colonies were then counted using a clicker and a microscope with results being recorded for CFU/ml calculation through the previously mentioned formula.

The procedures for this study are outlined in figure 2.3

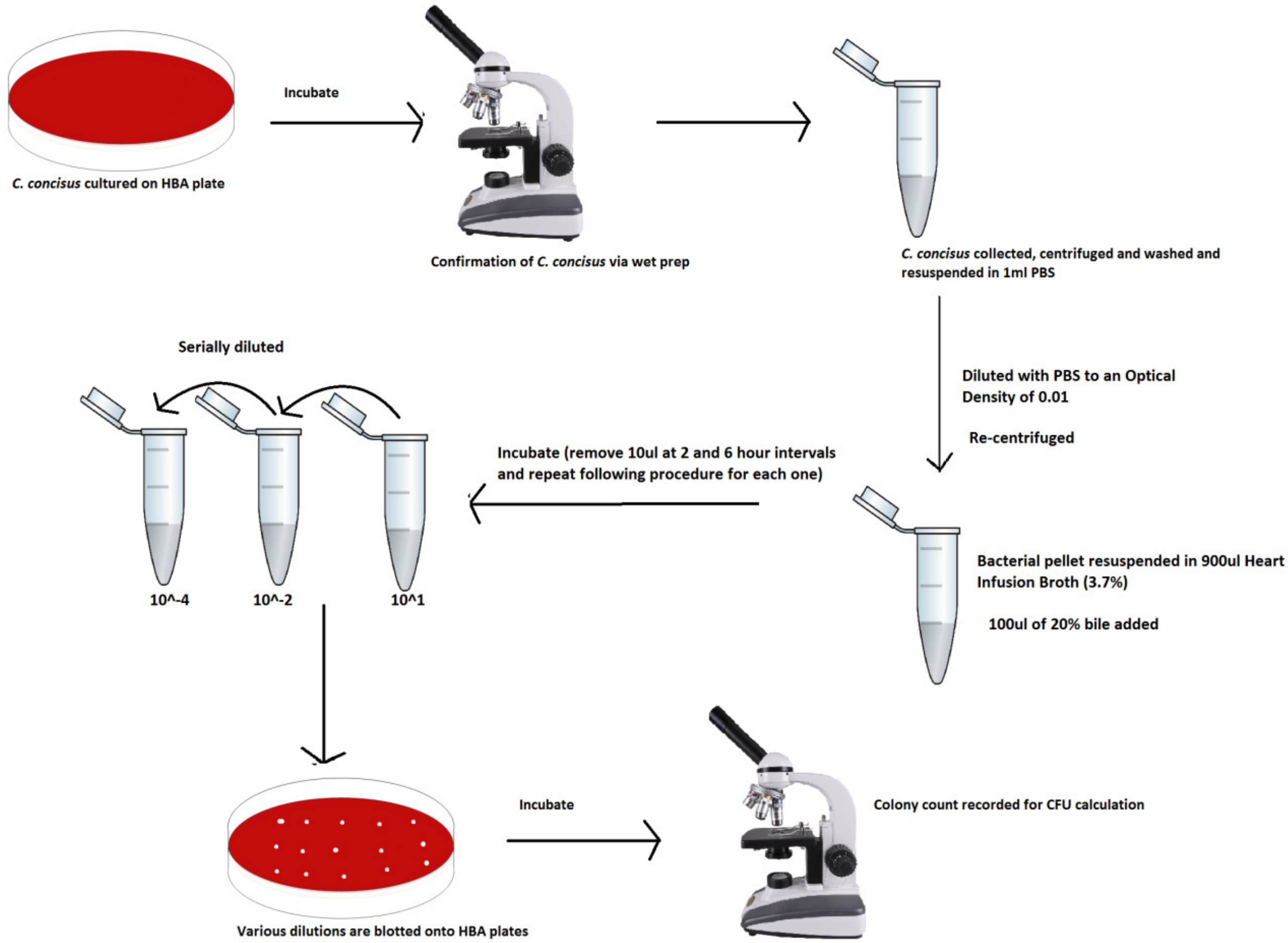

Figure 2.3: Diagrammatic illustration of the procedures used in examination of the impact of two and six hours of exposure to 2% bile on the growth of *C. concisus*. The detailed methods were described in 2.2.5

2.2.6 The effects of different concentrations of bile on *C. concisus* growth

This procedure is similar to the test outlined in 2.2.4 except varying concentrations of bile were introduced. For this test, three *C. concisus* strains isolated from patients with IBD were selected. After culturing, bacteria were collected in 1ml PBS. The solution was then centrifuged at 9,000rcf at 15°C and washed in 1ml PBS twice. OD_{600} was then read and diluted to a reading of 0.1 before plating 10µl on HBA-B plates with varying bile concentration (0% (control), 0.1%, 0.25%, 0.5%, 1% and 2%). Four plates were made in total for each concentration and were incubated in the same conditions for 48 hours.

Resultant bacteria were then collected, washed in PBS and serially diluted from a concentration of 10^{-3} to a concentration of 10^{-6}. Duplicate HBA plates were then blotted in 5µl aliquots (three blots per dilution) for each of the bacteria grown in each HBA-B plate. Plates were then incubated for another 48 hours under the same conditions.

A dissection microscope was used to count how many *C. concisus* colonies were present for each plate. Results were recorded and averaged and CFU/ml was calculated with the previously mentioned formula.

The procedure is illustrated in figure 2.2, except repeated for HBA-B plates containing differing concentrations of bile.

2.2.7 Data analysis

Chi squared tests were used to compare the percentage of bile resistance between *C. concisus* strains isolated from patients with UC, CD and healthy individuals.

A t-test was used to compare CFU/ml of *C. concisus* strains grown in the presence of different concentrations of bile.

2.3 Results

2.3.1 Growth of *C. concisus* strains in HBA plates containing 2% bile

Of the total 42 strains tested, 19 (45.2%) were resistant (R) to 2% bile, 16 strains (38.9%) were tolerant (T) and 7 strains (16.9%) were found to be sensitive (S).

Of the 13 strains isolated from patients with UC, five (38.46%) were resistant, five (38.46%) were tolerant and three (23.08%) were sensitive to 2% bile.

Of the 14 strains isolated from patients with CD, eight (57.14%) were resistant, four (28.57%) were tolerant and two (14.29%) strains were sensitive to 2% bile.

Of the 15 strains isolated from healthy individuals, six (40%) were resistant and nine (60%) were tolerant to 2% bile.

Table 2.1: Growth of *C. concisus* strains on 2% HBA-B plates

Strain	Diagnosis	R (Resistant)	T (Tolerant)	S (Sensitive)
P10CD-O-S2	CD			x
P10CD-O-S1	CD	x		
P1CD-O2	CD	x		
P1CDO-S2	CD	x		
P2CD-O-S2	CD	x		
P2CDO-S1	CD			x
P2CD-O6	CD	x		
P6CDO-S1	CD	x		
P11CD-S1	CD			x
P11CDO-S2	CD			x
P18CDO-S1	CD		x	
P4CD-S1	CD		x	
P4CDO-S3	CD	x		
P4CDO-S4	CD	x		

P12CDO-S2	CD	x		
P1CDB-S1	CD	x		
P7UC-S1	UC		x	
P8UCO-S1	UC			x
P14UCO-S2	UC	x		
P14UC-S1	UC			x
P16UCO-S2	UC		x	
P16UC-S1	UC		x	
P16UCO-S3	UC	x		
P15UC-S1	UC			x
P15UC-S2	UC	x		
P3UCLW-S1	UC	x		
P3UCLW-S2	UC		x	
P3UC-S1	UC		x	
H11O-S1	H		x	
H11O-S2	H		x	
H6O-S1	H		x	
H6O1-S1	H	x		
H10O-S1	H	x		
H12O-S1	H		x	
H13O-S2	H	x		
H17O-S2	H		x	
H14O-S1	H		x	
H15O1-S1	H	x		
H9O-S2	H		x	
H8O-S1	H		x	
H8O-S3	H		x	
H1O1-S1	H	x		

CD = Crohn's Disease, UC = Ulcerative Colitis, H= Healthy R/T/S = Resistant/Tolerant/Sensitive to 2% bile x= *C. concisus* strain falls under this category. HBA and 2% HBA-B plates were incubated in hydrogen enriched anaerobic conditions over 48 hours before observation of *C. concisus* colonies.

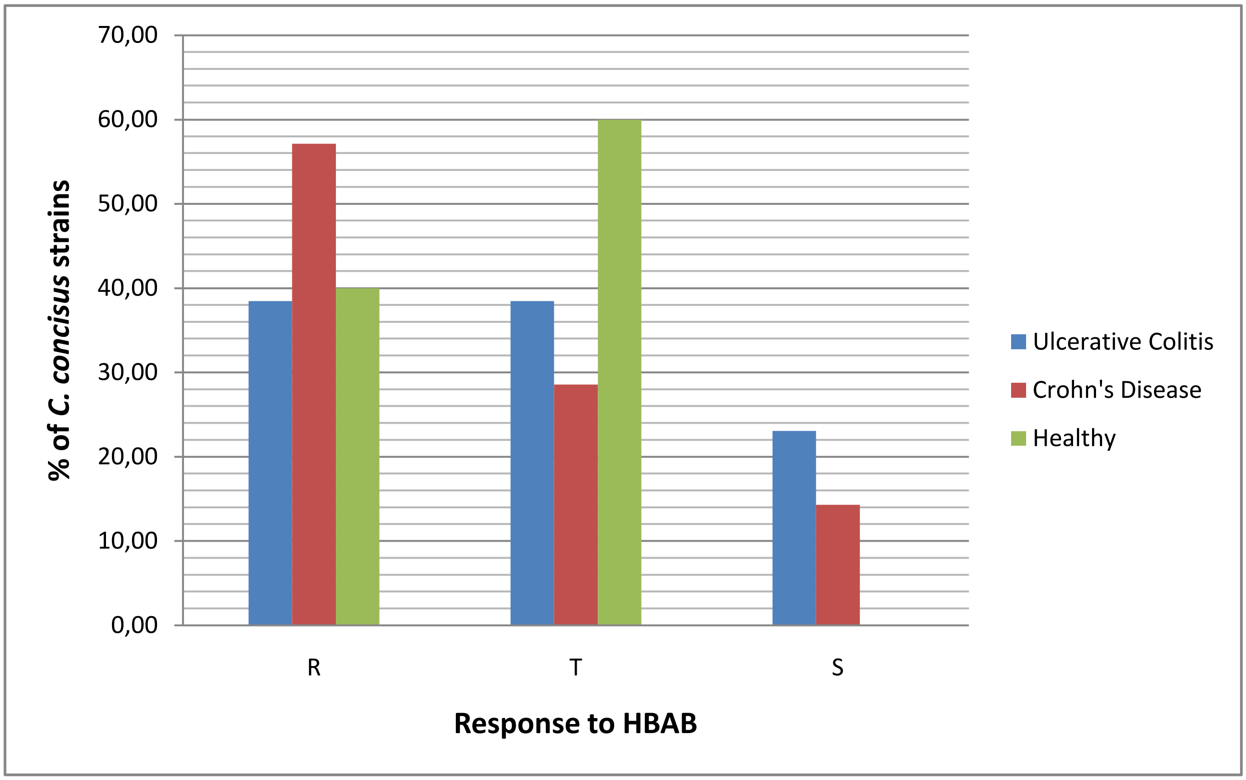

Figure 2.4 : Comparison of the growth of *C. concisus* strains isolated from patients with CD, UC and healthy controls on HBA plates containing 2% bile (HBA-B). A total of 42 strains were examined (15 strains from healthy controls, 13 strains from patients with UC and 14 strains from patients with CD). Strains were recorded as R (Resistant) if colonies were observed following 48 hours cultivation. Strains without colonies following 48 hours cultivation but had colonies when the same strains were re-streaked from HBA-B plates onto HBA plates were recorded as T (Tolerant). Strains with no colonies on both HBA-B plates and the re-streaked HBA plates were recorded as S (Sensitive). Data were presented as a percentage of strains of each category (R, T or S) in each diagnostic group. Results between each diagnostic group were not statistically significant (P>0.05).

2.3.2 Quantitative measurement of *C. concisus growth in the presence of 2% bile*

Of the 26 strains tested, nine were isolated from patients with CD, eleven from patients with UC and six from healthy individuals.

The percentages CFU/ml of 26 strains grown on HBA plates containing 2% bile relative to the CFU/ml of the same strains grown on HBA plates without bile were 0-6.34%. P2CD-O6, a strain isolated from a patient with CD, showed the most tolerance to bile, which was followed a strain isolated from a healthy individual (H1O1-S1). Three strains (P1CD-O2, P2CDO-S1 and P15UC-S1) did not grow on HBA plates containing 2% bile.

Table 2.2: Quantitative measurement of the growth of *C. concisus* in the presence of 2% bile.

Strain	**Diagnosis**	**CFU/ml(27)**	**CFU/ml (2% HBA-B)**	**HBA-B/HBA (%)**
P1CD-O2	CD	$1.06 \times 10^{6} \pm 4.11 \times 10^{5}$	0.00	0
P1CDO-S2	CD	$1.60 \times 10^{9} \pm 9.09 \times 10^{8}$	$6.67 \times 10^{7} \pm 1.15 \times 10^{7}$	4.17
P2CDO-S1	CD	$3.35 \times 10^{10} \pm 6.61 \times 10^{9}$	0.00	0
P2CD-O6	CD	$1.94 \times 10^{9} \pm 6.18 \times 10^{8}$	$1.23 \times 10^{8} \pm 9.02 \times 10^{6}$	6.34
P9CD-S1	CD	$3.53 \times 10^{9} \pm 8.06 \times 10^{8}$	$1.26 \times 10^{6} \pm 2.31 \times 10^{5}$	0.04
P11CD-S1	CD	$3.34 \times 10^{5} \pm 1.89 \times 10^{5}$	$1.34 \times 10^{3} \pm 2.31 \times 10^{3}$	0.40
P12CD-S1	CD	$7.86 \times 10^{9} \pm 6.18 \times 10^{8}$	$8.73 \times 10^{6} \pm 9.45 \times 10^{5}$	0.11
P12CDO-S3	CD	$2.12 \times 10^{9} \pm 1.89 \times 10^{8}$	$2.67 \times 10^{5} \pm 1.22 \times 10^{5}$	0.01
P4CD-S1	CD	$6.66 \times 10^{9} \pm 1.96 \times 10^{9}$	$2.00 \times 10^{5} \pm 8.00 \times 10^{4}$	0.003
H16O1-S1	H	$2.60 \times 10^{9} \pm 1.00 \times 10^{8}$	$3.50 \times 10^{6} \pm 3.00 \times 10^{6}$	0.13
H9O-S1	H	$1.02 \times 10^{9} \pm 1.07 \times 10^{8}$	$3.00 \times 10^{6} \pm 2.58 \times 10^{6}$	0.29
H9O-S3	H	$4.45 \times 10^{9} \pm 5.26 \times 10^{8}$	$2.90 \times 10^{6} \pm 5.77 \times 10^{5}$	0.07

H8O-S2	H	$4.53 \times 10^{9} \pm 5.25 \times 10^{8}$	$6.67 \times 10^{5} \pm 1.15 \times 10^{6}$	0.02
H1O1-S1	H	$1.50 \times 10^{9} \pm 9.02 \times 10^{8}$	$9.35 \times 10^{7} \pm 5.00 \times 10^{6}$	6.23
H7O1-S1	H	$2.80 \times 10^{9} \pm 0$	$3.40 \times 10^{4} \pm 0$	0.001
P15UC-S1	UC	$1.06 \times 10^{9} \pm 4.99 \times 10^{8}$	0.00	0
P15UC-S2	UC	$1.08 \times 10^{10} \pm 2.79 \times 10^{9}$	$5.67 \times 10^{6} \pm 5.03 \times 10^{5}$	0.05
P15UC-S3	UC	$1.51 \times 10^{9} \pm 3.77 \times 10^{7}$	$1.28 \times 10^{7} \pm 6.00 \times 10^{5}$	0.85
P3UC-S1	UC	$1.87 \times 10^{9} \pm 9.43 \times 10^{7}$	$6.67 \times 10^{5} \pm 1.15 \times 10^{3}$	0.35
P13UCO-S2	UC	$6.86 \times 10^{9} \pm 2.49 \times 10^{8}$	$2.20 \times 10^{7} \pm 1.25 \times 10^{7}$	0.32
P13UCO-S3	UC	$1.47 \times 10^{9} \pm 2.49 \times 10^{7}$	$9.47 \times 10^{6} \pm 1.10 \times 10^{6}$	0.64
UCF-1	UC	$8.33 \times 10^{8} \pm 2.49 \times 10^{7}$	$2.32 \times 10^{6} \pm 8.33 \times 10^{5}$	0.28
P14UCO-S3	UC	$4.00 \times 10^{9} \pm 9.93 \times 10^{8}$	$1.59 \times 10^{7} \pm 3.60 \times 10^{6}$	0.40
P16UCO-S2	UC	$9.34 \times 10^{8} \pm 2.49 \times 10^{8}$	$6.67 \times 10^{4} \pm 4.62 \times 10^{4}$	0.01
P16UCO-S3	UC	$1.86 \times 10^{9} \pm 4.11 \times 10^{8}$	$4.67 \times 10^{5} \pm 3.06 \times 10^{4}$	0.03
P8UCO-S1	UC	$1.64 \times 10^{10} \pm 1.88 \times 10^{9}$	$8.67 \times 10^{6} \pm 5.03 \times 10^{6}$	0.05

CD = Crohn's Disease, UC = Ulcerative Colitis, H= Healthy, CFU/ml = Colony Forming Units per millilitre, HBA = Horse Blood Agar, HBA-B = HBA plates containing 2% Ox bile, HBA-B/HBA (%)= the average CFU/ml of *C. concisus* strains grown on HBA/ the average CFU/ml of the same strains grown on HBA-B.

2.3.3 The impact of two and six hours bile exposure on the growth of bile sensitive *C. concisus strains*

Ten strains were used in this test. Two strains (P3UC-S1 and P14UC-S1) did not grow on HBA plates after exposure to 2% bile for two hours. The remaining eight strains grew on HBA plates following a two hour exposure to 2% bile. However, CFU/ml were much lower compared to the CFU/ml of the same strains without bile exposure (0.01-14.17%).

After six hours of bile exposure, four strains (H8O-S1, P2CDO-S1, P8UCO-S1 and P16UC-S1) grew on HBA plates and their CFU/ml were 0.01%, 0.16%, 0.44% and 0.16% respectively of CFU/ml of the same strains without bile exposure (Table 2.3). The remaining six strains did not grow.

Table 2.3: Impact of 2% bile on the growth of bile sensitive strains of *C. concisus* over two and six hours bile exposure

Strain	Diagnosis	0 Hour CFU/ml	2 Hour CFU/ml	2hr/0hr (%)	6 Hour CFU/ml	6hr/0hr (%)
H8O-S1	H	$4.95 \times 10^{7} \pm 1.43 \times 10^{7}$	$6.00 \times 10^{6} \pm 3.38 \times 10^{6}$	12.12	$2.38 \times 10^{3} \pm 7.44 \times 10^{3}$	0.01
H13O-S2	H	$2.23 \times 10^{7} \pm 9.83 \times 10^{6}$	$7.68 \times 10^{5} \pm 1.74 \times 10^{5}$	3.44	0	0
P10CD-O-S2	CD	$2.73 \times 10^{7} \pm 6.53 \times 10^{6}$	$2.50 \times 10^{3} \pm 7.07 \times 10^{3}$	0.01	0	0
P2CDO-S1	CD	$1.27 \times 10^{8} \pm 1.04 \times 10^{7}$	$1.80 \times 10^{7} \pm 7.41 \times 10^{6}$	14.17	$2.00 \times 10^{5} \pm 8.28 \times 10^{4}$	0.16
P11CDO-S2	CD	$3.56 \times 10^{7} \pm 9.10 \times 10^{6}$	$4.25 \times 10^{4} \pm 2.71 \times 10^{4}$	0.12	0	0
P8UCO-S1	CD	$1.14 \times 10^{8} \pm 2.06 \times 10^{7}$	$9.00 \times 10^{6} \pm 2.62 \times 10^{6}$	7.89	$5.05 \times 10^{5} \pm 1.45 \times 10^{5}$	0.44
P15UC-S1	UC	$2.53 \times 10^{7} \pm 9.00 \times 10^{6}$	$2.90 \times 10^{5} \pm 9.62 \times 10^{4}$	1.15	0	0
P3UC-S1	UC	$1.09 \times 10^{8} \pm 1.93 \times 10^{7}$	0	0	0	0
P14UC-S1	UC	$5.05 \times 10^{7} \pm 8.05 \times 10^{6}$	0	0	0	0
P16UC-S1	UC	$1.17 \times 10^{8} \pm 2.02 \times 10^{7}$	$6.50 \times 10^{6} \pm 3.96 \times 10^{6}$	5.56	$1.90 \times 10^{5} \pm 6.85 \times 10^{4}$	0.16

CD = Crohn's Disease, UC = Ulcerative Colitis, H= Healthy, CFU/ml = Colony Forming Units per millilitre. 2hr/0hr (%) = CFU/ml at two hours as a percentage of CFU/ml at 0 hours. 6hr/0hr = CFU/ml at 6 hours as a percentage of CFU/ml at 0 hours.

Ten strains were used in this test. Two strains (P3UC-S1 and P14UC-S1) did not grow on HBA plates after exposure to 2% bile for two hours. The remaining eight strains grew on HBA plates following a two hour exposure to 2% bile, however the CFU/ml numbers were greatly reduced compared to the CFU of the same strains without bile exposure (0.01-14.17%).

After six hours of bile exposure, four strains grew on HBA plates and the CFU/ml were 0.01%, 0.16%, 0.44% and 0.16% respectively compared to the CFU/ml of the same strains without bile exposure (Table 2.3).

2.3.4 The effects of different concentrations of bile on *C. concisus* growth

The CFU/ml of three *C. concisus* strains grown on HBA plates containing different concentrations of bile were shown in Table 2.4. The CFU/ml of all three strains grown on HBA plates containing 0.1%, 0.25%, 0.5%, 1% and 2% bile were significantly lower than the CFU/ml of the same strain grown on HBA plates without bile ($P<0.05$). Furthermore, CFU/ml showed a significantly decline as bile concentrations increased in *C. concisus* strains P2CDO-S1 and P1CDO-S2 ($P<0.05$). P4CD-S1 did not observe significant differences in CFU/ml between 0.1% and 0.25% bile ($P>0.05$) as well as CFU/ml between 1% and 2% bile ($P>0.05$). Aside from the mentioned exceptions, all tested strains of *C. concisus* found statistically significant differences in CFU between increments of bile concentration ($P<0.05$).

Strain P4CD-S1 was more sensitive to higher concentrations of bile (1% and over) than P2CDS1 but was affected by lower concentrations of bile (0-1%) in a more similar manner. Strain P1CDO-S2, on the other hand, recorded consistently higher levels of bile resistance when compared to the other two strains in all concentrations of bile (Table 2.4).

Table 2.4: CFU of three different *C. concisus* strains in HBA-B plates of varying bile concentrations.

Bile conc. In HBA (%)	**P4CD-S1(CFU/ml)**	**P2CDO-S1(CFU/ml)**	**P1CDO-S2 (CFU/ml)**
0	$3.35 x10^{10} ± 7.55 x10^{9}$	$1.8 x10^{10} ± 4.32 x10^{9}$	$4.05x10^{9} ± 6.34 x10^{8}$
0.1	$3.40x10^{8} ± 8.64 x10^{7}$	$1.25 x10^{8} ± 3.79x10^{7}$	$2.18x10^{9} ± 4.21 x10^{8}$
0.25	$2.05 x10^{8} ± 6.61 x10^{6}$	$7.50 x10^{7} ± 1.91x10^{7}$	$5.48x10^{8} ± 1.78x10^{8}$
0.5	$6.53 x10^{7} ± 1.48 x10^{7}$	$1.40x10^{7} ± 2.83x10^{6}$	$3.32x10^{8} ± 5.46x10^{7}$
1	$8.50 x10^{4} ± 1.91 x10^{4}$	$6.10x10^{5} ± 1.25 x10^{5}$	$2.07x10^{8} ± 3.83x10^{7}$
2	0.00	$4.42x10^{5} ± 2.87x10^{4}$	$1.23x10^{8} ± 3.23x10^{7}$

2.4 Discussion

This investigation exposed various strains of *C. concisus* isolated from both patients with IBD and healthy individuals to bile in different scenarios. This includes incubating strains on HBA plates containing 2% bile over 48 hours, incubating strains in HiB containing 2% bile for up to six hours and incubating strains in HBA plates containing varying concentrations of bile with the aim of determining whether or not there are major differences in bile resistance between strains isolated from patients with IBD and healthy individuals.

Ox bile is used because it readily contains conjugated and non-conjugated bile acids and its similarity in chemical constituents to human bile (14). In this investigation it is 2% concentrated, a common concentration often used in bile related studies; Vandamme *et al* looked differential characteristics between species of *Campylobacter* and recorded the percentage of tested strains that were able to grow in bile. Between 14-50% of tested *C. concisus* strains were able to grow in the presence of 2% bile (80). This concentration is most similar to what one would expect to find in the human jejunum, an area that *C. concisus* would encounter while it travels through the gastrointestinal tract (17, 23, 52, 64). Therefore, in order for any strains of *C. concisus* to survive long enough to colonize the intestinal tract in an *in vivo* situation, some degree of bile tolerance and resistance must be present.

However, results indicate that the growth of *C. concisus*, regardless of the strain, is strongly inhibited by the presence of bile, although varying degrees of resistance has been found between strains. Incubating 42 *C. concisus* strains in HiB containing 2% bile for 48 hours and observing the presence of colonies under dissection microscope found a range of results (resistant, tolerant and sensitive to 2% bile) in a pattern unrelated to the diagnosis of the person the strain was isolated from (differences in bile resistance between strains from IBD patients and strains from healthy individuals was insignificant). Therefore, if bile resistance was the sole determining factor for infection, results show there is no reason why strains isolated from a healthy individual couldn't also contribute to the onset of IBD. This indicates other differences between strains are more likely to contribute to successful intestinal colonization.

Quantitative results from a similar experiment revealed a range of bile resistance between strains with greater depth. Strains P1CDO-S2 and P2CD-O6 showing particularly high levels of resistance to HBA-B plates containing 2% bile (4.17% and 6.34% respectively) when compared to other strains (<0.85%).

Exposure time also had different impacts on different strains of *C. concisus* when incubated in HiB containing 2% bile for up to six hours; two hours of bile exposure, for example, had a greater impact on certain strains of bacteria than others. 3.43% of H13O-S2 survived after two hours compared to 0.0091% in P10CD-S2, despite the fact both were completely eliminated by six hours of exposure.

Another interesting piece of information was found in the exposure of *C. concisus* strains to varying concentrations of bile. As expected, growth was highest when bile concentration was low. Growth was then reduced proportionally with increasing concentrations of bile between the three tested strains, although there were differences in each strain's reaction to varying concentrations of bile. For example, at 0.5% concentration, 0.078% of strain P4CD-S1 survived (compared to control CFU/ml) as well as 0.19% of P2CDO-S1 while P1CDO-S2 showed a much higher level of bile tolerance (8.21%). By 2% bile concentration, strains P4CD-S1 and P2CDO-S1 were almost if not completely eliminated while 3.03% of strain P1CDO-S2 survived. The overall inhibitory effect indicates that *C. concisus* is sensitive to bile in a concentration dependent manner, implying that individual variations in bile release (either basal or in response to a meal) in hosts could possibly increase the likelihood of *C. concisus* colonizing their intestines.

These results have other implications; the fact that there was little difference in bile resistance between healthy and disease causing strains of *C. concisus* does not mean that bile resistance is only present in disease associated strains as the required factors for colonization of the intestines by *C. concisus* are still not fully known (85). Works by Gradel *et al*. noted that infection by *Campylobacter jejuni* can increase the likelihood of onset or relapse of CD, giving insight into mechanisms of IBD onset not explored in this investigation. In addition, *C. concisus* present in oral cavities also has the potential to cause gingivitis; yet despite being present in the oral cavities of humans, *C. concisus* has a low rate of disease onset (85). It is possible that many strains of *C. concisus* do not possess important virulence factors for a causative role in IBD or gingivitis. Also, these putative virulence factors, if present, may not be expressed in every *C. concisus* strain.

It can also be considered that *C. concisus,* unlike *C. jejuni,* targets the colon (46), where the majority of bile salts have been reabsorbed (52). Therefore, time can be a considerable factor; if enough bacteria are able to survive long enough to reach the colon (despite exposure to bile in the ileum), they may be more likely to cause infection. This could mean that while the standard time it takes for food to travel through the GIT would usually completely inhibit the growth of many strains of *C. concisus,* in situations where food travels through the intestines at a faster than usual, *C. concisus* could persist long enough to reach the colon where bile concentrations are much lower. The fact that *C. concisus* is inhibited by bile in a time and concentration dependent manner is similar to findings from other studies such as the response of *C. jejuni* to bile, where increased expression of *cmeABC* in response to bile occurs depending on concentration of bile and exposure time (40). However, as the exact role of *C. concisus* in IBD is not known, it need to be considered that the presence of *C. concisus* in the intestines of IBD patients could be a result of the disease instead of a factor in its onset. Diarrhoea is commonly observed in IBD patients, a symptom which would increase transit time of foods through the intestines. It is possible that this symptom allows *C. concisus* to quickly travel through the ileum to its preferred colonization site

It is also important to consider that the tests in this investigation do not accurately simulate an intestinal environment. They do not account for passive or active reabsorption of bile throughout the ileum and operate under the assumption that ileal bile is 2% concentrated. In reality, bile secretion varies between individuals, time of day and type of diet. This investigation also assumes that *C. concisus* strains isolated from patients with CD and UC originated from the oral cavity of the host and colonized the colon after travelling through the alimentary tract.

Studies involving other common intestinal pathogens and the impact of bile exposure on their growth and survival have been conducted with pathogens exhibiting bile resistance. It can be noted that these pathogens typically target the ileum, where bile concentrations would be high. With the information provided in this investigation about *C. concisus*, it is highly possible that bile sensitivity is the main reason for *C. concisus*' preferential targeting of the colon (46):

Table 2.5: The bile concentrations tolerated by *C. concisus* and other bacterial species.

Bacteria	**Maximum tolerable bile concentration (%)**	**Reference**
S. typhimurium	18-80	Lacroix *et al*., Velkinburgh *et al*. (33, 78)
B. fragilis	5	Turgeon *et al*. (76)
L. monocytogenes	5-30	Begley *et al*. (3)
C. jejuni	5	Fox *et al*. (14)
C. concisus	0-2	This study

When considering the above table, it should be considered that bile concentrations over 2% were not used in this investigation. However, due to the high inhibitory 2% bile has on the growth of all tested *C. concisus* strains and the fact that bile affects *C. concisus* in a concentration dependent manner, it is very likely that bile concentrations higher than 2% would have an even greater detrimental (if not completely bacteriocidal) effect on the growth and survival of *C. concisus*.

Works by Fox *et al*. on the response of *C. jejuni* to bile exposure found that CFU/ml experienced a steady decline with increasing concentrations of bile; CFU/ml was 87% of controls at 1.25% bile, 67.2% at 2.5% bile, 59.6% at 3.75% bile and 39.78% at 5% bile (14). The concentration-dependent bile sensitivity demonstrated by *C. jejuni* here is similar to what was found in this investigation except lower concentrations of bile had a much greater inhibitory effect on the growth of *C. concisus* compared to *C. jejuni*. For example, *C. concisus* strain P1CDO-S2 showed the strongest resistance to increasing concentrations of bile out of the three tested strains but only retained 5.11% CFU/ml in 1% bile compared to *C. jejuni* retaining 87% CFU/ml in 1.25% bile (14). This is likely because *C. jejuni* infects the ileum, giving a greater need for bile resistance.

C. concisus, on the other hand, is a pathogen of the colon, where bile concentrations are much lower (8).

When considering bile concentration and exposure time in an *in vivo* situation, it is considerable from the results of this investigation that a combination of reduced intestinal bile secretion as well as reduced food transit time can strongly increase the chance of *C. concisus* colonizing the large intestines. Both these factors are subjective to environmental influence; for example, bile is secreted in response to fat content in meals. Low fat meals, therefore, could reduce intestinal bile secretion leading to lower ileal bile concentrations. In addition, intestinal transit time of ingested foods can vary greatly between individuals depending on peristalsis rates or dietary, physical or medical influences. Examples can include conditions like IBD, Irritable Bowel Syndrome, an infection from an intestinal pathogen resulting in diarrhoea or simply having a high liquid or high fibre diet (39).

Finally, the fact that there is variance in bile resistance between strains could provide some insight as to why the intestines of all humans are not colonized by *C. concisus* despite it being commonly present in human oral cavities. Aabenhus *et al*. found that *C. concisus* virulence factors can vary between strains and subsequent ability to colonize intestinal tracts of mice (2). It is therefore possible that a combination of strain-dependent bile resistance, virulence factors and host susceptibility could contribute to the likelihood of *C. concisus* infection.

2.5 Conclusion

The hypothesis, which suggested that strains of *C. concisus* involved with IBD would have a greater ability to tolerate bile exposure when compared to strains isolated from healthy individuals was not supported by the results; strains isolated from healthy patients observed similar levels of bile resistance when compared to those isolated from patients with IBD. It is likely, therefore that there are other factors involved in the intestinal colonization of *C. concisus*.

Bile resistance may not the determining factor in potential infection but it is without doubt that the presence of bile inhibits *C. concisus* growth in a time and concentration dependent manner. In addition, it is clear that bile resistance abilities vary between strains of *C. concisus.* Further work is required to determine whether or not *C. concisus* infectivity in strains associated with IBD patients is in any way related to enteric bile; protein expression analysis may determine a further relationship between *C. concisus* growth and bile exposure.

Chapter 3: Protein secretion by *C. concisus* in response to bile exposure

3.1 Introduction

Previous studies have detected various virulence proteins expressed by *C. concisus* including invasins, adhesins, hemolysins and iron-related factors, supporting the pathogenic role of *C. concisus* (28)(28)(28)(28)(28)(28)(28)(28)(28) (27, 26, 25, 24).

A study by Kaakoush *et al*. on the secretome of *C. concisus* strain UNSWCD, a strain isolated from a cecal biopsy from a child with CD. The procedure identified 201 proteins, 25 of which were likely related to virulence including invasins, adhesins, hemolysins and iron-related factors (29). 86 of these proteins were secreted by *C. concisus* UNSWCD strain, some of which were known virulence proteins such as outer membrane fibronectin-binding protein, surface antigen CjaA and S-layer-RTX protein (27).

Bile salts have been found to have bacteriocidal effects on Gram negative bacteria (22). In humans, bile enters into the intestinal tract at the duodenum. The concentration of bile salts in the small intestine of different individuals varies greatly. Between 0.2 and 0.6 g/d of bile acids are synthesized daily in humans with most of the bile (95%) being reabsorbed before reaching the large intestine (9, 67). Studies in chapter 2 found that bile had time and concentration dependent inhibitory effects on the growth of *C. concisus,*

Exposure to bile in some bacterial species such as *Bacteroides fragilis* upregulated the expression of virulence factors (64). Little work has been done on proteins expressed by *C. concisus* in response to environmental stimuli such as bile salt. Studies included in this chapter examined the second hypothesis of this thesis, thus exposure to bile increases expression of virulence proteins in *C. concisus.* This study examined the proteins secreted by *C. concisus* in response to the presence of bile.

3.2 Material and methods

3.2.1 Identification of proteins secreted by *C. concisus* in the presence of bile

C. concisus strain, P2CDO-S1 (oral strain isolated from a patient with CD), was randomly selected. In preparation for tests, bacteria were aseptically streaked via platinum loop on HBA plates prepared using 20g of Oxoid Blood Agar Base (CM0271) and 30ml SR0050 defibrinated horse blood (6.0%) in distilled water (total volume of 500ml). Plates were incubated in hydrogen enriched anaerobic conditions over 48 hours at 37ºC. Bacteria were then collected via hockey stick in 1ml PBS and centrifuged for 2 minutes at 9,000rcf and 4ºC using the Eppendorf Centrifuge 5415 R. The bacterial pellet was resuspended in 500µL PBS. The optical density (OD) at 600nm was then measured through the SpectraMax 340 molecular device system managed by SoftMaxPro40 software. Bacterial inoculums (1ml with an OD_{600} of 1.0) were then transferred to two falcon tubes; one tube containing 25ml Heart Infusion Broth, (HiB) CM1135 (Oxoid) and one tube containing 25ml HIB supplemented with 0.1% ox bile salt (Sigma). The falcon tubes were subsequently incubated for 24 hours at 37 ºC with rapid agitation. The falcon tubes were then centrifuged using the Eppendorf Centrifuge 5810R at 3,220rcf for one hour and the supernatant preserved. Amicon ultra centrifugal filters (3K membrane) (Amicon, MA, USA) were then used to concentrate protein contents in the supernatant via 3K membrane. The resultant filtrate were removed and sent to the UNSW Biomass Spectrometry facility for identification of proteins using a Thermo Electron LTQ-FT Ultra mass spectrometer. The facility then returns search results (using Matrix Science) for any identified proteins.

3.2.2 Polymerase chain reaction (PCR) primers and plasmid vectors

In order to clone the gene encoding hypothetical protein CCC13826_1330, PCR primers were designed to amplify this gene. The full gene sequence of CCC13826_1330 was obtained from the *C. concisus* reference strain 13826 (Accession Number ASM1772v1) from the National Centre of Biotechnology Information (NCBI) database. Forward and reverse primers were designed using Primer-BLAST, an online tool provided by NCBI. The sequences of forward and reverse primers were modified to include restriction enzyme Xho1 and Nco1 digestion site respectively using NEBcutter V2.0 (81). This pair of primers is expected to amplify the full length of CCC13826_1330 gene with a PCR product of 717 bp.

Table 3.1: Primer sequence

Primers	Sequence
Forward	5'- GGC CTG CCA TGG TCC TTT TAA AAA GCT GGG AGG GAT -3'
Reverse	5' - GCA TGA CTC GAG AAA CTA AGG CTT GCT GCG TAG T - 3'

The underline indicates the restriction enzyme site. Nco1, CCA TGG; XhoI, CTC GAG

Plasmid vector pETBlue-2 cloning expression system (Novagen, Darmstadt, Germany) was used. The restriction enzyme digestion sites included in this vector are shown in Figure 3.1

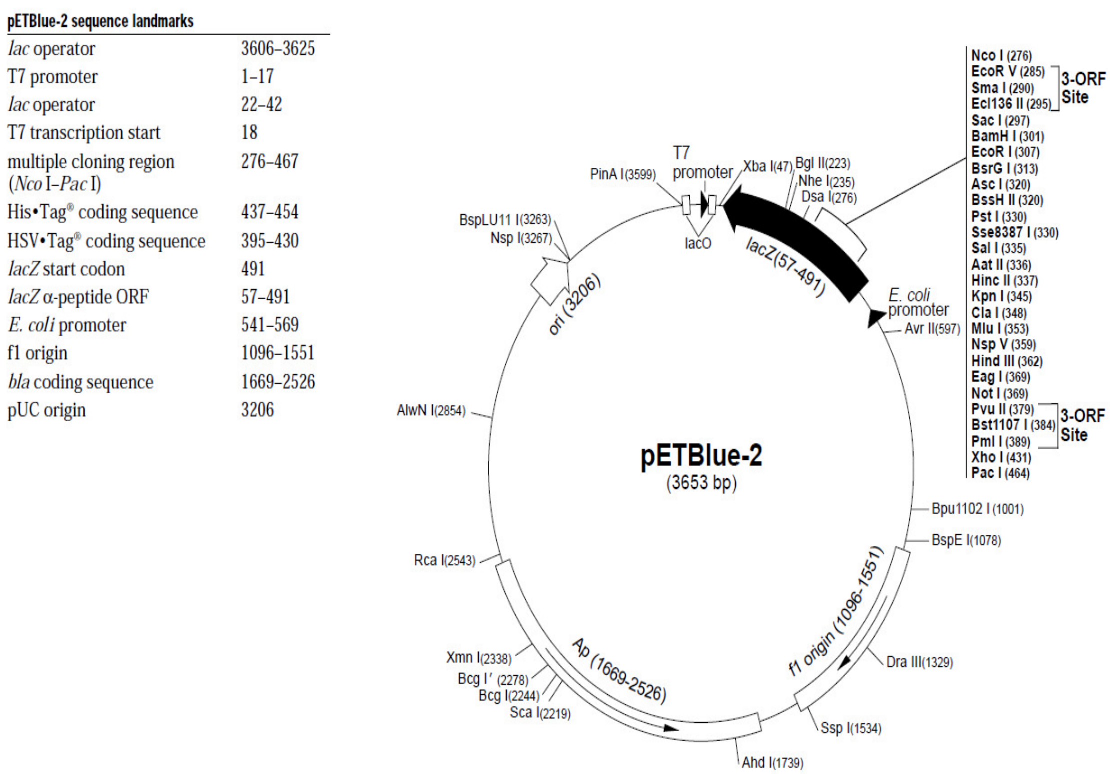

Figure 3.1: The gene map of the pETBlue-2 vector used in this study. This map was adapted from the Novagen petBlue-2 manual.

3.2.3 Amplification of CCC13826_1330 gene by polymerase chain reaction (PCR)

PCR was conducted to amplify the CCC13826_1330 gene. Genomic DNA was previously extracted from *C. concisus* strain 13826. The PCR reaction volume was 25 µl containing 12.5µl nuclease free water (Sigma), 2.5µl 10X reaction buffer (Thermo), 2.5µl 10X dNTPs (Thermo), 1µl 10pmol/µl forward and reverse primers, 1.5µl $MgCl_2$ (Thermo) and 2.0µl *Taq* DNA polymerase (Thermo). PCR was run through a thermal cycler (Thermo) for 35 cycles. Each cycle consisted of denaturing for 20 seconds at 94ºC, annealing at 55ºC for 20 seconds and extension at 72ºC for 40 seconds.

3.2.4 Purification of PCR product

The PCR product was briefly mixed with 40µl 80% ethanol and left for 30 minutes at room temperature. After centrifugation for 20 minutes at 9,200rcf, the supernatant was removed. The PCR pellet was then air-dried for 5 minutes and resuspended with 10 µl nuclease free water. Agarose gel electrophoresis was conducted to confirm the PCR result.

3.2.5 Sequencing PCR product

For DNA sequencing, 5X BigDye sequencing buffer Cat. No. 4336697 (Invitrogen) was used. This included a single reaction tube containing 2 µl PCR product, 12.5 µl nuclease free water, 3.5 µl 5X buffer, 1 µl BigDye and 1 µl primer. PCR product sequencing was performed at duplication.

The PCR thermo cycler was set at ten seconds at 96 ºC for denaturing, five seconds at 50 ºC for annealing and four minutes at 60 ºC for extension.

Ethanol/EDTA purification was performed prior to sequencing. Ethanol (480µl of 100% ethanol) and EDTA (30µl, 125mM) were added to the solution containing the PCR products before allowing it to stand for 15 minutes (for precipitation of proteins) and centrifuging at max speed for 20 minutes. Supernatant was then removed and 750µl of 70% ethanol was added for removal of salts. The solution was then centrifuged again at max speed for 10 minutes before supernatant was again removed. The solution was then heated at 90ºC to evaporate any remaining ethanol. BigDye® Terminator V3.1 (Invitrogen) was used for sequencing analysis.

3.2.6 Extraction of pETBlue-2 vector

For expression of bacterial proteins, the pETBlue2 vector (Novagen) was used. This expression system allows easy selection by blue/white colony screening and *bla* gene (ampicillin resistance gene) based selection, protein extraction through 6 His tag and a multi cloning site for easy primer design. *Escherichia coli* Novablue (Novagen) containing plasmid pETBlue2 vector were cultured on NA (Nutrient Agar) plates containing ampicillin (Sigma) and tetracycline (Sigma) at aerobic conditions at 37ºC. A single colony of *E. coli* was inoculated in 10ml Nutrient Broth (NB) with 12.5µg/ml tetracycline overnight through rapid agitation at 37ºC. Following this, the solution was centrifuged for five minutes at 9,000rcf. The supernatant was then removed and the pellet was resuspended in 500µl of GET buffer solution (5% Glucose/10mM EDTA/100mM Tris) and 1ml of solution containing 1% SDS and 0.2N NaOH solution was added. The solution was left to stand for five minutes before adding 750µl of 5M KOAc and left in ice for the same time. Centrifugation was then run again for five minutes at max speed, transferring 2ml of supernatant to a new tube. Another 2ml of cold 100% ethanol was added and the tube was left in a -20ºC freezer for 20 minutes. The solution was then centrifuged at 9200rcf for ten minutes, the supernatant removed and the tube heated at 80ºC for one minutes. Finally, the pellet was dissolved in 15µl of TE buffer.

3.2.7 Double digestion of PCR products and the pET-Blue2 vector

The purified PCR products of the CCC13826_1330 gene and pET-Blue2 vector were digested through enzymes NcoI and XhoI (New England Biolabs, MA, US). The digestion mixtures containing 5µl NEBuffer 3.1 ,1µl Nco1 and 0.5µl of Xho1 and 1µg of either the purified PCR products or pET-Blue2 vector DNA were placed into 1.5 ml eppendorf tubes in a total volume of 50 µl. The tubes were placed in a 37ºC water bath for 1 hour before stopping the reaction by heating the tubes to 80ºC for 20 minutes (denaturing enzymes Xho1 and Nco1). The digested PCR products and plasmid DNA were then purified through the previously mentioned method.

3.2.8 Ligation of digested PCR product and pETBlue2 vector

The digested PCR product and pETBlue2 vector were then ligated. NEB T4 ligase (1µl containing 20,000 units) was added to 2µl of 10X reaction buffer as well as 50ng of DNA insert and vector before the solution was made up to 20µl using nuclease free water. The solution was then incubated at room temperature for one hour before being prepared for transformation into competent *E. coli* cells.

3.2.9 Preparation of competent cells for plasmid transformation

The competent cells were prepared by streaking *E. coli* Novablue (Novagen) on NA plates containing tetracycline. The plates were incubated overnight at 37ºC before a single colony was picked using a platinum loop and inoculated in 10ml of Nutrient broth (Oxoid) containing 12.5 µg/ml tetracycline. The broth was incubated overnight at 37ºC and 1ml was transferred to 100ml of warm NB with 12.5 µg/ml tetracycline. The broth was incubated for two hours and split between four falcon tubes (25ml each). The tubes were iced for ten minutes and centrifuged at 4,000rcf/4ºC for 5 minutes. While keeping the tubes on ice, supernatants were discarded and bacterial pellets were suspended in 30ml of 0.1M cold calcium chloride solution. The tubes were then incubated a further 1.5 hours in ice before being centrifuged at 2,000rcf/4ºC for 15 minutes. The supernatant was again discarded and the pellet resuspended in 4ml of 85mM cold calcium chloride/15% glycerol. Finally, 500µl aliquots were distributed between eight 2ml cryogenic tubes and stored at -80ºC. The competency was tested following transformation method.

3.2.10 Transformation of competent cells

Transforming the competent cells with the new plasmid involved the preparation of three tubes; competent cells alone (negative control), competent cells with the vector of interest and competent cells containing the test plasmid containing the *bla* gene (positive control) (Novagen). 20µl of cell suspensions were mixed in a 1.5ml tube with 5ng of vector. Negative controls were instead mixed with TE buffer. The solutions were kept on ice for 5 minutes. In turn, heat shock was followed by incubating the tube in 42 ºC water bath for 20 seconds exactly and immediately cooled down on ice for at least 2 minutes. 80µl of SOC media was then added to each solution. Tubes were then incubated at 37ºC in water for one hour with occasional mixing every 15 minutes before spreading bacteria on NA plates containing 80uM IPTG and 700µg/ml Xgal. Plates were incubated overnight at 37ºC and resultant colonies were identified via blue/white colour screening; white colonies were considered recombinant while blue colonies were not. *lacl* is under the control of a transcriptional repressor. The presence of IPTG binds this repressor, allowing expression of *lacZ* and subsequent enzymatic digestion of X-gal, presenting blue colonies. White colonies indicate the disruption of the *lac* operon through the insertion of targeted sequences in the Multiple Cloning Site (MCS).

3.3 Results

3.3.1 Proteins secreted by *C. concisus* strain P2CD-S2 in the presence of 0.1% bile

Mass spectrometry analysis was performed in order to detect *C. concisus* proteins in the bacterial culture supernatant of *C. concisus* strain P2CDO-S1 in either the presence or absence of 0.1% bile salt. In the supernatant without bile salt, no *C. concisus* proteins were identified (Table 3.2). In contrast, in the supernatant of the same *C. concisus* strain cultured in media containing 0.1% bile for 24 hours, nine *C. concisus* proteins were identified (Table 3.2). Interestingly, the hypothetical protein CCC13826_1330 (Information obtained from KEGG database) showed a motif that is shared with plasminogen binding protein.

Table 3.2 A summary of proteins secreted by *C. concisus* in the absence of bile compared to proteins secreted in the presence of 0.1% bile

Proteins secreted by P2CDO-S1 without bile	**Proteins secreted by P2CDO-S1 in 0.1% bile**
No secreted *C. concisus* proteins were detected.	1) Trigger factor (gi 157165093) 2) 2-cys peroxiredoxin BAS1 (thiol-specific antioxidant protein) (gi 157164012) 3). Fumarate reductase flavoprotein subunit (gi 157165747) 4) Hypothetical protein CCC13826_0291 (gi 157164504) 5) Holo - (acyl-carrier-protein) synthase (gi 157164251) 6) Methyl-accepting chemotaxis protein (gi 157165447) 7) Fumarate hydratase (gi 157164497) 8) DNA-3-methyladenine glycosylase 1 (gi 157165393) 9) Hypothetical protein CCC13826_1330 (gi 157163941)

3.3.2 Ligation of PCR product of CCC13826_1330 and pETBlue2 vector

The gene of CCC13826_1330 was successfully amplified via PCR .The PCR product had an expected size of 717bp. Sequencing of the PCR products confirmed the identity of CCC13826_1330 gene.

On agarose gel electrophoresis, the digested PCR and pETBlue 2 vector showed a single band with correct sizes, being 692bp and 2686bp respectively. The pETBlue2 vector that ligated with the PCR products showed three bands. The top band was the digested pETBlue 2 vector and the bottom band was the digested PCR products. The third faint band (1392bp) may represent the pETBlue 2 vector that has ligated with the PCR products.

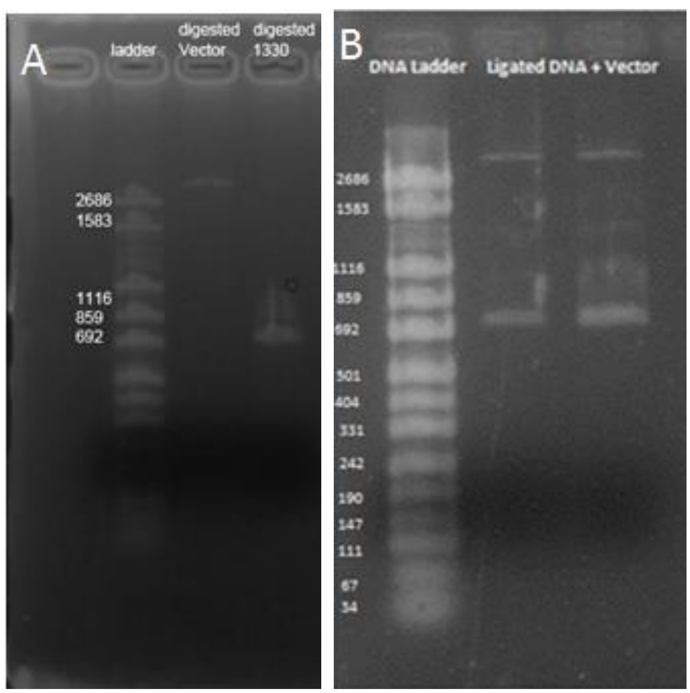

Figure 3.2: A : Enzyme digested PCR products and pETBlue2 vector. **B**: pETBlue2 vector ligated with the PCR products at ratios 1:6 (lane 2) and 1:9 (lane 3). The gel used contained 1.2% agarose and was stained with 60µl gel red in 200ml 0.1M NaCl . The sizes of PCR products and digested pETBlue 2 vector were 692 bp and 2686bp respectively.

3.3.3 Transformation of competent *E. coli*.

For transformation, a plasmid containing the *bla* antibiotic resistance gene was used as a positive control. After transformation, *E. coli* were streaked onto NA plate containing 80µM IPTG and 700µg/ml Xgal. Several blue colonies appeared in the positive control NA plate, indicating that the positive control plasmid has been successfully transformed into *E. coli*. However, neither blue nor white colonies were formed on the NA plate that was streaked with *E. coli* component cells transformed with pETBlue2 vector ligated with CCC13826_1330 PCR product. Out of a total of five repeats, three resulted in only blue colonies being found while two did not result in the growth of any colonies at all.

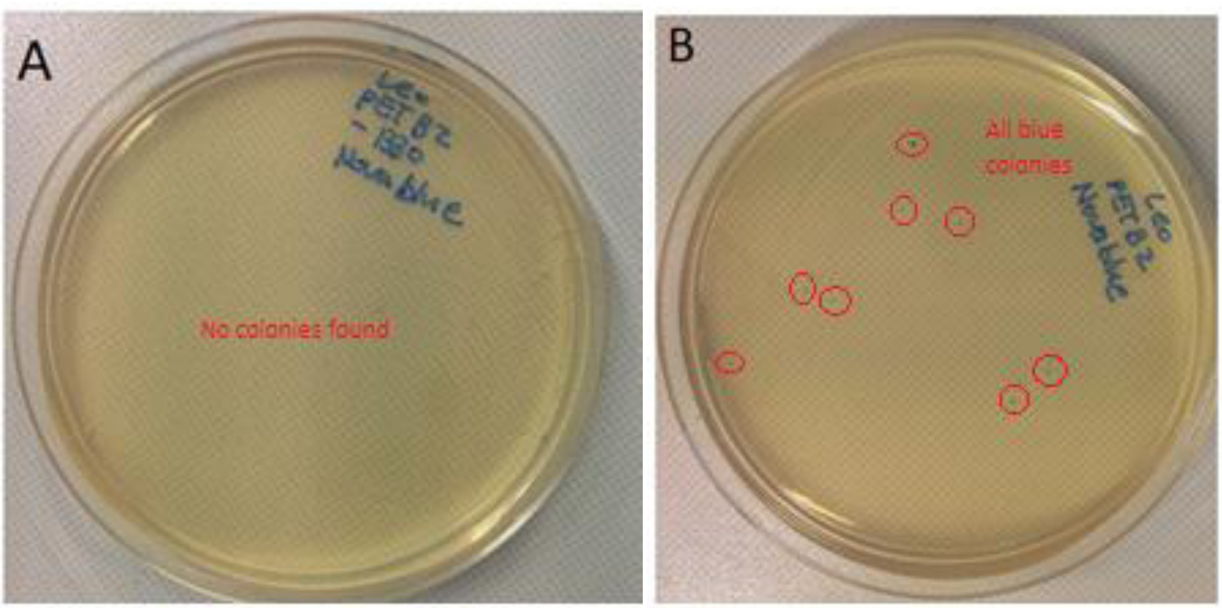

Figure 3.3: The transformation of the recombinant vectors into *E.coli* component cells.**A:** *E.coli* component cells transformed with pETBlue2 vector ligated with CCC13826_1330 PCR products . **B**: *E. coli* component cells transformed with the positive control plasmid.

3.4 Discussion

This study examined the proteins secreted by an oral *C. concisus* strain (P2CDO-S1) isolated from a patient with CD. Nine *C. concisus* proteins were identified in the presence of 0.1% bile. Of these nine proteins identified, two proteins were also identified in the secreted proteins of UNSWCD strain, a strain isolated from intestinal biopsies of a child with CD (28). These two proteins are a methyl-accepting chemotaxis protein and a hypothetical protein encoded by gene CCC13826_1330. Further examination of the information provided in the publically available databases showed that the hypothetical protein encoded by CCC13826_1330 shared a motif with plasminogen-binding protein (Plg-B). Plasminogen is the precursor of plasmin, plasmin is a protease that cleaves fibrin, fibronectin, laminin and vitronectin (70, 4) The formation of active plasmin catalyzes degradation of tissue matrix barrier, which facilitates the spread of bacteria. Plasminogen-binding is a strategy used by various pathogens, such as group A *Streptococci*, *Haemophilus*, *Neisseria* and *E. coli* (4, 62, 70). For example, Berge *et al*. showed that group A *Streptococci* serotypes that express M41 and M53 proteins, which were able to specifically absorb plasminogen from human plasma (68). The finding in this study that a Plg-B like protein (the protein encoded by CCC13826_1330) secreted by P2CDO-S1 strain in the presence of 0.1% bile suggests that exposure to bile may enhance expression of some virulence factors in some *C. concisus* strains.

In order to further characterize the function of the protein encoded by CCC13826_1330 gene. Studies were carried out in this project to express this protein in an *E. coli* system. Unfortunately, the attempt to clone the CCC13826_1330 gene into a plasmid vector was not successful. Due to time restraints (based on university policy, further extension of the study period is not allowed), no further laboratory work can be conducted. However, a number of possible technique issues related to the failure of the cloning of CCC13826_1330 gene are discussed below. These discussions may be helpful for future students to continue with this project.

The first factor that may have contributed to the failure of the cloning step may come from the step of PCR product digestion. It is difficult to know whether the PCR product has been sufficiently digested by the restriction enzymes. In this project, the PCR product was digested for one hour. Perhaps, in future studies, a longer digestion time can be used.

The second step that may have gone wrong was the ligation. As shown in figure 3.2 (B), most of digested PCR product and the vector still remained in their pre-ligation positions, suggesting that they were not ligated well. In future studies, various concentrations of PCR product and vector DNA should be used to find out the optimal ligation conditions. In addition to the ligation step itself, the insufficient digestion discussed above may also have has prevented the ligation of PCR product and vector.

The transformation process was successful, indicated by the appearance of blue colonies on the plate containing the positive control plasmid.

Finally, the likelihood of human error also cannot be ignored for this investigation; the student performing the investigation had little to no experience in biotechnology before beginning postgraduate studies and could have made fundamental errors in the investigation despite multiple repeat attempts.

In the experiment of identification of secreted *C. concisus* proteins, a concentration of 0.1% bile was used. It was impossible to investigate protein expression in higher concentrations of bile due to the great bacteriocidal effects of bile salts have on *C. concisus* based on the results from chapter 2. Since the test attempted to focus on secreted proteins only, minimizing cell lysis was important. However, from the tests on the effect of bile exposure on the growth of *C. concisus*, even 0.1% bile can have a severe inhibitory effect on certain strains of *C. concisus,* although complete elimination is unlikely. This negatively impacted the results of this investigation, where the amount of bacteria in 0.1% bile was very low, possibly reducing the reliability of identified secreted proteins as well as being unable to identify many potential secreted proteins due to high cell death.

The bile concentration used in this investigation does not necessarily replicate colonic concentrations. Ileal bile secretion varies between individuals (8, 40, 52) and bile is both passively and actively reabsorbed throughout the ileum (8) with secreted bile being almost completely reabsorbed before arrival at the colon, the rate of which may also vary between individuals (31).

It should also be noted that bacteria were incubated in 0.1% bile overnight. The prolonged exposure to 0.1% bile would have increased the bacteriocidal effect bile has on the *C. concisus* strain; it is therefore advisable that future researchers reduce the bile exposure time, minimizing bacterial death while still allowing them to respond to bile.

Fewer secreted proteins were found in this investigation when compared to the secretome identified by Kaakoush *et al*., possibly due to the use of different strains of *C. concisus* in this investigation and the bacteriocidal effect of bile on *C. concisus* (29).

3.5 Conclusion

While finding a secreted protein related to 0.1% bile exposure by *C. concisus* strain P2CDO-S1 (hypothetical protein CCC13826_1330) was interesting, it was unfortunate that cloning of this gene into a plasmid vector for protein expression in *E. coli* system was unsuccessful. Further work is needed to continue the investigation of the effects of bile on *C. concisus* virulence protein expression.

Chapter 4: General discussion and conclusion

Data generated from this project showed that *Campylobacter concisus* is much more sensitive to the bacteriocidal and bacterial inhibitory effects of bile compared to common enteric bacterial pathogens such as *Campylobacter jejuni*, *Salmonella typhimurium, Escherichia coli* and *Listeria monocytogenes* (3, 14, 33, 76, 78).

C. concisus is present in the human oral cavity (85) and can be introduced to the intestinal tract through swallowing of saliva or the movement of ingested food. Despite this, the colonization of *C. concisus* in the intestinal tract of healthy individuals is low (77). The finding that *C. concisus* has a low resistance to bile indicates that the majority of *C. concisus* is killed in the duodenum or jejunum during its journey from the oral cavity to the lower parts of the gastrointestinal tract on a daily basis, which possibly explains why *C. concisus* transported from the oral cavity to the intestines do not usually cause intestinal diseases. *C. concisus'* sensitivity to bile also helps to explain why the increased prevalence of *C. concisus* in IBD was often detected in large intestine, a site with low concentration of bile.

C. concisus has been associated with patients with inflammatory bowel disease (IBD) due to a significant higher prevalence of *C. concisus* in the intestinal tract of patients with IBD compared to healthy individuals (47, 50, 88). This project found that the abilities of oral *C. concisus* strains isolated from patients with IBD and controls to resist bile were similar, suggesting that other environmental factors may have contributed to the high intestinal prevalence of *C. concisus* in patients with IBD.

Exposure to 0.1% bile induced the secretion of a number of proteins by *C. concisus* P2CDO-S1 strain. Of the proteins secreted by this strain in response to bile, the hypothetical protein encoded by CCC13826_1330 is particularly interesting given its motif shared with plasminogen binding protein. Future studies are required to characterize this protein and investigate its relationship with *C. concisus* virulence.

Recent studies suggest that the zonula occludens toxin carried by some *C. concisus* strains may contribute to the initiation of IBD by increasing intestinal permeability, increasing the translocation of bacterial products from intestinal lumen to intestinal tissues (29). The *zot* gene in *C. concisus* was found to be carried by a putative prophage CON_phi2 (89). Future studies should be conducted to investigate whether exposure to bile induce the production of prophage proteins in *C. concisus* .

In conclusion, this project has investigated the resistance and protein secretion of *C. concisus* in response to bile. The data generated from this project help further understanding the pathogenic potential of *C. concisus*.

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