In vitro studies of *Brachyspira pilosicoli* pathogenesis

Ram Naresh  
BVSc & AH, MVSc (Medicine)

Division of Health Sciences  
School of Veterinary Biology and Biomedical Sciences  
Murdoch University, Perth 6150  
Western Australia

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This thesis is dedicated to

**My Late Father (Appa)**

*A Man of Moral Values*

You proved that an illiterate father and farmer can educate his son very well
You were always reluctant to send me away from home
But you never stopped me and I kept on crossing seas
You were my best teacher to introduce me in this world
You taught me at the very beginning to be respectful and kind
I am still living very happily on your philosophies
You were a man of big heart, no one ever saw you in tears or crying
Sorry I was not around at the last day of your life
Though you are immortal father

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*Great Animal Lover*

You were the best animal lover I could ever see in my life,
I still remember those broken leg dogs you used to bring home
You treated them as an ancient veterinarian
You fought several people for animal cruelties
You were skilled to communicate with animals
I always feel that I became a veterinarian to represent your traditional skills through university education
You were more than a mum for many children in the community
Perhaps you knew my future and asked me to learn cooking
I laughed at you. I learned cooking during my Australian PhD!

**You were not a quitter in any situation**

**My lovely daughters**

*Chelsi* (Anvesha) and *Khushi* (Aduesha)
Little puppies you were my rare emotional strength in Australia during this PhD
You both sacrificed in many ways

**Love You Both**
Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Ram Naresh
Abstract

*Brachyspira pilosicoli* is an intestinal spirochaete that colonizes the large intestine of a variety of species of birds and animals, including human beings. Colonization can lead to local inflammation and to diarrhoea in a condition known as “intestinal spirochaetosis”. This infection has been described in many countries throughout the world. In the colonization process the bacterium must cross the thick mucus blanket overlaying the colonic epithelium. Characteristically, *B. pilosicoli* then attaches by one cell end to the underlying epithelium, forming a dense “false brush border”. The mechanisms involved in moving through the mucus layer, attaching to enterocytes and inducing local cellular damage are poorly understood. The lack of *in vitro* models to study these events has been a major constraint to understanding the pathogenesis of *B. pilosicoli* infections.

The work described in this thesis deals with i) the development of an *in vitro* model of spirochaete attachment by using cells in suspension (erythrocytes) and cell monolayers (Caco-2), ii) the attraction of *B. pilosicoli* to mucin, and iii) the effects of norepinephrine exposure on expression of virulence traits by *B. pilosicoli*.

Attachment assays conducted with erythrocytes from different species at different ratios and time intervals identified one human isolate (WesB) that
adhered to goose and chicken erythrocyte at a 1:1000 ratio. This same strain, and an isolate from a pig (95/1000) also attached to Caco-2 cells. Transmission and scanning electron microscopy confirmed that the attachment resembled the in vivo situation. Exposure of the Caco-2 cells to B. pilosicoli resulted in actin rearrangements, damaged cell junctions and apoptosis. Caco-2 cells that were colonized with B. pilosicoli also demonstrated a significant up-regulation of interleukin-1β (IL-1β) and IL-8 expression, helping to confirm that the spirochaetes were inducing pathological changes in the cultured cells. Treatment of the monolayers with B. pilosicoli sonicates caused significant up-regulation of IL-1β, TNF-α, and IL-6, but culture supernatants and non-pathogenic Brachyspira innocens did not altered cytokine expression. Hence IL-8 expression was specifically associated with exposure to live B. pilosicoli cells.

For mucin attraction, 15 B. pilosicoli strains isolated from humans, pigs, chickens and dogs, and a control strain of Brachyspira hyodysenteriae, were analysed for their ability to enter solutions of hog gastric mucin in an in vitro capillary tube assay. Attraction started in a 2 % mucin solution, and then increased with increasing concentrations to peak at around 6 - 8 % mucin. Attraction varied from strain to strain. B. pilosicoli strain 95/1000 and B. hyodysenteriae strain B204 also were attracted to viscous solutions of polyvinylpyrillodone (PVP), in a manner mirroring the response to mucin. This suggested that as well as chemotaxis to mucin components, “viscotaxis” is involved in the attraction to mucin.
Finally, exposure of *B. pilosicoli* to norepinephrine enhanced the attachment to Caco-2 cells, chemotactic response to mucin, and spirochaete growth. Taken together, these *in vitro* studies have shed new light onto the pathogenic processes that are involved in intestinal spirochaetosis caused by *B. pilosicoli*. 
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Awards and publications from thesis work

Awards

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2. **Best Poster Award. Pathogens and Parasite.** Attraction of *Brachyspira pilosicoli* chemotaxis to mucin. Research Poster Day 2009, School of Veterinary and Biomedical Sciences, Murdoch University – 2009. Sponsored by Gene Works Pty Ltd.

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Presentations


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CHAPTER 1: LITERATURE REVIEW

1.1. Preface

This thesis is concerned with the anaerobic intestinal spirochaete *Brachyspira pilosicoli*, a helical shaped bacterium that colonizes the large intestine of various species. Specifically the thesis examines the development of *in vitro* attachment models for the spirochaete, the attraction of *B. pilosicoli* to mucin, and the effects of norepinephrine on *B. pilosicoli* in the model systems. These aspects previously have not been examined, or have received only minor attention.

There are seven officially named species in the genus *Brachyspira*, of which only *B. pilosicoli* and *B. aalborgi* are known to attach by one cell end to the intestinal mucosa of susceptible hosts. Unlike *B. aalborgi*, which has a host range restricted to humans and non-human primates, *B. pilosicoli* colonizes the large intestines of a wide range of species of animals and birds, as well as human beings. *B. pilosicoli* is an aetiological agent of three named diseases, these being porcine colonic (or intestinal) spirochaetosis, human intestinal spirochaetosis and avian intestinal spirochaetosis. Other than end-on attachment by the spirochaete, the pathogenesis of the associated diseases is poorly understood. In the case of other enteropathogenic bacteria *in vitro* attachment models have been widely used to study their cellular and molecular pathogenesis. A similar reproducible *in vitro* attachment model is not available for *B. pilosicoli*, and a description of attempts to develop such a
model, initially using red blood cells and later Caco-2 cell monolayers, forms an important part of the work in the thesis.

Uninterrupted mucus secretion is a characteristic feature of the goblet cell network that is densely dispersed in the large intestine. As such, enterocytes of the caecum and colon are covered with a 0.7 – 1 mm thick mucus blanket. *B. pilosicoli* needs to cross this physical mucus barrier to make an intimate end-on attachment to enterocytes of the caecum and colon, and the ability of the spirochaete to do this has not been adequately studied. Consequently aspects of the attraction of *B. pilosicoli* to mucin were investigated in this thesis.

According to one estimate, over half of all norepinephrine in the body is produced from neuronal cells in the gastrointestinal tract, and it seems likely that a large quantity of norepinephrine is present in the large intestine at any one time. There have been several reports on the role of norepinephrine in modulating virulence traits (growth, adhesiveness, motility and chemotaxis) of enteropathogenic bacteria, for example in *Escherichia coli* O157:H7, *Salmonella Typhimurium*, *Campylobacter jejuni* and *Shigella flexineri*. No work has been reported on the effects of norepinephrine on virulence traits of *B. pilosicoli* (or other intestinal spirochaetes), although when the spirochaetes come into contact with norepinephrine in the intestinal tract their behaviour potentially could be modulated. This possibility has been investigated in the current study.
The thesis has been prepared to cover these aspects sequentially. The first chapter provides a general review of the literature on intestinal spirochaetes, disease potential of members of the genus *Brachyspira*, and then more detail on *B. pilosicoli*, including its disease potential and pathogenesis. In order to prime the reader for the experimental work described later in the thesis, the first chapter then gives a brief background to Caco2 cells, some cellular changes induced in them by attaching bacteria, and some information about cytokines and how norepinephrine can alter bacterial phenotypes. More detailed specific literature is discussed later, in the respective chapters.

The second short chapter describes the general materials and methods used in the work, particularly the way the spirochaetes were grown and counted. Chapters 3, 4, 5, and 6 deal respectively with red blood cell attachment assays, Caco-2 cell attachment assays, mucin attraction assays and the effects of norepinephrine on *B. pilosicoli*. Each of these chapters contains a brief introduction, materials and methods, results and discussion. Chapter 7 presents some general discussion and concluding remarks.

### 1.2. Intestinal spirochaetes

Intestinal spirochaetes of the genus *Brachyspira* belong to the Subkingdom Negibacteria, Infrakingdom Glycobacteria, Division Spirochaete, Class Spirochaetes, Order Spirochaetales (Garrity et al 2001; Smith 2002). Some species currently classified in the genus *Brachyspira* previously have been included in the genera *Treponema, Serpula* and/or *Serpulina* (Paster and Dewhirst 1997). Morphologically the members of the genus *Brachyspira*
have similar unique characteristics. They are poorly Gram-negative, motile, non-spore forming and oxygen-tolerant anaerobic bacteria. They are broadly rod-shaped with a helical body and tapered cell ends, and possess a protoplasmic cylinder and periplasmic flagella covered by an outer envelope. The coiled body of the cell contains nucleic acid and cytoplasmic elements. *Brachyspira* species are variably motile and possess four to 14 flagella anchored at each cell end that run along the cell body in the periplasmic space and overlap near its center (Sellwood and Bland 1997). The *Brachyspira* species tend to be resistant to antibiotics like streptomycin, rifampicin, colistin, vancomycin and spectinomycin, which can then be used in selective media for their isolation (Songer et al 1976; Jenkinson and Winger 1981; Brooke et al 2003).

According to biochemical and phylogenetic studies, *Brachyspira* spp. can be divided into biochemical groups I, II, IIIa, IIIb, IIIc, and IV, and *B. pilosicoli* belongs to group IV (Fellström et al 1995). Their haemolytic properties vary from strong in *B. hyodysenteriae* to weak in the other species, including *B. pilosicoli* (Alexander and Taylor 1969; Harris et al., 1972a; Trott et al 1996a). Currently the genus *Brachyspira* contains seven officially named species, and several others that have been unofficially proposed. The seven named species and three of the suggested species and their associated diseases are shown in Table 1.1.
Table 1.1: *Brachyspira* species and their disease potential

<table>
<thead>
<tr>
<th>Intestinal Spirochaete</th>
<th>Disease</th>
<th>Host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. hyodysenteriae</em></td>
<td>Swine Dysentery</td>
<td>Pigs</td>
<td>Alexander and Taylor 1969; Harris et al 1972</td>
</tr>
<tr>
<td><em>B. innocens</em></td>
<td>Non-pathogenic</td>
<td>Pigs</td>
<td>Kinyon and Harris 1979</td>
</tr>
<tr>
<td><em>B. aalborgi</em></td>
<td>Human Intestinal Spirochaetosis</td>
<td>Humans</td>
<td>Hovind-Hougen et al 1982</td>
</tr>
<tr>
<td><em>B. pilosicoli</em></td>
<td>Intestinal Spirochaetosis</td>
<td>Humans, Pigs and Chickens</td>
<td>Trott et al 1996a &amp; b; Trott et al 1997a &amp; b</td>
</tr>
<tr>
<td><em>B. murdochii</em></td>
<td>Non-pathogenic</td>
<td>Pigs and Rats</td>
<td>Stanton et al 1997</td>
</tr>
<tr>
<td><em>B. intermedia</em></td>
<td>Mild diarrhoea</td>
<td>Birds</td>
<td>Stanton et al 1997</td>
</tr>
<tr>
<td>“<em>B. canis</em>”</td>
<td>Associated with diarrhoea</td>
<td>Dogs</td>
<td>Duhamel et al 1998</td>
</tr>
<tr>
<td><em>B. alvinipulli</em></td>
<td>Intestinal Disease</td>
<td>Birds</td>
<td>Stanton et al 1998</td>
</tr>
<tr>
<td>“<em>B. pulli</em>”</td>
<td>Pathogenic potential unknown</td>
<td>Birds</td>
<td>Stephens and Hampson 2001</td>
</tr>
<tr>
<td>“<em>B. suanatina</em>”</td>
<td>Diarrhoea</td>
<td>Ducks and Pigs</td>
<td>Råsbäck et al 2007</td>
</tr>
</tbody>
</table>

1.3 Ecology and disease potential of *Brachyspira* species

1.3.1 Early observations on intestinal spirochaetes

According to Escherich (1884), spirochaetes were first reported in 1719 by Van Leeuwenhoek, who observed vivaciously moving spiral “an-i-mal-cules” in his faeces. Subsequently other workers reported spirochaetes in the intestinal tracts of man and various animals (Werner 1909; Fantham 1916; MacFie 1917). At the beginning of the 20th century interest increased in the occurrence of colonization of humans by intestinal spirochaetes (reviewed by Korner and Gebbers, 2003). At this time the term “intestinal spirochaetosis”
was used to describe colonization of the large intestine (Parr, 1923). Intestinal spirochaetes were reported to be present in the faeces of 3.3% of soldiers in one army command where dysentery previously had occurred (Fantham, 1916), and in 27.7% of healthy people in Chicago (Parr, 1923). Later spirochaetes were isolated from the bovine intestinal tract, and were cultured and characterized as non-pathogenic entities (Bryant 1952). In the early 1970s *Treponema hyodysenteriae* (now *Brachyspira hyodysenteriae*), the aetiological agent of swine dysentery, was identified as an independent pathogenic bacterium of pigs by workers in two different laboratories (Taylor and Alexander 1971; Harris et al 1972b).

1.3.2 Disease potential for animals

Species in the genus *Brachyspira* range from being non-pathogenic (*B. innocens*) to being highly pathogenic (*B. hyodysenteriae*), with different distributions in different animal species. *B. hyodysenteriae* enjoyed the status of being the only known pathogenic intestinal spirochaete species during the 1970s, until the isolation and identification of the spirochaete that is now known as *B. pilosicoli* (Taylor et al 1980; Trott et al 1996a). Around this time *Treponema* (now *Brachyspira*) *innocens* from pigs (Kinyon and Harris 1979) and *B. aalborgi* from humans (Hovind-Hougen et al 1982) were isolated and named, but these were of uncertain pathogenic potential. A number of epidemiological studies subsequently were conducted investigating different aspects of intestinal spirochaetes, and information about their host range as well as their geographical distribution has increased significantly since 1972. For example, *B. hyodysenteriae* now is known not
only to be pathogenic for swine, but also to colonize and induce variable
degree of colitis in naturally and experimentally-infected rodents (Taylor and
Alexander 1971; Harris et al 1972; Joens and Kinyon 1982), game birds
(Jansson et al 2001), chickens (Sueyoshi and Adachi 1990; Trott et al 1995;
Trott and Hampson 1998; Feberwee et al 2008), farmed and wild mallards
(Jansson et al 2004), and dogs (Songer et al 1978).

1.3.3 *Brachyspira pilosicoli*

1.3.3.1 Distribution of *B. pilosicoli* in animals

*Brachyspira pilosicoli* is known to have the widest host range amongst the
intestinal spirochaetes (Hampson 2003). Among animals it has been shown
to colonize swine (Trott et al 1996a; Trott et al 1996b), poultry (Trott and
Hampson 1998; Stephens and Hampson 2002; Jamshidi and Hampson 2003;
Phillips et al 2005), wild birds and game birds (Webb et al 1997; Jansson et
al 2001), ducks (Trott et al 1997a) and dogs (Trott et al 1997a; Duhamel et al
1998; Fellström et al 2001; Johansson et al 2004; Manabe et al 2004; Munshi
et al 2004). Colonic spirochaetal infections and their association with *B.
pilosicoli* in non-human primates also have been reported (Duhamel et al
1997). In a survey of swine farms where colitis occurred, *B. pilosicoli* was
the most commonly isolated bacterial pathogen from faeces, and was
suggested to be the primary agent of colitis in 18% of outbreaks and as part
of a mixed infection in 24% of affected herds (Thomson et al 2001). *B.
pilosicoli* also has been isolated from the faeces of wild Sika deer in Japan
(Shibahara et al 2000) and from cases of chronic diarrhoea in foals
(Hampson et al 2006a).
1.3.3.2 Distribution of *B. pilosicoli* in humans

A high prevalence (~100%) of spirochaetes in the faeces of humans from developing countries such as Africa was reported at the beginning of the 20th century (Macfie 1917), but the species of the organisms that were involved was uncertain. Much later, examination of biopsy samples identified uncharacterized spirochaetes attached by one cell end to human colonic epithelium, to form a dense "false brush border" (Harland and Lee 1967: Figure 1.1).

Figure 1.1. End-on attachment of *B. pilosicoli* to underlying enterocytes to form false brush borders (arrows) in a chicken (panel A, from Trott et al 1995) and a human (panel B, from Marthinsen et al 2002).

This form of colonization in humans, known as “human intestinal spirochaetosis” (HIS), has been associated with various clinical disorders,
such as long-standing diarrhoea and rectal bleeding (Gad et al 1977; Crucioli and Busuttil 1981; Douglas and Crucioli 1981). Studies on the prevalence of human intestinal spirochaetosis have been reviewed by Kroner and Gebbers (2003). A high incidence (11-100%) of HIS has been reported from developing countries (Delladestima et al 1987; Mathan and Mathan 1985; Barrett 1990), whereas individuals in communities with high living standards in developed countries generally are at low risk of developing HIS. Infection with intestinal spirochaetes can occur in individuals of all ages, including children. For example, pediatric patients from southern Sweden with different gastrointestinal diseases were found to be histologically positive for intestinal spirochaetosis (Marthinsen et al 2002), and in another study from the USA HIS due to *B. aalborgi* and/or *B. pilosicoli* was reported in four children with clinical illness involving their large intestines (Koteish et al 2003).

Among groups of Indonesians living in Bali the prevalence of *B. pilosicoli* in faeces from unselected individuals ranged from 3 to 23.4% (Margawani et al 2004). In a survey in a North Eastern State of India more than 25% of the human population was found to be colonized with *B. pilosicoli* (Munshi et al 2004). The specific prevalence was associated with a number of factors including geographical location, demographic loads, water supply, eating habits and consistency of faeces. Other workers have reported a strong association of *B. pilosicoli* with clinical cases of human diarrhoea from various parts of the world. In Australia, histological evidence of HIS was found in 22 of 41 (53.7%) rectal biopsy specimens from homosexual men.
attending a sexually transmitted diseases clinic. *Serpulina* (now *Brachyspira*) *pilosicoli* was cultured from 11 of the positive biopsy specimens (50%) and from two specimens (10.5%) in which spirochaetes were not observed (Trivett-Moore et al 1998). The prevalence of HIS was determined histologically in 402 subjects from South Norway (Kristiansand) who had undergone colorectal resections during the 5-year period 1991-1996. The prevalence was 2.5% in mid-Norway and 3.0% in South Norway, and in both regions the majority of the cases were males (70% - 75%) (Lindboe 2001). Spirochaetes have also been associated with appendicitis in adult humans with or without HIV infection in the USA (Yang and Lapham 1997). Overall, *B. aalborgi* has been more commonly associated with HIS in biopsy samples from human populations from Western countries than has *B. pilosicoli* - with a prevalence rate of 56.4 to 86% (Mikosza et al 1999; Mikosza et al 2001; Kroner and Gebbers 2003). On the other hand, colonization of humans by *B. pilosicoli* occurs commonly in developing countries.

**1.3.3.3 Predisposing factors for *B. pilosicoli* infections**

There have been relatively few epidemiological surveys conducted on *B. pilosicoli*. In an important early study conducted in villages in the Highlands of Papua New Guinea, around one third of individuals were found to be colonized with *B. pilosicoli* at any one sampling time, and it was calculated that 93.6% of the population could expect to be colonized within a 12-month period (Trott et al 1997a). Other host related factors such as age and gender did not have any significant influence on colonization rates. In a more recent study conducted in Bali, Indonesia, the prevalence of *B. pilosicoli* was higher
in a peri-urban area than in an urban area and in rural areas, and drinking water from a well was a risk factor for colonization compared to using tap water (Margawani et al 2004). In Thailand, eating lizards, snakes and frogs was associated with *B. aalborgi* colonization (Munshi et al 2005). In Australia, the prevalence of *B. pilosicoli* was 9.9% in rural Australian Aboriginals and 0% in 142 non-Aboriginals Australian patients with various bowel disorders. Furthermore, in a random survey of healthy migrants to Australia from developing countries, around 10% had *B. pilosicoli* in their faeces (Brooke et al 2001).

Sexual orientation has been recognized as a major predisposing factor for intestinal spirochaetosis among males, with male homosexuality being strongly associated with the condition (Tompkins et al 1986; Korner and Gebbers, 2003; Cizek and Lobova 2004). For example, in one study 30% of homosexual men with different bowel associated problems were positive for intestinal spirochaetes in rectal biopsies (Surawicz, et al 1987). In a study at a Sexual Health Center in a Sydney Hospital, 39% of homosexual men who were sampled were found to be positive for spirochaetes in their rectal biopsies (Law et al 1994), and oral-anal contact was associated with its high prevalence. Subsequently these spirochaetes were shown mainly to be *B. pilosicoli* (Trivett-Moore et al 1998).

The combination of homosexuality and HIV infection has been shown to further increase the risks of clinical intestinal spirochaetosis in citizens of Western countries like the USA (Ruane et al 1989; Guccion et al 1995),
Germany (Kasbohrer et al 1990), Australia (Law et al 1994; Mikosza and Hampson 2001), France (Lafeuillade et al 1990) and other countries (Nathwani et al 1990; Dauzan et al 1990).

1.3.3.4 Molecular and cellular basis of B. pilosicoli virulence

A characteristic feature of colonization with B. pilosicoli is the intimate end-on attachment of spirochaete cells to the luminal surface of colonic and rectal epithelial cells (Hampson et al 2006). As previously mentioned, this description was first made in biopsy samples from humans where the associated dense layer of attached spirochaetes was described as a “false brush border” (Harland and Lee 1967). Subsequently, a similar condition was described in pigs (Taylor et al 1980), and eventually it was shown that strains of the same spirochaete species (now called B. pilosicoli) could cause the condition in both humans and pigs (Lee et al 1993a; Lee and Hampson 1994).

Very little is known about virulence determinants in B. pilosicoli. A DNA-DNA hybridization study demonstrated that the spirochaetes appeared to lack the attachment and invasion determinants encoded by the inv, ail and yadA genes of Yersinia enterocolitica, the eae gene from enteropathogenic Escherichia coli, and a virulence plasmid of Shigella flexneri (Hartland et al 1998). Progress in this area has been hampered by a lack of genomic information for this spirochaete, an absence of means for genetic manipulation, and a lack of in vitro models in which to study the pathogenesis of infection.
Pigs, chickens and mice have been used experimentally as models of *B. pilosicoli* infection, using spirochaete strains isolated from various species, including humans (Trott et al 1995, Muniappa et al 1996, Trott et al 1996a, Sacco et al 1997, Thomson et al 1997, Jensen et al 2000). In these models, as in the natural infections, one cell end of the spirochaetes often can be seen invaginating into the mature columnar cells without penetrating the host cell membrane.

Detailed knowledge about the mechanisms of *B. pilosicoli* colonization is not available. As previously stated, the attachment of the spirochaetes by one end to the colonic epithelium is a pathognomonic histological finding in pigs (Taylor et al 1980; Andrew and Hoffman 1982; Spearman et al 1988; Giard et al 1995), humans (Trivett-Moore et al 1998), non-human primates (Duhamel et al. 2003), dogs (Duhamel et al 1996), and domestic chickens (Trampel et al 1994, Stephens and Hampson 2001). In pigs naturally infected with *B. pilosicoli*, histological examinations have further revealed catarrhal, multifocal erosive or ulcerative typhlocolitis, with infected parts of the intestines showing oedema of the mucosa and mild inflammation of the lamina propria with infiltration of mononuclear and polymorphonuclear cells (Andrews and Hoffman 1982). Examination of infected intestinal tissues by electron microscopy has revealed polar attachment of large numbers of spirochaetes to the apical portions of columnar epithelial cells (Jacques et al 1989; Duhamel et al 1997). Trivett-Moore et al (1998) observed a 20 μm thick layer of spirochaetes attached to the mucosal layer of the rectal
epithelial cells of human patients. The spirochaetes were situated between and parallel to the microvilli, with little loss of the microvilli in the area. A depression or pocket was seen at the point of attachment between the ends of the spirochaetes and the mucosal cell membrane; this formed a small electron lucent pit, and some exhibited an electron dense cap in this area. In a few rare cases the spirochaetes have been seen penetrating the intestinal mucosal layer (Korner and Gebbers 2003). Scanning electron microscopy (SEM) examination of colonic tissues from experimentally infected pigs revealed focal erosions and other epithelial lesions with hyperaemia and degenerated and necrotic epithelial cells with sparse and disrupted microvilli, which were bulging from the surrounding epithelium (Jensen et al 2000). Numerous spirochaetes were seen in close contact to necrotic epithelial cells and were seen invading the intercellular space between necrotic and degenerated cells. In erosions with complete loss of epithelial cells between the crypt openings, the spirochaetes formed a mat of randomly oriented cells on the denuded basement membrane. However, this study could not verify the end-on attachment of *B. pilosicoli* to the colon enterocytes in spite of varying degrees of colonization.

In an experimental study, inoculation of chickens with human and canine isolates of *B. pilosicoli* caused microvilli effacement, disruption of terminal web microfilaments and cap like elevations at the point of attachment to the caecal epithelium (Muniappa et al 1996). In an experimental pig infection trial, *B. pilosicoli* (strain P43/6/78) crossed the epithelial cell layer into the lamina propria of the caecum and colon (Fossi et al 2005). Invasion on
occasion may be more extensive, as, in humans, there have been a number of reports of $B. \text{pilosicoli}$ being found in the bloodstream of critically-ill individuals who had underlying immunosuppressive disorders (Fournié-amazouz et al 1995; Trott et al 1997b; Kanavaki et al 2002; Bait-Merabet et al 2008; Zeeshan et al 2009). To date a similar invasive potential has not been reported in animals.

1.4 *In vitro* attachments assays

The development of *in vitro* attachment assays forms an important part of the work described in this thesis, and consequently this topic is briefly covered here.

1.4.1 Red blood cells

The investigation of bacterial adhesion began a century ago with studies of interactions between bacteria and mammalian erythrocytes, in a process originally termed hemagglutination (Guyot 1908). Thereafter, erythrocytes remained an easily-available commodity with which to study *in vitro* adhesions for a range of bacteria including *E. coli* (Salit and Gotschlich 1977; Salminen et al 2007), *Mycoplasma gallisepticum* (Razin et al 1980; Vogl et al 2008), *Ureaplasma urealyticum* (Saada et al 1991), *Streptococcus pneumoniae* (Hament et al 2003), *Staphylococcus aureus* (Shin et al 2005), and many others (Nelson 1953). However, to date, there have been no reports on the interactions of intestinal spirochaetes with erythrocytes. According to the available literature, lectins present on the outer membrane of many
bacteria are responsible for their haemagglutination properties, and these vary with cell types (Ofek et al. 2003).

In the current work it was considered useful to examine interactions between red blood cells from different species and different *B. pilosicoli* strains as an easy starting point to investigate adhesion with this spirochaete. Red blood cells were also of interest as there have been a number of reports of *B. pilosicoli* being found in the bloodstream of critically-ill humans who have underlying immunosuppressive disorders (Fournié-amazouz *et al* 1995; Trott *et al* 1997b; Kanavaki *et al* 2002; Bait-Merabet *et al* 2008; Zeeshan *et al* 2009). *Borrelia* and *Leptospira* also have well-defined spirochaetaemic phases, and *B. burgdorferi* has been reported to adhere to erythrocytes *in vitro*. Hence it was of interest to know whether or not *B. pilosicoli* attaches to red blood cells.

1.4.2 Attachment of intestinal spirochaetes to cell lines

Interaction between *Treponema* (Brachyspira) *hyodysenteriae* and isolated swine intestinal epithelial cells or mouse adrenal cells in culture have been examined (Knoop *et al* 1979). The extent of attachment was dependent on both incubation time and temperature. The interaction of spirochaetes did not alter the cellular morphology or the surface of the intestinal epithelial cells at the site of attachment. This publication did not present good images of attachment, except for 1 or 2 bacteria that were seen on intestinal cells by SEM. Another study characterized the attachment of *B. hyodysenteriae* to Henlé intestinal epithelial (HIE 407) cells (Bowden *et al* 1989). The
frequency of attachment depended on the motility and viability of the spirochaetes. Attachment was inhibited by pig and rabbit hyper-immune sera. The heavy sugars N-acetylneuraminic acid, D-glucuronic acid, and fetuin also inhibited attachment, however these findings were not verified microscopically. Furthermore, the relevance of this attachment remains uncertain, as \textit{B. hyodysenteriae} does not naturally attach to enterocytes in infected pigs.

To date there has only been one report of the use of an \textit{in vitro} attachment assay for \textit{B. pilosicoli} - using monolayers of intestinal cells to simulate the colonic environment (Muniappa et al 1998). In that study the attachment of four \textit{B. pilosicoli} isolates, one from a human and three from Rhesus monkeys, was greatest with human embryonic intestinal epithelial H407 cells, less with Caco-2 and HEp-2 cells, minimal with FaDu and HeLa cells, and absent with HT29 cells. The attachment was described as diffuse, non-polar, time-dependent, and did not involve actin rearrangements. This study did not demonstrated typical end on attachment of \textit{B. pilosicoli} to any of the cell lines. Other cellular responses, such as apoptosis, junctional damage and cytokine induction, were not investigated (Muniappa et al 1998).

1.4.3 \textit{In vitro} attachment assays for other pathogenic bacteria using Caco-2 cells

As the human colonic carcinoma cell line Caco-2 was used in the current study, this section provides some background to these cells and their application. The various epithelial properties of the cells have been described
(Grasset et al. 1984; Grasset et al. 1985; Ramond et al. 1985). Rousset (1986) studied 20 human colon carcinoma cell lines and proposed the use of the cell lines HT-29 and Caco-2 as *in vitro* models for the study of intestinal differentiation. In particular, the cell lines were studied for their ability to develop some of the characteristics of the normal intestinal epithelium, for example epithelial polarity, presence of the actin-binding protein villin, or the occurrence of an enterocytic differentiation either when cultured under standard conditions, as for Caco-2 cells, or when grown in a glucose-free medium, as for HT-29 cells. This survey showed that the Caco2 cell line most closely resembled the normal intestinal epithelium of the colon. The cells spontaneously underwent an enterocytic differentiation characterized by a polarization of the cell layer with the formation of domes and the presence of an apical brush border membrane which was endowed with hydrolases such as sucrase-isomaltase, lactase, amino-peptidase N, dipeptidylpeptidase IV and alkaline phosphatase (Chantret et al. 1988).

Thereafter Caco2 cell monolayers have been widely used as an *in vitro* attachment model for a number of different bacterial species. French researchers were the first to exploit Caco-2 cells as an *in vitro* attachment model for *Listeria monocytogenes* (Gaillard et al. 1987). Subsequently, enterotoxigenic *Escherichia coli* (ETEC) strains possessing colonization factor antigen I (CFA/I), CFA/II, CFA/III, and antigen 2230 were tested for their ability to adhere to a range of cell lines including HeLa, HEP-2, HRT 18, Hutu 80, MDBK, MDCK, Vero, and Caco-2, but they only adhered to the Caco-2 cell line (Darfeuille-Michaud et al. 1990).
In an important early study, Finlay and Falkow (1990) infected polarized Caco-2 cell monolayers with either *Salmonella Choleraesuis* or *Salmonella Typhimurium*. Both serovars penetrated the monolayer, and appeared in the basolateral medium after two hours. Both serovars caused a loss in transepithelial resistance by 3-4 hours, and the monolayer's integrity was completely disrupted by 6 hours. Scanning and transmission electron microscopy revealed that the bacteria interacted with well-defined apical microvilli and caused disruptions in the brush border, including elongation and denuding of the microvilli. The cytoplasm was also disrupted locally, with blebs protruding from the apical surface. The bacteria entered (invaded) these cells and were enclosed in membrane-bound vacuoles within the cytoplasm. These morphological observations correlated well with animal infection models, indicating that this *in vitro* system was useful for studying pathogens that interact with human intestinal epithelia. Subsequently, many such studies have been conducted with other bacterial species. For example, differentiated Caco-2 cells were used as a model for enteric invasion studies by *Campylobacter jejuni* and *C. coli* (Everest et al 1992) and *Shigella flexneri* (Mounier et al 1992). Invasion by the latter occurred through the basolateral pole of the cells. The few bacteria that interacted with the apical surface either bound to microvilli of the cell dome without causing detectable alterations, or bound at the level of intercellular junctions at which they demonstrated a limited capacity for paracellular invasion, which permitted subsequent entry through the lateral domain of the cells. *Salmonella Typhimurium* grown under oxygen-limiting conditions was found to enter
Caco-2 cells and elicit actin filament rearrangement and effect morphological changes in the cells (Francis et al 1992). Invasion of a confluent Caco-2 cell monolayer by *Yersinia* and *Salmonella* took place within 4 hours of contact, which was in marked contrast to *E. coli* which did not invade (Curfs et al 1995). Many such studies have since been undertaken, with each study providing specific information about the mechanisms used by enteropathogenic bacterial species to interact with the intestinal epithelium. Consequently, Caco-2 cells were selected for use in the current study.

1.5 Cellular changes caused by other bacteria

The next section of the review gives background information relating to some of the cellular features and cellular changes that were studied as part of the work described in the body of the thesis, including a brief introduction to cytokines and the effects of norepinephrine on bacteria.

1.5.1 Zonula Occludens (ZO)

Tight junctions are the closely associated areas of two cells whose membranes join together forming a virtually impermeable barrier to fluid. These intercellular junctions occur adjacent to the apical end of the lateral membrane surface. When the tight junction completely circumscribes the apex of the epithelial cell as a continuous structure, it is known as the zonula occludens (Gumbiner 1986). Tight junctions have a barrier (or gate) function and a fence function. The barrier function regulates the passage of ions, water, and various macromolecules through paracellular spaces, and is thus relevant to the occurrence of oedema and diarrhoea, amongst other things.
On the other hand, the fence function maintains cell polarity, preventing intermixing of molecules in the apical membrane with those in the lateral membrane (Sawada et al 2003). The tight junction is one of the major structures of absorptive and secretory epithelia: it contributes to a trans-epithelial permeability barrier by controlling the diffusion of ions and neutral molecules, thus working as a gate with variable permeability properties. Zonula occudens-1 (ZO-1) is a molecular component associated with tight junctions. It is a 220-kDa peripheral membrane protein, located at the cytoplasmic surface of the zonula occludens (Stevenson et al 1986). ZO-1 binds with junctional proteins (ZO2, occludin) and the cytoskeleton protein spectrin (Anderson and Van Itallie 1995). The occurrence of ZO-1 specific mRNA and protein expression during tight junction assembly in Caco-2 cells was first reported in the late nineteen eighties (Anderson et al 1989).

Bacterial attachment or invasion of cells can induce variable degrees of disruptions to ZO-1. For example, short-term infection of MDCK II monolayers with *Salmonella* Typhimurium SL1344 damaged ZO-1 integrity (Jepson et al 1995). In another study exposure of the human gut monolayer T84 with the vacuolating toxin of *Helicobactor pylori* relocalized several junctional proteins, including ZO-1 (Papini et al 1998). Similarly, the toxins released by *Clostridium difficile* dissociated ZO-1 and ZO-2 in the epithelial cell line T84 (Nusrat et al 2001). Hence, in the current study it was considered useful to monitor ZO-1 in the infected monolayers to determine whether the cell junctions were intact.
1.5.2 Actin cytoskeleton

The cellular cytoskeleton protein actin has been extensively investigated with regard to its role in bacterial pathogenesis. Eukaryotic cells possess three kinds of cytoskeletal elements, these being 5-9 nm diameter actin filaments, 24 nm diameter microtubules and 10 nm diameter intermediate filaments. The actin cytoskeleton is the primary determinant of eukaryotic cell shape, and provides the machinery of whole-cell movement. Epithelial cells use actin structures to maintain strong adhesive connections with each other and with their underlying extracellular matrix. There are two known forms of actin, G and F: monomeric globular actin (G – actin, 43 kDa) polymerizes through reversible non-covalent interactions into a 2-stranded helical polymer (F-actin). Several important functions of epithelial cells such as cell motility, morphogenesis or transport of organelles from one part of the cell to another depend on coordinated interactions between actin and membrane compartments.

The epithelial lining of the intestine is vulnerable to the action of bacteria within the lumen. These epithelial cells are capable of absorbing small protein particles, but cannot phagocytose bacterial cells. Therefore, in order to be taken up by intestinal epithelial cells, bacterial pathogens need to be able to induce their own phagocytosis. So far, two mechanisms by which pathogenic bacteria induce phagocytosis have been recognized: these are known as the zipper and trigger mechanisms. Both mechanisms occur due to actin polymerization and reorganization of actin filaments at the plasma
membrane induced by bacterial interactions with these cells (Cossart et al 2005). In general terms there are two forms of bacterial pathogenesis: extracellular bacteria adhere tightly to host cells to mediate their effects extracellularly, while intracellular bacterial pathogens find ways to invade and survive intracellularly. In both cases the bacteria exploit the host cell’s cytoskeleton. Details of cytoskeleton manipulations by *E. coli*, *Salmonella Typhimurium*, and *Shigella flexineri* have been reviewed (Goosney et al 1999).

To induce cytoskeletal changes, pathogenic microbes must ensure delivery of effector molecules onto or into host cells. Effectors are usually proteins that interface with and influence host-cell pathways, and can facilitate disease. Bacteria use several methods to deliver effector proteins to the host cell. Some effectors, such as toxins, are secreted by bacteria in the vicinity of the host cell, where they bind to specific receptors and are taken up by endocytosis. Other effector proteins can facilitate their own uptake with pore-forming subunits or autotransporter domains. Some Gram-negative pathogenic bacteria have acquired sophisticated ‘molecular syringes’, such as type III or type IV secretion systems, which are multi-subunit molecular machines that span the bacterial and host membranes and translocate effectors directly into host cells (Gruenheid and Finlay 2003; Nakano et al 2007; Bhavsar et al 2007). To date it is not known whether *B. pilosicoli* has similar methods to interact with enterocytes in the colon, and indeed whether the spirochaete can influence the actin cytoskeleton of the cells to which it attaches.
1.5.3 Apoptosis

Apoptosis and necrosis are two distinct mechanisms of cell death. In the case of apoptosis the cell’s own molecules are ultimately responsible for its death, whilst in the case of necrosis the molecules released by other cells damage the host cell. In a healthy tissue or organ both development and homeostasis are achieved through a balance between cell death and cell growth. Apoptosis or programmed cell death in multicellular organisms is defensive and can be activated when it is desirable for the well-being of the whole tissue or organism. Changes to cellular morphology and DNA fragmentation are the basic criteria used to distinguish apoptosis from necrosis. Morphological changes such as cell shrinkage with loss of cell-to-cell contacts, blebbing at the cell surface and intense cytoplasmic vacuolization, conservation of organelle structure, condensation of the chromatin (often at the perinuclear region) and loss of normal nuclear architecture are the features of apoptosis. In the case of necrosis the organelles are critically damaged, the plasma membrane is ruptured, the cytoplasmic elements are dispersed into the extracellular space and the shape of the nucleus is normally conserved, although flocculation of chromatin may be detected. Apoptotic cells break up their DNA into multimers of approximately 200 base pairs (Cossart et al 2005).

Examples of bacterial pathogens that induce apoptosis include \textit{L. monocytogenes} (Valenti et al 1999), uropathogenic \textit{E. coli} (Klumpp et al 2001) and enterohaemorrhagic \textit{E. coli} (Barnett et al 2000). To date no
observations have been made on the capacity of intestinal spirochaetes to induce apoptosis. However, the non-intestinal spirochaetes, *Leptospira interrogans* (Jin et al 2009), *Borrelia burgdorferi* (Ramesh et al 2003; Ramesh et al 2008) and *Treponema denticola* (Leung et al 2002) all have induced apoptosis in different types of eukaryotic cells. Hence it was of interest to determine whether *B. pilosicoli* could induce apoptosis in a colonized cell line.

1.5.4 Cytokines

Cytokines are small to medium size proteins (8-80 kDa) and glycoproteins that mediate very potent biological activities and affect many cell types. They generally act over very short distances and usually on the cells that produced them (autocrine) or cells close by them (paracrine), rather than acting on distant cells (endocrine). Cytokines are extremely potent and are capable of inducing biological changes at picomolar and sometimes even femtomolar levels. They are known to have critical roles in haematopoiesis, inflammatory responses and the development and maintenance of immune responses. A particular characteristic of cytokines is that they act in networks or cascades. Typical properties of cytokines in these networks are pleiotropy, redundancy, synergistic activity and antagonistic effects upon each other (Townsend and McKenzie 2000; Roitt et al 2001). Most cytokines target multiple cells at a time and may induce different responses in each cell; this is called pleiotropy. There are possibilities that many different cytokines may act on a single cellular target, which is called redundancy. Another quality of cytokines is that some work best in association with other cytokines in a
process called synergy (Tizard 2000). The role of cytokines has been studied
in the pathogenesis of many bacteria infections, including *E. coli* (Svanborg et al 1996; Zhou et al 2003), *Chlamydia pneumoniae* (Kaukoranta-Tolvanen et al 1996), *Salmonella* Dublin (Elhufy and Bost 1999), *Streptococcus pneumoniae* (Prinz et al 1999) and *Helicobacter pylori* (Yamaguchi et al 1999). There are numerous different cytokines (>30) (Borish and Steinke 2003), and a brief description of the functions of the nine that were studied in the current work is presented in Table 1.2.

The non-intestinal spirochaetes are known to induce interleukin production in different tissues and cells. For example, *Treponema pallidum* induced IL2 in syphilis patients (Podwińska et al 1995), *Treponema pectinovorum* induced IL-1alpha, IL-1beta, IL-6, IL-8, IL-10, and *Treponema denticola* induced IL-6 and IL-8 in fibroblast cells (Nixon et al 2000). In the case of intestinal spirochaetes, a butanol/water extract of *B. hyodysenteriae* has been reported to induce IL-1 and TNF production in murine peritoneal exudate cells (Greer and Wannemuehler 1989; Nibbelink et al 1997), but to date no work has been done with *B. pilosicoli*.

Caco-2 cells have been widely used to evaluate cytokine induction by different pathogenic bacteria, including *Salmonella* Dublin, *Shigella dysenteriae, Yersinia enterocolitica, L. monocytogenes* and enteroinvasive *E. coli* (Michalsky et al 1997; Steiner et al 1998). Hence these cells were used in the current study to evaluate how *B. pilosicoli* might mediate cytokine changes in the intestinal tract.
Table 1.2. Names and activities of the nine cytokines investigated in the current thesis.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Cell source</th>
<th>Target cells and Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Mononuclear phagocytic cells, Endothelial cell, Keratinocytes, Synovial cells, Osteoblasts, Neutrophils Others</td>
<td>Targets pancreatic cells, dendritic cells APC, Activates T lymphocytes by enhancing the production of IL-2, Augments B cell proliferation and increases immunoglobulin synthesis, Produces fever, anorexia and lethargy, Induces acute inflammation and delayed type hypersensitivity</td>
</tr>
<tr>
<td>IL-2</td>
<td>Th1 cells, Granular lymphocytes and others</td>
<td>Induces T Lymphocytes – (CD4 and CD8 differentiations), Interferon production B Lymphocytes - growth and polyclonal antibody production, Production of TNF Macrophages activation, NK cell growth and cytotoxicity, Production of IL-1, TNF and CSF</td>
</tr>
<tr>
<td>IL-4</td>
<td>TH2 CD4+ T cells, Mast cells and others</td>
<td>T and B cells, Macrophages activation and increased class II MHC, Co-stimulant with IL-3 for BM stem cells and mast cells.</td>
</tr>
<tr>
<td>IL-5</td>
<td>TH2 cells and others</td>
<td>T lymphocytes, B lymphocytes, Eosinophil growth and chemotactic for eosinphils (eosinophilia)</td>
</tr>
<tr>
<td>IL-6</td>
<td>Fibroblast, Macrophages, B lymphocytes, TH2 cells, Endothelial cells, Keratinocytes and others</td>
<td>B cell differentiation to plasma cells and polyclonal and antibody production. Production of acute phase proteins by hepatocytes. Tumor cell cytostasis</td>
</tr>
<tr>
<td>IL-8</td>
<td>Monocytes Fibroblasts Endothelial cells Keratinocytes Neutrophils Joint fluid Alveolar fluid Psoriatic tissue and others</td>
<td>Chemoattracts neutrophils, T cells and basophils. Augments keratinocyte proliferation. Activation of neutrophil degranulation, enzyme release and respiratory burst. Modulation of neutrophil adhesion and local infiltration.</td>
</tr>
<tr>
<td>IL-10</td>
<td>TH2 cells and others</td>
<td>Inhibit TH1 cell production of IL-2, and IFNγ Promote B cell growth and antibody production</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>B cells and others</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>Th1 cells (CD4+ and CD8+), NK cells, Cytotoxic T cells and granular lymphocytes and others</td>
<td>Suppression of IL-4 and IL-10 producing cells. Monocytes stimulation for antigen presentation, adherence, phagocytosis, secretion and respiratory burst.</td>
</tr>
<tr>
<td>Tumor Necrosis Factor-α (TNF-α)</td>
<td>Macrophages, T and B lymphocytes, neutrophils, fibroblast, smooth muscles, astrocytes and others</td>
<td>Tumour cell cytotoxicity (antitumor immunity). Polymorphnuclear cell activation. Induction adhesion molecules to endothelial cells Neutrophils activation Induction of acute inflammation</td>
</tr>
</tbody>
</table>

(Source: Oppenheim and Saklatvala 1993; Borish and Steinke 2003)

### 1.6 Mucus, motility and chemotaxis

The intestinal tract has numerous adaptations to allow it to maintain its internal environment. One of the properties of the surface layer is the continuous secretion of mucus from goblet cells that reside throughout the length of the tract. Goblet cells are responsible for the production and maintenance of a mucus blanket that covers the intestinal mucosa. The thickness of the mucus layer varies from region to region in the intestine; for example, the mucus layer is thicker in the caecum and colon than in the duodenum. Mucus is composed of high molecular weight glycoproteins known as mucins, which are filamentous proteins comprising complex O-linked glycosylated amino acids such as proline, threonine and serine. The major function of mucins in the gut is to form a gel-like mucus layer over the intestinal mucosa. Other than mucins, mucus contains a range of proteins, carbohydrates, lipids, nucleic acids and several other chemical constituents
that originated from other sources (Specian and Oliver 1991; Ofek et al. 2003; Laux et al. 2005). N-Acetylglucosamine, N-Acetylgalactosamine, fucose and galactose are the primary mucin oligosaccharides (Deplancke and Gaskins 2001). The mucus layer remains unstirred adjacent to the epithelial surface despite the tremendous shearing actions due to intestinal peristalsis (Cone 2009). The mucus layer works as a medium for protection, lubrication and transport between the intestinal contents and the epithelial surface (Deplancke and Gaskins 2001). Bacterial insults may result in enhanced mucin secretions by goblet cells (Dohrman et al. 1998; Mack et al. 1999; Belley et al. 1999).

Once bacteria enter the intestinal tract, they face a hostile environment around the surface of epithelial cells. Pathogenic bacteria need to penetrate the physical mucus barrier to establish direct contact with epithelial cells to allow their attachment, adhesion and invasion. Goblet cells maintain a continuous outward flow of mucin secretion, and bacteria need to move in the opposite direction to the flow to reach the epithelial surface. Bacterial movement is governed by the innate ability of the bacteria to move and the nature of the mucus present in the tract. For successful colonization bacteria such as *B. pilosicoli* must cross the physical mucus barrier. `On the one hand, the mucus layer acts as a physical barrier, and its components such as antibodies and complex sugars can protect from epithelial colonization, while on the other hand the bacteria can use chemical components of mucin as nutrients by releasing different degrading enzymes (Miller and Hoskins 1981; Hoskins et al. 1985). The bacteria can degrade mucins by synthesizing
and secreting hydrolytic enzymes such as glycosidases, proteases, peptidases, and sulfataseproteases (Macfarlane et al 2005; Tu et al 2008; Denève et al 2009). Not all bacteria can use the mucus molecules as a source of carbon, nitrogen and energy during and after invasion of the mucus layer (Cohen et al 1983; Macfarlane and Gibson 1991; Macfarlane et al 2001).

Theodor Engelmann and Wilhelm Pfeffer discovered that bacterial movement was not random and arbitrary, but was directed towards certain stimuli and away from others – a behaviour that they termed ‘chemotaxis’ (Engelmann 1883; Pfeffer 1884). In bacteria there are two groups of chemotaxis molecules, the surface methyl-accepting chemotaxis proteins that respond to chemical stimulation in the environment, and the chemosensory transducers that are linked to flagella motion, and hence bacterial movement (Stecher et al 2004; Butler and Camilli 2005; Wang et al 2005; Mariconda et al 2006). Assays of chemotaxis have been correlated with virulence potential of bacterial species such as E. coli and Salmonella Typhimurium, as well as spirochaete species (Alder 1966; Laux et al 2000; Charon and Goldstein 2002; Boin et al 2004; Lux and Shi 2004; Stecher et al 2008). Spirochaete cells have a corkscrew-like motility, making them particularly capable of advancing through highly viscous substances such as mucus (Li et al 2000). Furthermore, the non-intestinal spirochaetes are known to move more rapidly in viscous than in non-viscous environments (Kaiser and Doetsch 1975; Kimsey and Spielman 1990; Goldstein et al 1994; Ruby and Charon 1998), whereas externally flagellated bacteria cannot respond in the same way (Shoesmith 1960; Schneider and Doetsch 1974; Strength et al 1976).
Limited work has been done on motility and chemotaxis in intestinal spirochaetes. Virulent strains of *B. hyodysenteriae* have been reported to be chemotactic to mucin, the major component of the mucus gel, as well as to components of mucin such as L-serine (Kennedy and Yancey, 1996), whereas avirulent strains were found to have reduced chemotaxis to mucin compared to virulent strains (Milner and Sellwood, 1994). In contrast, and unexpectedly, in the latter study analysis of five porcine *B. pilosicoli* strains showed that these were not attracted to mucin under the same test conditions as *B. hyodysenteriae* (Milner and Sellwood 1994), despite *B. pilosicoli* clearly needing to be able to penetrate the mucous layers to access attachment sites. More recently, Witters & Duhamel (1999) investigated three *B. pilosicoli* strains, and found that one from a human being and one from a dog were slightly attracted to 1 % porcine gastric mucin, whilst a third porcine strain that had also been examined by Milner and Sellwood (1994) was not attracted to mucin, but it was mildly attracted to 10 mM D-L serine. Clearly additional work is needed to clarify the ability of *B. pilosicoli* strains to interact with mucin.

### 1.7 Effects of norepinephrine on bacterial pathogens

The human enteric nervous system contains approximately 100 million neurones of various subtypes, distributed differently depending on the intestinal region (Hansen 2003). Norepinephrine (NE) is a major neurotransmitter in this system. The mesenteric organs are densely innervated and are responsible for production of a large proportion of the
body’s NE. High concentrations of noradrenergic neurons are found throughout the intestine, terminating within the submucosal plexus and intestinal mucosa (Lundgren 2000). The importance of neurostress hormones in the pathogenesis of bacterial, viral and parasitic diseases was realized long ago (Peterson et al 1991), although the mechanism(s) remained unclear. The NE released by these mesenteric neurons escapes breakdown at the release site and spreads over into the mesenteric tissue circulation (Aneman et al 1996).

In the last decade a number of interesting investigations have been made into the effects of neurotransmitters on different virulence traits of pathogenic bacteria. Like norepinephrine, epinephrine and dopamine exposure has been shown to enhance the growth of $E. \text{coli}$, $Y. \text{enterocolitica}$ and $Pseudomonas \text{aeruginosa}$ (Lyte and Ernst 1992). Later it was reported that NE supplementation triggers the production of a different growth autoinducer in the case of $E. \text{coli}$ O157:H7 (Lyte et al 1996). Subsequently, a number of reports have been published showing that in vitro NE exposure has enhanced the growth of pathogens such as $Salmonella$ Choleraesuis (Nietfeld et al 1999), $Staphylococcus \text{aureus}$ (Belay and Sonnenfeld 2002), $Actinomyces naeslundii$, $Actinomyces \text{gerenscseriae}$, $Eikenella \text{corrodens}$, $Campylobacter \text{gracilis}$ and $Bacteroides \text{forsythus}$ (Roberts et al 2002), $Staphylococcus \text{epidermidis}$ (Lyte et al 2003), and $Klebsiella \text{pneumoniae}$, $Pseudomonas \text{aeruginosa}$, $Enterobacter \text{cloacae}$, $Shigella \text{sonnei}$, and $Staphylococcus \text{aureus}$ (O'Donnell et al 2006). On the other hand, there have been contradictory reports, where NE did not enhance the growth of
*Porphyromonas gingivalis, Bacteroides fragilis, Shigella boydii, Shigella sonnie, Enterobacter sp., and Salmonella Choleraesuis* (Belay et al. 2003). It also has been shown that norepinephrine augments *E. coli* O157:H7 induced enteritis and adherence in *in vivo* situations (Vlisidou et al. 2004).

*B. pilosicoli* is a fastidious and opportunistic pathogen, and colonizes the large intestines of susceptible hosts. Like other gut pathogens *B. pilosicoli* can also be exposed to NE in the large intestine during its colonization process. The potential impact of NE exposure on *B. pilosicoli* and other intestinal spirochaetes is currently unknown. Consequently, in the current study attempts were made to study the effects of NE exposure on the growth, chemotaxis and attachment properties of *B. pilosicoli*.

### 1.8 Aims and objectives

From the above literature review, it is evident that the mechanisms used by *B. pilosicoli* in moving through the mucus layer, attaching to enterocytes and inducing local cellular damage are poorly understood. The lack of *in vitro* models to study these events has been a major constraint to understanding the pathogenesis of *B. pilosicoli* infections. Furthermore, the effects of NE exposure on *B. pilosicoli* pathogenesis are not understood.

In view of these circumstances, the aim of the work described in this thesis was to develop *in vitro* models to study *B. pilosicoli* pathogenesis. Specific objectives were:
1. To develop an in vitro attachment model for B. pilosicoli by using either cells in suspension (red blood cells) or cell monolayers (Caco-2 cells).

2. To investigate the in vitro attraction of B. pilosicoli to mucin.

3. To examine the effects of norepinephrine exposure on the in vitro activity of B. pilosicoli.
CHAPTER 2: GENERAL MATERIALS AND METHODS

Details of the equipment used and the suppliers of the chemicals and reagents used in the work described in the thesis are listed in Appendix A.

2.1 *Brachyspira pilosicoli* and *Brachyspira hyodysenteriae* strains

The strains of *B. pilosicoli* and *B. hyodysenteriae* that were used in the study were obtained as frozen stock from the culture collection held at the Australian Reference Centre for Intestine Spirochaetes at Murdoch University, Perth, Western Australia. The names of the strains are listed in the appropriate chapters.

2.2 Growth of spirochaetes on solid medium

The strains obtained as frozen stock were grown first on Trypticase Soy agar containing defibrinated sheep blood (5%), colistin (25µg/ml), vancomycin (25 µg/ml) and spectinomycin (400 µg/ml) (Jenkinson and Winger 1981). The plates were placed in an anaerobic jar and an anaerobic environment (<1% oxygen and 9-13% CO₂) was generated inside the container by adding an anaerobic system envelope “AnaeroGen™”. The plates were incubated at 39°C for 3-5 days and were examined for the extent of growth from day 3 onwards.
2.3 Growth of spirochaetes in liquid medium

2.3.1 Preparation of broth medium

Kunkle’s pre-reduced anaerobic broth (Kunkle et al 1986) was used to grow spirochaetes in liquid medium. This broth contained Trypticase Soy broth 30 g/L, yeast extract 10g/L, glucose 5g/L, sodium hydrogen carbonate 3g/L, L-cysteine 1 g/L, cholesterol 20 mg/L, new born calf serum 5 ml/L, foetal calf serum 35 ml/L, and distilled water. Before boiling the pH was adjusted to 6.8 by adding 1.1 ml/L of absolute hydrochloric acid. One ml/L Resazurin (0.1%) was added as an anaerobic indicator. The mixture was heated until the disappearance of the red Resazurin colour. Heated broth was transferred to 20 ml glass test tubes (10 mL volume) and 500 ml glass bottles (200 mL volumes). The tubes and bottles were deoxygenated using a flow of compressed gas mix (H₂ = 4.88%, CO₂ = 10.2% in N₂). The medium in the tubes and bottles was bubbled with the gas mix to remove residual oxygen. The tubes and bottles were sealed with rubber bungs. The medium was autoclaved at 121°C for 15 minutes and was tested for any contamination or effective autoclaving by incubating at 37°C for 24 hours. The medium was stored in the dark at room temperature for up to 14 days.

2.3.2 Transfer of spirochaetes from solid medium to liquid medium

After assuring a good non-contaminated growth of spirochaetes on a plate, a small piece (1cm²) of agar was removed from the plate using a sterile surgical blade. The agar piece was placed inside the shaft of a sterile 5 mL syringe and the syringe piston was placed back into the syringe. One mL of broth culture was sucked into the syringe using a sterile 20-gauge needle.
After thoroughly shaking the syringe, the broth was inoculated into a 10 mL broth tube. Aseptic conditions were maintained all the time by flushing the tops of the stoppers with 70% alcohol. The tubes were placed in a metal rack and were incubated on a rocking platform at 39°C for 2-5 days until the concentration of bacteria reached to $10^8$/mL. Later the bacteria in the broth were transferred by syringe into 200 mL of broth in bottles.

2.4 Counting spirochaetes

As and when required, aliquots of the culture were diluted with phosphate buffered saline (PBS; 3.2 mM Na$_2$HPO$_4$, 0.5 mM KH$_2$PO$_4$, 1.3 mM KCl, 135 mM NaCl, pH 7.4) to count the spirochaetes accurately. Eight to ten µL of liquid culture was added under the coverslip of a Neubauer haemocytometer. Spirochaetes were counted in 48 squares and the number per ml was calculated using the formula:

$$\text{Cells/mL} = \frac{\text{Total number of counted spirochaetes}}{\text{Total number of squares}} \times \text{dilution factor} \times 4,000,000$$
3.1 Introduction

As discussed in the literature review, there is a real need to develop an in vitro attachment model for B. pilosicoli. At the start of the work described in this thesis it was decided to determine whether the spirochaete would attach to red blood cells. Red blood cells are relatively easy to obtain and to handle, and they have been used in attachment models with various species of pathogenic bacteria (see the literature review). It was recognized that the relevance of any such attachment to the in vivo situation would require careful consideration. An interesting consideration was the fact that B. pilosicoli, like other spirochaetes, can have a spirochaetaemic phase in certain immunosuppressed individuals - where the spirochaete would have ample opportunities to interact with erythrocytes.

Accordingly, the purpose of the work described in this part of the thesis was to investigate:

i) Whether strains of B. pilosicoli can attach to erythrocytes,

ii) Whether such attachment resembles the attachment seen with colonic enterocytes, and

iii) Whether there is specificity in such attachment related to the spirochaete strain and/or the host species from which the erythrocytes are derived.
3.2 Materials and Methods

3.2.1 *B. pilosicoli* strains and culture conditions

Australian *B. pilosicoli* strains 95/1000 and Cof-10 isolated from pigs, and WesB and Karlton isolated from humans, were obtained as frozen stocks, and were grown in Kunkle’s broth, as described in Chapter Two. Further details of these strains are given in Chapter Five.

3.2.2 Source of blood and processing

Heparinized pooled blood specimens from healthy sheep, horses, cows, pigs, dogs, chickens, geese and humans were purchased from a commercial source (appendix A). These were held at 4°C and used within 24 hours. The blood was centrifuged at 700 x g for 10 minutes, and the erythrocyte pellet was resuspended in normal saline. This washing process was repeated three times to remove any remaining anticoagulant. The final concentration of erythrocytes was adjusted to 1x10⁷ per mL in normal saline. The erythrocytes were tested for their tolerance to Kunkle’s broth by incubating them at 1x10⁷ per mL in the medium for 12 hours at 38°C.

3.2.3 Attachment assays

The attachment assays were conducted in sterile 48 well tissue culture plates with lids. Six replicates were used for each spirochaete strain at each erythrocyte concentration for each incubation period. Each well contained a 1 mL volume comprising 10⁷ actively motile spirochaetes in mid-log phase in Kunkle’s broth and a serial 10-fold dilution of erythrocytes from 10⁶ to 10⁴.
The plates were incubated on a rocking platform at 38°C for 2, 4, and 6 hour time intervals.

The contents of the wells then were aspirated and an aliquot examined using a phase contrast microscope at 40 X and 100 X magnification. A semi-quantitative scoring system for the extent of attachment at the different time points was used. The operator was blinded to the origin of the samples, and examined 12 fields and scored these using a system where: 0 represented no attachment; 1 represented attachment of 1-2 spirochaetes to < 10% of the erythrocytes; 2 represented attachment of 3-5 spirochaetes to >10 - <20% of erythrocytes, and; 3 represents attachment of >5 spirochaetes to >20% of erythrocytes.

Where attachment of spirochaetes to erythrocytes was seen, the cells were processed for electron microscopy. The erythrocytes were transferred to a 2 mL conical eppendorf tube, allowed to settle at 4°C for 6 hours, and then the fluid was carefully aspirated from the tube without disturbing the pellet. The pellet was resuspended and fixed overnight in 2.5% glutaraldehyde at 4°C. The glutaraldehyde was gently removed without disturbing the erythrocytes at the bottom of tube, and the pellet was washed five times with 0.07 M Sorensen’s buffer (3 parts 0.01 M Na₂HPO₄ and 1 part 0.01M KH₂PO₄). The cells were post fixed in 1% aqueous osmium tetroxide at 4°C for 1 hour, and washed three times with 70% ethanol before being dehydrated through an ethanol series. For scanning electron microscopy (SEM), the cells were resuspended in 100% alcohol and spread on an SEM specimen stub. They
were sputter-coated with gold to a thickness of 90 nm in a Balzers sputter coater, and examined using a Philips XL 20 scanning electron microscope. For transmission electron microscopy (TEM), the dehydrated cells were processed for infiltration with propylene oxide (2 changes over 20 minutes), then with a propylene/resin mix (60/40) for 1 hour at 4°C, and finally with absolute resin on a rotary mixer at 25°C overnight. The cells were embedded with pure resin at 60°C for 24 hours, and 90 nm sections were cut with an ultra-microtome and mounted on carbon-coated grids. The grids were stained with freshly prepared uranyl acetate and lead citrate and were examined using a Philips 1 CM -100 transmission electron microscope.

3.3 Results

Incubation of the erythrocytes in Kunkle’s broth did not result in lysis or any abnormal appearance of the cells. Of the four strains of *B. pilosicoli* tested, only human strain WesB adhered to erythrocytes, and only to those from chickens and geese. By phase contrast microscopy, attachment was first observed after 4 hours at an erythrocyte/spirochaete ratio of 1:1000, where scores per field were 1. At 6 hours the attachment was seen at both 1:100 and 1:1000 ratios, with scores of 2 at 1:100, and 3 at 1:1000. In the latter case large numbers of spirochaetes were seen attached to individual erythrocytes (Figure 3.1), sometimes with groups of erythrocytes forming agglutinated clumps bridged by the spirochaete cells (Figure 3.2). Viewing with the SEM revealed end-on attachment by individual *B. pilosicoli* cells, sometimes with apparent penetration of the erythrocyte membrane (Figure 3.3). Often large numbers of attached spirochaetes also were observed lying on their sides.
over the surface of the cells (Figure 3.4). Occasionally bacterial ends were found lying flat over the RBCs (Figure 3.5).

Figure 3.1: Cells of *B. pilosicoli* WesB (human isolate) attached to an erythrocyte from a goose (original magnification x 100)

Figure 3.2: Cells of *B. pilosicoli* WesB associated with the surface of nucleated goose erythrocytes, agglutinating them. Phase contrast microscopy (original magnification x 100).
Figure 3.3: Invaginated end-on attachment of a single cell of WesB to the surface of a goose erythrocyte. Scanning electron microscopy (original magnification x 10360)

Figure 3.4: Large numbers of cells of WesB attached to a goose erythrocyte. Scanning electron microscopy (original magnification x 4828)
Figure 3.5: Non-invaginated and flat end-on attachment of WesB to a goose erythrocyte. Scanning electron microscopy (original magnification x 11343)

By TEM, although end-on attachment of spirochaete cells was seen, no invagination into the cell membrane typical of that seen with colonic enterocytes was observed (Figure 3.6). Spirochaetes also were seen to have adhered along part of their length to the outer membrane of erythrocyte, with a close apposition suggestive of fusion of the spirochaete and erythrocyte membranes (Figure 3.7).
Figure 3.6: End-on attachment of a cell of WesB to a goose erythrocyte. Transmission electron microscopy (original magnification x 66000)

Figure 3.7: Flat side of a cell of WesB attached to a goose erythrocyte (arrow). Transmission electron microscopy (original magnification x 9700)
3.4 Discussion

In natural infections with *B. pilosicoli* the characteristic polar attachment of spirochaete cells to colonic enterocytes appears to be an important component of the colonization process. The basis of this attachment is not understood, although it has been suggested that spirochaete outer membrane proteins could be involved in interactions with the enterocytes (Trott et al 2001). To help study this process it would be extremely helpful to have an *in vitro* attachment system. In the current work erythrocytes were used for this purpose, as these cells are readily available and previously have been exploited as an easy tool to study *in vitro* adhesions for a variety of bacterial species, including spirochaetes (Mikx and Keulers 1992; Leong et al 1995).

An important observation in the study was that attachment to erythrocytes was strain specific: thus, despite all four strains being actively motile and in mid-log phase, only human strain WesB attached. By analogy with other bacterial-eukaryotic cell interactions, such strain specificity may reside with molecules such as lectins that are present in the bacterial outer membrane (Ofek et al 2003). For example, the spirochaete *Borrelia burgdorferi* expresses a lectin activity that promotes agglutination of erythrocytes, and bacterial attachment to glycosaminoglycans (Leong et al 1995). The specificity of the interaction was further emphasized by the fact that attachment only occurred with nucleated erythrocytes from chickens and geese. Attachment was time dependant, being first observed after four hours. Subsequently, the number of attached cells and the extent of agglutination of erythrocytes increased, particularly with a high ratio of spirochaetes to
erythrocytes. In future work it would be useful to determine what lectins and proteins are present on the surface of the different strains of *B. pilosicoli*, and to look for known associations with surface molecules on erythrocytes from different species. Blocking assays using different lectins, recombinant *B. pilosicoli* surface proteins and/or antiserum raised against the proteins would also help to identify the specific components involved in these interactions.

In this system, although spirochaetes occasionally appeared to be penetrating the erythrocyte membrane (Figure 3.3), this could not be confirmed by TEM. Instead of the spirochaetes being found invaginated into the erythrocyte surface, the surface of one cell end appeared to be attached to the surface of the erythrocyte membrane, or the spirochaetes lay on their sides with a clear and close association of the membranes (Figures 3.6 and 3.7). The apparent lack of invagination may have arisen as result of the erythrocytes not being immobilized, such that they were pushed away in the liquid medium as the ends of the motile spirochaetes thrust against the erythrocyte membrane. Alternatively, given the relatively small numbers of spirochaetes attached by one cell end, it is possible that invagination occurred but was not detected by TEM.

Although agglutination of erythrocytes is a simple and rapid assay, the relationship between the ability of a given strain to agglutinate erythrocytes and its *in vivo* attachment to different cell types, and pathogenicity, needs to be explored. The capacity of certain *B. pilosicoli* strains to attach to erythrocytes also may become relevant in cases of *B. pilosicoli*
spirochaetaemia. Although *B. pilosicoli* spirochaetaemias have not yet been described in avian species, if it occurred an agglutination of erythrocytes as seen in the current study could further exacerbate any clinical outcomes. It remains possible that strains of *B. pilosicoli* that attach to and agglutinate human erythrocytes may exist, and it would be useful to investigate this by screening a larger panel of *B. pilosicoli* strains with human erythrocytes.

Due to time constraints, and the need to develop an *in vitro* assay system that more closely mimicked the situation in the large intestine, further work examining interactions between *B. pilosicoli* and erythrocytes was not pursued as part of the current thesis work.
CHAPTER 4: ATTACHMENT ASSAYS WITH CACO-2 CELL MONOLAYERS

4.1. Introduction

As previously discussed, there is a pressing need to develop an in vitro attachment model to study the pathogenesis of B. pilosicoli infection. Previously Caco-2 cell lines have been used with other species of enteropathogenic bacteria to study such interactions (see the literature review). Consequently, the aims of the work described in this chapter were to establish an in vitro model of B. pilosicoli attachment with the Caco-2 cell line, and to use this to study aspects of the pathogenesis of the infection that have not been documented previously.

4.2 Materials and methods

4.2.1 Spirochaete strains and growth

The two Australian strains of B. pilosicoli isolated from pigs (95/1000 and Cof-10) and the two isolated from human beings (WesB and Karlton) that were used in the study described in Chapter Three, as well as the Brachyspira innocens type strain B256T, were obtained as frozen stock and grown in Kunkle’s anaerobic broth as described in Chapter Two.

4.2.2 Culture supernatants and sonicates

Culture supernatants were prepared by centrifuging adequate amount of broth culture containing actively motile mid-log phase spirochaete cells (10^9/mL) at 10,000 X g for 40 minutes and carefully aspirating the
supernatant. To prepare cell sonicates, desired amount of broths were centrifuged at 5,000 X g for 15 minutes, and the pellet was resuspended in PBS. This material was disrupted with an Ultrasonic Processor at 4°C with six bursts of 10 seconds each, centrifuged at 10,000 X g for 25 minutes, and the supernatant was aspirated and used as the source of sonicate.

4.2.3 Cell cultures
The human intestinal epithelial cell line Caco-2, derived from a colonic adenocarcinoma (HTB-37; ATCC), was grown in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% heat-inactivated foetal bovine serum, 1% L-glutamine, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 0.25 μg of amphotericin B/mL. The cells were grown at 37°C in a humidified atmosphere containing 5% CO2. The culture medium was changed every 2-3 days, and when appropriate the cells were passaged with 2 X trypsin-EDTA.

4.2.4 Attachment assays
For electron microscopy, the monolayers were grown in 48 well plates with sterile round glass (10 mm) or thermanox (13 mm) inserts in the bottom of each well. The wells were seeded with trypsinized Caco-2 cells at a concentration of 4 X 10^4 cells per well, and incubated at 37°C under 5% CO2 tension for 10-14 days. The growth of the monolayers was monitored and the supplemented DMEM was replaced as required. Well-grown, confluent and fully differentiated Caco-2 cells were used for the attachment assays. Actively motile cultures of the *B. pilosicoli* strains in mid-log phase (10^8
cells/mL) were used in the assays. The spirochaetes either were harvested from the broth culture by centrifuging at 800 X g for 20 minutes, and then the pellet was resuspended in the supplemented DMEM, or the broth culture was used directly. One mL of the respective suspensions containing 10^8 spirochaete cells was added per well. Control wells received 1 mL of sterile broth or supplemented DMEM. Incubation was for 2, 4 and 6 hours, with three replicates for each spirochaete strain at each time point. At the end of the incubation period the medium containing the bacteria was aspirated, and the wells were gently washed three times with PBS to remove any remaining unattached bacteria, before they were processed for electron microscopy.

4.2.5 Electron microscopy

The cells on the washed inserts were fixed with 2.5% glutaraldehyde at 4°C overnight, and then were washed five times with 0.07 M Sorensen’s buffer (three parts 0.01 M Na_2HPO_4 and 1 part 0.01M KH_2PO_4). The inserts were post-fixed in 1% aqueous osmium tetroxide at 4°C for 1 hour, and washed three times with 70% ethanol before being dehydrated through an ethanol series. For scanning electron microscopy (SEM), the inserts were removed from the wells, critically point dried on a Balzers Union critical point dryer with carbon dioxide as the exchange medium, and mounted on stubs using double-sided adhesive tape. Stubs were sputter coated with gold to a thickness of 90 nm in a Balzers sputter coater, and examined using a Philips XL 20 scanning electron microscope. A semi-quantitative scoring system for the extent of attachment at the different time points was used. The operator who was blinded to the origin of the samples examined 12 fields at a 2,000 X
magnification, and scored each field from 0 to 5, where 0 indicated no attached spirochaetes and 5 indicated that the surface of the whole field was covered with attached spirochaetes.

For transmission electron microscopy (TEM), the dehydrated cells were processed for infiltration with propylene oxide (two changes over 20 minutes), then with a propylene/resin mix (60/40) for 1 hour at 4°C, and finally with absolute resin on a rotary mixer at 25°C overnight. The cells were embedded with pure resin at 60°C for 24 hours, and 90 nm sections of the monolayers were cut with an ultra-microtome and mounted on carbon coated grids. The grids were stained with freshly prepared uranyl acetate and lead citrate and were examined using a Philips 1 CM -100 transmission electron microscope.

4.2.6 Preparation of Caco-2 monolayers for staining

400µl volumes of trypsinized Caco-2 cells (10^6 cells/mL) were added to Lab-Tek chamber slides, and were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed every 24 hours, and assays were conducted when the monolayers were confluent. A total of 10^8 cells of B. pilosicoli 95/1000 resuspended in 400µl supplemented DMEM was added to the slides, and they were incubated for 2, 4 or 6 hours. The assays were run in triplicate. Control slides were incubated for the same time with broth supernatant, with sterile uninoculated broth, or supplemented DMEM. The slides were washed three times with PBS before processing.
4.2.7 ZO-1 and Hoechst fluorescent staining

Staining for the tight junction protein zonula occludens-1 (ZO-1) with labeled antiserum, and for DNA using Hoechst staining, was performed on the washed Caco-2 monolayers. The cells were fixed and permeabilized for 20 minutes at 4°C by adding 400 µl of cold methanol to each well. For ZO-1 staining, after two washes with PBS 100 µL of primary antibody (rabbit anti-ZO1) diluted 1:100 in PBS containing 2% foetal bovine serum was added to each well, and these were incubated at 37°C in a humid chamber for 1 hour. The cells were washed twice with PBS, and 100 µL of secondary antibody (conjugated goat anti-rabbit) diluted 1:2,000 in PBS containing 2% foetal bovine serum was added to each well. After incubating for 1 hour at 37°C in a dark humid chamber, the cells were washed twice with PBS. For nuclear staining, 400 µL of a 1 µM Hoechst solution was added to each well, and these were incubated in the dark for 5 minutes at 25°C. After two washes with PBS, the coverslips were mounted in the dark using aqua polymount. The slides were stored in the dark until they were examined under an Olympus BX51 epifluorescent microscope with Green Excitation Filter UMWG2 for ZO-1 and Ultra Violet Excitation Filter UMWU2 for nuclear staining. The relative numbers of condensed and non-condensed nuclei were counted in six visual fields at a 100 X magnification, and the percentages for the different treatments were compared using Student’s *t*-test.
4.2.8 Staining of filamentous actin

The washed Caco-2 cells were fixed in 3% neutral buffered formalin for 20 minutes at 25°C. They were washed three times with PBS, and were permeabilized by treating with 0.1% Triton X-100 in PBS for 5 minutes. After three washes in PBS, the cells were treated with a 5µg/mL solution of fluorescein isothiocyanate-phalloidin in PBS for 20 minutes. The cells were washed three times with PBS and were mounted with glycerol-PBS (3:1). The monolayers were examined under an Olympus BX51 epifluorescent microscope (FITC filter, U-MWIB2).

4.2.9 Cytokine expression assays

Two experiments were conducted using quantitative reverse transcription PCR (RT q-PCR) to assess the expression of cytokine genes in Caco-2 monolayers in response to exposure to spirochaetes or their products. The Caco-2 cells were grown in 48 well plates and exposed to 95/1000 cells resuspended in supplemented DMEM, or other materials, as previously described. In the first experiment the expression of the genes encoding nine cytokines [interferon-γ, tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-8, and IL-10], as well as β-actin as the internal control, was assessed in triplicate after 2, 4, 8 and 12 hours exposure to cultures of B. pilosicoli 95/1000 or B. innocens 256T. In the second experiment only the expression levels of TNF-α, IL-1β, IL-6 and IL-8, and the internal control, were measured. In the second experiment six replicates of the Caco-2 cells were exposed for 12 hours either to sterile uninoculated broth, supplemented DMEM, broth supernatant from the 95/1000 culture, a
sonicate of 95/1000, 10<sup>8</sup> cells of 95/1000 in supplemented DMEM, or 10<sup>8</sup>
cells of <i>B. innocens</i> B256<sup>T</sup> in supplemented DMEM. In both experiments the
cells then were rinsed in PBS, trypsinized, and total RNA was isolated from
the treated samples using the High Pure RNA Isolation Kit, according to the
manufacturer’s instructions. Complementary DNA (cDNA) was synthesized
using the High Capacity cDNA Reverse Transcription Kit from 500ng RNA
in a 20µL reaction. Cytokine mRNA expression was measured using a hot
start master mix, according to the manufacturer’s instructions.

The primers used for all the cytokines except IL-8 were those described
previously by Vernal et al (2008), whilst the primers specific for IL-8 were
designed to target a 136 base pair conserved region of the molecule. The
primers are listed in Table 4.1.

Table 4.1. Forward and reverse primers used for cytokine amplification by
quantitative real-time PCR

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1β*</td>
<td>ctgtctctgcgttgtaaaga</td>
<td>tggtaatrtttgggatctaca</td>
</tr>
<tr>
<td>IL2*</td>
<td>aagttttatagtgcaccaagaagg</td>
<td>aagtgaaagtttttgctttgagc</td>
</tr>
<tr>
<td>IL4*</td>
<td>caccgagttgaccgaacacag</td>
<td>gccctgacgaaggttcc</td>
</tr>
<tr>
<td>IL5*</td>
<td>cttgaggattcctctctctgt</td>
<td>cagtacccctttgacagt</td>
</tr>
<tr>
<td>IL6*</td>
<td>gccgcgtatgaactctctctctctct</td>
<td>gaaggcagcaggcaacac</td>
</tr>
<tr>
<td>IL8**</td>
<td>acctctccacccaaatttttcctc</td>
<td>ttgcaccagttctttccttg</td>
</tr>
<tr>
<td>IL10*</td>
<td>tgggggacacagtgaagac</td>
<td>ccttgctttgacacaggg</td>
</tr>
<tr>
<td>TNFα*</td>
<td>cagctctctctctctctctctct</td>
<td>ggcagaaggtgaaggtgaga</td>
</tr>
<tr>
<td>IFNγ*</td>
<td>ggcattttgaagaatggagaag</td>
<td>ttgggtgcctgtttcacttt</td>
</tr>
<tr>
<td>β Actin***</td>
<td>tccctctacgcctctcctctct</td>
<td>tgggtgtagcgtctgtag</td>
</tr>
</tbody>
</table>

(* Vernal et al 2008, ** Designed in house, *** Song and Hampson 2008)
The primers for β-actin, a reference gene, have been described previously (Song and Hampson 2008). Amplification and detection of specific products were conducted on the Rotor-gene 6000 real time PCR system, with the following cycle profile: one cycle of 95°C for 10 minutes, and 40 cycles of 95°C for 20 seconds and 60°C for 60 seconds. The expression ratio of each cytokine gene in cells subjected to the specific treatment relative to those incubated with supplemented DMEM was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Within each experiment, fold difference values for the different treatments were compared using analysis of variance (ANOVA), and differences between groups were compared using the Tukey-Kramer Multiple Comparisons test in SPSS Statistics 17.0.

4.3 Results

4.3.1 Attachment to Caco-2 monolayers

The Caco-2 monolayers exposed to sterile broth or supplemented DMEM for six hours remained intact throughout the assays. Under the SEM the cells had a healthy appearance, and the tight junctions were visible (Figure 4.1). Following washing and processing, B. pilosicoli strains 95/1000 and WesB showed attachment to the monolayer, whilst no attachment was observed for strains Karlton or Cof-10. Similar attachment patterns were obtained whether the spirochaetes were resuspended in DMEM or used directly in Kunkle’s broth. The results of the semi-quantitative attachment scores for 95/1000 and WesB are presented in Table 4.2. For both strains the extent of attachment increased with time, but it was greater for 95/1000 than for WesB. At two
hours, attachment was limited, and mainly confined to the junctions of the Caco-2 cells (Figure 4.2). At four hours, the cell junctions were colonized with large numbers of spirochaetes, and more spirochaetes were observed overlying the rest of the cell surfaces (Figure 4.3). At six hours there was massive colonization covering all the cell surfaces, especially with strain 95/1000 (Figure 4.4). The adhering ends of the spirochaetes appeared to penetrate the outer membranes of the cells (Figure 4.5).

Table 4.2. Density of attachment of \textit{B. pilosicoli} cells to Caco-2 cells after 2, 4 and 6 hours incubation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hours post-infection</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>95/1000</td>
<td>2</td>
<td>2.5</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.0</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5.0</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>WesB</td>
<td>2</td>
<td>1.5</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.0</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.0</td>
<td>3.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Results at each time point are derived from 12 fields of view, where 0 represents no attachment observed and 5 represents the entire surface of the field covered with spirochaetes such that the Caco-2 cell surface was not visible.
Figure 4.1: Caco-2 cells exposed for six hours with sterile broth medium. The non-infected cells show intact tight junctions with clear boundaries. SEM, original magnification X 2100

Figure 4.2: Caco-2 cells exposed with *B. pilosicoli* culture for two hours. The *B. pilosicoli* 95/1000 cells mainly colonize the cell boundaries. SEM, original magnification x 2100
Figure 4.3: Caco-2 cells exposed to *B. pilosicoli* 95/1000 for four hours. Attachment is denser at the junctions. SEM, original magnification x 2100

Figure 4.4: Most of the Caco-2 cells surface is covered by *B. pilosicoli* 95/1000 after six hours exposure. SEM, original magnification x 2100
Figure 4.5: Cells of WesB can be seen penetrating the outer membrane of the Caco-2 cells (arrows). SEM, original magnification x 9800

Using the TEM, in the infected monolayers tangential sections and cross-sections of spirochaete cells were observed between the cell junctions and associated with the outer membrane of the Caco-2 cells (Figures 4.6 and 4.7). TEM further confirmed the typical end-on attachment of the spirochaete cells, invaginating into the membranes of the Caco-2 cells (Figure 4.8). In some cases an accumulation of electron-dense fibrillar material was seen beneath the attached end of the *B. pilosicoli* strains (Figure 4.9). Compared to the nuclei of cells in the uninfected monolayers (Figure 4.10), in the infected monolayers there was a time-dependant increase in the number of cells found that had nuclei showing chromatin condensation and fragmentation, consistent with apoptosis (Figure 4.11).
Figure 4.6: Cross-sections and tangential-sections (arrows) of *B. pilosicoli* can be seen at the cell junctions of Caco-2 cells. TEM, original magnification x 5,800

Figure 4.7: Cross-sections and tangential-sections (arrows) of *B. pilosicoli* under the Caco-2 cell membrane. TEM, original magnification x 7,900
Figure 4.8: Intact spirochaete cell invaginating into a pit-like structure (arrows) in the Caco-2 cell membrane. TEM, original magnification x 24,500

Figure 4.9: Accumulations of electron dense material around the site of attachment (arrows). TEM, original magnification x 33,800
Figure 4.10: Nuclei of non-infected Caco-2 cell with unique and regular distribution of chromosome all over the nucleus. TEM, original magnification x 5800

Figure 4.11: Nuclei of an infected Caco-2 cells with chromatin condensation and fragmentation (arrows), consistent with apoptosis. TEM, original magnification x 5800
4.3.2 ZO-1 distribution

The monolayers exposed to sterile broth or supplemented DMEM for six hours showed intact cell junctions with regular distribution of ZO-1 on the pericellular tight junctions of the Caco-2 monolayers (Figure 4.12). After two hours incubation with *B. pilosicoli* 95/1000, the junctions appeared irregular, were occasionally broken, and some ZO-1 was punctated and had migrated towards the cytoplasm of the cells from the junctions (Figure 4.13). After six hours exposure the junctions of many of the Caco-2 cells were disrupted either focally or completely, and the ZO-1 staining was punctated on the junctions. A large amount of ZO-1 had migrated from the junctions towards the centre of the Caco-2 cells (Figure 4.14), and overall there appeared to be considerable damage to the tight junctions of the cells. The supernatant from the *B. pilosicoli* culture did not induce notable changes to the distribution of ZO-1 (Figure 4.15).
Figure 4.12: Control, epifluorescent micrograph illustrating ZO-1 integrity in Caco-2 cell monolayers exposed to sterile autoclaved broth for six hours. No junctional damage can be seen. Original magnification x 100.

Figure 4.13: Epifluorescent micrograph illustrating ZO-1 integrity in Caco-2 cell monolayers exposed to *B. pilosicoli* 95/1000 broth-culture for two hours. ZO1 is not as regular on the junctions as it was in control. The junctions are
uneven and occasionally broken, and in some places the ZO-1 is punctuated and has moved towards the cytoplasm (arrows). Original magnification x 100

Figure 4.14: Epifluorescent micrograph illustrating ZO-1 integrity in Caco-2 cell monolayers exposed to B. pilosicoli 95/1000 broth-culture for six hours. The junctions are badly damaged and ZO-1 has migrated towards the cells’ cytoplasm (arrows). Original magnification x 100

Fig 4.15: Epifluorescent micrograph illustrating ZO-1 integrity in Caco-2 cell monolayers exposed to sterile autoclaved broth for 6 hours. Original magnification x 100
4.3.3 Hoechst staining of Caco-2 cell nuclei

The control Caco-2 monolayers exposed to sterile broth or supplemented DMEM for six hours exhibited characteristic uniform fluorescent nuclear staining throughout all nuclei (Figure 4.16). A two-hour exposure to the 95/1000 culture induced mild chromosomal condensation and nuclear fragmentation among some cells, with a few nuclei showing intense changes. After six hours exposure there was considerable condensation and fragmentation of the nucleic acid in many cells (Figure 4.17). At this time, the mean and standard deviation of the percentage of nuclei in the six fields showing condensation and/or nuclear fragmentation was $8.8 \pm 1.9$ in the controls and $35.6 \pm 5.8$ in the infected cells, and this difference was highly significant ($P < 0.001$).

![Figure 4.16: Epifluorescent micrograph showing Hoechst staining of DNA in Caco-2 cells exposed to sterile broth for six hours. Nuclei are normal with regular chromatin distribution. Original magnification x 100.](image)
4.3.4 Actin rearrangements

In the control cells stained with FITC-phalloidin there was regular distribution of actin filaments at the cell peripheries/junctions (Figure 4.18). After two hours exposure to *B. pilosicoli*, the distribution pattern of actin in the Caco-2 cells was regular, with some actin relocalized in a few places at the periphery of cells (Figure 4.19). The monolayers exposed for six hours exhibited an irregular distribution of round or oval concentrations of actin filaments, which was intense at many places on the Caco-2 cell peripheries/junctions, at the primary site of spirochaete attachment (Figure 4.20). The culture supernatant did not induce any rearrangement of filamentous actin (Figure 4.21).
Figure 4.18: Epifluorescent micrographs showing actin staining in Caco-2 monolayers exposed to sterile broth for six hours (control). There is regular distribution of FITC (phalloidin) all over the monolayers, including the junctions.

Figure 4.19: Epifluorescent micrographs showing actin staining in Caco-2 monolayers after two hours incubation with *B. pilosicoli*. There are a few spots of localized actin (arrows).
Figure 4.20: Epifluorescent micrographs showing actin staining in Caco-2 monolayers after six hours incubation with *B. pilosicoli*. The actin filaments are clearly mobilized on the junction of Caco2 cells where the bacteria attached. The actin rearrangement can be seen as round bodies distributed over the junctions (arrows).

Figure 4.21: Epifluorescent micrographs showing actin staining in Caco-2 monolayers after six hours incubation with broth. The actin distribution is regular all over the Caco-2 cells and junctions.
4.3.5 Cytokine expression

An example of the RT q-PCR output for the β-actin gene is shown in Figure 4.22. After amplification, each reaction was tested to ensure that it was specific by using gel electroporation and melting curve analysis (Figure 4.23).

![Image of quantitation data for cycling A.FAM/Sybr for the β-actin gene.]

Figure 4.22. Example of quantitation data for cycling A.FAM/Sybr for the β-actin gene.

![Image of melt data for Melt A.FAM/Sybr with the β-actin gene.]

Figure 4.23. Example of melt data for Melt A.FAM/Sybr with the β-actin gene.
In the first experiment, the RT q-PCR assays showed that exposure of Caco-2 cells with live *B. pilosicoli* 95/1000 cells but not *B. innocens* B256$^T$ cells significantly ($P < 0.05$) enhanced expression of the IL-1β and IL-8 genes in a time dependent manner, with expression being greatest at 12 hours (Figures 4.24 and 4.25). Expression of TNF-α and IL-6 also increased, but not significantly (Figures 4.26 and 4.27), while expression of the remaining five cytokines was unaltered.

Figure 4.24. The quantitative expression of IL1-β by Caco-2 cells at different time intervals after *B. pilosicoli* and *B. innocens* exposure. The expression remained unaltered in the case of *B. innocens* but increased significantly ($P < 0.05$) in the case of *B. pilosicoli* after 8 and 12 hours, and these values were significantly ($P < 0.05$) higher than those for *B. innocens*. 
Figure 4.25. The quantitative expression of IL-8 by Caco-2 cells at different time intervals after *B. pilosicoli* and *B. innocens* exposure. The expression did not differ in the case of *B. innocens* but changed significantly (*P* < 0.05) in the case of *B. pilosicoli* after 12 hours, and was significantly (*P* < 0.05) higher than *B. innocens* at this time.

Figure 4.26: The quantitative expression of IL-6 by Caco-2 cells at different time intervals after *B. pilosicoli* and *B. innocens* exposure. The expression did not differ significantly with time or between the species.
Figure 4.27: The quantitative expression of TNF-α by Caco-2 cells at different time intervals after *B. pilosicoli* and *B. innocens* exposure. The expression did not differ significantly with time or between species.

In the second experiment, examining four genes, expression of IL-1β and IL-8 was significantly up-regulated by incubation with live 95/1000 for 12 hours, whilst the sonicate caused a significant up-regulation of TNF-α, IL-1β and IL-6, and a non-significant numerical increase in expression of IL-8 (Table 4.3). No other treatments, including exposure to *B. innocens*, caused significant up-regulation of these genes.
Table 4.3. Changes in cytokine expression in Caco-2 cell monolayers exposed for 12 hours to sterile broth, supplemented DMEM, 
*B. pilosicoli* strain 95/1000 culture supernatant and sonicates, live *B. pilosicoli* 95/1000, and live non-pathogenic *B. innocens* B256ᵀ. The results are mean ± standard deviation of fold difference in gene expression measured by RT-qPCR.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Sterile broth</th>
<th>DMEM</th>
<th>Culture supernatant</th>
<th>Culture sonicate</th>
<th>Live <em>B. pilosicoli</em></th>
<th>Live <em>B. innocens</em></th>
<th>P value in ANOVA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>1.11ᵃ ± 0.52</td>
<td>1.05ᵇ ± 0.36</td>
<td>1.18ᵃ ± 0.57</td>
<td>21.25ᵇ ± 12.97</td>
<td>17.72ᵇ ± 4.12</td>
<td>2.24ᵇ ± 2.71</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.02ᵃ ± 0.25</td>
<td>1.06ᵇ ± 0.36</td>
<td>1.42ᵃ ± 0.49</td>
<td>24.92ᵇ ± 5.84</td>
<td>5.51ᵃ ± 3.46</td>
<td>4.61ᵃ ± 6.84</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.26ᵃ ± 0.72</td>
<td>1.32ᵇ ± 0.71</td>
<td>5.68ᵃ ± 3.07</td>
<td>37.12ᵇ ± 16.60</td>
<td>1.04ᵃ ± 0.81</td>
<td>2.83ᵇ ± 3.65</td>
<td>0.0005</td>
</tr>
<tr>
<td>IL-8</td>
<td>1.19ᵃ ± 0.67</td>
<td>1.05ᵇ ± 0.35</td>
<td>1.83ᵃ ± 0.79</td>
<td>6.75ᵇ ± 5.96</td>
<td>25.33ᵇ ± 19.05</td>
<td>1.62ᵃ ± 2.12</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

*For each cytokine, treatment means marked with a different superscript differ at least at *P* < 0.05.*
4.4 Discussion

The ability of pathogenic bacteria to adhere is one of the essential features that they require to successfully colonize the gastrointestinal tract (Cerda and Cossart 2006). In both natural and experimental infections with *B. pilosicoli* in humans, animals and birds, the spirochaete shows an unusual form of attachment to the surface of colonic enterocytes, whereby one cell end appears to push against and then to invaginate into the cell surface (Duhamel 2001). Subsequently, specific interactions with the adjacent host cell appear to occur, anchoring it in place within the pit-like structure that is formed (Trott et al 2001). A previous attempt to reproduce this form of attachment with cultured cell monolayers was unsuccessful; however, a diffuse and non-polar attachment occurred with H407 cells, a similar but lesser attachment occurred with Caco-2 and Hep-2 cells, minimal attachment occurred with FaDU and HeLa cells, and there was no attachment with HT-29 cells (Muniappa et al 1998). Similar patterns of cell associations were seen with four strains of *B. pilosicoli*, including one isolated from a human, and three from rhesus monkeys.

Despite this past lack of success, Caco-2 cells were used in the current study because they have many similarities to normal human colonic enterocytes, and have been used to study *in vitro* adhesion of various pathogenic enteric bacteria. Using this system, the characteristic *B. pilosicoli* polar interaction with enterocytes was reproduced. Of the four *B. pilosicoli* strains tested, only porcine strain 95/1000, and to a lesser extent human strain Wes B, attached to the Caco-2 cells. The other two strains failed to attach, and were
completely removed by the washing and fixing process. Hence, these differences between the strains used may help to explain the failure of the previous attempt to obtain polar attachment to Caco-2 cells (Muniappa et al 1998). The basis for there being differences in attachment with different *B. pilosicoli* strains is not known. Interestingly, however, Caco-2 cells are known to produce mucins (van Klinken et al 1996), and in later work I have shown that strain 95/1000 is highly attracted to mucin in a capillary tube assay, whilst Cof-10 (a motile, but non-attaching strain) was not attracted to mucin (see Chapter 5). This suggests a possible involvement of attraction towards mucus as part of the attachment process, perhaps helping to orientate the spirochaete so that it approaches the cell surface in a perpendicular fashion. Differences in other properties of the strains, such as their specific surface proteins, lipoproteins and/or glycans that may interact with receptors on the cell surface of enterocytes, also could help to account for their different attachment phenotypes. Such potential differences require further investigation. It will also be important to determine whether the *in vitro* activities of the different strains reflect how they behave *in vivo*, and whether this can be used to help predict the virulence potential of a given isolate.

Attachment of the two *B. pilosicoli* strains to the Caco-2 cells was time-dependant; it was clearly present after two hours, and there was extensive colonization by six hours. The site of attachment initially was mainly around the cell junctions, and then, with time, attached spirochaetes were observed over the rest of the cell surface. A similar pattern of attachment at the cell junction has been seen *in vivo*, and it has been suggested that this distribution
may facilitate penetration of the epithelial layer (Duhamel 2001). Hence *B. pilosicoli* may be able to translocate through the colonic epithelium, and enter the bloodstream by using an intercellular route (Trott et al. 1997b). The reasons for the subsequent spread of attachment from the junctions over the whole cell surface are not obvious, but it is possible that spirochaete-induced changes occurred in the Caco-2 cell membranes that made them more receptive to the spirochaetes.

Both transmission electron microscopy and Hoechst staining indicated that in the cells exposed to *B. pilosicoli* there was a time-dependant condensation and fragmentation of the nuclear material, consistent with apoptosis. In future work, pre-treatment with specific inhibitors, such as a caspase-3 inhibitor, could be used to help to confirm the occurrence of apoptosis (Chin et al. 2002). Similarly, using ZO-1 staining, a time-dependant disruption to the zonula occludens was observed. It was unclear whether the resultant changes in cellular permeability may have initiated apoptosis, or *visa versa* (Chin et al. 2002; Bojarski et al. 2004). The disruptions at the cell junctions occurred beneath the site of maximal *B. pilosicoli* attachment, and were not induced by the culture supernatant. Hence, the cellular damage appeared to be associated with the specific spirochaete attachment, rather than toxic products released into the medium from the growing cells. Currently nothing is known about how *B. pilosicoli* could induce such localized damage, but the spirochaete is known to possess membrane-associated serine proteases and other proteases that could induce local damage (Dassanayake et al. 2004). Equally, it is possible that the spirochaete produces and delivers toxic
molecules directly at the cell surface. Some examples of such bacterial toxins that act at the cell junction include the fragilysin toxin produced by *Bacteroides fragilis*, causing the degradation of the ZO-1 protein (Obiso et al 1997), and the *Clostridium difficile* toxins TcdA and TcdB that cause the dissociation of occludin, ZO-1, and ZO-2 (Nusrat et al 2001).

TEM identified the presence of electron-dense accumulations of material resembling microfilaments under the attached spirochaetes, and subsequent specific staining revealed a time-dependant accumulation of filamentous actin at the cell margins, under the area of greatest spirochaete attachment. This is the first report providing clear evidence for actin rearrangement associated with *B. pilosicoli* cellular attachment. An accumulation of actin was not previously seen in monolayers where the spirochaetes showed only a diffuse non-polar attachment (Muniappa et al 1998), and this observation supports the existence of a causal association between the polar attachment and these specific changes. The ability of certain bacteria to manipulate the host’s cytoskeleton in such a way is known to be important for adhesion and invasion (Bhavsar et al 2007, Goosney et al 1999, Gruenheid and Finlay 2003), and further work is required to elucidate the specific mechanisms involved in this unique interaction. The earlier work of Harland and colleagues (Hartland et al 1998) using gene probes suggests that the mechanisms involved are likely to be different to those that occur with enteropathogenic *E. coli*, *Y. enterocolitica* or *S. flexneri*. Further insight into possible effectors and mechanisms may become available once the full genome sequence of *B. pilosicoli* becomes available.
In order to help identify the responsiveness of the Caco-2 cells to the attachment by *B. pilosicoli*, assays were undertaken to assess the expression of selected cytokines by the monolayers. In the first experiment, exposure to live attaching 95/1000 cells induced a significant time-dependent increase in expression of the genes encoding IL-1β and IL-8, and some increases in TNF-α, and IL-6. The increase in IL-1β and IL-8 expression was confirmed in the second experiment, where a sonicate of 95/1000 also was shown to induce significant increases in expression of IL-1β, TNF-α, and IL-6. The fact that culture supernatants caused no change in cytokine mRNA expression is an important observation, since it suggests that cell-free *B. pilosicoli* toxins or by-products were not involved in the stimulatory effects. IL-1β is an important mediator in intestinal inflammation, promoting production of the pro-inflammatory chemokine IL-8, so it was interesting that both live cells and sonicate of *B. pilosicoli* stimulated its expression. On the other hand, only live (attached) *B. pilosicoli*, and not sonicate or live (unattached) *B. innocens* induced significant expression of IL-8, suggesting that induction of this gene following attachment may be important in the generation of the focal tissue damage and colitis that can occur *in vivo*. Taken together, these findings add further weight to the evidence that *B. pilosicoli* 95/1000 has pathogen potential. Many other enteric bacterial pathogens similarly induce IL-8 production by cultured enterocytes (Steiner et al 2000), with, for example, both bacterial motility and adherence being important for this induction in the case of *Vibrio cholerae* (Sarak and Chaudhuri 2004), and adherence and probably the presence of
lipopolysaccharide in the case of *Helicobacter pullorum* (Varon et al 2009). Currently it is unclear what *B. pilosicoli* mediators and Caco-2 cell surface receptors, transduction pathways and transcription factors are involved in generating the up-regulation, although a range of different bacterial products and corresponding Toll-like or other surface receptors on the Caco-2 cells could be involved. This could be investigated further using purified *B. pilosicoli* cell-surface components, together with antagonists of specific surface receptors or intracellular signaling cascades. Again it was interesting that the sonicate caused significant up-regulation of the pro-inflammatory cytokines IL-1ß, TNF-α, and IL-6, presumably in response to liberated materials present in the cellular debris. Interestingly, the fact that IL-8 was not significantly upregulated by the sonicate suggests that there is a specificity in the spirochaete attachment process that is involved in generating IL-8 expression.

In conclusion, an *in vitro* assay for investigating the interactions of *B. pilosicoli* with cultured enterocytes has been developed in which many features of the natural infection can be monitored. In future work, more extensive transcriptomics analysis and the use of specific antagonists may help to identify some of the pathways and processes involved in the interaction. The current study has demonstrated that strains of *B. pilosicoli* vary in their attachment phenotypes, and that the typical polar attachment is associated with cellular changes, including accumulation of actin under the site of attachment, alterations in the permeability of the cell membrane, apoptosis, and up-regulation of IL-8. Taken together, these results add to the
available evidence demonstrating that strains of *B. pilosicoli* have pathogenic potential, and provide a basis for explaining the focal colitis that may be seen in animals and humans that are infected with *B. pilosicoli*. 
CHAPTER 5

ATTRACTION OF B. PILOSICOLI TO MUCIN AND OTHER SUBSTRATES

5.1 Introduction

As explained in chapter one, B. pilosicoli needs to be able to penetrate and move through the mucous layer that overlies the caecal and colonic epithelium, before it can attach to the underlying enterocytes. Consequently, the aim of the current study was to investigate the extent to which B. pilosicoli is attracted to mucin, and to determine whether this property differs amongst strains of the species. Comparisons also were made with the behaviour of B. hyodysenteriae strain B204, and the underlying basis of the strong attraction to mucin that was found was investigated.

5.2 Materials and Methods

5.2.1 Animal ethics

Permission to use the pigs in this study was obtained from the Murdoch University Animal Ethics Committee.

5.2.2 Spirochaete strains and culture conditions

Brachyspira species strains were obtained as frozen stock from the culture collection held at the Australian Reference Centre for Intestine Spirochaetes at Murdoch University. They included 15 strains of B. pilosicoli (five isolated from humans, four recovered from pigs, and three each from chickens and dogs), and Brachyspira hyodysenteriae strain B204. Of these
strains, *B. pilosicoli* porcine strain P43/6/78\(^T\) previously had been examined for chemotaxis to porcine gastric mucin by both Milner and Sellwood (1994) and Witters and Duhamel (1999), and canine strain 16242-94 previously had been examined by Witters and Duhamel (1994). *B. hyodysenteriae* strain B204 had been examined for chemotaxis to mucin by Milner and Sellwood (1994) and by Kennedy and Yancey (1996). The names and origins of the 15 strains are listed in Table 5.1.

Table 5.1. Strains of *B. pilosicoli* used in the attraction assays.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species of origin</th>
<th>Description/Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wes B</td>
<td>Human</td>
<td>Isolated from the faeces of an Aboriginal child, Western Australia</td>
</tr>
<tr>
<td>Karlton</td>
<td>Human</td>
<td>Isolated from the faeces of an Aboriginal child, Western Australia</td>
</tr>
<tr>
<td>PE90</td>
<td>Human</td>
<td>Isolated from a spirochaetaemia in a patient from France</td>
</tr>
<tr>
<td>BR81/80</td>
<td>Human</td>
<td>Isolated from a spirochaetaemia in a patient from France</td>
</tr>
<tr>
<td>V2H5</td>
<td>Human</td>
<td>Isolated from the faeces of a healthy human being from Papua New Guinea</td>
</tr>
<tr>
<td>P43/6/78(^T)</td>
<td>Pig</td>
<td>Isolated from a case of porcine intestinal spirochaetosis, UK</td>
</tr>
<tr>
<td>95/1000</td>
<td>Pig</td>
<td>Isolated from a case of porcine intestinal spirochaetosis, Western Australia</td>
</tr>
<tr>
<td>Code</td>
<td>Organism</td>
<td>Isolation Details</td>
</tr>
<tr>
<td>------</td>
<td>----------</td>
<td>------------------</td>
</tr>
<tr>
<td>L72</td>
<td>Pig</td>
<td>Isolated from a case of porcine intestinal spirochaetosis, Western Australia</td>
</tr>
<tr>
<td>Cof 10</td>
<td>Pig</td>
<td>Isolated from the faeces of a healthy pig in Western Australia</td>
</tr>
<tr>
<td>Chicken</td>
<td>CSP-1</td>
<td>Isolated from a case of avian intestinal spirochaetosis, Queensland</td>
</tr>
<tr>
<td>Chicken</td>
<td>98.0026</td>
<td>Isolated from a case of avian intestinal spirochaetosis, Queensland</td>
</tr>
<tr>
<td>Chicken</td>
<td>97.006</td>
<td>Isolated from a case of avian intestinal spirochaetosis, Queensland</td>
</tr>
<tr>
<td>Dog</td>
<td>Boof</td>
<td>Isolated from the faeces of a healthy dog, Western Australia</td>
</tr>
<tr>
<td>Dog</td>
<td>16242.94</td>
<td>Isolated from the faeces of a dog, USA</td>
</tr>
<tr>
<td>Dog</td>
<td>D148</td>
<td>Isolated from the faeces of a dog, Western Australia</td>
</tr>
<tr>
<td>Pig</td>
<td>B204</td>
<td>Isolated from a pig with swine (B. hyodysenteriae) dysentery, USA</td>
</tr>
</tbody>
</table>

The spirochaete cells were thawed and grown in Kunkle’s pre-reduced anaerobic broth, as described in Chapter 2. Only actively motile mid-log phase spirochaete cells were used in the assays. Cells were counted using a Neubauer counting chamber under the 40 times objective of a phase contrast microscope, and final dilutions to 10^8 cells mL^-1 were made in freshly prepared pre-warmed anaerobic broth.
5.2.3 Capillary tube assays

The system used was based on the method of Milner and Sellwood (1994), with modifications. Porcine gastric mucin type II was dissolved in chemotaxis buffer (0.01 M potassium phosphate buffer [pH 7.0], 0.2 mM L-cysteine hydrochloride) (Greenberg and Canale-Parola 1977) to final concentrations of 10, 8, 6, 4, 3, 2, 1, 0.5 and 0.2% (w/v). D-serine was similarly dissolved in chemotaxis buffer to make 100, 10, 1 and 0.1 mM solutions, and polyvinylpyrrolidone (PVP) was dissolved in chemotaxis buffer to make solutions of 4.0, 3.0, 2.5, 2.0, 1.5, 1.25, 0.75 and 0.25% (w/v).

Only *B. pilosicoli* strain 95/1000 and *B. hyodysenteriae* strain B204 were tested in 8 and 10% mucin, whilst all strains were tested in the other seven dilutions of mucin. Strain 95/1000 also was tested in PBS, in chemotaxis buffer, in the D-serine solutions, and it and B204 were tested in the various dilutions of PVP, with 6% mucin used as a positive control. A suspension of strain 95/1000 that had been inactivated overnight in 4% formalin also was tested with chemotaxis buffer and 4% mucin. For each spirochaete strain to be tested, six 75 mm haematocrit capillary tubes (75 μL volume) were filled with the appropriate concentration of mucin or the various other solutions, and six paired tubes were filled with chemotaxis buffer. The top ends of the tubes were sealed with plasticine, and then they were hung vertically with their lower ends submerged in fresh bacterial broth culture (10^8 cells mL^-1) in 48 well round-bottomed tissue culture plates. These were incubated at 39°C in a CO_2 incubator. An appropriate incubation time was determined by
testing *B. pilosicoli* strain 95/1000 in 4% mucin in chemotaxis buffer with incubation times of 15, 30, 60, 90 and 120 minutes. Based on the results, a 90-minute incubation time was selected for testing all the strains. After incubation, each tube was carefully removed, the outside wiped dry with a sterile tissue, and then placed upright into a 200 μL eppendorf tube. The top of the capillary tube was gently broken and the contents were collected in the tube. The collected solution was serially diluted in PBS and the spirochaetes were counted under a phase contrast microscope, as described above.

### 5.2.4 Viscosity measurements

Six healthy pigs of ~30 kg body weight that had been fed on a commercial antimicrobial-free Australian grower diet based on wheat and lupins were killed using a captive bolt pistol and exsanguination, and their large intestines were removed and opened. The intestinal contents were expelled manually, the mucosa rinsed with PBS, patted dry with paper tissue, and approximately one gram quantities of the mucus layer immediately overlying the epithelium in both the mid-caecum and proximal-colon were carefully collected by gently scraping with sterile scalpel blades. The viscosities of these samples were measured in a Brookfield LVDV-II+ cone plate (CP40) rotational viscometer, as previously described (Hopwood et al 2002). Briefly, the material was diluted 1:1 (v/v) with distilled water, mixed and centrifuged at 12,000 x g for 8 minutes. The viscosities of 0.5 mL fractions held at 39°C in a water bath were measured, applying a shear rate of 60 s⁻¹. The viscosities of the dilutions of porcine gastric mucin and PVP used in the capillary tube assays were similarly tested.
5.2.5 Statistical analysis

For each replicate of each mucin concentration or other solution tested, attraction was expressed as the ratio \((R_{att})\) of the number of spirochaetes in the tube containing the test solution compared to the number in the paired tube containing chemotaxis buffer. The mean and standard deviation of the six replicates for each concentration were calculated. One-way analysis of variance (ANOVA) was used to compare the \(R_{att}\) for each of the 15 strains across the different mucin concentrations, to compare the \(R_{att}\) for each of the 15 strains at each of the seven mucin concentrations, and to compare the \(R_{att}\) at each of the mucin concentrations for the sets of isolates from humans, pigs, chickens and dogs. \(R_{att}\) values of greater than two were considered significant (Moulton and Montie 1979). \(R_{att}\) values of zero were obtained when no spirochaetes were found in the mucin solution, but spirochaetes were present in the buffer tubes. Comparison of the number of spirochaetes in the capillary tubes just containing chemotaxis buffer for the 15 strains were similarly analysed by ANOVA. Regression analyses were made across all the strains for the numbers of spirochaetes in the tubes containing chemotaxis buffer and the \(R_{att}\) values in 4 % and in 6% mucin.

5.3 Results

5.3.1 Optimizing test conditions

The response of \(B. pilosicoli\) strain 95/1000 and \(B. hyodysenteriae\) B204 to various mucin concentrations from 0.2 to 10% are presented in Figure 5.1. In both species the attraction ratio \((R_{att})\) increased with increasing mucin.
concentrations, to peak at around 6-8% mucin for 95/1000, and 6% for B204, after which it showed a steep decline for the *B. hyodysenteriae* strain. Based on these results, all the *B. pilosicoli* strains were tested in mucin concentrations from 0.2% to 6%. The results of incubating strain 95/1000 in 4% mucin for different time intervals are shown in Figure 5.2. Based on these findings, an incubation time of 90 minutes was selected for all further incubations.

![Figure 5.1](image_url)

Figure 5.1. Means and standard deviations of Ratt values for *B. pilosicoli* strain 95/1000 (filled circles) and *B. hyodysenteriae* strain B204 (open circles) incubated for 90 minutes in different concentrations of porcine gastric mucin in chemotaxis buffer.
Chemotaxis buffer supported the activity of *B. pilosicoli* better than did PBS, as significantly ($P < 0.001$) more cells of strain 95/1000 were found in the tubes containing chemotaxis buffer than in those containing PBS (2.97 and $1.56 \times 10^6$ cells mL$^{-1}$, respectively). Cells of *B. pilosicoli* 95/1000 that had been inactivated in formalin did not enter the tubes containing chemotaxis buffer or mucin.

When analysing the *B. pilosicoli* numbers in the tubes just containing chemotaxis buffer after 90 minutes incubation, significant differences in counts were found for the different spirochaete strains ($P < 0.001$; Table 5.2). The corresponding mean number of cells for *B. hyodysenteriae* B204 in chemotaxis buffer was (2.09 ± 0.28) $\times 10^6$. When the numbers of *B. pilosicoli* cells in chemotaxis buffer were compared by linear regression with their *Ratt*
values for the same strains in 4% and 6% mucin, no significant associations were found ($P = 0.738$ and $P = 0.401$, respectively).

### 5.3.2 Ratt values of different spirochaete strains with mucin

The mean and standard deviations of the *Ratt* values for the 15 *B. pilosicoli* strains at seven mucin concentrations are presented in Table 5.2. For each strain the different *Ratt* values across the mucin concentrations all showed significant differences ($P < 0.0001$). The results for individual strains varied considerably. Strain Cof-10, isolated from a pig, was not attracted to any concentration of mucin, and indeed it was unable to enter solutions of 1% mucin or more. Strain 16242.94, isolated from a dog, showed the greatest attraction. All comparisons of the *Ratt* for the 15 strains at all the mucin concentrations showed statistically significant differences ($P < 0.0001$). Comparisons of sets of strains based on the four different species of origin revealed no significant differences at any mucin concentration. For mucin concentrations up to and including 1%, no strains had a *Ratt* value $> 2$. At mucin concentrations of 2%, two human, one porcine and two canine strains of *B. pilosicoli* had *Ratt* values $> 2$. In 3% mucin all strains except Cof-10 and L72 had *Ratt* values $> 2$, and at 4% and 6% mucin only Cof-10 did not show evidence of attraction to mucin. For nearly all strains the attraction increased with increasing mucin concentrations, up to 6%. The *Ratt* values for some *B. pilosicoli* strains with 6% mucin were extremely high, and exceeded those for *B. hyodysenteriae* B204 (Figure 5.1).
Table 5.2. Mean and standard deviation of relative attraction index \((\text{Ratt})\) at seven different concentrations of porcine gastric mucin, and the number of spirochaete cells in chemotaxis buffer tubes, for 15 strains of \textit{Brachyspira pilosicoli} after 90 mins incubation. Cells with significant attraction to mucin \((\text{Ratt} > 2)\) are outlined.

<table>
<thead>
<tr>
<th>Species of origin</th>
<th>Strain</th>
<th>% mucin concentration (%)</th>
<th>0.2</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>Cell no. ((\times 10^6)^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>WesB</td>
<td></td>
<td>1.06 ± 0.39</td>
<td>1.32 ± 0.12</td>
<td>0.63 ± 0.08</td>
<td>1.17 ± 0.28</td>
<td>4.94 ± 0.28</td>
<td>2.91 ± 0.94</td>
<td>22.6 ± 2.52</td>
<td>2.01 ± 0.65</td>
</tr>
<tr>
<td>Human</td>
<td>Karlton</td>
<td></td>
<td>0.78 ± 0.05</td>
<td>0.56 ± 0.15</td>
<td>0.38 ± 0.07</td>
<td>1.19 ± 0.05</td>
<td>3.59 ± 0.62</td>
<td>4.44 ± 0.85</td>
<td>22.64 ± 2.95</td>
<td>1.67 ± 0.30</td>
</tr>
<tr>
<td>Human</td>
<td>PE-90</td>
<td></td>
<td>1.08 ± 0.16</td>
<td>1.16 ± 0.17</td>
<td>1.31 ± 0.11</td>
<td>8.27 ± 0.75</td>
<td>16.01 ± 1.23</td>
<td>21.38 ± 1.77</td>
<td>40.34 ± 2.45</td>
<td>4.26 ± 0.66</td>
</tr>
<tr>
<td>Human</td>
<td>BR 81/80</td>
<td></td>
<td>1.1 ± 0.14</td>
<td>1.30 ± 0.1</td>
<td>1.5 ± 0.11</td>
<td>9.32 ± 1.57</td>
<td>20.62 ± 1.39</td>
<td>26.63 ± 1.03</td>
<td>28.96 ± 1.71</td>
<td>2.82 ± 0.55</td>
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<td>Human</td>
<td>V2H5</td>
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<td>1.12 ± 0.13</td>
<td>0.99 ± 0.10</td>
<td>1.06 ± 0.09</td>
<td>4.38 ± 0.33</td>
<td>10.97 ± 0.70</td>
<td>16.04 ± 2.26</td>
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<td></td>
<td>0.63 ± 0.17</td>
<td>0.52 ± 0.06</td>
<td>0.40 ± 0.07</td>
<td>1.8 ± 0.12</td>
<td>6.32 ± 1.08</td>
<td>12.79 ± 1.01</td>
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<td>7.39 ± 1.01</td>
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<tr>
<td>Pig</td>
<td>95/1000</td>
<td>0.87 ± 0.16</td>
<td>1.01 ± 0.06</td>
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<td>5.22 ± 0.21</td>
<td>9.09 ± 0.81</td>
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<td>0.62 ± 0.04</td>
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<td>1.91 ± 0.53</td>
<td>5.86 ± 1.10</td>
<td>29.84 ± 2.43</td>
<td>5.82 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>Cof-10</td>
<td>0.27 ± 0.08</td>
<td>0.13 ± 0.12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.9 ± 0.09</td>
<td></td>
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<tr>
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<td>CSP-1</td>
<td>0.71 ± 0.58</td>
<td>0.58 ± 0.09</td>
<td>0.93 ± 0.05</td>
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<td>4.57 ± 0.37</td>
<td>9.52 ± 1.22</td>
<td>39.91 ± 1.41</td>
<td>7.62 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>Chicken</td>
<td>98.0026</td>
<td>1.07 ± 0.17</td>
<td>0.78 ± 0.05</td>
<td>0.67 ± 0.04</td>
<td>0.75 ± 0.14</td>
<td>2.60 ± 0.61</td>
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<td>35.96 ± 1.42</td>
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</tr>
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<td>1.07 ± 0.11</td>
<td>0.86 ± 0.05</td>
<td>0.59 ± 0.10</td>
<td>1.22 ± 0.05</td>
<td>5.23 ± 0.47</td>
<td>11.57 ± 0.46</td>
<td>27.91 ± 0.85</td>
<td>6.80 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>Boof</td>
<td>0.73 ± 0.11</td>
<td>0.65 ± 0.07</td>
<td>0.98 ± 0.12</td>
<td>2.91 ± 0.12</td>
<td>4.49 ± 1.0</td>
<td>8.03 ± 1.51</td>
<td>39.99 ± 1.0</td>
<td>3.32 ± 0.85</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>16242.94</td>
<td>0.52 ± 0.08</td>
<td>0.85 ± 0.33</td>
<td>0.93 ± 0.34</td>
<td>3.73 ± 0.37</td>
<td>13.52 ± 2.0</td>
<td>17.63 ± 1.17</td>
<td>51.24 ± 3.22</td>
<td>1.66 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>D 148</td>
<td>0.93 ± 0.03</td>
<td>0.44 ± 0.03</td>
<td>0.34 ± 0.11</td>
<td>0.45 ± 0.17</td>
<td>4.76 ± 2.2</td>
<td>16.31 ± 3.47</td>
<td>16.11 ± 0.44</td>
<td>2.79 ± 0.17</td>
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</tr>
</tbody>
</table>

*a*Spirochaete cell numbers in tubes just containing chemotaxis buffer
5.3.3 *Ratt* values of 95/1000 with D-serine

*B. pilosicoli* strain 95/1000 only showed a significant *Ratt* (2.58 ± 0.55) with the 100 mM solution of D-serine.

5.3.4 *Ratt* values of 95/1000 and B204 in relation to viscosity

The viscosities of the mucin and PVP dilutions are listed in Table 5.3. The *Ratt* results in PVP in relation to their viscosity for *B. pilosicoli* 95/1000 and *B. hyodysenteriae* B204 are shown in Figure 5.3. For 95/1000, the *Ratt* increased with increasing PVP concentrations to plateau from a viscosity of around 8 mPa.s. These *Ratt* values followed the same trend seen with mucin at the same viscosities, but at a given viscosity were only about 20% of the values obtained with mucin (Figure 5.4). For B204, the *Ratt* continued to increase with increasing viscosities of the PVP solutions, although the *Ratt* had declined in the mucin solutions at the (similar) high viscosities. As with 95/1000, the *Ratt* values obtained with B204 generally were much higher in the mucin solutions than in the PVP solutions; however, at the higher viscosities the *Ratt* in mucin declined to values similar to those in the PVP solutions.
Table 5.3. Viscosities of the dilutions of mucin and polyvinylpyrrolidone (PVP) used in the attraction assays.

<table>
<thead>
<tr>
<th>Mucin (%)</th>
<th>Viscosity (mPa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>0.5</td>
<td>1.3</td>
</tr>
<tr>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>2.0</td>
<td>1.6</td>
</tr>
<tr>
<td>3.0</td>
<td>1.9</td>
</tr>
<tr>
<td>4.0</td>
<td>2.9</td>
</tr>
<tr>
<td>6.0</td>
<td>7.2</td>
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<tr>
<td>8.0</td>
<td>12.4</td>
</tr>
<tr>
<td>10.0</td>
<td>13.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PVP (%)</th>
<th>Viscosity (mPa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>1.5</td>
</tr>
<tr>
<td>0.75</td>
<td>2.1</td>
</tr>
<tr>
<td>1.25</td>
<td>3.5</td>
</tr>
<tr>
<td>1.5</td>
<td>4.3</td>
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<tr>
<td>2.0</td>
<td>5.8</td>
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<td>2.5</td>
<td>7.8</td>
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<tr>
<td>3.0</td>
<td>10.8</td>
</tr>
<tr>
<td>4.0</td>
<td>16.9</td>
</tr>
</tbody>
</table>

5.3.5 Viscosity of caecal and colonic mucus

The mucus layer overlying the epithelium in the porcine caecum and colon had viscosities of 7.53 ± 1.89 and 7.16 ± 2.21 mPa.s, respectively. These values were similar to those of the 6% mucin concentration (Table 5.3).
Figure 5.3: $Ratt$ values obtained for *B. pilosicoli* 95/1000 (filled circles) and *B. hyodysenteriae* B204 (open circles) in dilutions of PVC plotted against measured viscosities.

Figure 5.4: $Ratt$ values obtained for *B. pilosicoli* 95/1000 (filled circles) and *B. hyodysenteriae* B204 (open circles) in dilutions of mucin plotted against measured viscosities.
5.4 Discussion

In optimizing the capillary tube assay, the chemotaxis buffer supported the motility of the spirochaetes better than did PBS, and this is in agreement with previous findings (Milner and Sellwood 1994). On the other hand, an incubation period of 90 minutes was preferable to the incubation period of 60 minutes that has previously been used in similar experiments (Milner and Sellwood 1994; Witters and Duhamel 1999), and hence this was selected for use throughout the study. The use of a shorter incubation time by previous investigators might have underestimated the extent of the attraction to mucus that the previous workers recorded for a given strain.

A striking and important finding early in this study was the observation that the attraction of *B. pilosicoli* strain 95/1000 and *B. hyodysenteriae* strain B204 increased with increasing mucin concentrations. Previous studies on attraction to mucin by *B. pilosicoli* and *B. hyodysenteriae* have used 1% mucin, and it is clear that this concentration is not optimal for assessing how the spirochaetes are attracted to mucin. Indeed, in the current study no strains showed attraction to 1% mucin. These differences in the assay conditions help to explain the discrepancies in the results in the current study with those of the previous studies, where *B. pilosicoli* strains showed little or no attraction to mucin.

A factor potentially influencing the viability of the spirochaetes and their attraction to mucin is the medium and conditions under which they are grown. For example, Witters and Duhamel (1999) found that two *B.*
*pilosicoli* strains showed attraction to mucin only if they were grown in a defined medium either containing pig faecal extract, or 0.1% mucin. The influence of the culture medium was not investigated in the current study, as clear responses were obtained under the standard culture conditions that were used.

Another important finding in the study was that attraction to mucin varied considerably between *B. pilosicoli* strains, with one of the 15 strains (Cof-10) not showing any attraction. Furthermore, the spirochaete strains varied in their capacity to enter the chemotaxis buffer, and this was taken to indicate that they also varied in their intrinsic motility under a given set of environmental conditions. Although Cof-10 was the least motile strain, and it was not attracted to mucin, over all the strains greater or lesser “basal” motility did not necessarily equate with greater or lesser attraction to mucin. *In vivo*, the capacity to efficiently colonize and cause disease presumably requires both motility and strong attraction to mucin. Cof-10 was isolated from an Australian pig as part of a prevalence survey, but its ability to cause disease has not been investigated experimentally. On the other hand, *B. pilosicoli* strains WesB, P43/6/78T, 95/1000 and CSP-1 as well as other strains have been used successfully to experimentally infect pigs and/or chickens (Taylor et al 1980; Trott et al 1995; Trott et al 1996b; Stephens and Hampson 2002). These strains were all found to be motile and to be attracted to mucin in the current study, although they differed in the extent of this activity. Ideally strains with known attraction and motility properties should be tested simultaneously in an animal model to determine whether the *in*
vitro measurements equate with in vivo colonization capacity, and pathogenic potential. It was interesting that the human strains PE-90 and BR 81/80, isolated from the bloodstream of French patients (Trott et al 1997b), were strongly attracted to mucin. This attribute may have facilitated their colonization of the large intestine in these patients, and increased opportunities for their translocation through the colonic epithelium.

In this study there was no significant correlation between the species from which a given strain of B. pilosicoli was isolated and the extent of its attraction to mucin, or motility. This is consistent with previous observations that strains of B. pilosicoli from humans do not form a genetically distinct group from strains recovered from animals (Hampson et al 2006). Strains from all sources varied in their properties, and this supports the likelihood that cross-species transmission of B. pilosicoli strains can occur.

Mucin is a serine-threonine-proline rich protein with covalently bound polysaccharides, and such amino acids and polysaccharides can bind to chemotaxis receptors on the surface of various bacteria, including spirochaetes (Laux et al 2000). Previously B. pilosicoli strain P43/6/78\textsuperscript{T} has been shown to be chemotactic to DL-serine (Witters and Duhamel 1999), a component of mucin, and a similar response to D-Serine was obtained in the current study with strain 95/1000. Hence it appears likely that the attraction to mucin by B. pilosicoli includes an element of chemotaxis. Other potential attractants that would be present in mucin and the mucous gel would include a range of simple sugars, and further work is required to identify these.
Previously *B. hyodysenteriae* has been shown to be chemotactic towards a variety of mucin components (Kennedy and Yancey 1996), and *B. hyodysenteriae* is known to have a large number of genes encoding methyl-accepting chemotaxis proteins (46) and chemosensory transductor molecules (17) (Bellgard et al 2009). Less is known about *B. pilosicoli*, although one putative glucose-galactose chemoreceptor has been described (Zhang et al 2000). It is not clear how chemotactic molecules would be released from the mucin solutions that were used in the *in vitro* assay in the current study, although *B. pilosicoli* is known to possess membrane-associated serine proteases and other proteases that could be involved in their degradation and release (Dassanayake et al 2004).

It was noted that the higher concentrations of mucin that were used were highly viscous (Table 5.3), and these concentrations had similar viscosities to those that were found in the mucus layer overlying the epithelium in the porcine caecum and colon. Hence, the behaviour of the strains at the higher mucin concentrations is likely to reflect their activities *in vivo*, when they encounter the mucus barrier. This relationship led to an investigation of the potential involvement of the viscosity of the mucin solutions in their attractiveness to the spirochaetes. Previously, a strain of the spirochaete *Leptospira interrogans* (*biflex*) was shown to possess a “viscotaxis” response, whereby it was attracted to a viscosity gradient made up of different concentrations of the viscous linear polymer PVP (Petrino and Doetsch 1978). When *B. pilosicoli* 95/1000 was tested against dilutions of
PVP with different viscosities, it showed a response that resembled its response to mucin, although the \textit{Ratt} values were numerically considerably less than those achieved with mucin at a given viscosity. Hence, it appears that at least part of the response of \textit{B. pilosicoli} to mucin is likely to be a form of viscotaxis. In future work it would be interesting to add chemotactic materials from mucin to the PVP solutions, to see whether the combinations would increase the overall \textit{Ratt} to levels similar to those achieved with mucin. It is not clear how a viscotaxis response could function, but \textit{B. pilosicoli} strain NK1f has been shown to develop improved motion efficiency with increasing viscosity of a motility medium (Nakamura et al 2006). Hence it is possible that the spirochaete cells that entered the viscous solutions through normal motility developed improved motion efficiency in the solutions, and then travelled up the tubes. In this way, at least part of the observed “viscotaxis” may be simply due to increases in motion efficiency in response to increasing viscosity of the medium.

It was interesting that \textit{B. hyodysenteriae} strain B204 showed a somewhat different pattern of “attraction” to mucin, with this phenomenon declining at higher mucin concentrations. In explanation, initially it was thought that \textit{B. pilosicoli} and \textit{B. hyodysenteriae} might differ in their changes to motion efficiency relative to the viscosity, perhaps related to the fact that \textit{B. pilosicoli} strains have 8-12 periplasmic flagella per cell, whilst \textit{B. hyodysenteriae} strains have 22-28 (Stanton 2006). When B204 was tested with the PVP solutions, however, its \textit{Ratt} values continued to increase as the viscosity increased. It appears therefore that factors other than viscosity may
affect the behaviour of *B. hyodysenteriae* in high concentrations of mucin. For example, at high mucin concentrations the response to the chemoattractant properties of mucin may be switched off, such that only the viscotaxis effects still operate. This would then explain why, at a viscosity of around 14 mPa.s, the *Ratt* for B204 in mucin had declined to a value similar to that found in PVP (Figures 5.3 and 5.4).

The apparent viscotaxis of the spirochaetes was of practical interest and application, as different dietary ingredients and/or the addition of carboxymethylcellulose to experimental pig diets have been shown to influence the viscosity of the contents in the large intestine, with higher viscosities being associated with greater colonization by *B. pilosicoli* (Hampson et al 2000; Hopwood et al 2002; Lindecrona et al 2002). Hence there may be a link between viscosity of the colonic contents and the propensity for colonization with *B. pilosicoli* and *B. hyodysenteriae*. Such a link would also be important in disease states, where the composition or viscosity of the mucus layer might change. For example, in swine dysentery there is a great outpouring of mucus from the colonic crypts in response to the presence of *B. hyodysenteriae*. Depending on the composition and viscosity of this material, it might either enhance or inhibit colonization by this spirochaete species. The differences in response to mucin by the two pathogenic species also may help to account for the different behaviours and colonization sites for the two species. *B. hyodysenteriae* is found in the lumen of the colon and deep in the colonic crypts, where it enters goblet cells. Although *B. pilosicoli* also can enter the crypts, unlike *B.
*B. hyodysenteriae* it specifically penetrates the dense mucus layer at the crypt shoulders, and attaches to the underlying colonic enterocytes (Jensen et al 2000). Presumably, as the mucus concentration increases towards the enterocyte surface, the attraction to mucin and the motion efficiency of *B. pilosicoli* would continue to increase, and this may be the means by which the subsequent specific attachment to the cell surface is facilitated. On the other hand, the densest part of the mucus barrier at the cell surface might be less attractive to *B. hyodysenteriae*, such that it does not undergo polar attachment.
6.1 Introduction

Norepinephrine (NE) has been shown to have important effects on a range of different pathogenic bacterial species, but, despite the presence of NE in the gastrointestinal tract, to date no work has been reported on potential modulating effects of NE on *B. pilosicoli* (or other intestinal spirochaetes). Consequently, the aim of the work described in this chapter was to investigate potential effects of NE exposure on growth, motility, attraction and Caco-2 cell attachment of *B. pilosicoli*.

6.2 Materials and Methods

6.2.1 Preparation of norepinephrine (NE) stock solution

Stock solutions (0.01 M) of norepinephrine bitartrate salt were prepared in PBS and were sterilized by passing them through a 0.22 µm filter. The stock solutions were prepared just before the start of each experiment, and were held in a dark brown glass vessel to avoid exposure to light.

6.2.2 Effect of NE on the growth of *B. pilosicoli* 95/1000

A set of 20 mL glass tubes each containing nine mL of Kunkle’s broth medium was prepared and autoclaved at 121°C for 15 minutes, as described in Chapter Two. The tubes were wrapped with aluminium foil to keep them dark, and each was seeded with 0.5 mL of *B. pilosicoli* 95/1000 broth-culture (10⁷/mL). A fresh stock solution of NE was prepared in a dark glass bottle as described earlier, and a sufficient amount of the solution was added to the
broth to achieve concentrations of 0.05, 0.1, 0.5 and 1 mM NE. An equal amount of sterilized PBS was added to the control tubes. There were six replicates of each NE concentration and the NE-free control. The tubes were incubated on a rocking platform at 37°C for four days. On day four, the spirochaetes in the tubes were counted, as described in Chapter Two. One-way analysis of variance (ANOVA) was used to compare the means from the different groups.

6.2.3 Effect of NE on *B. pilosicoli* attraction to mucin or buffer

These experiments were conducted using the general methods described in Chapter Five. The NE was added either to the *B. pilosicoli* culture prior to the mucin attraction assay, added to the chemotaxis buffer, or added to the mucin solution in the haematocrit tubes.

6.2.3.1 NE added to the broth culture

A fresh culture of *B. pilosicoli* 95/1000 was harvested at mid log-phase (10⁸/mL). A stock solution of NE was prepared as described earlier, and, once the assay was ready, the NE was added to the culture to obtain final concentrations of 0.05, 0.1, 0.5 and 1 mM. Four percent hog gastric mucin, type II was dissolved in chemotaxis buffer. Six 75 mm haematocrit capillary tubes were filled with 4% mucin concentration for each NE concentration, and six paired tubes were filled with 4% mucin without NE. The attraction assays were conducted for 90 minutes at 37°C in a CO₂ incubator, and samples were processed for bacterial counting as described in Chapter 5.
6.2.3.2 NE added to chemotaxis buffer
In this experiment NE was added to chemotaxis buffer to final concentrations of 0.05, 0.1, 0.5 and 1 mM. Six haematocrit capillary tubes were filled with buffer and each of the NE concentrations. The assays were conducted using *B. pilosicoli* 95/1000 for 90 minutes at 37°C in a CO₂ incubator, as described above.

6.2.3.3 NE added to 4% mucin
In this experiment NE was added to final concentrations of 0.05, 0.1, 0.5 and 1 mM in 4% hog gastric mucin. Six haematocrit capillary tubes were filled with the mucin solution and each of the NE concentrations. The assays were conducted using *B. pilosicoli* 95/1000 for 90 minutes at 37°C in CO₂ incubator, as described above.

6.2.4 Effect of NE on *B. pilosicoli* attachment to Caco2 cells
The attachment assays were conducted as described in Chapter Four. The NE was either added to the *B. pilosicoli* culture at the time of the assay, or spirochaetes that had been grown in the presence of NE were tested for their ability to attach to Caco-2 cells.

6.2.4.1 Immediate effects of NE addition
A fresh mid-log phase broth culture of *B. pilosicoli* strain 95/1000 (10⁸ cells/mL), prepared as described in Chapter Two, was harvested. When the assay was ready, NE was added to the culture to give a final dilution of 0.1 mM. One mL of this culture was added to two-week-old confluent Caco-
2 cells grown on 10 mm round glass coverslips in 48 well plates (as described in Chapter 4), and incubated for 2, 4 and 6 hours. A minimum of three wells was allocated for each time interval. The wells then were washed three times with PBS and the coverslips were processed for SEM as described in Chapter Four.

6.2.4.2 Effect of cultures grown with NE

The stock solution of NE was prepared as described above. *B. pilosicoli* 95/1000 was inoculated into 200 mL of broth in a 500 mL glass bottle. The bottle was wrapped with aluminum foil to protect the NE from light degradation, and sufficient NE stock solution was added to the broth to bring the NE level to 0.1 mM. The control culture did not have NE added. The broths were incubated on a rocking platform at 37°C for 2 days until the bacteria were in mid-log phase with a cell count of ~10^8/mL, and then they were harvested by centrifugation. Two week-old, confluent Caco-2 cells grown on 10 mm round coverslips in 48 well plates were exposed with 1 ml of the culture for six hours. The cells then were processed for SEM as described above.

6.2.4.3 Quantitative scoring of attachment and statistical analysis

The degree of attachment was scored and analyzed in the same way as described in Chapter Four.
6.3 Results

6.3.1. Effect of NE on growth of *B. pilosicoli*

The addition of NE to the *B. pilosicoli* culture resulted in a significant (*P* > 0.002) increase in growth only with the lowest concentration of 0.05 mM NE (Figure 6.1). With this concentration the number of spirochaetes had increased from approximately $5 \times 10^7$ per mL to just over $8 \times 10^7$ per mL after four days incubation. The numbers of bacteria also were higher with the 0.1 mM NE concentration than with the control, but the difference was not statistically significant. The number of bacteria in the two remaining NE concentrations (0.5 and 1 mM) did not differ significantly from the control.

![Figure 6.1: Effect of Norepinephrine on the growth of *B. pilosicoli* 95/1000 in liquid medium](image)

Figure 6.1: Effect of Norepinephrine on the growth of *B. pilosicoli* 95/1000 in liquid medium
6.3.2 Effect of NE on the attraction of *B. pilosicoli* to mucin or buffer

6.3.2.1 NE added to the spirochaete culture

The results of the assays where *B. pilosicoli* was exposed to different concentrations of NE at the time they were added to the assay are summarized in Figure 6.2. The *B. pilosicoli* culture with the lowest NE concentration (0.05 mM) showed the greatest attraction to 4% mucin. The number of spirochaetes in the mucin solution was significantly higher \((P > 0.02)\) at 0.05 mM NE than in the control without NE, and all other differences were not significant.

![Figure 6.2. Effect of NE addition on the attraction of *B. pilosicoli* to 4% mucin.](image)

6.3.2.2 NE added to 4% mucin

In this experiment, different NE concentrations were mixed in 4% mucin. NE levels of 0.05 \((P > 0.07)\), 0.1 \((P > 0.03)\) and 0.5 mM \((P = 0.005)\) attracted
significantly more bacteria than the control. The highest NE concentration (1mM) was not effective in attracting the spirochaete (Figure 6.3).

Figure 6.3. *B. pilosicoli* attraction to different NE concentrations in 4% mucin

### 6.3.2.3 NE added to the chemotaxis buffer

The effect of NE on *B. pilosicoli* numbers in chemotaxis buffer alone is shown in Figure 6.4. All except the lowest (0.05 mM) NE concentrations in chemotaxis buffer attracted significantly higher numbers of bacteria than were present in the plain control chemotaxis buffer (*P* > 0.004). The number of bacteria at the lowest NE level (0.05 mM) was significantly less (*P* > 0.03) than in the other three NE concentrations. The bacterial count did not differ significantly at NE levels of 0.1, 0.5 and 1 mM.
6.3.4 Effects of NE on attachment to Caco-2 cells

The effects of NE on attachment of \textit{B. pilosicoli} were studied in two ways. In the first the NE was added immediately before adding the \textit{B. pilosicoli} culture to the Caco-2 cells, and in the second \textit{B. pilosicoli} was exposed to NE during its growth.

6.3.4.1 Attachment assays with cultures of \textit{B. pilosicoli} immediately exposed to NE

The \textit{B. pilosicoli} cultures that had and had not been exposed to 0.1 mM NE immediately prior to adding to the attachment assay both showed a time dependent increase in attachment to the Caco-2 cells, but at all time points more of the NE-treated spirochaetes were attached (Figures 6.5 through 6.10). The NE-exposed \textit{B. pilosicoli} cells tended to clump and tangle in their attachment to the Caco-2 cells (Figure 6.11).

Figure 6.4: Effect of NE on \textit{B. pilosicoli} numbers in chemotaxis buffer
Figure 6.5: *B. pilosicoli* 95/1000, not exposed to NE, after two hours (control). Relatively low numbers of bacteria were attached.

Figure 6.6: *B. pilosicoli* 95/1000, exposed to NE, after two hours. More spirochaetes are attached than with the control.
Figure 6.7: Control *B. pilosicoli* 95/1000, not exposed to NE, after four hours. Relatively fewer bacteria were attached than with the NE-exposed culture.

Figure 6.8: *B. pilosicoli* 95/1000, exposed to NE, after four hours. More spirochaetes are attached than with the control (the layer of spirochaete is thicker).
Figure 6.9: Control *B. pilosicoli* 95/1000 culture, not been exposed to NE, after six hours. Good attachment to Caco-2 cells. Original magnification x 4000.

Figure 6.10: *B. pilosicoli* 95/1000, exposed to NE, after six hours. More spirochaetes are attached than with the control. Original magnification x 4000.
Figure 6.11: Extensive attachment with clumping and tangling of *B. pilosicoli* following exposure to NE for 6 hours (original magnification x 10500).

### 6.3.4.2 Attachment assays with culture grown with NE

After six hours the *B. pilosicoli* cells grown with 0.1 mM NE showed extensive colonization of the Caco-2 cells, with thick mats of spirochaetes over the cells, obscuring them (Figure 6.12). The attachment was also good in the case of the control (Figure 6.13), but was not as extensive, and the Caco-2 cells were visible. The NE-grown culture showed clumping and tangling of the *B. pilosicoli* cells, resulted in a cobweb-like formation over the Caco-2 cells.
Figure 6.12: *B. pilosicoli* grown with 0.1 mM NE for 2 days, showing extensive colonization of the Caco-2 cells after six hours. Original magnification x 1000.

Figure 6.13: *B. pilosicoli* grown without NE for two days, showing less attachment than the NE-grown culture after six hours (some cell surfaces are still visible). Original magnification x 1000.
6.4 Discussion

This study provided evidence that exposure to NE can modulate some properties of *B. pilosicoli*. This spirochaete is a slow-growing anaerobe, and it was encouraging to find that a concentration of 0.05 mM NE significantly enhanced the growth rate. A concentration of 0.1 mM also resulted in greater growth than in the control, but it was interesting that concentrations greater than this were not effective at enhancing growth. As mentioned in the literature review, it has been reported that the growth of many, although not all, bacterial species is enhanced by NE, and generally a concentration of 0.1 mM has been most effective (eg for *Campylobacter jejuni*; Cogan et al 2007).

The mechanism(s) whereby NE enhanced the growth rate of *B. pilosicoli* are uncertain. Examples of some suggested mechanisms for other bacterial species are listed in Table 6.1. In the case of *Salmonella* spp., the effects of catecholamine are thought to be mediated via iron uptake, and the involvement of cell surface siderophore-receptor proteins also has been suggested (Williams et al 2005). *C. jejuni* showed a 50-fold increase in growth due to NE exposure (Cogan et al 2006), and this bacterium is also known to express surface siderophore-receptor proteins (van Vliet et al 2002). The mechanism of modulation by NE may differ from one bacterial species to another. In the case of *C. jejuni*, NE did not enter into the bacterial cells but it enhanced iron internalization (Cogan et al 2006), whilst in the case of *E. coli* there was NE internalization along with internalization of iron (Freestone et al 2000). NE also facilitates iron acquisition by *E. coli* from the
Table 6.1. Some suggested mechanisms by which NE may affect the growth of different bacterial species

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Growth pattern</th>
<th>Suggested Mechanism</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli strain E2348/69</td>
<td>NE exposure supplies iron from mammalian transferrin and lactoferrin for bacterial growth</td>
<td>Freestone et al 2000</td>
<td></td>
</tr>
<tr>
<td>Porphyromonas gingivalis Bacteroides fragilis Shigella boydii Shigella sonnie Enterobacter Sp Salmonella choleraesuis E. coli - control</td>
<td>There was no increase in the growth of any of these bacteria</td>
<td>NA Belay et al 2003</td>
<td></td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>2 log increase</td>
<td>Increased iron uptake by bacterial cells exposed with NE</td>
<td>Cogan et al 2006</td>
</tr>
<tr>
<td>Klebsiella pneumoniae Pseudomonas aeruginosa Enterobacter cloacae Shigella sonnei Staphylococcus aureus</td>
<td>6-9 log increase 5 log increase 1-3 log increase 1-8 log increase &gt; 1 log increase</td>
<td>Transferrin involved in NE-induced growth</td>
<td>O’Donnell et al 2006</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>Significant increase</td>
<td>NE stimulated enterobactin synthesis and TonB-dependent iron uptake by bacterial cells. Mutations in these genes prevent growth in the presence of NE</td>
<td>Methner et al 2008</td>
</tr>
</tbody>
</table>
mammalian iron binding proteins transferrin and lactoferrin (Freestone et al 2000). In the case of *Salmonella enterica*, the NE growth-stimulating effects have been shown to depend on enterobactin synthesis and TonB-dependent iron uptake, as mutations in these genes prevented growth in the presence of NE (Methner et al 2008). It would be of interest to determine whether genes associated with iron acquisition are upregulated in *B. pilosicoli* cells that are exposed to NE.

In addition, NE may affect some of the pathways that are involved in quorum sensing in bacteria, including growth stimulation and pathogenesis. An example can be seen with enterohaemorrhagic *E. coli* (EHEC). In these important enteric pathogens expression of the locus of enterocytes, effacement (LEE) genes that are involved in production of attaching and effacing lesions involves the *luxS* quorum sensing system, which regulates the autoinducer -3 (AI-3) (Jarvis et al 1995). Exposure to NE also activated the LEE genes in a manner similar to that of AI-3 in a study where mutants and wild strains of EHEC were exposed to NE or epinephrine (Walters and Sperandio 2006; Kendall et al 2007). In *E. coli* O157:H7, NE has been reported to trigger the production of a different growth autoinducer (Lyte et al 1996). Similarly, the presence of NE and some other quorum sensing molecules has been correlated with *in vitro* and *in vivo* pathogenesis of *Salmonella enterica* serovar Typhimurium (Bearson and Bearson 2008; Moreira et al 2009).
The increase seen in *B. pilosicoli* cell numbers associated with NE exposure after four days incubation was not large, being just over $8 \times 10^7$ cells per mL compared to $5 \times 10^7$ per mL in the controls. Other bacterial species have shown far greater increases in growth after NE exposure. For example, as previously mentioned, *C. jejuni* has shown a 50-fold increase in growth due to NE exposure (Cogan et al 2006). Nevertheless, any increase in growth rate for *B. pilosicoli* could be helpful when isolating or preparing cultures of the spirochaetes in the laboratory, and consideration should be given to adding NE to such media.

In this study the potential influence of NE on the motility and movement of the spirochaete also was investigated in several ways. Addition of 0.05 M NE to the culture just prior to starting the mucin attraction assay resulted in significantly more spirochaetes entering the 4% mucin solution than occurred with the controls or with the other NE concentrations. This demonstrated the occurrence of a rapid change in *B. pilosicoli* behaviour in an NE dose-dependent fashion. Presumably the NE-induced changes increased the spirochaete’s motility or motion efficiency, and/or possibly changed its responsiveness to chemotactic signals from the mucin. In order to determine the mechanism(s) involved in this change in behaviour, it could be informative to look at transcriptomics profiles of the spirochaete immediately after addition of 0.05 M NE to see what genes have altered expression. For example, in the case of enteropathogenic *E. coli*, microarrays analysis before and after NE exposure revealed that the NE exposure upregulated the expression of several genes, including motility clusters (Bansal et al 2007).
The addition of NE to the chemotaxis buffer resulted in a dose-dependent response, whereby spirochaete numbers increased in 0.05 M NE, then peaked at 0.1 M and then showed a plateau in numbers. In this case the most likely explanation for the results was that the NE acted as a chemoattractant for the spirochaete. Similar chemoattractant responses to NE have been seen with other bacteria (Bansal et al 2007; Bearson and Bearson 2008; Hughes et al 2009). When the NE was added to the 4% mucin tubes, spirochaete numbers again increased in a NE dose-dependent manner, but this time peaked at 0.5 M NE, and then declined at 1 M NE to a level similar to the controls. Again, in this assay the NE may have been acting as a chemoattractant – although it is possible that once the spirochaetes entered the mucin solutions and encountered the NE, their motility and chemoattractant response to other mucin components may have been stimulated, resulting in greater movement into the mucin. It is unclear why the 1 M NE failed to act as a chemoattractant in mucin, whilst this concentration was apparently still attractive when presented in chemotaxis buffer.

It was of interest to know whether the changes in motility/chemotaxis induced by the NE would influence attachment of the spirochaetes to Caco-2 cells. Therefore, an experiment was conducted where NE was added to a final concentration of 0.05 M to a B. pilosicoli culture just before conducting an attachment assay. Subjectively, more NE-exposed spirochaetes appeared to attach, and they appeared denser and more tangled than with the control.
culture that was not exposed to NE. Attachment also occurred more rapidly with the NE-exposed cells. Similarly, when the attachment assay was performed with a culture grown with 0.05 mM NE for two days, more spirochaetes appeared to be attached to the Caco-2 cells than with the control culture that had not been exposed to NE. The interpretation of this second experiment was complicated by the fact that the greater apparent attachment could have been due to there being more bacteria in the NE-grown culture than in the control, as previously it was shown that NE exposure enhances *B. pilosicoli* growth. It was an over right not to establish the cell numbers.

The resulted from the two experiments do suggest that exposure to NE enhanced colonization, but the subjective nature of the measurement of attachment was a recognised weakness in this assay. In future work it would be useful to quantitated the attached bacteria, for example using quantitative-PCR, or specific labelling and counting of bacterial cells, as has been done for quantifying adhesion of *Bifidobacterium, E. coli, Salmonella* and *Yersinia* to Caco-2 cells (Candela et al 2005). In other cases attached bacteria have been removed by treating cell monolayers with 0.1% Triton X-100 in PBS and then inoculating them into a suitable medium to count colony forming units (Bansal et al 2007; Cogan et al 2007).

Others have reported that NE can enhance attachment of bacteria species. For example, Bansal et al (2007) found that exposure to 0.05 mM NE enhanced the attachment of *E. coli* O157:H7 to HeLa cells 5.2 fold. In another study there was a ten-fold increase in attachment to Caco-2 cells with a *C. jejuni*
culture exposed to NE compared to a non-NE exposed culture (Cogan et al 2007). Furthermore, the NE exposed culture of *C. jejuni* induced more cytotoxic effects, including occludin disruption and decreased transepithelial resistance in Caco-2 cells than the non-treated cultures. In future work it would be informative to investigate whether cultures of *B. pilosicoli* exposed to NE showed an increased cytopathic effect with the Caco-2 cells. These *in vitro* effects are likely to have relevance to the whole animal, as catecholamines have been shown to increase the adherence of *E. coli* O157 to intestinal mucosa in different *in vivo* models of infection (Chen et al 2003; Green et al 2004).

Even though the concentration of NE has not been measured in the intestinal contents around the mucosa of infected and non-infected pigs, poultry and human beings, it is reasonable to assume that *B. pilosicoli* encounters NE during its colonization of the intestinal mucosa. It has been reported that a very high local concentration of norepinephrine exists in the GI tract, which can arise from leakage into the intestinal lumen through diffusion driven by a concentration gradient (Lyte and Bailey 1997). Evidence for this can be seen from the fact that addition of 0.01 mM NE to the contraluminal side of mucosal explants from the caecum and colon increased enterohaemorrhagic *E. coli* adherence on the luminal side (Chen et al 2006).

In conclusion, exposure to low concentrations of NE (0.05 mM) promotes the growth of *B. pilosicoli*. NE also appears to increase spirochaete motility, attraction to mucin and the rapidity and possibly the extent of attachment to
Caco-2 Cells. Different concentrations of NE appeared to have different effects, but this is not unexpected given the complex pathways by which NE is likely to mediate its various effects. Further more detailed studies need to be carried out to record gene expression changes due to NE exposure. NE levels also should be monitored in infected and non-infected animals. Furthermore, *in vivo* studies could be conducted to determine the relevance of the *in vitro* changes that have been observed; for example, mice or other species could be infected with NE-exposed and non-exposed *B. pilosicoli* cultures, to determine whether the exposure to NE makes them more pathogenic. Unfortunately there was insufficient time to conduct such experiments within the constraints of my PhD programme.
In many ways the intestinal spirochaete *Brachyspira pilosicoli* can be regarded as a “neglected” pathogen. Despite the fact that the spirochaete has now been recognised as colonizing the large intestines of vast numbers of people in developing countries, and to be common in other species – notably in pigs and chickens, where it is a recognized pathogen – there remains relatively little study being undertaken on this micro-organism. Reasons for this may include difficulties in culturing and working with this slow-growing anaerobic species, but also a failure to establish a clear pathogenic role for the spirochaete in human beings. This is made worse by the distribution of the micro-organism, as it is largely a “third-world” problem, or confined to minority groups in Western countries, so that it is difficult to obtain research funding to work on the spirochaete. Even though the species is recognised as a pig pathogen, the associated disease is not severe, and it is relatively easily controlled using antimicrobials. In relation to poultry, many in the egg industry remain largely ignorant of the occurrence and potential costs of the infection, and they are unwilling to invest in research. Again this lack of knowledge reflects difficulties with diagnosis, but also a lack of data showing that the spirochaete has a “real” pathogenic potential, and hence can affect health, production and profits.

The work of this PhD project was carried out to try to delve deeper into some aspects of the biology of *B. pilosicoli* that previously have not been
addressed. To date, many of the simple *in vitro* methods (as well as more complex genomics-based studies) that have been applied to other enteropathogenic bacteria have not been used with *B. pilosicoli* to obtain further information about the pathogenic potential of the spirochaete. Accordingly, as described in Chapter One, the aim of the work in this thesis was to develop simple *in vitro* models that could be used to study *B. pilosicoli* pathogenesis. The specific objectives were:

1. To develop an *in vitro* attachment model for *B. pilosicoli* by using either cells in suspension (red blood cells) or cell monolayers (Caco-2 cells).

2. To investigate the *in vitro* attraction of *B. pilosicoli* to mucin.

3. To examine the effects of norepinephrine exposure on *in vitro* traits of *B. pilosicoli*.

To a greater or lesser extent, all these objectives were achieved in the study.

Attachment of *B. pilosicoli* by one cell end to the apical surface of colonic enterocytes is a pathognomonic feature of the infection in many species. Of the other spirochaetes, only *B. aalborgi* also attaches in this way, and that species seems to be confined to humans and non-human primates. It is not clear whether this attachment is an absolute requirement for the development of disease, as there have been a number of reports where individual pigs have had *B. pilosicoli* positive faecal cultures and hence appear to be colonized, but end-on attachment was not necessarily observed at post-mortem (eg Thomson et al. 1997). Nevertheless, in the absence of any evidence for the
release of soluble toxic molecules from the spirochaete that could cause diarrhoea and colitis without attachment, it is intuitively likely that attached spirochaetes may have the potential to cause cellular changes that result in local pathological changes in the structure or function of the enterocytes. Such changes can be sought in colonic samples obtained from naturally or experimentally infected animals, but the availability of a simple *in vitro* model of attachment would greatly facilitate studies on these interactions (even though it is recognised that they can never totally equate with the *in vivo* situation).

When looking for a means of studying the attachment process, it was decided to start by focusing on using red blood cells, as these are easy to access and to work with. Other bacterial species (including spirochaetes) are known to interact with and attach to these cells, sometimes causing agglutination. The major initial problem I encountered was growing the spirochaetes, as they have fastidious requirements and grow slowly. Hence it took a considerable amount of patience and practice to be able to grow the spirochaetes to an appropriate concentration for use in the assays. Only four strains of *B. pilosicoli* were used at this stage, due to these practical difficulties. Despite using red blood cells from a range of species, and using various ratios of spirochaetes and cells, attachment was only achieved using nucleated avian cells from geese and chickens, and only with one of the *B. pilosicoli* strains (human strain WesB). The extent of attachment varied depending on the incubation time and the ratio of both cells, with a six hour time interval and 1:1000 (RBC:bacteria) ratio giving the maximum amount of attachment.
In this system one end of the bacterial cells tended to be interacting with the red blood cells, with the spirochaetes either lying flat on the cell membrane of the red blood cells, or occasionally with one end apparently attached to the surface. Despite an extensive search using the TEM, invagination of spirochaete into the membrane of the red blood cells was not observed. It is possible that it did occur, but was too rare to detect, or it may be that the fact that the red cells were able to move freely in the liquid medium meant that it was difficult for the spirochaetes to push into the cell membrane of the mobile cell. As a result of these limitations, and the fact that the surface of the red blood cells may not accurately mirror the situation with enterocytes, it was decided not to try to develop this system further, but to examine cultured enterocytes. The work that had been done was useful, as it clearly indicated that there was specificity in the interaction between the spirochaetes strains and the cells: thus only one strain attached, and only to avian cells. The basis of this specificity was unclear, but it presumably involved interactions between surface molecules on the spirochaete and the cells. These may have involved specific surface proteins, lipoproteins and/or glycans.

Initially when seeking an alternative system for studying attachment of *B. pilosicoli* to colonic cells, explants of colons obtained from foetal pigs were considered. These would be sterile, and the overall architecture of the colon should be preserved. Nevertheless this was considered too difficult for the current project, as there were numerous issues about the availability of
experimental material as well as likely problems in keeping the material sterile and viable for prolonged periods to allow attachment to occur. There had been one previous study where monolayers of a number of different types of intestinal epithelial cells had been used to try to study attachment with *B. pilosicoli* (Muniappa et al 1998). The extent of the interactions varied with the cell type, and no specific end-on attachment of spirochaetes or pathological changes such as actin rearrangement were observed. Nevertheless, it was decided to try one of these cell lines (Caco-2 cells) in this study, as such cells do more closely represent the sorts of cells with which the spirochaete interacts *in vivo*.

Working with this system was quite challenging, as it required coordinating the growth of the Caco-2 cells with the growth of the strains of the fastidious spirochaete - so that they could both be ready for the assays at the same time. Optimising and maintaining the experimental conditions that allowed both the cells and the bacteria to be viable for the assays also was technically difficult. Nevertheless, the system was established, and the resultant study was highly successful, providing important new information about the interaction of *B. pilosicoli* with colonic enterocytes. Attachment of the spirochaetes occurred, but again there was specificity in the interaction, with only one human strain (WesB) and one porcine strain (95/1000) attaching to the Caco-2 cells. The basis of the specificity was not explained, but one possibility is that the two attaching strains were expressing specific surface molecules that interacted with the Caco-2 cell surface, and which were not expressed by the other two *B. pilosicoli* strains. In future work it will be
important to compare the composition of the outer membrane of these four spirochaete strains, to try to identify specific components that may be involved in the attachment process. Not unexpectedly, the attachment that occurred increased with time, with a peak at 6 hour. It was difficult to ensure normal cell viability beyond this time, although incubation periods of up to 12 hours were still achieved and used for some of the cytokine assays.

Scanning and transmission electron microscopy confirmed that the ends of the attached spirochaetes were invaginated into the cell membrane of the Caco-2 cells in a manner resembling that seen in vivo. Initially the junctions of the Caco-2 cells were the primary targets for attachment, but later the whole Caco-2 surface was covered with huge numbers of spirochaetes. Phalloidin staining revealed a time dependent relocalization of filamentous actin over the junctions of the Caco-2 cells. ZO-1 staining showed the presence of severe disruption to the Caco-2 cell junctions, and the cells exposed to B. pilosicoli also underwent apoptotic changes in a time dependent manner. Furthermore, the monolayers recognized the colonization, and significantly up-regulated IL-1β and IL-8 in response to it. Treatment of the monolayers with sonicates of B. pilosicoli caused significant up-regulation of IL-1β, TNF-α, and IL-6, but culture supernatants and non-pathogenic Brachyspira innocens did not induce changes. The occurrence of these time-dependent changes clearly demonstrates that B. pilosicoli has pathogenic potential in this in vitro system, and strongly suggests that it is likely to have similar potential in the colon. Although the mechanisms involved in these pathological changes were not studied in detail, the
availability of this model system now permits further more detailed studies to understand the molecular mechanism of these changes, and ultimately should help in devising measures to control the infection.

The separate issue of how *B. pilosicoli* isolates may penetrate the mucin layer in the large intestine to gain access to the underlying enterocytes also was addressed in this thesis. Previous work had suggested that, unlike *B. hyodysenteriae*, *B. pilosicoli* either was not attracted to 1% mucin (Milner and Sellwood 1994), or that some strains had only a weak chemotactic response to mucin components (Witters and Duhamel 1999). The results of the current study provided a very different and new understanding about the interaction of *B. pilosicoli* (and *B. hyodysenteriae*) with mucin.

The study showed that most (but not all) *B. pilosicoli* strains are highly attracted to mucin concentrations with viscosities that are similar to those that are found in the mucous layer overlying the porcine colon. The attraction appears to involve an element of chemotaxis to mucin components, but there is also a strong “viscotaxis” response whereby the attraction and/or swimming efficiency of the spirochaete increases with increasing viscosity of the mucin solution. This was demonstrated by the attraction of *B. pilosicoli* (and *B. hyodysenteriae*) to the non-biological viscous material PVP. The lack of attraction to mucin by the porcine *B. pilosicoli* strain Cof-10 may help to explain why this strain would not attach to red blood cells or Caco-2 cells. Interestingly, the control strain of *B. hyodysenteriae* also showed an increasing attraction to mucin as the concentration (and viscosity) increased.
to 6% mucin; however, unlike *B. pilosicoli*, in 8% and 10% mucin this attraction decreased. This result suggested that different mechanisms of chemotaxis and viscotaxis exist for the two spirochaete species, and these differences may help to explain the different behaviours and colonization sites of the two species in the colon.

In the final part of the work, a preliminary attempt was made to see whether norepinephrine (NE) could modulate the behaviour of *B. pilosicoli*, as it does to other enteric bacterial pathogens. It was gratifying to find that low concentrations of NE (0.05-0.1 mM) did enhance growth, motility/attraction and attachment responses of *B. pilosicoli*. A higher concentration (0.5 mM) was required to maximize entry of spirochaetes into 4% mucin. The mechanisms involved in these modulatory effects of NE on *B. pilosicoli* were not studied and they deserve further investigation. One simple approach would be to compare gene expression in cultures that either have or have not been exposed to NE, and to try to identify pathways with changes in gene expression. It would also be relatively easy to compare the behaviours of such treated and untreated cultures in an *in vivo* model, to determine their clinical relevance, but importantly there was insufficient time to do this in the context of the whole PhD programme. Nevertheless, the current observations make a good starting point for further more detailed work on how NE may influence the pathogenicity of *B. pilosicoli* in colonized animals or humans. For example, it could be postulated that stress or co-infections with other pathogens could increase NE levels in the gastrointestinal tract, which in turn could enhance the pathogenicity of *B.*
*pilosicoli*, if it is present. In this context, it is interesting to note that all the human cases of spirochaetaemia with *B. pilosicoli* that have been reported have occurred in individuals with concurrent disease.

In conclusion, the work described in this thesis has added to the available knowledge about *B. pilosicoli*. Useful *in vitro* models of attachment and attraction to mucin were developed; pathological changes that have not previously been observed were identified; and an unusual viscotaxic behaviour was observed that may help to explain how *B. pilosicoli* strains may vary in their ability to penetrate mucus layer and colonize the colonic epithelium. Finally, the *in vitro* behaviour of *B. pilosicoli* was shown to be modified by NE, and this observation may in part help to explain why individuals show different degrees of disease following colonization by the spirochaete.
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## Appendix A

### List of chemicals and major equipments and their manufacturers

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<th>Chemicals and reagents</th>
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