Cell Biology of the Interaction between

*Listeria monocytogenes* and *Colpoda* spp.

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Abstract

Intracellular bacterial pathogens, such as *Listeria monocytogenes*, survive and multiply within mammalian cells. However, interaction with protozoans in the external environment may protect this pathogen from harsh conditions. In particular, if *L. monocytogenes* is able to survive within these protozoans, these cellular hosts may act as a vehicle that links contamination of food processing environments to contamination of foods. Though *Colpoda* ciliates are a very common type of protozoa, current knowledge on the interaction between intracellular bacterial pathogens and *Colpoda* at cellular level is limited. The interaction of *L. monocytogenes* and *Colpoda* ciliates was investigated in the present study.

Co-cultures of planktonic and biofilm *L. monocytogenes* DRDC8 with *Colpoda* sp. strains RR (isolate of natural environment) and strain MLS-5 (isolate of food processing environment) at 25°C were used to examine this interaction. Bacteriological counts and microscopy (fluorescence and transmission electron microscopy (TEM)) were used to track the fate of internalized *L. monocytogenes* within the ciliates. TEA chloride was used to inhibit phagocytosis to determine if *L. monocytogenes* induce its own uptake into *Colpoda*. Grazing of *Colpoda* on *L. monocytogenes* biofilms and changes in biofilm structures were evaluated by crystal violet assay and scanning electron microscopy (SEM). Mechanisms utilized by *Colpoda* RR in killing and degradation of DRDC8 were investigated by using chemical inhibitors of phagosome-lysosome fusion (NH₄Cl), vacuolar acidification (bafilomycin A1 and monensin), proteases (protease inhibitor cocktail) and nitric oxide (L-NMMA). In addition, the ability of *Colpoda* to secrete faecal pellets containing bacteria following co-cultures with DRDC8 was examined.

Co-culture of DRDC8 with *Colpoda* RR and MLS-5 provided direct evidence that these ciliates were able to actively phagocytose and kill planktonic and biofilm forms of DRDC8. *L. monocytogenes* was unable to initiate its own uptake into either *Colpoda* RR or MLS-5 and the level of expression of Listeriolysin O did not influence the outcome of co-culture. The increase in viable counts of *Colpoda* following feeding with DRDC8 together with a concomitant reduction in viable counts of intra-ciliate DRDC8 within a 4 h period, indicated *Colpoda* used *L. monocytogenes* as a food source. This was confirmed by observations that internalized DRDC8 were confined within tight vacuoles the presence of
large food vacuoles containing many electron-dense bacteria-sized particulates within *Colpoda* cytosol. *Colpoda* RR also effectively phagocytosed and degraded *S. Typhimurium* C5, as well as several non-pathogenic bacteria such as *B. subtilis* and *E. coli* DH5α.

An important and novel outcome was the observation that induction of encystment of DRDC8-fed *Colpoda* RR, lead to the entrapment of bacterial cells within cyst outer walls and the cytosol. Furthermore, co-cultures of DRDC8 with *Colpoda* RR and MLS-5 resulted in the secretion of faecal pellets containing intact, viable and respiring DRDC8 cells. Bacteriological counts confirmed that faecal pellet-located DRDC8 were resistant to concentrations of gentamycin (up to 100 µg mL⁻¹) and sodium hypochlorite (up to 10%), that were well above concentrations that are otherwise lethal to suspensions of DRDC8.

Fluorescence microscopy of acidotrophic stains Lysosensor™ Blue DND 167 and acridine orange treated Colpoda cells showed lysosomes fused with DRDC8-containing vacuoles within *Colpoda* RR. Following treatment of co-cultures with NH₄Cl, bafilomycin A1 and protease inhibitor cocktail, viable intra-ciliate bacterial counts and TEM showed evidence of survival without replication of DRDC8 within inhibitor-treated *Colpoda* RR for up to at 24 h post feeding. These outcomes indicated that *Colpoda* RR employs phagosome-lysosome fusion, vacuole acidification and proteases as mechanisms to kill intra-ciliate *L. monocytogenes*. However, nitric oxide was not involved in the killing of DRDC8 within *Colpoda*.

Both *Colpoda* strains used, actively phagocytosed and killed *L. monocytogenes*. *L. monocytogenes* were unable to escape the ciliate phagocytic vacuole and establish an intracellular lifestyle within *Colpoda*. This conclusion is in stark contrast to observations of the fate of *L. monocytogenes* cells within mammalian cells in which these bacteria escape into the cytosol in a Listeriolysin O dependent manner and then spread from cell to cell. However, the release of *Colpoda*-derived faecal pellets containing viable *L. monocytogenes*, indicated that these encapsulated forms of bacteria may provide a reservoir and a mechanism for transmission of pathogens. Considering that a faecal pellet-location endows bacteria with resistance to disinfectants and cleaning agents used in food manufacturing and preparation facilities, this may explain why *L. monocytogenes* is difficult to eradicate from food processing facilities.
Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Rethish Raghu Nadhanan and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Rethish Raghu Nadhanan

Thursday, December 6, 2012
# Abbreviations

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Acknowledgements

Let us be grateful to people who make us happy; they are the charming gardeners who make our souls blossom.
Marcel Proust

Praise the bridge that carried you over.
George Colman

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Rethish Raghu
Chapter 1: Literature Review

1.1 Introduction to *Listeria monocytogenes*

A 1981 fatal outbreak from the consumption of contaminated coleslaw in Canada (Schlech *et al.* 1983), together with epidemics in the following years that were associated with dairy foods such as pasteurized milk (Fleming *et al.* 1985) and Mexican-style cheese (James *et al.* 1985), highlighted major concerns about the survival and growth capabilities of the pathogen *Listeria monocytogenes* in contaminated foods, most importantly during storage of foods at refrigeration temperatures. Since the occurrence of these epidemics, *L. monocytogenes* has been widely acknowledged both as an important hazard in the food industries, in addition to its significance as a medically significant pathogen.

*L. monocytogenes* is a Gram positive, intracellular bacterial pathogen, facultatively anaerobic, rod-shaped (0.4 - 0.5 µm in diameter and 0.5 - 2 µm in length) and non-spore-forming. Biochemical analysis demonstrated that *L. monocytogenes* are oxidase-negative, catalase-positive and hydrolyse esculin, are methyl red and Vogus-Proskauer positive, do not hydrolyse urea and is incapable of producing H$_2$S or indole (Feresu and Jones 1988). Typically *Listeria* spp. have a low % [G+C] content (< 50%).

As one of the seven species of the genus Listeria, *L. monocytogenes* belongs to a genus of the Corynebacteriaceae family that comprises of *L. monocytogenes*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. murrayi*, *L. seeligeri*, and *L. welshimeri*. While *L. monocytogenes* is pathogenic to both humans and animals, *L. ivanovii* is only pathogenic to animals, mainly sheep and cattle. The rest of the *Listeria* species are known not to cause any disease (Cossart 2007).

From a food safety perspective, *L. monocytogenes* is resistant to environmental conditions that normally halt growth of other pathogenic bacteria. For example, it can grow in foods containing up to 10% NaCl, a wide pH range (4.5 - 9) as well as low temperatures (Vázquez-Boland *et al.* 2001). *L. monocytogenes* is also able to colonize and persist within the high pH environment of the gallbladder (Begley *et al.* 2009). This finding suggests the occurrence of both long-term and chronic infections, as well as the ability of these bacteria to survive within the microenvironments of the gastrointestinal tract. Though the optimum
growth temperature range for *L. monocytogenes* growth is between 30°C - 37°C, growth has not only been shown to occur between 3°C - 45°C (Junttila et al. 1988), the bacteria has also been demonstrated to grow at temperatures between 1°C - 4°C (Farber and Peterkin 1991). This adaptability of *L. monocytogenes* to a variety of temperatures suits it for survival and growth in either processed or refrigerated food items.

Although *L. monocytogenes* is considered to be ubiquitous, this it is most commonly isolated from decaying vegetation and soil (Weis and Seeliger 1975). Following ingestion of *L. monocytogenes* by a susceptible person, the bacteria can make the transition from being a saprophyte to a parasite that promotes survival and replication within host cells (Freitag 2006). *L. monocytogenes* has been demonstrated to be capable colonizing a variety of inert surfaces, as well as able to form biofilms on food-processing surfaces (Roberts and Wiedmann 2003). The presence of *L. monocytogenes* in numerous environments such as farms, soil, water, silage produced from contaminated grasses and also food processing facilities, indicates that there *L. monocytogenes* has many opportunities to contaminate the food production process (Cossart and Bierne 2001).

### 1.2 Listeriosis

Though human listeriosis outbreaks, following ingestion of *L. monocytogenes* contaminated food items, have been reported previously, it is only recently that this organism been recognized as a major cause of human infections (Lecuit 2007). This is due to increased numbers of susceptible, immuno-compromised individuals, the increase in large-scale agro-industrial plant development, as well as an increased reliance on refrigerated food items.

Fresh vegetables are an example of origins of contaminants. Vegetables can be contaminated via soil or from manure used as fertilizer. Animals may also carry the bacteria asymptomatically and contaminate foods of animal origin. Other examples of food items that are most linked to listeriosis outbreaks include ready-to-eat meats, undercooked meats, cold cuts, pâté (McLauchlin et al. 1991), salads, dairy products, especially soft cheeses (Linnan et al. 1988) and milk that is either inadequately pasteurized or contaminated post-pasteurization (Fleming et al. 1985, Jackson et al. 2011).
1.2.1 Listeriosis in Humans

A previous study has demonstrated that asymptomatic carriage of *L. monocytogenes* does occur within the intestinal tract of 5% or more of healthy humans (Griř et al. 2003). However extremely rare infections also occur in healthy adults and young children, where there is increasing evidence in recent years to prove that listeriosis may also occur in healthy individuals within just 24 h post ingestion of highly contaminated food (Swaminathan and Gerner-Smidt 2007).

Those most at risk from listeriosis are generally immuno-compromised individuals such as diabetics, AIDS patients, those with renal failure, organ transplant patients, cancer patients and also elderly adults. Diarrhoea is usually an early symptom of the infection. Advanced symptoms in these susceptible individuals include septicemia or meningoencephalitis. The main clinical features of *L. monocytogenes* meningitis are abnormal movements, seizures, as well as alteration of consciousness. *L. monocytogenes* meningitis cases have been shown to have the highest mortality rate (22%) in comparison to all types of bacterial meningitis (Lecuit and Cossart 2001). Certain patients are known to experience rare and localized infections, for example due to direct inoculation of the bacterium (Schlech 2000, Lecuit and Cossart 2001).

Others at high risk of the disease also include pregnant women, foetus and also newborns (Vázquez-Boland et al. 2001). Due to the fact that pregnant women have a naturally depressed, cell-mediated immune system (Weinberg 1984), pregnant women are more likely to acquire listeriosis upon post consumption of contaminated food compared to other individuals. By gaining access to the maternal circulation, *L. monocytogenes* colonizes the placenta to induce placentitis, infecting the defenseless foetus. As opposed to maternal illness, the severity of infection within foetal and neonatal is much higher. The common fatal symptoms include pre-term labour, amnionitis, spontaneous abortion, stillbirth as well as early onset sepsis (Vázquez-Boland *et al.* 2001).

The occurrence of fatality due to listeriosis is continuing to decrease in industrialized countries in recent years. This has been attributed to the stricter implementation of food quality and safety standards (Allerberger and Wagner 2010).
1.2.2 Listeriosis in Animals

Various species of animals can be infected with *L. monocytogenes*, but clinical disease is rare. The bacterium can also live within the intestines of healthy animals without causing any infections. Though most cases of animal listeriosis are generally seen in ruminants, this disease can also occur in poultry and other birds, pigs, dogs, cats, domestic and wild rabbits, and many other small mammals. Infected ruminants have been shown to experience encephalitis, septicemia, and even abortions (Schoder *et al.* 2003). The course of disease in sheep and goats is more rapid and death may occur 24 - 48 h upon the onset of symptoms. In cattle however, the course of disease is less acute.

Many *L. monocytogenes*-infected animals excrete the bacterium in faeces and milk. This is a common source of animal to animal spread of infection. Grass silage is presumed to be the source of infection, as it can be contaminated with large numbers of *L. monocytogenes*. Furthermore, the high pH of low-quality silage is insufficient to control growth of *L monocytogenes* cells (Pham 2006). Besides silage, the bacterium has also been isolated from other sources such as water troughs, manure, soil and animal feeds. *L. monocytogenes* infection may also cause mastitis in cattle and sheep (Wagner *et al.* 2005).

In ruminants such as sheep, infections that lead to lesions in the brain stem, result in characteristic clinical symptoms (Rebhun and deLahunta 1982). Typical symptoms of listeriosis in ruminants include turning or twisting of the head to one side and walking in circles, drooping of the eyelid and ear caused by paralysis of the unilateral facial nerve. The infected ruminant may also drool saliva as a result of partial pharyngeal paralysis (Rebhun and deLahunta 1982).

Animals that excrete *L. monocytogenes* cells in faeces have been suggested as the primary cause of entry of this pathogen into food-processing plants (Schonberg and Gerigk 1991). The growth and multiplication of *L. monocytogenes* cells is usually promoted by not only the high humidity, but also the nutrient rich waste present within certain food production plants. Hence, it is not surprising that animal listeriosis does pose a serious contamination risk for the food industry in general.
1.3 Pathophysiology of \textit{L. monocytogenes}

\textit{L. monocytogenes} is usually ingested with contaminated food (Figure 1.1). In immuno-compromised individuals, \textit{L. monocytogenes} invades the epithelial cells of the intestines and spreads to other parts of the body by cell-to-cell spread. \textit{L. monocytogenes} secretes invasins (InlA + InlB) to enable it to penetrate cells of the intestinal epithelial lining (Gaillard \textit{et al.} 1987, Mengaud \textit{et al.} 1996). \textit{L. monocytogenes} cells that cross the intestinal epithelial barrier are then carried by the lymph or blood to the mesenteric lymph nodes, the spleen, and also the liver (Marco \textit{et al.} 1992, Pron \textit{et al.} 1998). Entry into the host's monocytes, macrophages, or polymorphonuclear leukocytes promotes growth of \textit{L. monocytogenes}, and the infection becomes blood-borne (septicemic) dissemination.

\textit{L. monocytogenes} then enters the liver after the intestinal translocation and carriage by the bloodstream (Marco \textit{et al.} 1992, Dramsi \textit{et al.} 1998). Hepatocytes are generally the main site of \textit{L. monocytogenes} multiplication within the liver (Vázquez-Boland \textit{et al.} 2001). When there is an inadequate immune response by the host, \textit{L. monocytogenes} usually multiplies within the liver parenchyma. Release of the bacteria into blood causes bacteremia. Blood-borne \textit{L. monocytogenes} cells are then able to cross the blood-brain barrier (Kirk 1993). High levels of \textit{L. monocytogenes} cells in the brain accompanied by bacteremia will generally result in meningoencephalitis (Tunkel and Scheld 1993, Tuomanen 1996).

In pregnant women, \textit{L. monocytogenes} usually gains access to the foetus by entering the endothelial layer of the placental barrier (Gray and Killinger 1966). The bacterial cells will reach the bloodstream of the foetus by firstly colonizing the trophoblast layer. The bacteria then will reach the bloodstream of the foetus by translocating across the endothelial barrier. This will usually result in infection and the possible subsequent death of the foetus within the uterus, or occasionally even the premature birth of a severely infected neonate (Vázquez-Boland \textit{et al.} 2001).

1.3.1 Virulence Factors of \textit{L. monocytogenes}

A wide array of virulence factors is wielded by \textit{L. monocytogenes} to assist the bacterium to interact and manipulate the host cells. Virulence genes of \textit{L. monocytogenes} are known to be optimally expressed at 37°C, but expressions almost does not occur at 30°C (Freitag \textit{et al.} 1996).
al. 2009). The key transcriptional activator of *L. monocytogenes* virulence factor genes, known as PrfA, is also known to be thermo-regulated. PrfA is usually activated upon the ingestion of *L. monocytogenes* contaminated foods (Figure 1.2). PrfA is also known to regulate a variety of the bacterium’s virulence genes (Camejo *et al.* 2011, Stavru *et al.* 2011) as well as other core genome genes.

Internalins (InlA and InlB), which are *L. monocytogenes* surface proteins, have been previously shown to involve in the invading of the host cells (Seveau *et al.* 2007). InlA is known to bind E-cadherin, which is the host cell’s adhesion molecule, whereas InlB binds to Met, which is the hepatocyte growth factor (HGF) receptor. By the binding of internalin proteins to E-cadherin and Met, *L. monocytogenes* cells are able to gain entry into the host cells; this is done by taking advantage of the endocytic machinery of the host cells (Pizarro-Cerda and Cossart 2006).

Once internalized within the host cell, *L. monocytogenes* mediates escape from membrane-bound vacuoles through the secretion of Listerialysin O (a pore-forming haemolysin) (Gaillard *et al.* 1987), as well as two phospholipases: phosphatidylinositol (PI) phospholipase (PLC-A) (Camilli *et al.* 1993) and phosphatidylcholine (PC) phospholipase C (PLC-B) (Grundling *et al.* 2003). Together, these proteins assist in breaking down the host phagosome that contains *L. monocytogenes* cells. This is done to allow the bacterium to escape into the host cytosol (Kathariou *et al.* 1987, Camilli *et al.* 1991, Mengaud *et al.* 1991, Vázquez-Boland *et al.* 1992, Schnupf and Portnoy 2007, Scortti *et al.* 2007). Upon entering the host cell’s cytosol, *L. monocytogenes* cells begin to replicate (O’Riordan *et al.* 2003, Joseph and Goebel 2007), and then with the assistance of actin polymerization mediated cell-cell spread, the bacterium moves through the host cell for the purpose of migrating into the neighbouring host cells. The actin polymerization mediated cell-cell spread process is directed by ActA. The ActA protein binds and activates Arp2/3, which is a seven-protein host complex. Arp2/3 has been shown to induce actin polymerization as well as generate actin filaments (Pizarro-Cerda and Cossart 2006). Upon entry into the adjacent host cell, *L. monocytogenes* cells secrete both Listerialysin O and also PC-PLC to assist the bacteria in escaping from the double-membrane secondary vacuoles, known as listeriopods, which were formed as a result of cell-to-cell spread (Freitag *et al.* 2009).
1.3.2 Invasion of Mammalian Cells by *L. monocytogenes*

*L. monocytogenes* has evolved a number of strategic methods to evade or resist killing by the innate immune response of mammalian phagocytic cells that are usually known to phagocytose and degrade most pathogens that invade the host cells (Ryter and De Chastellier 1983). The bacterium is able to multiply in a variety of mammalian cell types such as professional phagocytic cells, for example, J774 macrophage-like cells (Portnoy *et al.* 1992), as well as non-professional phagocytes such as epithelial cells (Rácz *et al.* 1972), endothelial cells (Drevets *et al.* 1995) and hepatocytes (Conlan and North 1992). Marco *et al.* (1992) previously demonstrated that in mice that were infected with *L. monocytogenes* cells, the bacterium first infected the macrophage cells, followed by infection of the hepatocytes in the liver. *L. monocytogenes* has also been shown in a separate study to be able to efficiently invade hepatocytes *in vitro* (Wood *et al.* 1993).

Within mammalian host cells, *L. monocytogenes* is internalized within membrane-bound phagosomes upon adhering to host cells. The bacterium then escapes into the host cytosol from the phagosome by disrupting the phagosomal membrane. Within the host cytosol, *L. monocytogenes* grows and multiplies, and then proceeds to infect neighbouring host cells (Freitag *et al.* 2009). Gaillard *et al.* (1987) showed *L. monocytogenes* was able to initiate entry into human colon carcinoma cell line Caco-2, and multiply within the host cytosol. That same study also provided evidence to show that *L. monocytogenes* was able to induce phagocytosis by Caco-2 cells. Francis and Thomas (1996) demonstrated recovery of a higher numbers of *L. monocytogenes* cells of hemolytic strains from both HeLa and Caco-2 cell lines, in comparison to non-hemolytic strains. Furthermore, the extensive morphological changes that the host cells exhibited not only included loss of confluence and host cell lysis, but also the presence of very high counts of *L. monocytogenes* cells within the host cells were detected (Francis and Thomas 1996).

1.4 Is there an Environmental Reservoir for *L. monocytogenes*?

Although *L. monocytogenes* causes severe disease in human and animal hosts, unlike other Gram negative intracellular pathogens, this pathogen has no recognized animal reservoir. Several studies have suggested that interactions with soil-borne organisms such as protozoa, provide the selection pressures required to allow a number of intracellular

While *Acanthamoeba* spp. is known to harbour a number of bacterial pathogens, *L. monocytogenes* has been recently demonstrated to be phagocytosed and rapidly degraded by the host amoeba within just 2 h of ingestion (Akya et al. 2010). In view of this result, the question arises whether protozoa could act as a potential reservoir for *L. monocytogenes*.

### 1.5 Interactions between Bacteria and Protozoa

Protozoa are unicellular eukaryotic microorganisms that are ubiquitously present in diverse habitats. They feed heterotrophically and are generally recognised as the major consumers of bacteria in the environment. Protozoan cells can be present either singly or as colonies of cells (eg. *Volvox* spp.), may swim freely (*Paramecium* spp.), or are parasitic for other animals (eg. *Trypanosoma* spp.).

Briefly, there are three main groups of bacterivorous protozoa: amoebae, ciliates and flagellates. Amoebae feed on algae, bacteria, plant cells, and smaller protozoans. Amoebae move by forming pseudopods (temporary foot-like structures) with diverse morphologies. Ciliates can be found almost everywhere there is water, such as lakes, rivers, oceans and also soil. They are characterized by large numbers of hair-like organelles (called cilia) that are involved in movement of the cells, chemotaxis, as well as predation of bacteria (Fenchel 1987). Flagellates have whip-like appendages (called flagella) for the main purposes of locomotion as well as to direct food particles or cells into its mouth-like opening.

The major cause of bacterial mortality in the environment is suggested to be caused by feeding of bacteria by protozoans (Pernthaler 2005). A majority of protozoa feed by phagocytosis, a process by which they engulf bacteria and digest them within a food vacuole. Briefly, once the bacterial prey is captured, it is packaged into a food vacuole. Once inside the protozoan food vacuole, the process of digestion commences (Fenchel
This is carried out through the release of host proteases and lysozymes into the food vacuole in order to break down the bacteria within the food vacuole. This will supply the protozoa with energy and nutrients for its growth. The acidic environment within the food vacuoles assists the protozoa in disabling the bacterial prey for digestion. The products of the digestion process are then released into the cytoplasm. However, previous studies have clearly demonstrated that not all bacteria are digested as food source. Some types of bacteria survive within the protozoa in order to persist and utilize those protozoan cells as a host. The major outcomes in a bacteria/protozoa interaction (Figure 1.3) include:

1. Phagocytosed bacteria multiply to high numbers within the vacuoles, resulting in massive enlargement of these vacuoles that will eventually cause lysis of the host, releasing free bacteria into the extracellular environment, e.g. *Legionella pneumophila* (Rowbotham 1983).

2. The same process as (1), except that following lysis of host, free bacteria is released alongside intact vacuoles containing infectious bacteria, e.g. *L. pneumophila* (Rowbotham 1983).

3. Phagocytosed bacteria multiply within the host but not able to cause lysis of the host, e.g. *Coxiella burnetti* (La Scola and Raoult 2001).

4. Phagocytosed bacteria survive within encysted forms of protozoa, e.g. *L. pneumophila* (Rowbotham 1983).

A number of important studies have previously shown that pathogenic bacteria that are able to survive within protozoans can be protected from external stresses such as chemical disinfectants and antibiotics (King *et al.* 1988, Berk *et al.* 1998, Brandl *et al.* 2005, Bichai *et al.* 2008). It is likely that the ability of a number of intracellular bacterial pathogens to resist killing by its host protozoan cells may have resulted in their evolution as pathogens of the mammalian kingdom. Indeed it is possible that protozoan cells are the link between bacteria that inhabits the environment and the bacteria that cause diseases in mammals such as humans.
1.6 Protozoa as Model Organisms for Study of Pathogenesis

Protozoan cells have been previously utilized as model organisms for studies in various fields such as evolution and ecology (Friman et al. 2008), population and community biology (Holyoak and Lawler 2005), the role of organelles (Smith et al. 2007) as well as toxicity studies (Stefanidou et al. 2008). The use of protozoa in the study of host-pathogen interactions has its advantages and has increasingly become more common in recent years, most importantly in infectious diseases studies. Infection studies generally utilized mammalian species such as mice and sometimes even humans as the host systems. By using the mammals as the host system, the analysis is not only expensive, prolonged and subject to extensive ethical review, it is also technically challenging and complex. In contrast, similar studies in protozoans are more convenient, quicker and also cost-effective (Montagnes et al. 2012).

As model systems, protozoan cells can also help in understanding better the mechanisms of infectious diseases within mammalian cells (Montagnes et al. 2012). Intracellular bacterial pathogens have been previously shown to escape the phagolysosomes of protozoa and mammalian phagocytic cells by utilizing similar mechanisms. Hence, protozoa are useful models for studying the pathogenesis of opportunistic, human pathogens. In terms of evolution, single-celled organisms such as protozoans are older than multi-celled organisms such as mammals. This suggests that several bacteria pathogens of mammals may have evolved from intracellular pathogens within protozoan cells (Montagnes et al. 2012). For example, the fact that interactions involving L. pneumophila with mammalian and protozoan cells share a number of phenotypic and molecular features, indicates a common mechanistic basis for the observed parasitism (Barker and Brown 1994, Fields 1996).

It is now clear that protozoan-bacterial pathogen interactions play an important role in transmission of human disease. This was especially evident when protozoa harbouring L. pneumophila were identified as the cause of a Legionnaires’ disease outbreak during an American Legion convention in Philadelphia in 1976. A total of 34 fatalities out of 221 cases were reported during that outbreak. Therefore, it is inevitable that studies on bacteria/protozoa interactions can provide crucial steps into possible prevention of infectious diseases.
The amoeba *Dictyostelium discoideum* is an example of a useful model in the study of human pathogens, including *L. pneumophila* (Solomon *et al*. 2000), *Neisseria meningitidis* (Colucci *et al*. 2008) as well as *Salmonella enterica* serovar Typhimurium (Annesley and Fisher 2009). Other protozoan models commonly studied include the ciliate *Tetrahymena* spp. (Friman *et al*. 2008), the marine flagellate *Oxyrrhis* spp. (Montagnes *et al*. 2011), and also the choanoflagellate *Monosiga* spp. (Behringer *et al*. 2009). There are a number of other advantages with using protozoa as a model system, including the ease with which protozoans can be grown in large amounts as well as the simple storage and maintenance techniques. In general, protozoa cultures can be maintained on simple, inexpensive media, such as bacterial suspensions. Protozoa cultures may be easily isolated from a variety of natural and artificial environments. Protozoan cells can also be stored as stock cultures over a long period of time, for example by suspending concentrated suspensions of protozoan cells in DMSO with storage at -20°C. These stock cultures can be revived with only a little effort.

### 1.7 Interactions between Pathogenic Bacteria and Protozoa

Intracellular bacterial pathogens of humans that parasitize protozoans exist within a privileged environment, protected from external stresses. Thus bacteria-protozoa interactions are likely to have important ecological as well as public health consequences.

The association of pathogens and protozoa may have contributed to the survival and persistence of bacterial pathogens in various natural and artificial environments. Encapsulation of bacterial pathogens within protozoan cells and provides a protective effect against environmental stress, such as predation, starvation, disinfectants and high temperatures. A number of pathogens can survive for extended periods of time within cysts of protozoan cells, and cannot be detected by methodologies based in culture and cannot be killed by the normal anti-bacterial methods and other adverse environmental conditions (Greub and Raoult 2004). A number of studies have shown that following internalization within protozoans, the pathogens have increased virulence and demonstrate increase pathogenicity following infection of mammalian cells (Rasmussen *et al*. 2005, Steinberg and Levin 2007, Adiba *et al*. 2010). The definite reasons for this occurrence remain to be explained.
1.7.1 Interactions between Gram negative Pathogens and Protozoa

1.7.1.1 *Legionella pneumophila*

This bacterium is the causative agent of Legionnaires’ disease. It has long been established that it can grow within mammalian cells such as macrophages, monocytes and epithelial cells (Horwitz 1983). Ever since it was demonstrated that *L. pneumophila* can infect *Acanthamoebae* spp. (Rowbotham 1980), the interaction between *L. pneumophila* and protozoa, especially amoeba, has been widely researched at both the cellular and molecular levels. Since then, *L. pneumophila* has been studied as a model organism for bacteria/protozoa interaction studies and their role in pathogenesis to mammalian cells. The protozoans *Acanthamoeba* spp., *Balamuthia mandrillaris* (Shadrach et al. 2005), *Dictyostelium discoideum* (Solomon et al. 2000) and *Hartmannella vermiformis* (Abu Kwaik 1996) have been most commonly utilized in *in vitro* experiments with *L. pneumophila*.

*Acanthamoebae* spp. has been previously shown to protect *L. pneumophila* against external stresses, such as heat and biocides (Harb et al. 2000). Furthermore, the ability of *L. pneumophila* to survive and multiply within *Acanthamoebae* spp. signifies the possible role of *Acanthamoebae* spp. in transmission of *L. pneumophila*. Although intra-amoebic replication of *L. pneumophila* can result in the lysis of host *Acanthamoeba* spp., a study has demonstrated that *Acanthamoebae* spp. are capable of allowing the intra-amoebic survival of *L. pneumophila* for prolonged periods of even up to several months, without experiencing cell lysis (Winiecka-Krusnell and Linder 1999). The outcome of that study suggested that the host amoebae and bacteria have adapted very well with each other resulting in the ability of both microorganisms to survive for longer than usual periods in the environment.

Interestingly, Berk et al. (1998) showed that when *Acanthamoebae* spp. was co-cultured with *L. pneumophila*, the amoeba secreted faecal pellets encapsulating viable bacterial cells. *L. pneumophila* within these faecal pellets appeared to be resistant to treatment with cooling tower biocidal agents. An earlier study demonstrated that *L. pneumophila* cells within cysts of *Acanthamoebae* spp. are able to survive treatment
with high concentrations of free chlorine (Kilvington and Price 1990). Both studies implicate the amoeba in the dissemination of \textit{L. pneumophila}.

Similarities between the interactions of \textit{L. pneumophila}-amoeba and \textit{L. pneumophila}-mammalian cell interactions have been noted previously. This includes the uptake of \textit{L. pneumophila} by a coiling phagocytosis followed by the presence of the bacteria within host phagosomes. \textit{L. pneumophila}-containing phagosomes are generally surrounded by host organelles such as mitochondria, vesicles, and rough endoplasmic reticulum (Bozue and Johnson 1996, Gao \textit{et al.} 1997, Abu Kwaik \textit{et al.} 1998, Hilbi \textit{et al.} 2001). Further, it has been demonstrated that in amoeba and mammalian hosts, expression of virulence factors by \textit{L. pneumophila} was responsible for the inhibition of phagosome-lysosome fusion within the host (Bozue and Johnson 1996). These similarities have been taken to suggest that amoeba-derived \textit{L. pneumophila} are primed to cause infection within mammalian cells.

Similarities aside, there are a number of important differences in the interaction of \textit{L. pneumophila} with mammalian and protozoan hosts systems. Firstly, \textit{L. pneumophila} does not require host protein synthesis in macrophages, but it does in \textit{H. vermiformis} (Abu Kwaik \textit{et al.} 1994). Secondly, the bacterium is capable of inducing apoptosis in mammalian cells, but does not do so in \textit{A. polyphaga} (Harb \textit{et al.} 2000). By using mutant strains of \textit{L. pneumophila} that are defective for cytotoxicity, intracellular survival, and replication in both macrophage-like cells as well as \textit{A. polyphaga}, evidence provided by Gao \textit{et al.} (1997) demonstrated that although these mutants were not able to attach to \textit{A. polyphaga}, attachment to macrophages-like cells was only slightly impaired. By utilizing different mechanisms in order to infect different hosts, this implies that \textit{L. pneumophila} has evolved these mechanisms to widen its choice of hosts so that the bacterium is able to survive in a variety of environments.

Garcia \textit{et al.} (2007) reported that \textit{A. polyphaga} protected intra-amoebic \textit{L. pneumophila} from killing by NaOCl. The resuscitation of viable but non-culturable (VBNF) \textit{L. pneumophila} in NaOCl-treated water occurred with the assistance of \textit{A. polyphaga} hosts. Beside that, encystation of \textit{A. polyphaga} can be prevented by the presence of intra-amoebic \textit{L. pneumophila} (Garcia \textit{et al.} 2007).
1.7.1.2 *Escherichia coli*

*E. coli*, mostly pathogenic strains such as O157:H7 and K1 have been widely studied for their interaction with protozoa. One of the earliest works on *E. coli*-protozoa interaction showed that the non-pathogenic *E. coli* B/r was preyed upon by the ciliate *Colpoda steinii* (Drake and Tsuchiya 1976). A separate study by Gourabathini *et al.* (2008) showed that *C. steinii* viable counts increased following feeding on the pathogenic *E. coli* O157:H7, indicating the ciliate utilized this pathogen as a food source to grow. This is a unique finding, as other similar studies have demonstrated the ability of pathogenic strains of *E. coli* to survive within other types of protozoa such as *Acanthamoeba* spp. (Barker and Brown 1994). This observation could either be an indication that the pathogenic *E. coli* O157:H7 do not have the ability to escape killing by *C. steinii*, or that *Acanthamoeba* spp. do not have a killing mechanism in place for *E. coli* O157:H7. Schlimme *et al.* (1995) demonstrated that *E. coli* K12 resisted digestion by *Tetrahymena* spp. and were secreted from *Tetrahymena* spp. as viable cells within faecal pellets. Barker *et al.* (1999) demonstrated that *E. coli* O157 was able to survive and multiply within *A. polyphaga*, following a co-culture period of up to 35 d. Another study showed that *E. coli* cells that were present within the drinking water reservoir isolate ciliate *Cyclidium* sp. demonstrated high resistance to free chlorine; suggesting that *E. coli* can persists in chlorine-treated water through this method (King *et al.* 1988).

A study showed that invasive *E. coli* K1 associated with *A. castellanii* better than the non-invasive *E. coli* K12 (Alsam *et al.* 2006). Gentamycin protection assay by that study also showed *E. coli* K1 invaded *A. castellanii* better than *E. coli* K12. Besides that, *E. coli* K1 was also able to survive and multiply within *A. castellanii*, whereas *E. coli* K12 was phagocytosed and degraded by the amoeba. These outcomes are identical to that of a separate study that involved mammalian cells and *E. coli* K1 and K12 (Sukumaran *et al.* 2003). *E. coli* K1, but not K12, was able to invade, survive and multiply within both murine and human macrophages (Sukumaran *et al.* 2003). The similarity in the response of *Acanthamoeba* spp. and mammalian cells to *E. coli* K1 and K12 highlights the importance of using protozoan cells as an alternative model system to study the pathogenesis of intracellular bacterial pathogens.
1.7.1.3 *Salmonella enterica*

*S. enterica,* is a well-known intracellular bacterial pathogen that can use a number of protozoa as an environmental reservoir (King *et al.* 1988, Gaze *et al.* 2003, Tezcan-Merdol *et al.* 2004). This bacterium is known to interact with *Acanthamoeba* spp. and ciliates such as *T. pyriformis* (Brandl *et al.* 2005). Brandl *et al.* (2005) examined the interaction of Green Fluorescent Protein (GFP) labelled *S. enterica* serovar Thompson with a soil-borne isolate *Tetrahymena* sp. During co-culture with *S. Thompson, Tetrahymena* sp. released faecal pellets containing bacteria. Interestingly, that study also showed that in comparison to free *S. Thompson* cells, a significantly higher number of pellet-located *S. Thompson* cells remained viable (Brandl *et al.* 2005). Huws *et al.* (2008) however, found no proliferation of *S. enterica* serovar Typhimurium within *A. polyphaga*. Furthermore, counts of *A. polyphaga* even increased to up to 120% following co-cultures, indicating that *S. Typhimurium* was ingested and killed and used as a food source. This is a rare outcome in an interaction that involves this well-known intracellular bacterial pathogen. This result contrasts sharply with outcomes of other studies *Acanthamoeba - S. Typhimurium* interactions in which the bacterial cells exhibited the ability to survive and replicate intracellularly within its amoeba hosts (Gaze *et al.* 2003, Tezcan-Merdol *et al.* 2004, Akya *et al.* 2010). Survival of *S. Typhimurium* was also demonstrated to occur within *T. pyriformis* (King *et al.* 1988). The lack of *S. Typhimurium* proliferation in the study by Huws *et al.* (2008) was attributed by the authors to the difference in the strains of *S. Typhimurium* and *A. polyphaga* used in the study in comparison to the strains used in other studies that have shown survival and proliferation of *S. Typhimurium* within *A. polyphaga* hosts.

Choice of bacterial strains may also influence experimental outcomes. For example, Enteropathogenic *E. coli* (EPEC) does not survive predation by *A. polyphaga* (Huws *et al.* 2008), whereas a different study showed that another pathogenic isolate, *E. coli* O157, was able to survive predation by amoeba (Barker *et al.* 1999). In a separate study that utilized five different isolates of *Acanthamoeba* spp., *S. enterica* serovar Dublin was internalized more efficiently than *S. enterica* serovar *Enteritidis* or *S. Typhimurium* for each of the five amoeba isolates tested, although *A. rhysodes* demonstrated to be the most efficient host (Tezcan-Merdol *et al.* 2004). A study by Gaze *et al.* (2003) reported that *S. Typhimurium*
was used as food source by *A. polyphaga*, a similar outcome to Huws *et al.* (2008). However, Gaze *et al.* (2003) observed a number of *S. Typhimurium* cells replicated within phagocytic vacuoles of the amoeba host. Interestingly, the study also reported a rare evidence of a significant growth of *S. Typhimurium* inside the host contractile vacuoles of a few *A. polyphaga* cells (Gaze *et al.* 2003). The ability of *S. enterica* to survive and multiply within the intracellular environment of amoebae and other types of protozoa could be crucial for *S. enterica* to survive long term in the environment and may contribute to its transmission and prevalence within mammalian cells.

1.7.1.4 *Helicobacter pylori*

*H. pylori* is the most common cause of gastric ulcers and gastritis. To date, there is only a single study dedicated to the interaction between *H. pylori* and protozoa. *H. pylori* has been detected within *A. castellanii*, but intracellular replication of the bacteria was not observed (Winiecka-Krusnell *et al.* 2002). That study also demonstrated that 7 d co-cultures of *H. pylori* and *A. castellanii* resulted in a number of outcomes: ca. 100 fold increase of *H. pylori* counts, bacterial viability sustained for up to 8 weeks, as well as the presence of intact and viable *H. pylori* cells within *A. castellanii* vacuoles (Winiecka-Krusnell *et al.* 2002). This study suggested a possible role for *Acanthamoeba* spp. in transmission of *H. pylori*.

1.7.1.5 *Campylobacter jejuni*

*Campylobacter jejuni* is the major cause of enteritis, a food-borne disease in developed countries (Altekruze *et al.* 1998, Allos 2001). There are only a few published accounts regarding interactions between *C. jejuni* and protozoa. Bui *et al.* (2012) has shown *C. jejuni* is rapidly killed following phagocytosis by *A. castellanii*. However a number of other studies have shown a contrasting outcome.

The interaction between *C. jejuni* and protozoa within the context of the broiler chicken industry has been investigated by both in *vitro* and in *vivo* experiments. Snelling *et al.* (2005) has shown in *in vitro* studies that the presence of *C. jejuni* within *T. pyriformis* and *A. castellanii* can increase the pathogen’s survival period to up to 36 h in comparison to free *C. jejuni* cells. This outcome suggests that interaction of *C. jejuni* with protozoans may possibly increase the opportunities for *C. jejuni* cells to colonize the broilers. The
same study also demonstrated that *C. jejuni* present within *T. pyriformis* and *A. castellanii* was more resistant to Virudine, a common disinfectant used in the poultry industry, than free *C. jejuni* cells (Snelling *et al.* 2005).

In the *in vivo* experiments of a separate study by Snelling *et al.* (2008), broiler chickens were inoculated with either *C. jejuni* within amoeba or free *C. jejuni* cells. Intra-amoebic *C. jejuni* and free *C. jejuni* cells were then recovered from the chickens and treated with NaOCl. A significantly higher number of viable intra-amoebic *C. jejuni* cells were recovered following treatment with NaOCl, in comparison to free *C. jejuni* cells which were either killed or inactivated by NaOCl treatment (Snelling *et al.* 2008).

*C. jejuni*-protozoan interactions may also explain why Stern *et al.* (2002) discovered that treatment of broiler drinking water with chlorine had no effect on *Campylobacter* spp. colonization of the broiler chickens. Evidence from an earlier study supports this outcome, as it showed that a strain of *C. jejuni* were protected from free chlorine by growing the pathogens in cultures of amoeba or ciliates (King *et al.* 1988). As broiler chickens are reared solely for meat production, the outcomes of these studies have significant implications to the poultry as well as health industry. This is because these protozoans could act as reservoirs of *C. jejuni* in broiler drinking water.

In a separate study, four different strains of *C. jejuni* strains were shown to able to survive and multiply within *A. polyphaga* (Axelsson-Olsson *et al.* 2005). The study also demonstrated that intra-amoebic *C. jejuni* survived for longer periods in comparison to free *C. jejuni* cells, an outcome that mirrored the evidence of Snelling *et al.* (2005). Axelsson-Olsson *et al.* (2005) also showed that when non-culturable *C. jejuni* cells were inoculated into *A. polyphaga* cells and incubated at 37°C, these bacterial cells were resuscitated to the culturable state.

1.7.1.6 *Burkholderia* spp.

*B. cepacia* and *B. pseudomallei* are the well-known pathogenic species of the *Burkholderia* genus. *B. cepacia* is a significant threat to cystic fibrosis patients by causing pneumonia in these individuals, whereas *B. pseudomallei* is the etiological agent of melioidosis. Following 24 h co-culture with amoeba, *B. cepacia* and *B. vietnamiensis* cells exhibited intra-amoebic survival without replication within acidic vacuoles of the amoeba (Lamothe
et al. 2004). B. cepacia have been previously shown to survive within Acanthamoeba spp. for up to 17 d in the absence of any intracellular replication (Marolda et al. 1999). Though Lamotte et al. (2004) concluded that B. cepacia does not replicate intracellularly within the amoeba hosts, another study showed that the bacterium replicated to very high numbers in the amoebae-conditioned medium (Marolda et al. 1999). Interestingly, only a single study has demonstrated an increase in viable counts of intra-amoebic B. cepacia cells following co-cultures with host Acanthamoeba sp. (Landers et al. 2000). However it was not mentioned in that study whether the increase in counts of intra-amoebic B. cepacia cells was a result of intracellular replication, or whether it was just an increase in uptake of bacteria cells by Acanthamoeba sp.

In a study by Inglis et al. (2000a), B. pseudomallei was shown to survive in vacuoles of Acanthamoeba spp. Furthermore, prior to migration out of the amoeba, B. pseudomallei cells were shown in a separate study to be able to escape from amoeba vacuoles into the cytosol (Vandal 2008). Among the acute melioidosis cases during an outbreak in Western Australia, B. pseudomallei was identified as the causative agent within a number of the cases, and the bacterium was isolated during the outbreak from potable water in the affected community (Inglis et al. 2000b). Interestingly, a number of Acanthamoeba isolates were also recovered from the same potable water samples. It is likely that these Acanthamoeba spp. cells might have played a key role in the dissemination of B.pseudomallei.

1.7.1.7 Vibrio cholerae

This causative agent of cholera is known to be able to survive within amoeba such as Acanthamoeba spp. and Naegleria spp. (Thom et al. 1992, Abd et al. 2007, Sandstrom et al. 2010, Shanam et al. 2011), even at the low temperature of 4°C (Li et al. 2006). The presence of A. polyphaga or N. gruberi was shown to enhance the growth of V. cholerae (Thom et al. 1992). V. cholerae was also shown by the same study to be able to survive encystment within N. gruberi cysts (Thom et al. 1992). Similar work with A. castellanii showed V. cholerae was able to survive within cysts of the amoeba (Abd et al. 2005). In a separate study, both V. cholerae and Acanthamoeba spp. were isolated from natural water samples from different cholera endemic areas in Sudan (Shanan et al. 2011), however no evidence was provided to establish a link between the bacteria and Acanthamoeba spp. The
majority of studies of *V. cholerae*-protozoa interactions have utilized strains of the bacteria that cause epidemic and pandemic outbreaks of cholera. All of these studies confirmed the survival of these bacteria within the protozoan hosts. This highlights the importance of *V. cholerae*-protozoa studies in understanding the spread of epidemic cholera. For example it may be possible to develop ways of reducing the risk of cholera by targeting the interaction between the bacterium and its protozoan host.

1.7.2 Interactions between Gram positive Pathogens and Protozoa

1.7.2.1 *Mycobacterium avium*

Typically, *M. avium* is the etiological agent of tuberculosis in birds and swine. However, infection with *M. avium* usually causes systemic bacterial infection in susceptible individuals such as late-stage AIDS patients. Miltner and Bermudez (2000) have provided evidence to show that *A. castellanii* may act as reservoir for *M. avium*. Steinert et al. (1998) found that *M. avium* could survive within the outer walls of *A. polyphaga* cysts. Co-cultures of *M. avium* and *A. polyphaga* also led to bacterial growth (Steinert et al. 1998). In another study, *M. avium* was shown to inhibit lysosomal fusion to the bacteria-containing vacuoles within infected *Acanthamoeba* hosts, allowing the replication of *M. avium* within vacuoles within the amoebae (Cirillo et al. 1997). The presence of *M. avium* within the outer walls of cysts of *A. polyphaga* (Steinert et al. 1998) also signifies the possible role of the cysts as a reservoir for *M. avium* when extracellular conditions become less conducive, for example in the presence of external stressors, such as disinfectants.

1.7.2.2 *Staphylococcus aureus*

*S. aureus* causes a range of illnesses as diverse as minor skin infections to life-threatening diseases such as pneumonia and meningitis. However this bacterium is not easily degraded by either *A. castellanii* or *H. vermiformis* following phagocytosis (Nakazato and Hatano 1991). This phenomenon has been attributed to either the thick bacterial cell wall of *S. aureus*, typical of Gram positive bacteria, or carotenoids, which is a post ingestion defence that is known to protect this bacterium from oxidation. Previous work has demonstrated the ability of carotenoids in protecting *S. aureus* from destruction by leucocytes (Rossi 1986). Nevertheless, intact *S. aureus* were found present within faecal
pellets of amoeba, though it was suggested that only a small number of S. aureus cells managed to escape digestion by the amoeba host (Nakazato and Hatano 1991).

Healthcare-associated methicillin-resistant S. aureus (MRSA) infection is of major concern worldwide. Huws et al. (2006) was the first to demonstrate that isolates of epidemic MRSA replicate in the presence of A. polyphaga. About 50% of A. polyphaga cells harboured viable MRSA within the amoeba phagolysosomes, while only 2% of A. polyphaga had high numbers of intracellular MRSA. It is highly likely that these MRSA cells within the protected environment of amoeba are shielded from antibiotics that target MRSA. Whether this association of MRSA with amoebae is responsible for the increase in MRSA cases worldwide despite the continuous invention of anti-MRSA medications is not clear. In a separate study, the co-existence of Acanthamoeba sp. and MRSA was identified as the cause of corneal keratitis (Barsig and Kaufmann 1997).

1.7.2.3 L. monocytogenes

Interactions between L. monocytogenes and protozoa, especially ciliates, have received scant attention. Results of these studies are often conflicting. Ly and Müller (1990) suggested that L. monocytogenes is able to multiply intracellularly following ingestion by Acanthamoeba sp. following co-cultures at 37°C. Within the first 8 d of co-cultures, counts of extracellular L. monocytogenes decreased continually whereas numbers of Acanthamoeba sp. increased. Extracellular L. monocytogenes then increased to high numbers from day 8 onwards. Following 8 d of co-cultures, viable intracellular L. monocytogenes replicated to high numbers, eventually causing lysis of the protozoa hosts and releasing the bacteria into the extracellular medium. The paper concluded that L. monocytogenes could survive and multiply within Acanthamoeba sp. Experiments by Akya et al. (2009, 2010) however, obtained a starkly different outcome to that of Ly and Müller (1990). Co-cultures of various strains of L. monocytogenes, including clinical isolates, with A. polyphaga, A. castellanii and A. lenticulata at 22°C resulted in the phagocytosis and killing of the bacteria by the host amoeba; though the rates of killing were different for each of the amoeba. In general, L. monocytogenes were found confined singly within tight vacuoles located within the cytoplasm of amoeba, and the bacteria were later degraded within phagolysosomes of the amoeba within 4 - 5 h of co-cultures. No evidence of L. monocytogenes survival or replication within any of the three amoeba
species tested was observed. This outcome is consistent with data that demonstrated the phagocytosis and degradation of *L. monocytogenes* by the ciliate *Colpoda* sp. RR. (Raghu Nadhanan 2008) A more recent study has also shown that *L. monocytogenes* is unable to survive phagocytosis and digestion by *Acanthamoeba* spp. (Doyscher *et al.* 2012).

A separate study by Huws *et al.* (2008) showed that when *A. polyphaga* was used as the host system, the presence of amoeba resulted in an increase in the counts of extracellular *L. monocytogenes* compared to control experiments without amoebae (Huws *et al.* 2008). This outcome is similar to that reported by Ly and Müller (1990). Huws *et al.* (2008) also reported absence of both survival and replication of *L. monocytogenes* cells within *A. polyphaga* following a 24 h co-culture. Although this outcome is consistent with the outcome of 24 h co-culture by Akya *et al.* (2009), the work by Huws *et al.* (2008) did not consider the potential outcomes of co-culture within 5 h of infection, as other co-cultures of *L. monocytogenes* with *A. polyphaga* showed these amoebae were capable of actively phagocytosing and killing the bacteria within 2-5 h post infection (Akya *et al.* 2010). In contrast, a separate study reported that *L. monocytogenes* survived within *A. castellani* (Zhou *et al.* 2007). Evidence of *L. monocytogenes* present within *A. castellani* was provided by TEM micrographs (Zhou *et al.* 2007). This finding contrasts with outcome of the longitudinal study by Akya *et al.* (2009) that demonstrated both qualitatively and quantitatively that *L. monocytogenes* was phagocytosed and used as food source by *A. castellani* (Akya *et al.* 2009). It is possible that the *ad libitum* method of co-culture utilized by Zhou *et al.* (2007) resulted in the presence of *L. monocytogenes* within *A. castellani* even following 24 h of co-culture. In the *ad libitum* feeding method, the amoebae will continuously phagocytose bacterial cells and consequently, there will always be intact bacteria within amoeba cells in order to replenish digested bacteria. The pulse feeding method adopted by Akya *et al.* (2010) allowed tracking the fate of internalized bacteria from beginning to end at various stages of ingestion without the interference of other, newly phagocytosed bacteria. In this method, the bacteria were co-cultured with amoeba cells for a fixed period of time, and then removed from the co-culture suspensions by a combination of gentamycin treatment and differential centrifugation, followed by further incubation to different time-points. Though intra-amoebic survival of
*L. monocytogenes* was observed, there was no evidence that *L. monocytogenes* replicated within *A. castellanii* (Zhou et al. 2007).

A recent study using co-cultures with a *Colpoda* isolate has confirmed the results published by Akya et al. (2009). Raghu Nadhanan (2008) examined the interactions between *L. monocytogenes* and a *Colpoda* sp. (strain RR), an environmental isolate of a ciliated protozoan. This isolate was co-cultured with different strains of *L. monocytogenes* as well as a Listeriolysin O mutant. Co-cultures at 25°C showed these ciliates were able to feed equally well on cells of all strains of *L. monocytogenes* tested, irrespective of pathogenicity or Listeriolysin O status. Phagocytosed bacteria were rapidly degraded within food vacuoles. Bacteriological counts and TEM studies confirmed that *L. monocytogenes* strains were actively phagocytosed by *Colpoda* RR cells. *L. monocytogenes* present within vacuoles were not able to escape into the ciliate cytosol. Counts of *Colpoda* cells pulse fed *L. monocytogenes* typically increased 10-fold within 24 h post feeding. When *Colpoda* RR phagocytosis was inhibited by suspension in buffer containing a low Ca²⁺ concentration or treatment with TEA chloride, the uptake of *L. monocytogenes* cells was inhibited. These results indicated that *L. monocytogenes* was unable to initiate its own uptake into the ciliates.

An interesting, recent study provided evidence of *L. monocytogenes* aggregates as ‘backpacks’ on the exterior surfaces of *Acanthamoeba* spp. in co-cultures (Doyscher et al. 2012). Following a certain period of co-culturing, these backpacks were phagocytosed by the amoeba as food source. Importantly, the authors speculated that: (1) only *L. monocytogenes* cells expressing a motile phenotype formed the backpacks, and (2) *Acanthamoeba* spp. developed the backpacks to prevent bacteria from utilizing its motile phenotype in order to escape grazing (Doyscher et al. 2012). This unique phenomenon of assembling bacterial prey on exterior surface of *Acanthamoeba* spp. followed by endocytosis has been documented for other bacteria-*Acanthamoeba* spp. interactions (Ray 1951, Bottone et al. 1994). Bottone et al. (1994) concluded that the suitability of a bacterial food source was correlated directly with ability of the bacterial prey to adhere to the surface of the amoeba. This may explain the suitability of *L. monocytogenes* as a good food source for *Acanthamoeba* spp. (Akya et al. 2010).
The role of amoeba-derived faecal pellet containing bacterial cells has been documented for bacteria such as *L. pneumophila*, *E. coli* O157:H7 and *S. enterica* (Rowbotham 1986, Berk et al. 1998, Brandl et al. 2005). Since these secreted structures are made up of multiple pieces of membrane that are not simply cytoplasmic contents enclosed by a continuous membrane (which is what defines a vesicle), they are for the remainder of the thesis termed a faecal pellet (S. Berk, personal communication). Importantly, bacterial cells encapsulated within faecal pellets are resistant to biocidal agents and can potentially cause serious infections when inhaled as an aerosol.

Gourabathini et al. (2008) provided evidence that *Tetrahymena* sp., *C. steinii* and *A. palestinensis* did not secrete faecal pellets containing *L. monocytogenes* following co-culture. This observation is consistent with the outcome of a different study that showed the ciliate *Tetrahymena* sp. also rarely expelled *L. monocytogenes*-containing faecal pellets (Brandl et al. 2005). It is unknown why the protozoa cells used in these studies did not secrete faecal pellets containing *L. monocytogenes* following co-cultures with the bacteria, whereas they could secrete other species of bacteria. Whether *L. monocytogenes* inhibited the faecal pellet production pathway following internalization by these protozoa, or if this is a characteristic of all species of protozoa co-cultured with *L. monocytogenes*, is yet to be resolved.

1.8 Summary

*L. monocytogenes* causes a severe disease in susceptible mammalian hosts, but it has not been recognized as natural reservoir to date. The ability of *L. monocytogenes* to persist in natural environments indicates the presence of environmental reservoirs of this pathogen. It is likely that *L. monocytogenes* cells interact with a number of types of protozoan cells. However, there is little evidence of survival of *L. monocytogenes* following predation by protozoans. Akya et al. (2009, 2010) demonstrated the phagocytosis and killing of *L. monocytogenes* by a number of *Acanthamoeba* spp. A study by Gourabathini et al. (2008) showed that the ciliate *Colpoda steinii* did not secrete faecal pellets containing *L. monocytogenes* following feeding with the pathogen. No viable *L. monocytogenes* were detected within the cysts of *C. steinii* either (Gourabathini et al. 2008).
To date, there are very few *L. monocytogenes-*protozoa interaction studies that have utilized ciliated protozoans as the model host organism, despite the widespread prevalence of ciliates in environments where various pathogenic bacteria are also abundantly present. It is crucial to better understand the role and ability of ciliates in allowing the survival and replication of *L. monocytogenes* within the ciliates. In particular, the question of whether protozoa isolates from natural and artificial environments could act as a reservoir for *L. monocytogenes* remains to be satisfactorily answered.

### 1.9 Hypotheses and Aims

#### 1.9.1 Hypothesis 1

*L. monocytogenes* phagocytosed by the natural environmental isolate ciliate *Colpoda* strain RR and food processing environment isolate ciliate *Colpoda* strain MLS-5 are able to establish an intracellular lifestyle within these ciliated protozoans.

**Aim 1.1:** Isolate and identify protozoan isolate from food processing environment.

**Aim 1.2:** Investigate the outcomes of co-cultures of *Colpoda* RR and MLS-5 with *L. monocytogenes* and other bacteria.

**Aim 1.3:** Investigate the effects of phagocytosis inhibition on *Colpoda* RR and MLS-5 on co-cultures with *L. monocytogenes* and other common pathogenic and non-pathogenic bacteria.

**Aim 1.4:** Investigate the outcomes of co-cultures of *Colpoda* RR and MLS-5 with *L. monocytogenes* and other common pathogenic and non-pathogenic bacteria by TEM.

**Aim 1.5:** Investigate the effects of levels of Listeriolysin O expression on the outcomes of co-cultures of *Colpoda* RR with *L. monocytogenes*.

**Aim 1.6:** Investigate the survival of *L. monocytogenes* within encysted *Colpoda* RR.

**Aim 1.7:** Investigate the grazing of *Colpoda* RR and MLS-5 on biofilms of different strains of *L. monocytogenes* and other common pathogenic and non-pathogenic bacteria.
1.9.2 Hypothesis 2

Host defence mechanisms employed by *Colpoda* RR are involved in the killing and degradation of internalized *L. monocytogenes*.

**Aim 2.1**: Examine the effect of inhibition of phagosome-lysosome fusion on survival of *L. monocytogenes* within *Colpoda* RR.

**Aim 2.2**: Determine whether ciliate vacuolar acidification influences survival of *L. monocytogenes* within *Colpoda* RR.

**Aim 2.3**: Investigate the effect of inhibition of protease activity on survival of *L. monocytogenes* within *Colpoda* RR.

**Aim 2.4**: Investigate the production of intracellular superoxide and nitric oxide by *Colpoda* RR following phagocytosis of *L. monocytogenes*.

1.9.3 Hypothesis 3

*Colpoda* RR and *Colpoda* MLS-5 secrete faecal pellets containing *L. monocytogenes* cells.

**Aim 3.1**: Investigate the production of faecal pellets by *Colpoda* RR and MLS-5 following co-cultures with *L. monocytogenes*.

**Aim 3.2**: Determine the viability of *L. monocytogenes* within secreted faecal pellets of *Colpoda* RR and MLS-5.

**Aim 3.3**: Investigate the ability of *Colpoda* RR to secrete faecal pellets containing bacteria following co-cultures with other common pathogenic and non-pathogenic bacteria.

**Aim 3.4**: Investigate the viability of *L. monocytogenes* within secreted faecal pellets of *Colpoda* RR and MLS-5 following exposure to commonly used biocides.
1.10 References


Table 1.1: Pathogenic human bacterial species associated with free-living protozoa.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Protozoan Host</th>
<th>Interaction Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td><em>Acanthamoeba</em> spp.</td>
<td>Phagocytosis and digestion by hosts.</td>
<td>Aky et al. 2010.</td>
</tr>
<tr>
<td><em>B. cepacia</em></td>
<td><em>A. polyphaga</em></td>
<td>Survival within hosts but no intracellular replication.</td>
<td>Lamothe <em>et al.</em> 2004.</td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td><em>A. castellanii</em></td>
<td>Survival within hosts.</td>
<td>Jeong <em>et al.</em> 2007.</td>
</tr>
<tr>
<td></td>
<td><em>T. pyriformis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> K5</td>
<td><em>A. castellanii</em></td>
<td>Survival and multiplication within host trophozoites, and survival within host cysts.</td>
<td>Matin, 2011.</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td><em>A. polyphaga</em></td>
<td>Survival and intracellular within amoeba hosts.</td>
<td>Sandstrom <em>et al.</em> 2010.</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td><em>Acanthamoeba</em> spp. and <em>Naegleria</em> spp.</td>
<td>Survival within amoeba hosts and survival within <em>Naegleria</em> spp. cysts</td>
<td>Snelling <em>et al.</em> 2006.</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td><em>A. polyphaga</em></td>
<td>Survival of <em>M. avium</em> within outer walls of <em>A. polyphaga</em> cysts.</td>
<td>Steinert <em>et al.</em> 1998.</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td><em>A. polyphaga</em></td>
<td>Amoeba killing by replicating <em>M. smegmatis</em>.</td>
<td>Lamrabet <em>et al.</em> 2012.</td>
</tr>
</tbody>
</table>
Figure 1.1: Pathophysiology of *L. monocytogenes*. 
Figure 1.2: Steps in the invasion of cells and intracellular spread by *L. monocytogenes.*
Figure 1.3: Possible outcomes in bacteria/protozoa interactions.

Other possible interactions and outcomes are discussed by Rowbotham (1986).
Chapter 2: Co-culture of Colpoda spp. with planktonic and biofilm bacteria

2.1 Abstract

A balanced relationship between bacteria and protozoa is crucial for survival and persistence of pathogens in both natural and artificial environments. Intracellular bacterial pathogens such as Listeria monocytogenes and Salmonella enterica serovar Typhimurium thrive within mammalian cells but also certain protozoan cells. However, current knowledge on the interaction between these pathogens and the common free-living soil and freshwater ciliate, Colpoda, at the cellular level is not known.

Colpoda sp. strain RR, isolated from a natural environment, together with Colpoda sp. strain MLS-5, isolated from a food processing environment, were used to investigate the interaction with L. monocytogenes DRDC8 during co-culture with planktonic and biofilm cultures. During co-culture, both isolates of Colpoda actively fed on DRDC8 cells, resulting in a concomitant increase in ciliate numbers. No evidence of growth repressing molecules expressed by bacterial cells was obtained. Furthermore, the Colpoda isolates fed on L. monocytogenes, irrespective of the level of expression of Listeriolysin. Treatment of Colpoda RR and MLS-5 with the phagocytosis inhibitor, TEA chloride, eliminated uptake of DRDC8 and confirmed that DRDC8 was unable to initiate its own uptake into Colpoda ciliates. Counts of intra-ciliate bacteria together with TEM confirmed that Colpoda RR and MLS-5 actively degraded phagocytosed DRDC8 cells. However, artificial induction of encystment of Colpoda RR during co-culture resulted in partitioning of L. monocytogenes into the outer cyst walls.

Counts of intra-ciliate bacteria and TEM sections of Colpoda RR cells from co-cultures of with DRDC8, Salmonella enterica serovar Typhimurium C5, Bacillus subtilis and Escherichia coli DH5α showed that Colpoda RR processed Gram negative bacteria more rapidly than Gram positive bacteria. TEM also revealed that Gram positive cells were ultimately partitioned into large food vacuoles. By contrast, co-cultures with Gram negative bacteria resulted in partitioning of bacterial cells into food vacuoles that were smaller and more densely packed with bacterial cells.
2.2 Introduction

The role of protozoans as possible reservoirs of pathogenic bacteria has been widely studied, yet little is understood about the true nature of the interaction at cellular level (Barker and Brown 1994). Besides acting as a potential reservoir for the maintenance of pathogenic bacteria in the environment, these eukaryotic organisms are also vectors for the transmission of human and animal disease. How this occurs and the role of that process in evolution of these pathogens is not fully known. Following uptake by protozoa, several well-known intracellular bacterial pathogens of humans have been shown to evade digestion by the host and multiply within vacuoles (Rowbotham 1983, Barker and Brown, 1994, Tezcan-Merdol et al. 2004). Maintenance of virulence genes in environmental pathogens has been increasingly understood to be a result of interactions between pathogenic bacteria and soil-borne microorganisms, such as protozoans (Brown and Barker 1999, Adiba et al. 2010, Lamrabet et al. 2012).

*L. monocytogenes* is a facultative intracellular pathogen that is reported to be isolated from a wide range of environments, including processed foods (Vázquez-Boland et al. 2001). Thus consumption of *L. monocytogenes*-contaminated food results in human listeriosis, a disease associated with a high mortality rate, particularly in immune-compromised individuals (Schlech 2000). For this reason, food products contaminated by *L. monocytogenes* are a major concern for the food industry and public health authorities alike. However, while it is accepted that *L. monocytogenes* is ubiquitous, little is known of the ecology of this organism in natural environments. In particular, the role of protozoans as a natural reservoir for *L. monocytogenes* is not well studied, even though this bacterium has evolved an array of virulence factors that are critical for uptake and establishment of an intracellular lifestyle within mammalian cells (Cossart et al. 2003).

Protozoans are known to feed on bacteria through ingestion by phagocytosis (Barker and Brown 1994). This interaction yields different outcomes depending on the type of bacteria involved. For example, Rowbotham’s (1980) landmark study demonstrated that *Acanthamoeba polyphaga* could harbour replicating *Legionella pneumophila*. Since that study, a number of published reports have examined the relationship between *L. pneumophila* and a wide number of free-living protozoans. The virulence of *L. pneumophila* was enhanced by intracellular growth in *A. castellanii* (Cirillo et al. 1999).
Furthermore, resuscitation of viable but non-culturable *L. pneumophila* cells was triggered following ingestion by *A. castellanii* (Steinert *et al.* 1997). The ability of *L. pneumophila* to multiply intracellularly within mammalian cells has also been well-documented (Horwitz and Silverstein 1980).

In a separate study, co-culture of *Helicobacter pylori* with *A. castellanii* not only resulted in a 100 fold increase of bacterial counts, intact and metabolically active *H. pylori* were also found located within amoebic vacuoles (Winiecka-Krusnell *et al.* 2002). Brandl *et al.* (2005) later showed survival of Green Fluorescent Protein (GFP) labelled *S. enterica* serovar Thompson cells located in vesicle-like structures secreted by both starved and fed *Tetrahymena* cells. Similarly *Mycobacterium avium* was shown to be able to survive within the outer walls of *Acanthamoeba polyphaga* cysts (Steinert *et al.* 1998). Interestingly, *S. enterica* and *E. coli* O157:H7 cells encapsulated within expelled protozoan vesicles are able to multiply and exit from these structures (Gourabathinii *et al.* 2008). These observations suggested that the vesicle-like structures may provide a protected environmental reservoir for pathogenic bacteria.

Zhou *et al.* (2007) showed that *L. monocytogenes* had no predatory effect on *A. castellanii*, and the presence of amoebae can actually enhance the growth of the bacteria. However, a study by Akya *et al.* (2009) provided data that showed co-cultures of *L. monocytogenes* with *Acanthamoeba* spp. resulted in active phagocytosis and killing of the bacteria by these amoebae within 2-5 h of feeding. This outcome is consistent with data described by Raghu Nadhanan (2008) that demonstrated *Colpoda* ciliates were able to phagocytose and degrade *L. monocytogenes* as food source within just 4 h post feeding.

In both natural and artificial habitats such as rivers, drainage systems and water distribution systems, bacteria are typically present in planktonic and biofilm communities (Barker and Brown 1994). Biofilms represent a mode of growth for bacteria including mammalian bacterial pathogens. This environment comprises extracellular products, and inorganic and organic debris that promote biofilm bacteria survival during exposure to stressors, such as UV exposure (Espeland and Wetzel 2001), dehydration and salinity (LeMagre-Debar *et al.* 2000), antibiotics (Mah and O’Toole 2001) as well as grazing by protozoa (Snelling *et al.* 2006). Natural, bacterial biofilms are typically colonized by amoebae, flagellates and ciliates (Weitere *et al.* 2003, Parry 2004). However, it is not
known whether different strains of bacteria respond differently at the physiological level to grazing by protozoa. Huws et al. (2005) showed that A. castellanii and the ciliate Colpoda maupasi, were able to graze on biofilm material. Grazing protozoa can have a significant impact on the integrity and composition of biofilm communities. Grazing, for example, results in reduction of biofilm biomass and as a result, may cause rapid changes in morphological and taxonomical community composition in areas where planktonic bacteria are present (Hahn and Hofle 2001, Jurgens and Matz 2002). In this context, Jackson and Jones (1991) showed that amoebae were able to graze biofilm bacteria to a point where the biofilm disruption was accompanied by sloughing of the biofilm structure.

When protozoa are not present, L. pneumophila has been shown to persist and remain viable for up to 15 d within artificial biofilms that were made using filter-sterilized tap water or distilled water (Lau and Ashbolt 2009). However, other studies have provided evidence that 30% - 40% of biofilm samples isolated from sources such as hospital water supplies, dental units and taps showed presence of Acanthamoeba spp. (Barbeau and Buhler 2001, Carlesso et al. 2007). Though biofilms have generally been shown to provide protection against predation by protozoans, one study has demonstrated that the effect of protozoan grazing on the biofilm community is a crucial factor that controls the biofilm composition in aquatic ecosystems (Pedersen 1990). Furthermore, biofilms may also be able to restrict predation by protozoans as biofilm cells have been shown to secrete defensive factors that inhibit predation by either protozoan or bacterial competitors (Matz and Kjelleberg 2005).

Most studies on the interaction between protozoans and bacteria have focused on the overall fate of either the predator or the prey, with little detail describing outcomes at the cellular level. Whether the protozoan host employs different cellular mechanisms to process Gram positive and Gram negative bacteria is not known.

Ciliates other than Tetrahymena spp. has had limited use as model host systems to study the prey-predator interactions with pathogenic bacteria. The work described in this study employed Colpoda, a common freshwater ciliate, as the host cell for co-cultures. The objective of the study was to: (1) determine the nature of the interaction between Colpoda isolates and planktonic and biofilm cells of common intracellular bacterial pathogens such as L. monocytogenes; (2) assess whether Colpoda isolates from natural and artificial
environments process phagocytosed Gram positive and Gram negative bacteria in different ways, and (3) to compare the ability of *Colpoda* cells to graze on biofilms of *L. monocytogenes*.

### 2.3 Materials and Methods

#### 2.3.1 Ciliates and Culture Conditions

The *Colpoda* sp. isolate RR (GenBank accession number GQ_475427) was previously isolated from a Lotus pond at the Adelaide Botanical Gardens (Raghu Nadhanan 2008). *Colpoda* sp. isolate MLS-5 (see Appendix A), was isolated from a floor drain in a food processing plant (see Section 2.4.2). Clonal populations of each strain were established and maintained at 22°C in 250 mL volumes of aerated water containing washed suspensions of *E. coli* DH5α as a food source. Every 3 - 4 weeks, 10 mL of ciliate cultures were transferred to fresh 250 mL volumes of MQ water containing washed *E. coli* DH5α cells (10⁸ cells mL⁻¹) and incubated at 22°C with aeration. These cultures were incubated at 25°C and routinely monitored for ciliate growth. For co-cultures, ciliates were harvested by gentle centrifugation and resuspended in sterile, modified Neff’s Amoeba Saline (AS buffer) (pH 7.0) (Smirnov and Brown 2004).

Encystment of *Colpoda* RR was induced by first pulse feeding ciliate cultures with washed suspensions of bacteria (10⁸ cells mL⁻¹) for 1 h at 25°C, followed by washing in AS buffer to remove extracellular bacteria. Encystment was induced by incubation in encystment medium containing 1 mM CaCl₂, 1 mM KCl and 5 mM Tris-HCl (pH 7.2) for 12 h at 25°C as described by (Yamaoka *et al*. 2004).

#### 2.3.2 Isolation of *Colpoda* MLS-5 from a Food processing Environment

The MLS-5 strain of *Colpoda* was isolated as follows. Samples of effluent from floor drains were collected from a cheese production facility in South Australia. Up to 5 samples of the effluents were screened in order to obtain a protozoa isolate. These samples were added to washed cell suspensions of *E. coli* DH5α (10⁹ cells mL⁻¹) and the mixture incubated at room temperature with gentle aeration. Growth of ciliates was monitored daily by observation of samples of culture under a dissecting microscope. Individual cells were separated from cultures via glass capillary tubes into drops of sterile water. The selected
cells were repeatedly transferred to fresh drops of water to eliminate contaminants followed by co-culture with a suspension of *E. coli* DH5α (10⁹ cells mL⁻¹) as a food source. The culture was incubated at room temperature and monitored daily for growth of ciliates.

Pure cultures of ciliates were identified using a molecular method described by Brandl *et al.* (2005). Briefly, PCR was used to amplify a 634 bp fragment (97 bp - 731 bp) of the 18S ribosomal RNA (18S rRNA) gene from isolated protozoan genomic DNA. Genomic DNA was isolated from 1 mL of a pure culture of ciliates. Ciliates were harvested by centrifugation (450 × g, 2 min) and genomic DNA extracted and purified using a Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, USA) according to the manufacturer’s instruction. The 18S rRNA gene was PCR amplified using primer pair 18SF (5’-GAAACTGCGAATGGCTC-3’) and 18SR (5’-TGATCCTTCTGCAGGTT-CACCTAC-3’) (Brandl *et al.* 2005). The PCR reaction mixture consisted of MQ water (7 μL), forward and reverse primers (1 μL each), template DNA (1 μL), and GoTaq® Green Master Mix (Promega, Madison, USA). Each PCR reaction was carried out in a Bio-Rad MyCycler thermocycler as follows: Denaturation at 94°C for 3 min, followed by 35 cycles of 30 seconds denaturation at 94°C, 45 seconds annealing, and 90 seconds extension at 72°C with a final extension step of 7 min at 72°C and cooling to 4°C. Amplification of the 634 bp fragment of DNA was confirmed by separation by agarose gel electrophoresis, followed by staining with Gel Red. PCR products were then purified using the QIAquick PCR Purification Kit (QIAGEN, Doncaster, Victoria, Australia) according to the manufacturer’s instruction.

The 634 bp amplicon was prepared for sequencing as follows. Briefly, the sequencing reaction mixture consisted of 200 ng μL⁻¹ of purified DNA (1 μL), forward primer (4μL), Big Dye Terminator Ready Reaction Mix (1 μL), 5× Big Dye Buffer (3 μL), and MQ water (11 μL). The thermo-cycling process used was as follows: 95°C for 4 min preceded 30 cycles of 96°C for 30 seconds, 53°C for 15 seconds and 60°C for 4 min, with a final extension at 72°C for 7 min. The sequencing reaction extension products were precipitated in a mixture of 20 μL of MQ water, 60 μL of 100% isopropanol and 20 μL of sequencing reaction. The mixture was vortexed and incubated at RT for 15 min, followed by centrifugation at 4°C (4000 × g, 25 min). Precipitated DNA was washed with 50 μL of
70% (v/v) ethanol, followed by centrifugation (450 × g, 15 min) to recover the DNA. The DNA pellet was then dried in a Speed Vac SVC1000 (Savant Instruments Inc., Farmingdale, New York, USA) and submitted to the DNA Sequencing Facilities at the Institute for Medical and Veterinary Science (IMVS) (Adelaide, South Australia, Australia).

Sequence data obtained was compared to other sequence in the GenBank non-redundant nucleotide database using BLAST software available from the NCBI website (http://www.ncbi.nlm.nih.gov/). A dendrogram that graphically displayed the relationship of sequence data to other 18S rRNA gene sequence was prepared using a Maximum likelihood analysis. Maximum likelihood analysis was performed with the program PhyML v3, using the best of NNI and SPR moves for tree topology searching and aLRT to establish the statistical support of internal branches (Fenchel 1987). The TN93+G6 substitution model was selected using ModelGenerator v0.85 by comparison of Bayesian Information Criterion scores (Begley et al. 2009).

2.3.3 Bacteria and Culture Conditions

*L. monocytogenes* strain DRDC8 (O serotype 1, 4), an Australian dairy isolate known to invade and multiply within mammalian cells (Francis and Thomas 1996), *L. monocytogenes* strain EGD Kaufmann (Laboratory collection, University of Adelaide), and *L. monocytogenes* strain KE925 (a clinical human isolate from King Edward Hospital, Perth, Western Australia) were routinely cultivated in Listeria Enrichment Medium (LEM) (Oxoid Code CM862), or on LEM agar plates with incubation at 37°C overnight with aeration. To enhance Listeriolysin O secretion, *L. monocytogenes* was cultured in LEM containing 0.2% (w/v) activated charcoal (Sigma-Aldrich). The GFP-expressing *L. monocytogenes* strain ADGA (a derivative of DRDC8) was routinely cultivated in either LEM or LEM agar supplemented with 10 μg mL⁻¹ erythromycin (Akya 2007).

*E. coli* DH5α, *B. subtilis* and *S. Typhimurium* C5 (Laboratory collection, University of Adelaide) were routinely cultured in Luria broth (Lennox formulation) with incubation at 37°C. Where required, washed cell suspensions of *E. coli* DH5α in 1.5 mL microcentrifuge tubes were killed by heating for 45 min at 100°C.
2.3.4 Co-culture of Colpoda and Bacteria in Liquid Medium

Ciliates from actively grown cultures fed *E. coli* DH5α were harvested by gentle centrifugation (106 × g, 2 min). The cell pellet was washed once in AS buffer and resuspended in fresh AS buffer at 22°C and adjusted to final concentrations of 10⁴ cells mL⁻¹ or 10⁵ cells mL⁻¹. Overnight broth cultures of bacteria incubated at 37°C were harvested by centrifugation (4000 × g, 20 min) and the cell pellet washed once in AS buffer followed by re-suspension in fresh AS buffer to a final concentration of 10⁹ cells mL⁻¹.

Washed suspensions of ciliates (200 μL) at concentrations of ca. 10⁴ cells mL⁻¹ were then pulse fed with 200 μL of washed bacterial suspensions for 1 h at 22°C. Post feeding, the co-culture suspensions were washed free of any extra-ciliate bacteria, and the ciliates are incubated as required to determine growth of ciliates and survival of ingested bacteria. Suspensions of ciliates incubated in sterile AS buffer at 25°C were used as a negative control. Counts of viable ciliates present after 2, 4 and 24 h incubations were determined by a direct microscopic method using a haemocytometer slide. Viability refers to actively motile, intact ciliates. All counts were done in triplicates and mean concentrations of viable ciliates were determined.

To determine the counts of intra-ciliate bacteria following co-culture, samples of co-cultures were incubated at 25°C for 0.5, 1, 2 and 4 h. Post feeding, the co-culture suspensions were washed free of any extra-ciliate bacteria, following which 100 μL of the washed suspensions were serially diluted and plated out on agar plates to confirm the absence of any extra-ciliate bacteria. *Colpoda* cells were then lysed by addition of 0.3% Triton X-100 solution. Serial, decimal dilutions of lysates were then plated out on LEM agar (for *L. monocytogenes* DRDC8) and LB agar (for *S. Typhimurium* C5, *E. coli* DH5α and *B. subtilis*) followed by incubation overnight at 37°C. Unlysed *Colpoda* ciliates were used as washing control. Following incubation, the number of CFU mL⁻¹ was determined.

2.3.5 Phagocytosis inhibition assay

Washed cells suspensions of DRDC8 and GFP-expressing *L. monocytogenes* ADGA, *S. Typhimurium* C5, *E. coli* DH5α, *B. subtilis* and *Colpoda* ciliates were incubated separately in 5.0 mM TEA chloride (a K⁺ efflux inhibitor) at 25°C for 15 min to block phagocytosis
by Colpoda (Peck and Duborgel 1985). TEA chloride-treated Colpoda were pulse fed with the bacteria for a period of 1 h at 25°C. The co-culture suspensions were then washed to remove any extra-ciliate bacteria and the washed Colpoda cells were incubated for a further 0.5, 2, 4 and 24 h.

Phagocytosis of the GFP-expressing ADGA was monitored directly with an Olympus 1X70 fluorescence microscope to detect vacuoles containing this strain. Phagocytosis of other strains of bacteria was monitored by plate counts of intra-ciliate bacteria as follows. At each time point, Colpoda ciliates were washed free of remaining extra-ciliate bacteria, and the ciliates were then lysed by addition of 0.3% Triton X-100 solution. Diluted and undiluted lysates were then plated out on LEM agar (for DRDC8) and LB agar (for S. Typhimurium C5, E. coli DH5α and B. subtilis) followed by incubation overnight at 37°C. Samples of intact Colpoda ciliates were used as a wash control to confirm that any growth of bacteria present in lysates were not residual extracellular bacteria that remained after washing. Following incubation, counts of CFU mL⁻¹ per sample were determined.

2.3.6 Biofilm Grazing Experiments

To establish bacterial biofilms, overnight cultures of the bacteria strains were diluted (1:100) in LEM, and 200 μL samples were added into 96-well tissue culture plates. The plates were incubated separately at 4°C, 25°C and 30°C without shaking for 72 h. At 24 h intervals, the culture medium was replaced with fresh medium. After incubation for 72 h, the growth medium in the wells was removed, and the wells washed three times with fresh LEM. Colpoda cells (200 μL of a 10⁵ cells mL⁻¹ suspension) were added to each of the wells. As a control, Colpoda cells were added into wells without biofilm. Three replicates were used for each sample. The plates were then incubated separately at 4°C, 25°C and 30°C without shaking for 0, 4, 12, 24, 36 and 48 h. Counts of viable Colpoda in wells at 0, 4, 12, 24, 36 and 48 h were monitored by direct microscopy.

Biofilms biomass was quantified by a crystal violet staining assay. Briefly, the supernatant phase was removed from each 72 h well, and all wells were washed three times with LEM. The plates were air-dried, and 40 μL of 0.5% (w/v) crystal violet was added to each well. The plates were incubated for 45 min and rinsed repeatedly with LEM before crystal violet-stained biofilms were solubilized in 95% ethanol. 100 μL of the
solubilised crystal violet was transferred to a 96-well microtitre plate, and the absorbance was determined with a PowerWave XS2 Microplate Spectrophotometer (BioTek Instruments, Inc.) at 595 nm.

2.3.7 Haemolysis assay

The haemolytic activity of *L. monocytogenes* DRDC8 cultured in the presence or absence of 0.2% (w/v) activated charcoal was assessed by stab inoculation into 5% (v/v) sheep blood agar. Clear zones (indicating β-haemolysis of erythrocytes) surrounding the bacterial colonies were determined after incubation overnight at 37°C. To quantify haemolytic activity, washed cells derived from cultures grown in LEM with or without activated charcoal were incubated separately in 1% (v/v) sheep erythrocytes for 1 and 24 h at 25°C. Haemolysis was measured by absorbance at 545 nm, corresponding to the amount of haemoglobin released into the supernatant. The absorbance of sterile LEM was measured as a negative control.

2.3.8 Scanning Electron Microscopy (SEM)

Biofilms samples were fixed in PBS containing 4% (w/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde - overnight at 4°C. The samples were dehydrated through a graded ethanol series (70%, 90% and 100%, three times for 10 min each), then immersed in hexamethyldisilazane (HMDS) (three times for 5 min each), and air-dried. Following coating by gold sputter, the biofilms were observed using Philips XL30 scanning electron microscopy.

2.3.9 Transmission Electron Microscopy (TEM)

To study the fate of bacterial cells internalized within *Colpoda*, co-cultures of the ciliates and bacteria incubated to different time-points were prepared for TEM. *Colpoda* that were harvested from co-cultures by centrifugation were fixed in PBS containing 4% (w/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde overnight at 4°C, embedded in 1% (w/v) agarose and then post-fixed in 2% (w/v) OsO₄ in distilled water. After post-fixation, specimens were dehydrated in an ethanol series, the ethanol exchanged with propylene oxide and the samples embedded in Pro Scitech Epoxy resin. Ultra-thin sections of resin-
embedded specimens were stained with uranyl acetate and lead citrate and examined in Philips CM 100 transmission electron microscope.

2.3.10 Statistical analysis

All data reported are the means obtained from three independent experiments. Each experimental data point was the mean of three replicate samples. Tukey’s Multiple Comparison Test was used to compare the data, and \( p \)-values of < 0.05 was considered statistically significant. All statistical tests were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, California).

2.4 Results

2.4.1 Isolation and identification of a ciliate isolate from a food processing environment

Ciliates are a diverse and important group of protozoans. They have been detected and identified most commonly through careful microscopic observation of morphological properties. This approach can be difficult, is time-consuming and requires trained personnel. It is not a reliable means of detection or identification for many protozoan species, since the distinction of a given morphological feature can be problematic. The extremely rapid movement of some ciliates is an additional complicating factor that makes observation of live cells difficult. Consequently, molecular techniques based upon analysis of small-subunit RNA gene sequences and PCR are considered to be more reliable method of identification of protozoan species. Other published studies have successfully identified species of protozoans based on molecular identifications by PCR amplifications of the protozoan 18S rRNA (Brandl et al. 2005).

A ciliate isolated from a food-processing facility in South Australia was cultured by growth on \( E. coli \) DH5\( \alpha \). Morphologically distinct ciliates present in enrichment cultures were then micro-manipulated into fresh suspensions of \( E. coli \) DH5\( \alpha \). One clone of ciliates had a kidney-shaped appearance with cilia uniformly decorating the cell body. The ciliate size was approximated to be around 40 - 80 \( \mu \)m. Numerous food vacuoles were observed inside the cells. Movement was moderately rapid and irregular. Isolates formed a densely populated clonal culture within 3 – 5 d post isolation when fed with \( E. coli \) DH5\( \alpha \). These
ciliates were morphologically similar to *Colpoda* species based on the *An Illustrated Guide to the Protozoa* (Lee *et al.* 2000).

The identity of the ciliate clonal population was confirmed by PCR amplification of ciliate genomic DNA that encodes nuclear small-subunit rRNA gene (18S rRNA). The primers used were designed to amplify nucleotide bases 97 to 1731 bp of the 18S rRNA gene. Separation of the PCR products by agarose gel electrophoresis showed the presence of a single amplicon corresponding to a fragment of approximately 1.8 kbp in all four reactions (Figure 2.1). No amplicon was produced from the no template DNA negative control sample.

The 1.8 kb fragment was purified using a QIAquick PCR Purification Kit (QIAGEN) and the purified DNA was sequenced (Appendix A). BLAST analysis of the sequence data obtained from the ciliate isolate showed a high similarity with small-subunit ribosomal RNA gene sequences for a number of known *Colpoda* strains. The three most similar matches were *Colpoda* sp. PRA-118 (GenBank Accession AY905498) (97% similarity), *Colpoda* sp. LH20100323B12 (GenBank Accession JN251157) (96% similarity), and *C. steinii* strain Sp1 (GenBank Accession DQ388599) (96% similarity). The high sequence similarities obtained indicated that the axenic culture of kidney shaped ciliates was most likely to be that of the genus *Colpoda*. Phylogenetic analysis of the sequence data (Figure 2.2), indicated the new strain is part of a monophyletic group in a clade comprising of *Colpoda* sp. PRA118, *Colpoda* sp. LH20100323B12 and *C. steinii* (high similarity of 100%), and is most closely related to both *Colpoda* sp. PRA-118 and *Colpoda* sp. LH20100323B12 (similarity of 92%). This monophyletic group of four taxa is sister to another clade that comprises of *Colpoda* sp. RR and *C. aspera*, and also has high similarity with this clade (similarity of 100%). The clade comprising the newly isolated strain, *Colpoda* sp. PRA-118 and *Colpoda* sp. LH20100323B12 is unresolved, as there is no phylogenetic similarity (0%) for the grouping of the newly isolated strain and *Colpoda* sp. PRA-118. On the basis of this analysis, the ciliate was tentatively identified as a *Colpoda* and in this thesis is referred to as *Colpoda* MLS-5.
2.4.2 Co-culture of *Colpoda* RR and MLS-5 with bacteria

Data in Table 2.1 and Table 2.2 present counts of viable *Colpoda* RR and MLS-5 post feeding with different strains of bacteria at 25°C. Overall, the pattern of growth of *Colpoda* RR and MLS-5 during co-culture with DRDC8 was similar. Counts of *Colpoda* RR for the first 12 h of co-culture were not significantly different (*p* > 0.05) and remained at levels of ca. 1.2 × 10⁵ cells mL⁻¹, then increased to ca. 4.9 × 10⁵ cells mL⁻¹ (*p* < 0.05) by 24 h of co-culture (Table 2.1). Similarly, counts of viable *Colpoda* MLS-5 increased from ca. 1.3 × 10⁵ cells mL⁻¹ at 0 h post feeding to ca. 5.2 × 10⁵ cells mL⁻¹ by 12 h post feeding (*p* < 0.05) with DRDC8, followed by a reduction (*p* < 0.05) in counts to ca. 1 × 10⁵ cells mL⁻¹ by 24 h of co-culture (Table 2.2).

To confirm that the results obtained was an indication of killing of DRDC8 by the ciliates and not due to the incubation method used, suspensions of *Colpoda* RR and MLS-5 were incubated in AS buffer without addition of bacteria (no bacteria control). *Colpoda* RR viable counts decreased by ca. 10-fold from ca. 1.4 × 10⁵ cells mL⁻¹ at 0 h post incubation to ca. 2.3 × 10⁴ cells mL⁻¹ at 24 h post incubation. Concomitantly, viable counts of *Colpoda* MLS-5 decreased from ca. 1.3 × 10⁵ cells mL⁻¹ at 0 h post feeding to ca. 6.1 × 10⁴ cells mL⁻¹ (*p* < 0.05). The observed decrease in viable counts of *Colpoda* ciliates following 24 h in the absence of bacteria confirmed that the increase in viable counts of *Colpoda* RR and MLS-5 following co-culture with DRDC8 is the direct effect of *Colpoda* ciliates feeding on DRDC8 as food source.

To determine whether *Colpoda* isolates were also able to utilize other types of bacteria as a food source, co-cultures with *E. coli*, *S. Typhimurium* and *B. subtilis* were performed. During co-cultures of *Colpoda* RR with DH5α, counts of *Colpoda* RR increased (*p* < 0.05) from ca. 1.5 × 10⁵ cells ml⁻¹ to ca. 5.4 × 10⁵ cells mL⁻¹ in viable ciliate counts (Table 2.1). A similar increase (*p* < 0.05) in counts of *Colpoda* MLS-5 was recorded from ca. 1.2 × 10⁵ cells ml⁻¹ at 0 h post feeding to ca. 5.2 × 10⁵ cells ml⁻¹ at 12 h post feeding, followed by a reduction in counts to ca. 1.0 × 10⁵ cells ml⁻¹ at 24 h post feeding (*p* < 0.05) (Table 2.2). Co-cultures of *Colpoda* RR and *S. Typhimurium* C5 and *B. subtilis* were also performed (Table 2.1). Overall, counts of *Colpoda* RR following co-cultures with either C5 or *B. subtilis*, increased significantly following 24 h post feeding. Following co-culture with C5, counts of viable *Colpoda* RR increased from ca. 1.4 × 10⁵ cells ml⁻¹ at 0 h post feeding...
to ca. $5.1 \times 10^5$ cells ml$^{-1}$ at 24 h post feeding ($p < 0.05$). Similarly, counts of viable \textit{Colpoda} RR following co-culture with \textit{B. subtilis} increased from \textit{ca.} $1.4 \times 10^5$ cells ml$^{-1}$ at 0 post feeding, to \textit{ca.} $5.1 \times 10^5$ cells ml$^{-1}$ at 24 h post feeding.

Taken together, the above data indicated that both \textit{Colpoda} RR and MLS-5 were able to feed on DRDC8 as food source, resulting in an increase in viable counts of the \textit{Colpoda} ciliates (Figure 2.3). The fact that \textit{Colpoda} also fed on DH5α, C5, and \textit{B. subtilis}, indicated that these ciliates are able to utilize a range of Gram positive and negative bacteria as a source of nutrients. Furthermore, feeding on these bacteria resulted in a concomitant increase in ciliate numbers irrespective of the bacteria’s pathogen type.

Given that \textit{Colpoda} ciliates were able to grow on cultures of bacteria, it was of interest to quantitatively study the fate of bacteria internalized following co-culture with \textit{Colpoda} RR and MLS-5. Viable counts of intra-ciliate DRDC8, \textit{B. subtilis}, C5 and DH5α were determined at different times post feeding to \textit{Colpoda} RR at 25°C (Figure 2.4 and Figure 2.5) and MLS-5 (Figure 2.8). These data confirmed that co-culture for up to 4 h typically resulted in a reduction of intra-ciliate bacterial counts to below detectable levels. When co-cultured with \textit{Colpoda} RR, the viability of both intra-ciliate DRDC8 and \textit{B. subtilis} reduced from \textit{ca.} $6.0 \times 10^3$ CFU mL$^{-1}$ at 0 h post feeding to \textit{ca.} $8.0 \times 10^2$ CFU mL$^{-1}$ at 2 h post feeding ($p < 0.05$), and were below detectable levels at 4 h post feeding. Similarly, counts of intra-ciliate DRDC8 following co-culture with \textit{Colpoda} MLS-5 decreased from \textit{ca.} $7.0 \times 10^3$ CFU mL$^{-1}$ at 0 h post feeding to \textit{ca.} $8.0 \times 10^2$ CFU mL$^{-1}$ at 1 h post feeding ($p < 0.05$), and were below detectable levels within the ciliates by 2 h post feeding. A similar result was obtained for \textit{Colpoda} RR pulse fed with \textit{B. subtilis}. Furthermore the trends of viability of intra-ciliate C5 and DH5α following co-culture with \textit{Colpoda} RR were almost identical. Viable counts reduced from \textit{ca.} $6.0 \times 10^3$ CFU mL$^{-1}$ at 0 h post feeding to \textit{ca.} $8.0 \times 10^2$ CFU mL$^{-1}$ at 1 h post feeding ($p < 0.05$), but were below detectable levels at 2 h post feeding. Most notably, counts of viable intra-ciliate Gram negative bacteria however were reduced to undetectable levels 2 h post feeding, whereas viable intra-ciliate Gram positive bacteria were only eliminated by \textit{Colpoda} ciliates within a 4 h period of post feeding.
2.4.3 Inhibition of Colpoda Phagocytosis prevents Uptake of Bacterial Cells

Professional intracellular pathogens such as *L. monocytogenes* are able to mediate uptake into mammalian non-professional phagocyte cell lines (Gaillard *et al.* 1987, Vázquez-Boland *et al.* 2001). To determine whether (1) host phagocytosis is required for internalization of bacteria by *Colpoda* or (2) if the bacterial cells are able to mediate host phagocytosis-independent invasion of *Colpoda*, co-cultures were performed with ciliates and bacteria treated with the phagocytosis-inhibitor TEA chloride at 25°C. Counts of intra-ciliate bacteria present in lysates prepared from co-cultures with TEA-treated *Colpoda* RR or MLS-5 are presented in Figure 2.4 (DRDC8 and *B. subtilis*), Figure 2.5 (C5 and DH5α), whereas Figure 2.6 shows similar data for DRDC8 post feeding with TEA-treated *Colpoda* MLS-5. For all samples of co-cultures with TEA-treated preparations of *Colpoda* RR and MLS-5, counts of viable intra-ciliate bacteria were below detectable levels in lysates prepared 1 h post feeding, but increased slightly to ca. $1 \times 10^2$ CFU mL$^{-1}$ at 2 h post feeding, then reduced to below detectable levels by 4 h post feeding. This data contrasts significantly with counts of viable intra-ciliate bacteria obtained with co-cultures with untreated, control preparations of *Colpoda* cells. As expected, counts of viable bacteria in these cells decreased from about $6 \times 10^3$ CFU mL$^{-1}$ to undetectable levels by 4 h post feeding (for DRDC8 and *B. subtilis*) and 2 h post feeding (for C5 and DH5α). Taken together, this data indicated that active phagocytosis by the ciliates is required for bacteria to be internalized within vacuolar structures. Further, the data shows that professional intracellular pathogens like *Salmonella* and *L. monocytogenes* are unable to initiate uptake into *Colpoda* cells, as is the case for mammalian cells.

To confirm the microbiology data, treated *Colpoda* MLS-5 and GFP-expressing cells of ADGA were co-cultured for observations by fluorescence microscopy (Figure 2.7). GFP-expressing bacterial cells were not detected in samples of TEA-treated *Colpoda* MLS-5 at 0.5 h post feeding (Figure 2.7A), although these bacteria were detected in ciliates from untreated control co-cultures. Some GFP-expressing bacterial cells were found within located within vacuoles of the ciliates after 2 h post feeding (Figure 2.7B). As expected, untreated control *Colpoda* MLS-5 cells contained numerous GFP-expressing bacterial cells within vacuoles at 0.5 post feeding (Figure 2.7C), but not after 2 h post feeding (Figure
2.7). This outcome closely corresponds with the quantitative microbiology data on presented as Figure 2.6.

The data presented in this section, indicated that phagocytosis is required for internalization of DRDC8 within both *Colpoda* RR and MLS-5, as well as for internalization of C5, DH5α and *B. subtilis* within *Colpoda* RR. Apparently, DRDC8 is not able to invade *Colpoda* RR and MLS-5 independently of phagocytosis by these ciliates.

### 2.4.4 TEM of Co-cultures of *Colpoda* RR and MLS-5 with Bacteria.

TEM was used to qualitatively study the fate of *L. monocytogenes* DRDC8 (Figure 2.8 and Figure 2.9), *E. coli* DH5α (Figure 2.10), *B. subtilis* (Figure 2.9) and S. Typhimurium C5 (Figure 2.8 and Figure 2.10) phagocytosed by *Colpoda* RR at 25°C, as well as DRDC8 phagocytosed by *Colpoda* MLS-5 (Figure 2.11). Examination of ultra-thin sections of infected ciliates 1 h post feeding showed that intact bacterial cells were confined individually within tight vacuoles of DRDC8-fed *Colpoda* RR and *Colpoda* MLS-5 (Figure 2.8A and Figure 2.11A respectively). Sections of bacterial cells within vacuoles had well-defined cell wall structures with a thick peptidoglycan layer typical of Gram positive cell walls (Figure 2.8B and Figure 2.11A). Typically DRDC8 cells were present within tight vacuoles in close juxtaposition with large food vacuoles containing particulates of bacterial cell size (that are presumably the remains of intact bacterial cells), as seen in DRDC8-fed *Colpoda* MLS-5 (Figure 2.11B).

At 2 h post feeding with DRDC8, sections of *Colpoda* RR were dominated by large food vacuoles containing particles of bacterial cell size, adjacent to apparently intact bacterial cells with typical cell wall morphology. Separate DRDC8 cells within tight vacuoles were also observed in sections of ciliates 2 h post feeding (Figure 2.9A and Figure 2.9B). In some sections of *Colpoda* RR cells co-cultured with DRDC8, food vacuoles were observed to be in close juxtaposition with the ciliate contractile vacuole. (Figure 2.9A and Figure 2.9B). These contractile vacuoles contained apparently intact bacterial cells and a number of bacteria-sized particulates that were presumed to be the remains of digested bacterial cells.

Sections of *Colpoda* RR from co-cultures with DRDC8 for up to 4 h post feeding were also characterised by a cytoplasm filled with large food vacuoles containing degraded
particulates of bacterial cell size (Figure 2.9C). These electron-dense bacterial-sized particulates lacked a distinct cell wall structure typical of intact bacteria. No bacterial cells with intact cell wall structures were identified in any sections examined. By contrast, sections of *Colpoda* MLS-5 examined did not contain intact bacterial cells within tight vacuoles or large food vacuoles (Figure 2.11C) typical of *Colpoda* RR at 4 h post feeding with DRDC8. None of the sections of bacteria within *Colpoda* cells from 24 h co-cultures exhibited well-developed septa typical of dividing cells (data not shown).

TEM of sections of *Colpoda* RR co-cultured with *B. subtilis* mirrored observations of co-cultures with DRDC8. At 2 h post feeding with *B. subtilis*, the ciliate cytoplasm contained bacterial cells with intact cell walls located in tight vacuoles. These phagosomes were usually adjacent to large food vacuoles containing particulates that lacked an intact cell wall (Figure 2.9D and Figure 2.9E). Figure 2.9F shows a section of bacterial cells with intact cell walls, thick peptidoglycan layer typical of Gram positive bacteria.

Interestingly, sections of *Colpoda* RR from co-cultures with C5 at 0.5 h post feeding were predominated by food vacuoles that were densely packed with apparently partially degraded bacterial cells characterised by loss of structure typical of these cells. Very few sections of intact bacterial cells within tight vacuoles were observed at this stage of co-culture (Figure 2.8C and Figure 2.8D).

Surprisingly, unlike co-cultures with Gram positive bacteria (DRDC8 and *B. subtilis*), TEM sections of *Colpoda* RR co-cultured with DH5α cells at 2 h post feeding did not contain any intact bacterial cells within tight vacuoles. Instead, sections of these cells contained small food vacuoles densely packed with particulates of bacterial cell size, suggesting that phagocytosed DH5α (Figure 2.10A) and C5 (Figure 2.10C) were degraded by *Colpoda* RR within 2 h post feeding. Each food vacuole contained closely packed, electron-dense, bacteria-sized particles. All of the sections of bacterial cells within food vacuoles had a curled morphology and appeared to be enclosed within a membrane-like structure atypical of the rod-shaped appearance of normal, growing cells (Figure 2.10B and D). Mitochondria were distributed throughout the cytoplasm of the ciliates, but were especially concentrated at the periphery of food vacuoles.
2.4.5 Co-culture of *Colpoda* RR and activated charcoal-cultured *L. monocytogenes* DRDC8

2.4.5.1 Haemolytic activity of activated charcoal-cultured *L. monocytogenes* DRDC8

Activated charcoal mediated stimulation of expression of Listeriolysin O (Ripio 1996, Ermolaeva 1999) by *L. monocytogenes* DRDC8 was tested using LEM cultures prepared in the presence/absence of activated charcoal. Stabs on plates of 5% (v/v) sheep blood agar (data not shown) as well as haemolysis of 1% sheep erythrocytes (Figure 2.12) confirmed that the levels of Listeriolysin O expressed by activated charcoal-cultured DRDC8 were higher than LEM-cultured DRDC8.

To determine whether the increased levels of Listeriolysin O expressed by activated charcoal-cultured DRC8 affected the outcome of co-culture of DRDC8 and *Colpoda* RR, counts of viable *Colpoda* RR (Table 2.1) and viable intra-ciliate activated charcoal-cultured DRDC8 (Figure 2.13) were determined for co-cultures at 25°C. Counts of viable *Colpoda* RR increased from ca. 1.4 × 10^5 cells ml\(^{-1}\) at 0 h post feeding to 5.2 × 10^5 cells ml\(^{-1}\) at 24 h post feeding (p < 0.05), similar to the increase in the counts of viable *Colpoda* RR following co-cultures with LEM-cultured DRDC8 (Table 2.1). Counts of intra-ciliate activated charcoal-cultured DRDC8 decreased from ca. 10^3 CFU mL\(^{-1}\) at 0 h post feeding to ca. 10^2 CFU mL\(^{-1}\) at 2 h post feeding (p < 0.05), followed by reduction to undetectable levels at 4 h post feeding (Figure 2.13), presumably as a result of predation by *Colpoda* RR. Overall, the decrease in intra-ciliate activated charcoal-cultured DRDC8 viable counts was similar to counts obtained for LEM-cultured DRDC8 (p > 0.05).

TEM of activated charcoal-cultured DRDC8 phagocytosed by *Colpoda* RR at 25°C are presented in Figure 2.14. As was the case for co-cultures with LEM-cultured DRDC8 (Figure 2.8A and B), sections of activated charcoal-cultured DRDC8-fed *Colpoda* RR at 1 h post feeding contained many intracellular bacteria confined individually within tight vacuoles (Figure 2.14A). These bacterial cells had intact cell wall structures and the thick peptidoglycan layer typical of Gram positive bacteria was clearly visible (Figure 2.14B). *Colpoda* RR sections examined contained only a few large food vacuoles filled with particulates of digested bacteria (Figure 2.14A). After 4 h co-culture post feeding, *Colpoda*
RR contained many large food vacuoles filled with electron dense particles of bacterial cell size (Figure 1.14C) that did not have any obvious cell wall structure (Figure 2.14D). None of the sections of Colpoda RR from 4 h co-cultures contained any intact bacterial cells, nor was there any evidence of cell division or survival within infected Colpoda RR co-cultures incubated for 4 – 24 h post feeding (data not shown).

These observations correlated with the counts of viable intra-ciliate activated charcoal-cultured DRDC8 following co-culture with Colpoda RR (Table 2.1). Furthermore, the outcomes of co-cultures of Colpoda RR with activated charcoal-cultured DRDC8 were indistinguishable from that of LEM-cultured DRDC8.

2.4.6 Survival of L. monocytogenes DRDC8 within Colpoda RR Cysts

Previous work has reported encapsulation of L. pneumophila within amoebic cysts (Kilvington and Price 1990). To determine whether Colpoda cells co-cultured with L. monocytogenes DRDC8 could encapsulate these bacteria within cysts, cyst formation was induced by suspension of the ciliates in encystment medium. Washed cells of DRDC8 were incubated for 12 h at 25°C in either encystment medium (Yamaoka et al. 2004) or AS buffer (control) to confirm that the uptake of the encystment medium by Colpoda RR did not affect the viability of DRDC8 within the ciliates. These treated bacterial suspensions were then serially diluted and plated out on LEM agar, and incubated at 37°C overnight. The number of CFU mL⁻¹ was then determined. Counts of untreated DRDC8 were used as a control. Figure 2.15 presents viable counts that indicate the survival of DRDC8 is not affected by the encystment medium. Viable counts obtained were almost identical to those obtained for the untreated control ($p > 0.05$).

By observation using light microscopy, none of the Colpoda RR pulse fed with DRDC8 encysted within a 4 h period post feeding (data not shown). After 4 h incubation post feeding, Colpoda RR ciliates were observed to round up and by 12 h had formed mature cysts (as determined by the presence of a thick endocyst layer). TEMs of Colpoda RR cysts are presented on Figure 2.16. The sections of cysts shown in Figure 2.16A and Figure 2.16C are of a young cyst based on the presence of the thin cyst wall layers compared to that of the more mature cyst shown in Figure 2.16D. Figure 2.16A and C show bacterial cells within the ectocyst layer of a young cyst wall. None of the sections of
intact *Colpoda* cysts examined contained bacterial cells within the cytoplasm. Although the cyst shown in Figure 2.16B contains many bacterial cells, this cyst is also characterised by a loss of cytoplasmic content, suggesting that this structure may not be viable. However, this micrograph also shows bacterial cells located within the double ectocyst layers. The thick bacterial cell wall of the bacterial cells (Figure 2.16E) is typical of Gram positive bacteria.

All sections of intact cysts were characterised by mitochondria within the cytoplasm (Figure 2.16A). The outermost layer (ectocyst) of the sections examined, appeared to be made of thin myelin-like material, that was irregular in structure and separated from the inner smooth layer (endocyst). In Figure 2.16D, the ectocyst layer of the mature cyst is divided and encloses a bacterial cell. Bacterial cells were not observed within the cytoplasm of mature cysts.

### 2.4.7 Biofilm grazing by *Colpoda* RR and MLS-5

Bacterial biofilms were developed by extended culture of bacteria in the wells of 96 well plates with incubation at 4, 25 or 30°C. The biomass of biofilms developed by a number of *L. monocytogenes* isolates (DRDC8, EGD Kaufmann and KE925) as well as *B. subtilis*, *E. coli* DH5α and *S. Typhimurium* C5 are presented on Figure 2.17. Biofilm formation at 25°C and 30°C of all bacteria strains (except *S. Typhimurium* C5) was observed as early as 24 h post inoculation and as expected was much greater than that observed at 4°C (data not shown). Even with extended incubation times for up to 72 h post inoculation at 4°C, biofilm formation of all bacteria strains tested never reached the levels observed at 25°C and 30°C. The three isolates of *L. monocytogenes* tested formed highest biofilm biomass when incubated at 30°C and lowest biomass at 4°C (Figure 2.17). However, when incubated at 25°C and 30°C, cultures of EGD Kaufmann formed a higher (*p < 0.05*) biofilm biomass than the either DRDC8 or KE925. Nevertheless, the biofilm biomass produced by all three strains of *L. monocytogenes* was higher (*p < 0.05*) than other bacteria strains at all three temperatures. Though *B. subtilis* and *E. coli* DH5α had a generally lower biofilm biomass than *L. monocytogenes* at all growth temperatures tested, *S. Typhimurium* C5 produced the least amount of biofilm in comparison to other bacteria strains tested (*p < 0.05*). Crystal violet staining of biofilms within 96-well microtitre plates revealed that the morphology of the biofilm is dependent on the temperature of incubation, irrespective
of the bacteria strains (data not shown). Based on direct observations (data not shown), biofilms at 4°C were formed as a ring around the bottom of the wells, biofilms at 25°C were formed as both a ring around the bottom of the wells as well as a dense mass in the centre of the wells, whereas biofilms at 30°C were formed as only a dense mass in the centre of the wells.

When the bacterial biofilms were incubated with *Colpoda* RR or MLS-5 cells and these co-cultures examined by light microscopy, marked reductions in ciliate motility, viability and encystment were observed during incubation at 4 and 37°C, but not at 25°C (data not shown). Hence, all subsequent biofilm grazing experiments were performed at 25°C. The pre-grazing and post-grazing estimates of biofilm biomass and counts of viable ciliates are presented in Figure 2.18 and Figure 2.19 (*Colpoda* RR), Figure 2.20 and Figure 2.21 (*Colpoda* MLS-5). The controls used in this experiment were biomass of biofilms without the addition of *Colpoda*, as well as *Colpoda* only (not grazing on biofilm). Biofilms of six strains of bacteria were tested: DRDC8, EGD Kaufmann, KE925, *B. subtilis*, *S. Typhimurium* C5 and *E. coli* DH5α.

As expected, in the absence of added *Colpoda* RR, the biomass of the control biofilm of DRDC8 remained relatively unchanged for up to 48 h post incubation (*p* > 0.05) (Figure 2.18A). By 48 h post grazing by *Colpoda* RR, DRDC8 biofilm biomass estimates had steadily decreased (*p* < 0.05) compared to estimates at the commencement of grazing. A concomitant increase (*p* < 0.05) in counts of viable *Colpoda* RR (ca. $1 \times 10^5$ cells mL$^{-1}$ at the commencement of grazing to ca. $5.8 \times 10^5$ cells mL$^{-1}$ at 24 h post grazing) was observed when these ciliates grazed on the DRDC8 biofilm. This increase in counts of ciliates was similar to that noted following feeding on planktonic DRDC8 (Table 2.1). However, counts of viable *Colpoda* RR reduced from ca. $5.8 \times 10^5$ cells mL$^{-1}$ at 24 h post grazing to ca. $1 \times 10^5$ cells mL$^{-1}$ by 48 h post grazing (*p* < 0.05). The observed reduction in the biomass of the DRDC8 biofilm from 24 h to 48 h post grazing is due to grazing by the remaining vegetative *Colpoda* RR cells. As expected, there was a decrease in the viable counts of ungrazing *Colpoda* RR (in the absence of biofilm) from ca. $1 \times 10^5$ cells mL$^{-1}$ at 0 h post incubation to ca. $1 \times 10^4$ cells mL$^{-1}$ at 48 h post incubation. In general, similar outcomes were observed for biofilms of other bacteria strains tested (Figure 2.18B - D and Figure 2.19) and for co-cultures with *Colpoda* MLS-5 (Figure 2.20 and Figure 2.21)
The significant increase in viable counts of both isolates of *Colpoda* ciliates following grazing on *L. monocytogenes* biofilms together with the decrease in the viable counts of the ungrazing control *Colpoda* ciliates (suspensions of *Colpoda* ciliates in the absence of biofilm) demonstrated that *Colpoda* RR and MLS-5 are able to graze on *L. monocytogenes* biofilms as a food source. The ability of *Colpoda* RR and MLS-5 to also graze on biofilms of other strains of bacteria also suggested that *Colpoda* will graze a variety of bacterial types.

SEM was used to examine the impact of grazing of DRDC8 biofilms by *Colpoda* RR. Biofilms were collected 72 h post inoculation and subjected to grazing by *Colpoda* RR. SEMs of biofilms prior to grazing are presented as Figure 2.23A - C. The biofilm covered large surface areas of the coverslip (Figure 2.23A) and comprised about two layers of bacterial cells in depth (Figure 2.23B). The cells within the biofilm were distinctly rod-shaped and overall appeared to be devoid of any visible extracellular matrix (Figure 2.23B and Figure 2.23C), although the inter-cellular space between each DRDC8 cells was partly filled by some material. After 48 h post grazing by *Colpoda* RR (Figure 2.23D), most of the bacterial cells in the biofilm were removed. Other than the very few remaining ungrazed DRDC8 biofilm cells, only debris, probably from the grazing activities, was present. This observation corresponds with the observation (Figure 2.18A) a significant reduction (*p* < 0.05) in biofilm biomass had occurred following 48 h grazing by *Colpoda* RR compared to control, ungrazed biofilms.

Harmsen (2010) has previously shown that *L. monocytogenes* eDNA may be the only component of the extracellular matrix of biofilm formed by these bacteria. This observation may explain the lack of the typical extracellular matrix as seen by SEM analysis in Figure 2.23. To determine whether eDNA contributed to biofilm development by *L. monocytogenes* strains DRDC8, EGD Kaufmann and KE925, biofilm development in 96-well microtitre plate biofilm assays was assessed in the presence or absence of DNase I. DNase I was added to biofilms in two ways: (1) into wells during inoculation as well as at every 24 h during medium change for up to 72 h at 25°C, and (2) into wells of 72 h biofilms and incubated for up to 3 h at 25°C. Control biofilms were not treated with DNase I. Estimates of biofilm biomass for cultures incubated with or without DNase I are
presented on Figure 2.24. DNase I completely eliminated formation of a biofilm irrespective of the strain of *L. monocytogenes* used, or the method of DNase I used.

To determine whether biofilm cultures of *L. monocytogenes* strains expressed any compounds that inhibited *Colpoda* cells, biofilm supernatants were collected and tested for inhibitory effects on suspensions of *Colpoda* RR and MLS-5. Counts of *Colpoda* ciliates in the presence of biofilm supernatants supplemented with heat-killed *E. coli* DH5α are presented in Figure 2.25. Counts of control suspensions incubated in the presence of heat-killed *E. coli* DH5α alone are also shown. Suspensions incubated in the presence of supernatants prepared from *B. subtilis* biofilms were also included as a control. None of the supernatants prepared from the three *L. monocytogenes* strains tested had any effect on the viability of either *Colpoda* RR (Figure 2.25A) or *Colpoda* MLS-5 (Figure 2.25B). The counts of ciliates obtained, was similar to each of the controls used. Compared with the counts of control ciliates (without biofilm supernatant), there was no significant difference in the counts of viable ciliates incubated with biofilm supernatants irrespective of strains of *L. monocytogenes* tested (*p* > 0.05). Both strains of ciliates fed on the supplemented heat-killed *E. coli* DH5α as food source and increased in numbers during incubation.

### 2.5 Discussion

Bacteria and free-living protozoans are commonly found in freshwater and marine aquatic environments. Grazing activity by protozoans is a major cause of bacterial mortality in the environment. Hence, grazing by protozoans exerts a strong selection pressure on bacterial biomass and community composition in the environment (Hahn and Hofle 2001). In this context, the major outcomes of a bacteria-host cell interaction include:

(a) the host cells are able to phagocytose and degrade bacterial cells within the vacuoles in host cell cytosol, or

(b) the bacterial cells are able to initiate uptake into the host cell, multiply and lyse the host cell or

(c) the bacterial cells are able to invade the host cell cytosol and multiply without lysing the host cell.
Protozoans other than Acanthamoeba spp. and Tetrahymena spp. have had limited use as host systems to study prey-predator interactions with pathogenic bacteria. During this study, two independent isolates of the ciliate Colpoda were used as an alternative host for predator-prey studies during co-culture with bacteria. Colpoda ciliates feed on bacteria and are able to multiply rapidly under favourable conditions and these characteristics give this organism an advantage over other ciliates as an experimental system. The ease with which Colpoda ciliates can be cultivated under laboratory conditions also made it a desirable organism for this type of study.

Previous studies of co-cultures between Colpoda and L. monocytogenes have determined that ciliate-to-bacteria ratios of ca. 1:400 (or moi) allow growth and maintenance of these ciliates (Raghu Nadhanan 2008). Typically, the most effective concentration of bacteria used for co-cultures in this study was ca. $10^9$ cells mL$^{-1}$. At this concentration, sufficient bacteria were present to maintain the viability of Colpoda RR and MLS-5. In order for the ciliates to feed, the bacteria prey cells must be present at a sufficient cell density for ciliates to contact potential prey cells. Excessively high moi may lead to rapid ciliate death. Conversely, a low moi may require the ciliates to move around more in order to search for food, resulting in loss of energy in the ciliates.

Due to the widespread prevalence of ciliated protozoan cells and L. monocytogenes in natural environments, a primary aim of this study was to determine whether Colpoda ciliates could play a role in survival of L. monocytogenes, especially as the habitats of these ciliates are likely to overlap those of L. monocytogenes. While it is well known that L. monocytogenes cells are able to effectively parasitize mammalian cells by escape from phagosomal vacuoles into the host cell cytoplasm, the evidence for the same outcome between protozoan-L. monocytogenes interaction is less well defined. Evidence from studies with Acanthamoeba spp. indicated protozoans rapidly killed L. monocytogenes cells (Huws et al. 2008, Akya et al. 2009). Whether this fate is common for interactions with other protozoan species such as ciliates, is unclear. For this reason, co-culture experiments with Colpoda ciliates were conducted to gain an insight of possible bacteria-ciliate interactions. In order to better understand the interaction between Colpoda ciliates and L. monocytogenes, this study also included co-cultures with S. Typhimurium strain C5 (a well-established intracellular bacterial pathogen), as well as two non-pathogenic
bacteria: *E. coli* DH5α and *B. subtilis*. Together, these four bacterial strains were used to compare the responses of *Colpoda* ciliates to different types of Gram negative and Gram positive bacteria.

For this work, two isolates of *Colpoda* ciliates from a natural water body and a food processing plant were co-cultured primarily with *L. monocytogenes* strain DRDC8. This strain of *L. monocytogenes*, originally isolated from a dairy processing environment in New South Wales, has been previously characterized as pathogenic for mice (Francis and Thomas 1996) and used as a test organism in co-cultures with *Acanthamoeba* spp. (Akya et al. 2009). Raghu Nadhanan (2008) showed that following separate co-cultures of *Colpoda* sp. RR with either pathogenic strains of *L. monocytogenes* such as DRDC8 or EGD Kaufmann, or a non-pathogenic, listeriolysin O mutant strain (LLO17), the outcomes of the co-cultures were consistent, regardless of the strain of *L. monocytogenes* used. All strains of *L. monocytogenes* were actively phagocytosed and degraded by the host ciliate (Raghu Nadhanan 2008). Hence for the present study, only the DRDC8 strain was used for co-culture experiments.

A unique aspect of the work described was the methodology adopted for feeding bacteria to *Colpoda* cells. Typically published co-culture studies have fed protozoans with target bacteria for the entire duration of the experiment. Given the voracious appetite of ciliates for bacterial cells, the ciliates will continuously process the bacteria as a food source. This means it is difficult to dissect the individual processes involved. In this study an alternative feeding strategy, involving pulse feeding bacteria to *Colpoda*, was adopted. By sampling ciliates at different time points post feeding, it was hoped that discrete phenotypic and molecular events that determine the fate of ingested bacterial cells could be more easily tracked.

### 2.5.1 *Colpoda* kill Intra-ciliate *L. monocytogenes*

Surprisingly, the results of co-cultures demonstrated that *Colpoda* RR and MLS-5 were able to effectively phagocytose *L. monocytogenes* and other bacterial cells as a food source resulting in concomitant growth of the ciliates. The combination of bacteriological counts and microscopical observation of *Colpoda* ciliates in co-culture with DRDC8 and other bacteria proved useful in the investigation of the fate of intra-ciliate bacteria. Observations
obtained by light and electron microscopy were consistent changes in counts of intra-ciliate bacteria and ciliate during co-cultures with DRDC8. Given that the number of ciliates remained almost unchanged within a 4 h period immediately post feeding, whereas counts of intra-ciliate bacteria declined rapidly, provided compelling evidence that *Colpoda* cells are not parasitized and killed by DRDC8 cells internalized within the ciliates, but are in fact used by the ciliates as a source of nutrients. This conclusion is supported by TEM studies that showed intra-ciliate *L. monocytogenes* cells did not escape the *Colpoda* phagocytic vacuoles and multiply within host cytoplasm. The corollary of these observations is that DRDC8 cells are not able to evade the intracellular killing mechanisms of *Colpoda*. The outcomes are contrary to events following *L. monocytogenes* parasitism of mammalian cells, for example DRDC8 invasion and persistence in HeLa and Caco-2 cell lines, as well DRDC8 pathogenicity in mice (Francis and Thomas 1996). Guzman and colleagues showed that besides effectively achieving intracellular and cell-to-cell spread, *L. monocytogenes* persisted in infected mouse dendritic cells for at least 24 h (Guzman *et al.* 1995).

Importantly, for the first time a description of the sequence of events that lead to killing of *L. monocytogenes* DRDC8 by *Colpoda* ciliates following co-culture is possible. Immediately following feeding, *L. monocytogenes* cells are internalized either singly within small, tight vacuoles, or in larger food vacuoles that ultimately contain many partially degraded bacterial cells. Whether the bacterial cells are initially internalized singly within tight vacuoles and then fused to form larger food vacuoles is not known. What is clear is that during the first 2 h post feeding, the number of small vacuoles containing single bacterial cells declined rapidly, while the number of larger food vacuoles increased to the point where these filled the ciliate cytoplasm. Thereafter, the count of food vacuoles steadily declined. These observations indicated that vacuoles containing single bacterial cells may fuse together to form food vacuoles. Alternatively these small vacuoles may be shunted out of *Colpoda* cells by exocytosis, or released as part of faecal pellets. Careful microscopy using tagged bacterial cells will be required to resolve the discrete events involved in formation and processing of bacteria within these tight vacuoles.

Micrographs that showed a temporal increase in large food vacuoles containing electron-dense bacteria-sized particulates within the ciliate cytosol over the first 4 h post
feeding, provided compelling evidence that *L. monocytogenes* cells are degraded and used as a source of nutrients. This observation is similar to that reported for *Acanthamoeba* spp. In that work, *L. monocytogenes* cells were unable to survive within the amoebic compartments and degradation of DRDC8 cells occurred within 5 h after phagocytosis (Akya, *et al.* 2010).

Phagocytosis involves the uptake of particulate matter by cells and this process is the primary means by which ciliates feed. Internalization of *L. monocytogenes* cells by *Colpoda* cells was confirmed to be dependent on phagocytosis by *Colpoda* RR and MLS-5, based on the outcomes of experiments designed to examine the requirement of phagocytosis for uptake of bacteria. In this study, phagocytosis by *Colpoda* isolates was inhibited by addition of the inhibitor TEA chloride as described by Peck and Duborgel (1985). In that study, the ciliate *Pseudomicrothorax dubius* was used to establish the dependence of phagocytosis on K⁺ ion concentration. Low concentrations of K⁺ were shown to favour phagocytosis, whereas in media containing high concentrations of K⁺ inhibit phagocytosis. However, phagocytosis is also strongly inhibited by the K⁺ channel blocker, TEA chloride (Peck and Duborgel, 1985). These observations were confirmed for *Colpoda* RR by Raghu Nadhanan (2008) and validated in more detail by the present study using a combination of bacteriological counts and electron microscopy. Co-cultures of *L. monocytogenes* DRDC8 with *Colpoda* RR and MLS-5 ciliates pre-treated by suspension in media containing TEA chloride confirmed that phagocytosis was required for internalization of *L. monocytogenes* cells. Identical results were also obtained for co-cultures with *S. Typhimurium* C5, *E. coli* DH5α and *B. subtilis*. The effectiveness of TEA chloride as an inhibitor is not long lasting. Small numbers of bacterial cells are internalized by treated *Colpoda* ciliates after *ca.* 2 h incubation post feeding.

An interesting benefit of these inhibition studies was the observation that in the absence of phagocytosis, *L. monocytogenes* is apparently unable to initiate its own uptake into *Colpoda* ciliates. This indicated that the Internalin dependent pathways of invasion of mammalian cells do not operate in the *Colpoda* system. The inability of *S. Typhimurium* C5 to initiate its own uptake into *Colpoda* RR also starkly contrasts with outcomes of infection of mammalian cells, where for example, the bacteria actively induce their own uptake into specialized epithelial M cells using the *Salmonella* pathogenicity island 1-
TTSS (Jones et al. 1994). Salmonella cells also promote entry into macrophages by stimulating the process of macropinocytosis in the absence of host phagocytosis (Alpuche-Aranda et al. 1994).

It is unclear why L. monocytogenes are able to effectively parasitize mammalian cells, but are unable to parasitize Colpoda. There are several possibilities that may explain this outcome. As the co-culture experiments were done at 25°C, a temperature known to negatively affect the expression of the PrfA required for upregulation of virulence factors required for invasion and parasitisation of mammalian cells, it is possible that the phagocytosed L. monocytogenes cells were not competent to initiate escape from the vacuole into the ciliate cytoplasm. In this circumstance, the bacteria would not be able to escape the phagosome before destruction by the host. In order to assess whether the level of Listeriolysin O expression was a factor that limited parasitism of Colpoda ciliates, L. monocytogenes was cultured under conditions known to enhance Listeriolysin O expression prior to co-culture. Ripio et al. (1996) demonstrated that growth of wild-type strains of L. monocytogenes in media containing activated charcoal-treated resulted in expression of Listeriolysin O up to levels similar to that of constitutive, hyperhaemolytic variants. The same study also showed bacteria cultured in medium containing charcoal, resulted in induction of expression of virulence factors to levels similar to that expressed by mutant strains of L. monocytogenes constitutive for expression of those virulence factors. These observations were confirmed by a separate study (Ermolaeva et al. 1999). However, the fact that Colpoda RR was able to feed on activated charcoal-cultured L. monocytogenes DRDC8 equally well as LEM-cultured DRDC8 indicated that Listeriolysin O expressed by the bacterium does not ultimately influence the fate of the internalized bacteria.

The inability of S. Typhimurium C5 to survive and multiply within Colpoda RR is highly unusual and inconsistent with previous reports on host cell - S. Typhimurium interactions that document S. Typhimurium as an efficient intracellular bacterial pathogen of a variety of cell types including protozoans. S. Typhimurium has been previously shown to grow and survive within A. polyphaga during plate co-culture (Gaze et al. 2003, Akya et al. 2010). Similar to the present study, Akya et al. (2010) utilized S. Typhimurium C5, whereas Gaze et al. (2003) utilized S. Typhimurium (strain SL1344). Growth of
S. Typhimurium was also observed within the contractile vacuoles of A. polyphaga (Gaze et al. 2003). S. Typhimurium serovar Thompson cells are able to resist killing by the ciliate Tetrahymena sp. and furthermore, its survival was enhanced within the ciliate faecal pellets (Brandl et al. 2005). S. Typhimurium use TTSS to secrete molecules that inhibit lysosomal fusion with phagosomes containing bacterial cells, thereby avoiding the killing mechanisms utilized by professional eukaryotic phagocytes. Nevertheless, S. Typhimurium C5 cells are efficiently killed and digested by Colpoda RR within 0.5 h post feeding. Clearly, molecular mechanisms employed by S. Typhimurium C5 to parasitize mammalian cells, amoebae and Tetrahymena spp., have little or no effect in preventing killing by Colpoda RR.

Though this study, as well as several other published studies (Huws et al. 2008, Akya et al. 2010), has shown that L. monocytogenes are unable to survive within host protozoans, other studies have reported this organism survives co-culture (e.g. Ly and Muller 1990). Factors that might promote survival include traits of the bacteria as well as of the host. Other factors may be serotype specific. It is known that different serotypes of L. monocytogenes exhibit different levels of pathogenicity. Of the 13 serotypes of L. monocytogenes that can cause disease, more than 90% of human isolates belong to only three serotypes: 1/2a, 1/2b, and 4b. Serotype 4b isolates are responsible for 33% - 50% of sporadic human cases worldwide (Ward et al. 2004). Sandgren et al. (2005) has shown that following inoculation of mice models with six serotypes of Streptococcus pneumoniae known to cause invasive disease in humans, differing levels of host immune response was observed, suggesting that invasive disease caused by different serotypes may result in different degrees of host response. Different serotypes of S. pneumoniae even exhibit variations in resistance to phagocytosis (Guckian et al. 1980) as well as vary in the activation of the alternative complement pathway (Fine 1975). The nature of the disease caused by S. Typhimurium is suggested to be largely dependent on the specific serotype-host combination (Watson et al. 1999). Another study has shown that even among strains of S. pneumoniae of the same multilocus sequence type (ST) and serotype, differences in the virulence of these strains in a mouse infection model were observed; and these differences were attributed to the genetic differences between the bacterial strains of the same serotype (Silva et al. 2006). These may indicate that different serotypes of
L. monocytogenes have different properties that may influence its fate during interaction with a host protozoan cell, and probably some serotypes may even be host specific.

2.5.2 Differential processing of Gram positive and Gram negative Cells

Interestingly, viable intra-ciliate S. Typhimurium C5 and E. coli DH5α were eliminated from co-cultures with Colpoda RR within ca. 2 h post feeding compared with ca. 4 h for co-cultures with either L. monocytogenes or B. subtilis. The reduction in counts of bacteria corresponded with rapid formation and turnover of food vacuoles. It remains unclear why the food vacuoles of Colpoda ciliates that formed during co-culture with Gram positive bacteria were distinct in appearance from those formed during co-cultures with Gram negative bacteria. This phenomenon has not been previously described. In addition our understanding of the way different bacteria are processed by ciliates is equally limited. The data suggest that the internalization and degradation of Gram negative bacteria by Colpoda occurs more rapidly than for Gram positive bacteria. Indeed the different morphology of Gram negative cells within food vacuoles compared with Gram positive cells, indicated that these two different groups of bacteria may be differentially processed by Colpoda cells. Whether the thick peptidoglycan layer of the Gram positive bacteria cell wall is more recalcitrant to degradation is unclear. Certainly the cell walls of Gram negative bacteria are dimensionally thinner and less compact than that of Gram positive bacteria. It is possible that this difference increases the time required for phagolysosomal enzymes to degrade the thick peptidoglycan layer of Gram positive bacteria prior to fully digesting the internalized bacteria. Nevertheless, there is some precedent for this conclusion. González et al. (1990a, b) examined differential rates of digestion of E. coli and Enterococcus faecalis by freshwater and marine phagotrophic protozoans. Their work showed that E. faecalis persisted longer than E. coli in the presence of natural microbiota and suggested that this observation was due to differences in digestion rate as well as preferences for different bacteria as a primary food source. Furthermore, Iriberrri et al. (1994) confirmed that different bacteria are eliminated at different rates by flagellates and ciliated protozoans. Importantly, they showed that Gram positive bacteria (Staphylococcus aureus and E. faecalis) were digested at low rates compared to Gram negative bacteria such as E. coli and Aerobacter aerogenes.
In addition to the presence of intact DRDC8 cells and a number of bacteria-sized particulates within the contractile vacuole, the indistinct boundary membranes of new food vacuoles and the contractile vacuole most likely demonstrates that there is a relationship between the contractile vacuole and formation of food vacuoles. This feature was only observed for sections of ciliates from co-cultures of Colpoda ciliates and DRDC8. The contractile vacuole, an organelle solely known for its role in maintaining osmotic pressure and ionic balance, has not been shown by any previous studies to have any other function.

A number of studies have demonstrated that mitochondria are recruited to vacuoles containing intracellular pathogens (Horwitz 1983, Sinai et al. 1997, Chong et al. 2009). Recruitment of mitochondria to macrophage phagosomes resulting in an upregulation of mitochondrial reactive oxygen species (mROS) for bacterial killing by the mitochondrial pathway was shown in a previous study (West et al. 2011). These reports support TEM observations that mitochondria are located at the periphery of Colpoda food vacuoles. This indicates that mitochondria are likely to be recruited to assist the ciliate cells to degrade ingested bacteria.

2.5.3 L. monocytogenes Cells associated with Colpoda Cysts

A novel aspect of this work was the finding that L. monocytogenes DRDC8 cells were observed within the outer walls (ectocyst) of Colpoda RR cysts. Exactly how these bacteria became entrapped within the outer walls during the encystment process is unclear. Interestingly, Gourabathini et al. (2008) was not able to show that L. monocytogenes became occluded within Colpoda steinii cysts following co-culture. Whether this different result is a reflection of species differences or related to differences in experimental procedure, is unclear. However bacterial cells within cysts are likely to be protected from environmental stressors for example enzymes, disinfectants and sanitisers etc. If this is the case, then these encapsulated cells may well have an impact on transmission of L. monocytogenes from environment to food products. It is also possible that these entrapped bacterial cells will be ingested by Colpoda RR cysts when the surrounding environment is conducive enough for the cysts to convert into a vegetative state.

Although the discovery that L. monocytogenes may be entrapped within cysts is novel, it is by no means unique. Other published studies have shown a similar presence of
bacteria within the outer walls of host protozoans. Cyst forms of the ciliate *T. pyriformis* has been shown to harbour viable and virulent *L. monocytogenes* cells (Pushkareva and Ermolaeva 2010). Kilvington and Price (1990) showed *A. polyphaga* cysts can contain viable cells of *L. pneumophila* and that this environment protected the entrapped bacteria from biocidal agents. A separate study has shown that *M. avium* is located within the double walls of *A. polyphaga* cysts (Steinert et al. 1998). That study reported that these outer walls of cysts may provide a reservoir for the bacteria during unfavourable environmental conditions. Another study showed that cysts of *Acanthamoeba* spp. have high levels of resistance to a variety of disinfection methods including biocidal agents (Coulon et al. 2010). However it is not known if these entrapped bacteria within the outer walls of *Colpoda* RR are protected from external stress such as disinfecting agents.

### 2.5.4 Interaction of *Colpoda* with Biofilm Cultures of *L. monocytogenes*

An unexpected finding of this study was the observation that *L. monocytogenes* biofilm cultures of were eliminated as rapidly as planktonic cultures by *Colpoda* ciliates. Indeed, the ability of *Colpoda* RR and MLS-5 to graze on biofilms of pathogenic bacteria such as *L. monocytogenes* and *S. Typhimurium* as efficiently as biofilms of the non-pathogenic *B. subtilis* and *E. coli DH5α* indicated that the grazing activity of *Colpoda* ciliates is probably independent of the type of biofilm bacteria. Previous studies have demonstrated the ability of protozoans to graze on biofilms, resulting in the alteration of the biofilm’s population dynamics (Sibbald and Albright 1988, Starink et al. 1994). However, some types of biofilm have been demonstrated to be resistant to grazing by predators. For example, biofilms of *Yersinia pestis* resist grazing by the nematode *Caenorhabditis elegans* (Darby et al. 2002), and *Vibrio cholerae* biofilms are resistant to grazing by flagellates because they induce cell lysis of the flagellate grazers (Matz et al. 2005). However, for *L. monocytogenes* biofilms, grazing by *Colpoda* lead to elimination of biofilm biomass. The loss of biofilm then leads to death of ciliates, although encystment of *Colpoda* RR likely explains the reduction in the numbers of viable *Colpoda* ciliates.

The rapid loss of *L. monocytogenes* biofilm cells caused by grazing *Colpoda* is extraordinary. The extracellular matrix of bacterial biofilms has been known to play a very important role in shielding biofilm cells from grazing protozoans (Matz 2007). The extracellular matrix functions as a physical barrier against the protozoan grazers by
limiting the ability of grazing protozoans to penetrate and phagocytose biofilm cells, as well as interfering with antibacterial components secreted by protozoan grazers.

Vestby et al. (2009) has shown that S. Typhimurium is an inefficient producer of biofilm, a finding confirmed by the present study. Other studies have shown that biofilms of S. Typhimurium, E. coli and B. subtilis do comprise an extracellular matrix (Zogaj et al. 2001, Beloin et al. 2008, Vlamakis et al. 2008). Harmsen et al. (2010) however, suggested eDNA is the major component of L. monocytogenes extracellular matrix. That study showed that in the absence of extracellular matrix, biofilm cells of L. monocytogenes are held together by a combination of N-acetylglucosamine (a peptidoglycan component) and high molecular weight DNA (Harmsen et al. 2010). In the present study, this was confirmed by demonstration that DRDC8 biofilms did not form when exposed to DNase I. The reliance by L. monocytogenes on eDNA for adhesion and biofilm development could be a consequence of organism lacking the potential to produce structural components, such as extracellular polysaccharides, that are normally associated with mature biofilms.

Previous studies have provided evidence to show that grazing by protozoans can result in the formation of predation-resistant characteristics, such as microcolonies (Matz et al. 2004, Weitere et al. 2005). However this was not the case in the present study, as microscopy observations showed absence of any biofilm cells following 48 h post grazing by Colpoda ciliates. Hence it is possible that L. monocytogenes biofilm cells did not produce any microcolonies following grazing by Colpoda.

Another feature of bacterial biofilms is the expression of compounds that inhibit biofilm grazers. For example, incubation of the flagellate Rhynchomonas nasuta within cell-free supernatants of Vibrio cholerae biofilms resulted in a ca. 80% decrease of viable counts flagellate (Matz et al. 2005). However, L. monocytogenes biofilm seemed not to produce any anti-protozoal factors; L. monocytogenes-free biofilm supernatants did not impair the viability and feeding activity of Colpoda ciliates.

2.5.5 Conclusions

The outcomes of this study showed that the inability of DRDC8 to parasitize Colpoda RR and MLS-5 is an indication that the virulence of these intracellular bacterial pathogens does not adversely affect the viability of the ciliates. This intracellular bacterial pathogen is
not capable of leading an intracellular lifestyle within these *Colpoda* strains under the conditions tested in this study. Although the level of expression of Listeriolysin O seems not to be a factor involved in failure to parasitize *Colpoda* cells, this can really only be confirmed through the use of *L. monocytogenes* mutants that are constitutive for expression of virulence genes such as Listeriolysin O and the PrfA regulatory protein required for expression of virulence factors. Host phagocytosis is required for internalization of DRDC8 within *Colpoda* ciliates. Apparently, *L. monocytogenes* DRDC8 cells are not able to invade *Colpoda* ciliates independently of phagocytosis by this ciliate, although Gram positive and Gram negative bacteria are all efficiently internalised by *Colpoda* RR and *Colpoda* MLS-5.

- This study also provided evidence to show that *L. monocytogenes* DRDC8 can become entrapped within the outer walls of *Colpoda* RR cysts. However, whether these bacterial cells are viable or non-viable, or indeed the eventual fate of these bacterial cells is not known.

- Both *Colpoda* RR and MLS-5 are able to readily graze *L. monocytogenes* biofilm communities. Hence, growth of *L. monocytogenes* as biofilms is not likely to provide a complete protection against grazing *Colpoda* ciliates.

- The present study also provides the first evidence that *Colpoda* RR process Gram positive bacterium in a distinctly different manner than Gram negative bacteria. However, the different cellular mechanisms employed by *Colpoda* RR to process these two different groups of bacteria are not yet known. Whether this differential outcome is unique to *Colpoda* RR, or if all protozoans express this trait is yet to be elucidated.

### 2.6 Acknowledgements

We thank Ruth Williams and Lynette Waterhouse of Adelaide Microscopy for their invaluable assistance with the TEM. RRN was supported by the University of Adelaide.
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Figure 2.1: PCR amplification of DNA encoding protozoan 18S rRNA.

DNA was extracted from a clonal population of ciliates. The extracted DNA was PCR amplified using primers complementary to strongly conserved motifs that allow amplification of bases 97 through 1731 of the nuclear small-subunit rRNA gene. The primer annealing temperatures used were varied to optimise amplification of DNA encoding 18S rRNA. The DNA was analysed by electrophoresis in a 1% (w/v) agarose gel. The 2-Log DNA ladder was used as the size marker. The negative control lacked template DNA in PCR reaction mixture.
Figure 2.2: Maximum likelihood tree for Colpoda MLS-5.

This tree shows the placement of the Colpoda MLS-5 isolate from the food processing environment among Colpoda species based on the sequence of their 18S rRNA gene. 0.008 is the scale for the genetic distance represented by the branch length. The best of NNI and SPR moves were used for tree topology searching and aLRT to establish the statistical support of internal branches. The TN93+G6 substitution model was selected using ModelGenerator v0.85 by comparison of Bayesian Information Criterion scores.
Table 2.1: Growth of *Colpoda* RR in co-culture with different bacteria.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Incubation time (h)</th>
<th>Number of viable <em>Colpoda</em> RR (cells mL(^{-1} \times 10^5))</th>
<th>% Increase over 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em> DRDC8</td>
<td>0</td>
<td>1.23</td>
<td>298 *</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.90</td>
<td></td>
</tr>
<tr>
<td>Activated charcoal-cultured L. <em>monocytogenes</em> DRDC8</td>
<td>0</td>
<td>1.46</td>
<td>256 *</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.20</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> DH5(\alpha)</td>
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<td>1.56</td>
<td>248 *</td>
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<tr>
<td></td>
<td>2</td>
<td>1.46</td>
<td></td>
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<td>24</td>
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</tr>
<tr>
<td><em>S. Typhimurium</em> C5</td>
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<td>259 *</td>
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<td>2</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
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<td></td>
<td>12</td>
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<tr>
<td></td>
<td>24</td>
<td>5.13</td>
<td></td>
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<tr>
<td><em>B. subtilis</em></td>
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<td>1.46</td>
<td>249 *</td>
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<tr>
<td></td>
<td>4</td>
<td>1.43</td>
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<tr>
<td>No bacteria control</td>
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<td>1.43</td>
<td>-84 *</td>
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<td>2</td>
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<tr>
<td></td>
<td>24</td>
<td>0.23</td>
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</tbody>
</table>

Table shows changes in numbers of viable ciliates over 24 h at 25°C. Each value represents the mean of three replicate experiments. Data was compared using Tukey’s Multiple Comparison Test (* Significant at \( p < 0.05 \)). Viable counts at 24 h incubation time are compared with viable counts at 0 h. Viable *Colpoda* RR counts at 2, 4 and 12 h are not significantly different to viable counts at 0 h (\( p > 0.05 \)).
Table 2.2: Growth of *Colpoda* MLS-5 in co-cultures at 25°C with different bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Incubation time (h)</th>
<th>No. of viable <em>Colpoda</em> MLS-5 (cells mL(^{-1}) × 10(^5))</th>
<th>% Increase in 12 h</th>
<th>% Increase in 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td>0</td>
<td>1.31</td>
<td>298 *</td>
<td>-417 *</td>
</tr>
<tr>
<td>DRDC8</td>
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<td>1.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.42</td>
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<td>12</td>
<td>5.22</td>
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<tr>
<td></td>
<td>24</td>
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<td></td>
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</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>0</td>
<td>1.28</td>
<td>312 *</td>
<td>-402 *</td>
</tr>
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<td></td>
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<td>24</td>
<td>0.61</td>
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</table>

Each value represents the mean of three replicate experiments. Data was compared using Tukey’s Multiple Comparison Test (* Significant at \(p < 0.05\); ns Not Significant at \(p > 0.05\)). Viable counts at 12 h incubation times are were compared with viable counts at 0 h, whereas viable counts at 24 h incubation times are were compared with viable counts at 12 h. Viable *Colpoda* MLS-5 counts at 2 and 4 h are were not significantly different to viable counts at 0 h (\(p > 0.05\)).
Figure 2.3: Counts of viable ciliates during co-culture with *L. monocytogenes* DRDC8.

Washed suspensions of *Colpoda* RR and MLS-5 were pulse fed separately with *L. monocytogenes* DRDC8 cells for 1 h at 25°C. Pulse fed ciliates were then washed to remove any extra-ciliate bacteria, and incubated for up to 24 h at 25°C. Counts of viable *Colpoda* RR and MLS-5 were measured by direct cell counts by means of haemocytometer. Results presented are the means of three counts ± standard deviations.
Figure 2.4: Inhibition of phagocytosis of DRDC8 and B. subtilis by Colpoda RR.

Colpoda RR, L. monocytogenes DRDC8 and B. subtilis cells were treated separately with TEA chloride for 1 h at 25°C. TEA chloride-treated Colpoda RR was then pulse fed with either L. monocytogenes DRDC8 or B. subtilis for 1 h at 25°C, without removing the TEA chloride from the suspensions. Pulse fed Colpoda RR cells were then washed to remove any extra-ciliate bacteria and TEA chloride, followed by incubation for up to 24 h at 25°C. Co-cultures not treated with TEA chloride were used as a control. Counts of intra-ciliate bacteria were determined by plating ciliate lysates. Extra-ciliate bacteria were eliminated by washing prior to lysis of the ciliates. Results are the means of CFU ± standard deviations for triplicate samples.

(A) L. monocytogenes DRDC8

(B) B. subtilis
Colpoda RR and S. Typhimurium C5, and E. coli DH5α cells were treated separately with TEA chloride for 1 h at 25°C. The TEA chloride-treated Colpoda RR was then pulse fed with either S. Typhimurium C5 or E. coli DH5α for 1 h at 25°C, without removing the TEA chloride from the suspensions. Pulse fed Colpoda RR were then washed to remove any extra-ciliate bacteria and TEA chloride, followed by incubation for up to 24 h at 25°C. Co-cultures not treated with TEA chloride were used as a control. Counts of intra-ciliate bacteria were determined by plating Colpoda RR lysates. Extra-ciliate bacteria were eliminated by washing prior to lysis of the ciliates. Results are the mean of CFU ± standard deviations for triplicate samples.

(A)  S. Typhimurium C5
(B)  E. coli DH5α
Figure 2.6: Inhibition of phagocytosis of DRDC8 by *Colpoda* MLS-5.

*Colpoda* MLS-5 and *L. monocytogenes* DRDC8 cells were treated separately with TEA chloride for 1 h at 25°C. The TEA chloride-treated *Colpoda* MLS-5 was then pulse fed with *L. monocytogenes* DRDC8 for 1 h at 25°C, without removing the TEA chloride from the suspensions. Pulse fed *Colpoda* MLS-5 was then washed to remove any extra-ciliate bacteria and TEA chloride, followed by incubation for up to 24 h at 25°C. Co-cultures not treated with TEA chloride were used as a control. Counts of intra-ciliate bacteria were determined by plating ciliate lysates. Extra-ciliate bacteria were eliminated by washing prior to lysis of the ciliates. Results are the means of CFU ± standard deviations for triplicate samples.
Figure 2.7: Fluorescent microscopy of phagocytosis inhibition of *Colpoda* MLS-5.

*Colpoda* MLS-5 and GFP-expressing *L. monocytogenes* ADGA were treated with 5.0 mM TEA chloride for 15 min, and MLS-5 cells pulse fed with ADGA for 1 h at 25°C. Extra-ciliate bacteria eliminated by washing. *Colpoda* MLS-5 cells were then incubated for 0.5 and 2 h. Untreated samples were used as a control.

(A) Time = 0.5 h. Treated with 5.0 mM TEA chloride. No bacterial cells are located within vacuoles of *Colpoda* MLS-5. Bar = 7.5 µm.

(B) Time = 2 h. Treated with 5.0 mM TEA chloride. Note the several vacuoles containing GFP-expressing cells. Bar = 7.5 µm.

(C) Time = 0.5 h. Untreated control. Note the clusters of GFP-expressing cells in vacuoles of *Colpoda* MLS-5. Bar = 5 µm.

(D) Time = 2 h. Untreated control. No bacterial cells are located within vacuoles of *Colpoda* MLS-5. Bar = 7.5 µm.
Figure 2.8: TEM of Colpoda RR co-cultured with L. monocytogenes DRDC8 or S. Typhimurium C5.

(A) Bacterial cells (black arrows) within vacuoles of Colpoda RR at 1 h post feeding. Bar = 1.0 µm.

(B) As for (A). Note the apparently intact bacterial cell walls at 1 h post feeding. Bar = 0.5 µm.

(C) As for (A) except co-culture with S. Typhimurium C5. Bacterial cells (black arrows) within vacuoles of Colpoda RR at 0.5 h post feeding. Bar = 1.0 µm.

(D) As for (C). Note the close juxtaposition of bacterial cells with a food vacuole (FV) containing degraded bacterial cells. Bar = 0.5 µm.
Figure 2.9: TEM of *Colpoda* RR co-cultured with *L. monocytogenes* DRDC8 or *B. subtilis*.

(A) Bacteria (black arrows) within vacuoles of *Colpoda* RR at 2 h post feeding. Food vacuoles (FV) containing degraded bacteria are present. Bar = 0.5 µm.

(B) As for (A). Note the apparently intact bacterial cell walls. Bar = 0.25 µm. Contractile vacuole (CV). Inset shows a bacterial cell within *Colpoda* RR. Bar = 0.1 µm.

(C) As for (A) except co-culture for 4 h. Note the food vacuoles (FV) of *Colpoda* RR, containing degraded bacterial cells that lack an intact cell walls. Mitochondria (mt). Bar = 0.3 µm.

(D) As for (A) except co-culture with *B. subtilis*. Bacterial cells (arrows) within vacuoles of *Colpoda* RR. Note the food vacuoles (FV), containing degraded bacteria at 2 h post feeding. Bar = 0.8 µm.

(E) As for (D). Food vacuoles (FV) containing degraded bacteria. Bar = 0.3 µm.

(F) As for (D). Note the intact cell walls of bacteria located in vacuoles. Bar = 0.25 µm.
Figure 2.10: TEM of *Colpoda* RR co-cultured with *E. coli* DH5α or *S. Typhimurium* C5.

(A) *Colpoda* RR co-cultured with *E. coli* DH5α for 2 h post feeding. Ciliate cell structure dominated by food vacuoles (FV) containing partially destroyed bacterial cell particulates. Note the absence of any intact bacterial cells. Mitochondria (mt). Bar = 0.3 μm.

(B) As for (A). Bar = 0.2 μm.

(C) As for (A) except co-culture with *S. Typhimurium* C5. Mitochondria (mt). Bar = 0.3 μm.

(D) As for (C). Bar = 0.25 μm.
Figure 2.11: TEM of *Colpoda* MLS-5 co-cultured with *L. monocytogenes* DRDC8.

(A) Bacterial cells (black arrows) within vacuoles of *Colpoda* MLS-5 at 1 h post feeding. Note the mitochondria (mt). Bar = 1 µm.

(B) As for (A). A food vacuole, containing degraded bacterial cells. Note the close juxtaposition of single bacterial cells (black arrows) with the food vacuole (FV) containing apparently degraded bacteria. Bar = 1 µm. Inset shows a high magnification of a bacterium apparently in the midst of loss of intact bacterial cell wall. Bar = 0.25 µm.

(C) As for (A) except co-culture after 4 h post feeding. Note the absence of any intact bacterial cells and food vacuoles. Note the mitochondria (mt). Bar = 1 µm.
Figure 2.12: Listeriolysin expression by \textit{L. monocytogenes} DRDC8.

\textit{L. monocytogenes} DRDC8 cultures were grown overnight at 37°C in LEM supplemented with 0.2% activated charcoal (final concentration). Washed suspensions of overnight cultures of 0.2% activated charcoal-treated and untreated \textit{L. monocytogenes} DRDC8 cells were incubated separately with 1% sheep erythrocytes for up to 24 h at 25°C. The extent of haemolysis was measured by determining the absorbance at 545 nm. Each value represents the mean of three replicate experiments.
Figure 2.13: Counts of intra-ciliate viable activated charcoal-cultured *L. monocytogenes* DRDC8 within *Colpoda* RR.

*L. monocytogenes* DRDC8 were grown overnight in LEM supplemented with 0.2% (w/v) activated charcoal. Activated charcoal-cultured and LEM-cultured *L. monocytogenes* DRDC8 cells were pulse fed to *Colpoda* RR for 1 h at 25°C. Pulse fed *Colpoda* RR were washed to remove any extra-ciliate bacteria, followed by incubation for up to 24 h at 25°C. Counts of intra-ciliate bacteria were determined by plating *Colpoda* RR lysates. Extra-ciliate bacteria were eliminated by washing prior to lysis of the ciliates. Results are the means of CFU ± standard deviations for triplicate samples.
Figure 2.14: TEM of *Colpoda* RR co-cultured with activated charcoal-cultured *L. monocytogenes* DRDC8.

(A) Bacterial cells within *Colpoda* RR at 1 h post feeding. Food vacuoles (FV). Bar = 1 µm.

(B) As for (A). Note the apparently intact bacterial cell wall. Bar = 0.1 µm.

(C) As for (A) except 4 h post feeding. Food vacuoles (FV). Bar = 1 µm.

(D) As for (C). Note the bacterial cells that lack intact walls. Bar = 0.25 µm.
Figure 2.15: Viable counts of *L. monocytogenes* DRDC8 in encystment medium.

*L. monocytogenes* DRDC8 cells (10⁷ cells mL⁻¹) were suspended in encystment medium for 12 h at 25°C. These treated bacterial suspensions were then serially diluted and plated out on LEM agar, and incubated at 37°C overnight. The number of colony-forming units of bacteria (CFU mL⁻¹) was then determined. Counts of untreated *L. monocytogenes* DRDC8 suspended in AS buffer were used as a control. Results presented are the means of CFU ± standard deviations for triplicate samples.
Figure 2.16: TEM of encysted *Colpoda RR* following co-culture with DRDC8.

(A) Bacterial cells (black arrows) entrapped within the ectocyst (ec) of a young cyst at 4 h. Note the numerous mitochondria (mt) within cyst cytoplasm. Bar = 5 µm.

(B) Bacterial cells (black arrows) entrapped within the cytoplasm and ectocyst (ec) of a mature cyst at 4 h. Note the loss of cyst cytoplasmic contents indicating the cyst may not be viable. The ectocyst (ec) is divided and encloses bacterial cells. Bar = 2 µm.

(C) As for (A). Note the ectocyst (ec) and endocyst (en). Bar = 0.5 µm.

(D) A bacterial cell entrapped within the ectocyst (ec) of a mature cyst at 12 h. The ectocyst (ec) is divided and encloses the bacterial cell. Note the thick endocyst (en) layer. Bar = 0.5 µm.

(E) As for (B). Note the bacteria within the cyst cytoplasm. Bar = 0.5 µm.
Figure 2.17: Biofilm biomass of different strains of bacteria at 72 h post inoculation.

Overnight cultures of the bacteria strains were diluted (1/100 \( v/v \)) in LEM, and were added into 96-well microtitre plates. Replicate plates were incubated separately at 4, 25 and 30°C without shaking for 72 h. At every 24 h, the growth medium was changed every 24 h to allow the extension of the experiments. After 72 h incubation, the medium in the wells was removed, and the wells were washed three times with LEM. Biomass of biofilms was quantified by a 0.5% crystal violet staining assay. The microtitre plates were incubated stained for 45 mins and rinsed repeatedly to remove unbound stain. The crystal violet-stained biofilms were solubilized in 95% ethanol and the absorbance was determined with a plate reader at 490 nm. Results presented are the means of three measurements \( \pm \) standard deviations.
Figure 2.18: Biofilm biomass and counts of viable *Colpoda* RR following grazing.

Biofilms (72 h) of *L. monocytogenes* strains and *B. subtilis* were prepared at 25°C in 96-well microtitre plates. Washed suspensions of *Colpoda* RR were allowed to graze on the biofilms for up to 48 h. Growth rates of grazing ciliates and control ungrazing ciliates (suspensions of *Colpoda* RR in the absence of biofilms) were measured by direct cell counts by means of haemocytometer. Biomass of grazed and ungrazed (control) biofilms were quantified by a 0.5% crystal violet staining assay. The microtitre plates were incubated stained for 45 mins and rinsed repeatedly to remove unbound stain. The crystal violet-stained biofilms were solubilized in 95% ethanol and the absorbance was determined with a plate reader at 490 nm. Results presented are the means of three replicates ± standard deviations.

(A) *L. monocytogenes* DRDC8 biofilm.

(B) *L. monocytogenes* EGD Kaufmann biofilm.

(C) *L. monocytogenes* KE925 biofilm.

(D) *B. subtilis* biofilm.
Figure 2.19: Biofilm biomass and counts of *Colpoda* RR grazing on bacterial biofilms.

Biofilms (72 h) of *S. Typhimurium* C5 and *E. coli* DH5α were prepared at 25°C in 96-well microtitre plates. Washed suspensions of *Colpoda* RR were allowed to graze the biofilms for up to 48 h on. Growth rates of grazing ciliates and control ungrazing ciliates (suspensions of *Colpoda* RR in the absence of biofilms) were measured by direct cell counts by means of haemocytometer. Biomass of grazed and ungrazed (control) biofilms were quantified by a 0.5% crystal violet staining assay. The microtitre plates were incubated stained for 45 mins and rinsed repeatedly to remove unbound stain. The crystal violet-stained biofilms were solubilized in 95% ethanol and the absorbance was determined with a plate reader at 490 nm. Results presented are the means of three counts ± standard deviations.

(A) *S. Typhimurium* C5 biofilm.

(B) *E. coli* DH5α biofilm.
Figure 2.20: Biofilm biomass and counts of *Colpoda* MLS-5 grazing on bacterial biofilms.

Biofilms (72 h) of *L. monocytogenes* strains and *B. subtilis* were prepared at 25°C in 96-well microtitre plates. Washed suspensions of *Colpoda* MLS-5 were allowed to graze the biofilms for up to 48 h. Growth rates of grazing ciliates and control ungrazing ciliates (suspensions of *Colpoda* MLS-5 in the absence of biofilms) were measured by direct cell counts by means of haemocytometer. Biomass of grazed and ungrazed (control) biofilms were quantified by a 0.5% crystal violet staining assay. The microtitre plates were incubated stained for 45 mins and rinsed repeatedly to remove unbound stain. The crystal violet-stained biofilms were solubilized in 95% ethanol and the absorbance was determined with a plate reader at 490 nm. Results presented are the means of three counts ± standard deviations.

(A) *L. monocytogenes* DRDC8 biofilm.
(B) *L. monocytogenes* EGD Kaufmann biofilm.
(C) *L. monocytogenes* KE925 biofilm.
(D) *B. subtilis* biofilm.
Figure 2.21: Biofilm biomass and counts of viable *Colpoda MLS-5* following grazing.

Biofilms (72 h) of *S. Typhimurium* C5 and *E. coli* DH5α were prepared at 25°C in 96-well microtitre plates. Washed suspensions of *Colpoda MLS-5* were allowed to graze the biofilms for up to 48 h. Growth rates of grazing ciliates and control ungrazing ciliates (suspensions of *Colpoda MLS-5* in the absence of biofilms) were measured by direct cell counts by means of haemocytometer. Biomass of grazed and ungrazed (control) biofilms were quantified by a 0.5% crystal violet staining assay. The microtitre plates were stained incubated for 45 mins and rinsed repeatedly to remove unbound stain. The crystal violet-stained biofilms were solubilized in 95% ethanol and the absorbance was determined with a plate reader at 490 nm. Results presented are the means of three counts ± standard deviations.

(A) *S. Typhimurium* C5 biofilm.

(B) *E. coli* DH5α biofilm.
Figure 2.22: Growth of *Colpoda* RR and MLS-5 on biofilms of DRDC8.

Washed suspensions of *Colpoda* RR and MLS-5 were allowed to graze separately for up to 48 h on 72 h biofilms at 25°C in 96-well microtitre plates. Growth rates of grazing *Colpoda* RR and MLS-5 were measured by direct cell counts by means of haemocytometer. Results presented are the means of three counts ± standard deviations.
**Figure 2.23: SEM of *L. monocytogenes* DRDC8 biofilm.**

Biofilms samples were fixed in PBS containing 4% (w/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde - overnight at 4 ºC. The samples were dehydrated through a graded ethanol series (70%, 90% and 100%, three times for 10 min each), then immersed in hexamethyldisilazane (HMDS) (three times for 5 min each), and air-dried. Following coating by gold sputter, the biofilms were observed using Philips XL30 scanning electron microscopy.

(A) 0 h post-grazing at low magnification.

(B) 0 h post-grazing at higher magnification. Note the absence of any visible extracellular matrix.

(C) 0 h post-grazing at high magnification. Note the sticky material that connects the bacteria cells (white arrows).

(D) 48 h post-grazing by *Colpoda* RR.
Figure 2.24: Effect of DNase I on *L. monocytogenes* biofilm biomass.

DNase I was added to each well culture at a final concentration of 100 µg.mL⁻¹ per well. DNase I was added in two ways:

Method 1: DNase I added into wells of 72 h biofilms and incubated for up to 3 h at 25°C.
Method 2: DNase I added into wells during biofilm formation cultures at every 24 h for up to 72 h at 25°C.

The control biofilms were not treated with DNase I. Biomass of biofilms was quantified by a 0.5% crystal violet staining assay. The microtitre plates were stained incubated for 45 min and rinsed repeatedly to remove unbound stain. The before crystal violet-stained biofilms were solubilized in 95% ethanol and the absorbance was determined with a plate reader at 490 nm. Results presented are the means of three counts ± standard deviations.
Figure 2.25: Toxicity of biofilm supernatants for *Colpoda* ciliates.

*Colpoda* ciliates were incubated in biofilm supernatants supplemented with heat-killed *E. coli* DH5α for up to 48 h at 25°C. The control was ciliates incubated with heat-killed *E. coli* DH5α only, without biofilm supernatant. Counts of viable *Colpoda* ciliates were measured by direct cell count. Results are the means of three counts ± standard deviations.

(A) *Colpoda* RR.

(B) *Colpoda* MLS-5.
Chapter 3: Host Cell Processes Required for Inactivation of *L. monocytogenes*

3.1 Abstract

Phagosome-lysosome fusion, phagosome acidification and proteases are important components of the microbicidal response by infected eukaryotic cells. Thus, intracellular pathogens that reside within phagosomes must block these host cell processes in order to survive and replicate. In this work, the effects of these processes on the survival of the intracellular pathogen *Listeria monocytogenes* strain DRDC8 within *Colpoda* sp. strain RR ciliates were investigated. NH$_4$Cl, monensin, bafilomycin A1, protease inhibitor cocktail and L-NMMA were used to inhibit host cell mechanisms of defence against bacteria within vacuoles. NH$_4$Cl was used to inhibit phagosome-lysosome fusion; monensin and bafilomycin A1 were used to inhibit acidification of bacteria-containing vacuoles following fusion with lysosomes; and a protease inhibitor cocktail was used to inhibit the function of endogenous proteases. L-NMMA was used as a nitric oxide synthase inhibitor.

Fluorescence microscopy in combination with the acidotrophic stains Lysosensor$^\text{TM}$ Blue and acridine orange showed lysosomes fused with DRDC8-containing vacuoles, but not within NH$_4$Cl pre-treated *Colpoda* RR. Bacterial counts together with TEM were used to assess the fate of DRDC8 cells within treated *Colpoda* RR. Untreated controls preparations of *Colpoda* RR degraded all phagocytosed DRDC8 cells within 2 - 4 h post feeding. However, intact DRDC8 cells were detected within inhibitor-treated *Colpoda* RR at 4 h and 24 h post feeding, although there was no sign of replication of bacteria within the treated ciliates. Levels of nitrite production by *Colpoda* RR during co-cultures with DRDC8 were similar to starved *Colpoda* RR and control co-culture with *E. coli* DH5α. Bacterial counts and TEM showed that pre-treatment of *Colpoda* RR with L-NMMA resulted in phagocytosis and degradation of DRDC8, similar to untreated samples. *Colpoda* RR produced less superoxide following co-cultures with DRDC8 as compared to control co-cultures with heat-killed DRDC8 and *E. coli* DH5α.

Overall, the data showed that phagosome-lysosome fusion, vacuolar acidification and protease activity, but not nitric oxide, are necessary for the killing of *L. monocytogenes*.
within *Colpoda* RR. This work also indicated that unlike mammalian professional and non-professional phagocytic cells, *Colpoda* RR has a powerful mechanism to kill phagocytosed *L. monocytogenes*.

### 3.2 Introduction

*Listeria monocytogenes* is a facultative intracellular pathogen capable of survival and proliferation within mammalian cells such as macrophages and epithelial cells (Vázquez-Boland *et al*. 2001). As a food-borne pathogen, *L. monocytogenes* is the causative agent of human listeriosis that occurs following consumption of contaminated foods such as dairy and processed food. Upon entering a mammalian host cell via a passive or host-induced phagocytosis, *L. monocytogenes* is enclosed within phagosomes. At this stage, the host acidifies the phagosome to eliminate invading bacteria. In this acidic environment, Listeriolysin O secreted by the bacterial cells is activated, interacts with the phagosomal membrane. This interaction leads to destabilization of the membrane and eventual escape into the nutrient-rich cytosol of the host cell. In the host cytosol, *L. monocytogenes* begins to divide and express ActA (Tilney and Portnoy 1989), which in turn leads to recruitment of host cell proteins that are essential in the formation of actin tails and actin-based motility (Vázquez-Boland *et al*. 2001). Actin-based motility within the cytoplasm results in migration of bacterial cells to adjacent uninfected host cells via listeriopods (double-membraned phagosome). An infection cycle is completed when *L. monocytogenes* escapes from listeriopods within the new host cell (Vázquez-Boland *et al*. 2001).

Mammalian hosts employ a wide array of anti-microbial mechanisms to kill intracellular pathogens. Examples of these mechanisms include phagosome-lysosome fusion and vacuole acidification (Flannagan *et al*. 2009), proteases (Pham 2006) and production of nitric oxide (Vouldoukis *et al*. 1995, Nathan and Shiloh 2000, Bekker *et al*. 2001). Although most types of bacterial cells are successfully internalized and eliminated by host mammalian cells, several intra-cellular pathogens have evolved an array of methods to escape host defence mechanisms in order for intracellular survival within host mammalian cells. For example, *Legionella pneumophila* is known to survive and replicate within professional phagocytes by redirecting the maturation of phagosomes to create an intracellular environment that is conducive for intracellular bacterial replication (Hart *et al*. 2001).
1987), whereas several species of mycobacterium are capable of preventing phagosome-lysosome fusion within macrophages (Brüggemann et al. 2006). Furthermore there is evidence that *L. monocytogenes* inhibits host-induced apoptosis in macrophages (Barsig and Kaufmann 1997), induces a delay in host phagosome maturation (Henry et al. 2006), as well as inhibiting phagosome-lysosome fusion within macrophages (Bouvier et al. 1994). These reports indicate that *L. monocytogenes* engages several protective mechanisms to avoid, or delay, killing by host cells.

However, *L. monocytogenes* apparently experiences a completely different fate within host protozoan cells. Several studies have indicated that *L. monocytogenes* is phagocytosed and rapidly degraded by *Acanthamoeba polyphaga* (Huws et al. 2008, Akya et al. 2010). More recently, studies have shown that ingestion of *L. monocytogenes* cells by the soil and freshwater ciliate *Colpoda* RR leads to killing within 4 h post feeding (Chapter 2). The reason for the different responses observed for protozoans vs mammalian cells is not clear. Very little is understood about defense mechanisms employed by *Colpoda* ciliates to kill *L. monocytogenes* DRDC8. The principal aim of the work presented in this chapter is to identify whether phagosome-lysosome fusion, vacuole acidification, proteases activity and nitric oxide production are involved in killing of internalized DRDC8 cells.

### 3.3 Experimental Procedures

#### 3.3.1 Organisms and Culture Conditions

The *Colpoda* sp. isolate RR (GenBank accession number GQ_475427) was previously isolated from a Lotus pond at the Adelaide Botanical Gardens (Raghu Nadhanan 2008). Clonal populations were established and maintained in aerated, water containing washed suspensions of *E. coli* DH5α as a food source. These cultures were incubated at room temperature and routinely monitored for ciliate growth.

*L. monocytogenes* DRDC8, an Australian dairy isolate known to invade and multiply within mammalian cells (Francis and Thomas 1996) was routinely cultivated in Listeria Enrichment Medium (LEM) or on LEM agar plates with incubation at 37°C overnight with aeration. GFP-expressing *L. monocytogenes* ADGA was routinely cultivated in either LEM or on LEM agar plates that were supplemented with 10 µg mL⁻¹ erythromycin with incubation at 37°C overnight with aeration. *E. coli* DH5α (Laboratory collection,
University of Adelaide) were grown in LB broth (Lennox) with incubation at 37°C. Where required, washed cell suspensions of bacteria in 1.5 mL microcentrifuge tubes were killed by heating for 45 min at 100°C.

3.3.2 Co-culture Assays

*Colpoda* RR from actively grown cultures fed *E. coli* DH5α were harvested by gentle centrifugation (106 × g, 2 min). The cell pellet was washed once in modified Neff’s amoeba saline (AS) buffer (pH 7.0) (Smirnov and Brown 2004) and resuspended in fresh AS buffer at 22°C to final concentrations of 10⁴ cells mL⁻¹ or 10⁵ cells mL⁻¹. Overnight broth cultures of bacteria incubated at 37°C were harvested by centrifugation (4000 × g, 20 min) and the cell pellet washed once in AS buffer followed by resuspension in fresh AS buffer to a final concentration of 10⁹ cells mL⁻¹.

Washed suspensions of ciliates (200 μL) at concentrations of ca. 10⁴ cells mL⁻¹ were then pulse fed with 200 μL of washed bacterial suspensions for 1 h at 25°C. Post feeding, the co-culture suspensions were washed free of any extra-ciliate bacteria, and the ciliates were incubated as required to determine survival of ingested bacteria.

3.3.3 Enumeration of Intracellular Bacteria

To determine the concentrations of intra-ciliate bacteria following co-culture, samples of inhibitor-treated and untreated co-cultures were incubated at 25°C for 0, 0.5, 1, 2, 4 and 24 h. Post feeding, these co-culture suspensions were washed free of any extra-ciliate bacteria, and *Colpoda* RR were then lysed by addition of 0.3% Triton X-100 solution. Serial dilutions of lysates were then plated out on LEM agar followed by incubation overnight at 37°C. Unlysed *Colpoda* RR were used as washing control. Following incubation, the numbers of CFU mL⁻¹ were determined.

3.3.4 Inhibitors of Vacuolar Acidification, Phagosome-lysosome Fusion and Proteases

The final concentrations of inhibitors used were as follows: 12 mM ammonium chloride (NH₄Cl), 10 μM monensin, 0.08 μM bafilomycin A1 and 0.0625 × protease inhibitor cocktail. NH₄Cl was dissolved as a stock solution in water, monensin in absolute methanol and bafilomycin A1 in DMSO. These concentrations did not affect the viability of either
*Colpoda* RR (as determined by direct microscopy) or *L. monocytogenes* DRDC8 (as determined by plate counts). For inhibition experiments, washed suspensions of *Colpoda* RR were pre-treated with an inhibitor for 1 h, followed by co-culture with DRDC8. Co-cultures that were not pre-treated with inhibitors were used as controls.

Intracellular superoxide formation in *Colpoda* RR following co-culture with DRDC8 was measured by the reduction of NBT (Rook *et al*. 1985). Washed cell suspensions of *Colpoda* RR were pulse fed individually with washed suspensions of overnight cultures of *E. coli* DH5α and *L. monocytogenes* DRDC8 for 1 h at 25°C. Pulse fed *Colpoda* RR was then washed with AS buffer to remove any extra-ciliate bacteria and treated with NBT (final concentration of 1.25 mg ml⁻¹) ± DPI chloride (10 µM final concentration) (NADPH oxidase inhibitor) for 0, 1, 4 and 24 h post feeding. Reduced NBT deposits were then solubilised with 240 µL of 2 M KOH and 280 µL DMSO and absorbance was measured at 570 nm. The absorbance values obtained from wells with the DPI chloride were subtracted from the corresponding well minus the DPI chloride in order to calculate the amount of NADPH oxidase-dependent NBT reduction. Background values were determined by incubation of uninfected *Colpoda* RR. A standard curve was established by measuring the absorbance of serial dilutions of 1 mg mL⁻¹ NBT solution (in 2 M KOH/DMSO, at a ratio of 1.2 (KOH) : 1.4 (DMSO) ). Results are the means of CFU ± standard deviations for triplicate samples.

The production of nitric oxide was detected by quantitating the amount of nitrite released by *Colpoda* RR during co-cultures with DRDC8, using Griess reagent (Sigma-Aldrich) (Stuehr and Marletta 1985). 100 µL of co-culture suspensions were reacted with 100 µL of Griess reagent (Sigma-Aldrich) for 10 min in the dark at 25°C. Absorbance was measured at 550 nm in a microplate reader. Absorbance of sodium nitrite (NaNO₂) was used as a standard by diluting 0.1 M NaNO₂ solution in AS buffer to a concentration range of 0 - 100 µM. As a negative control, the nitric oxide synthase inhibitor L-NMMA (Sigma-Aldrich) was added. Co-cultures of *Colpoda* RR and *E. coli* DH5α were also used as a control to determine if production of reactive nitrogen species by *Colpoda* RR is dependent on pathogen type. The results were expressed as µM of nitrite.
3.3.5 Fluorescence microscopy

To view lysosome fusion with *L. monocytogenes*-containing phagosomes, *Colpoda* RR were pre-treated separately with either 10 µM LysoSensor Blue DND-167 (Molecular Probes, Inc.) or 6.4 µg mL⁻¹ acridine orange (Sigma-Aldrich) for 1 h at 25°C, followed by pulse feeding with *L. monocytogenes* for 1 h at 25°C, while still retaining LysoSensor™ Blue DND-167 or acridine orange in the suspension. Extra-ciliate bacteria were then removed by washing, and ciliates were incubated for a further 0.5 and 1 h at 25°C. All fluorescent samples were visualized with Olympus 1X70 microscope. The images were taken using Metamorph software version 6.3r7 (Molecular Devices).

3.3.6 Transmission electron microscopy (TEM)

To study the fate of bacterial cells internalized in *Colpoda* RR, various inhibitor-treated and untreated co-cultures of *Colpoda* RR and *L. monocytogenes* DRDC8 were prepared for TEM. Ciliates harvested from co-cultures by centrifugation were fixed overnight at 4°C in PBS containing of 4% paraformaldehyde and 1.25% (v/v) glutaraldehyde. The cell pellet was then embedded in 1% (w/v) agarose and post-fixed in 2% (w/v) OsO₄ in distilled water. After post-fixation, specimens were dehydrated in an ethanol series. The ethanol was replaced by propylene oxide and the specimens embedded in Pro Scitech Epoxy resin. Ultra-thin sections of resin-embedded specimens were stained with uranyl acetate and lead citrate and examined in Philips CM 100 transmission electron microscopy.

3.3.7 Statistical Analysis

Viable counts of intra-ciliate bacteria were counted by calculating the CFU mL⁻¹ of serially plated lysates of bacteria-fed *Colpoda* RR. All data reported are the means obtained from three independent experiments. Each experimental data point was the mean of three replicate samples. Tukey’s Multiple Comparison Test was used to compare counts of viable intra-ciliate bacteria at different time-points. *p*-values of < 0.05 were considered statistically significant. All statistical tests were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, California).
3.4 Results

3.4.1 Co-cultures of *L. monocytogenes* DRDC8 and *Colpoda* RR

Counts of intra-ciliate *L. monocytogenes* DRDC8 post feeding with *Colpoda* RR are presented in Figure 3.1A. Counts of intra-ciliate DRDC8 decreased from ca. $6 \times 10^3$ cells mL$^{-1}$ to ca. $7 \times 10^2$ cells mL$^{-1}$ within a 2 h period post feeding ($p < 0.05$). No viable intra-ciliate *L. monocytogenes* DRDC8 were detected within *Colpoda* RR at 4 h post feeding. These data were corroborated by examination of TEM of sections of *Colpoda* cells from co-culture with DRDC8. Sections of *Colpoda* cells from co-cultures up to 4 h post feeding, showed the cytoplasm was filled with large food vacuoles containing particulates typical of partially degraded bacterial cells that lacked an intact cell membrane or cell wall (Figure 3.1B). The food vacuoles typically contained the remains of 5 to 30 bacterial cells. However, sections of *Colpoda* cells at 24 post feeding (Figure 3.1C) contained only a few, small food vacuoles, indicating that *L. monocytogenes* cells were completely degraded. None of the sections of bacterial cells examined had septa or cell membrane invaginations typical of cell division, suggesting that intra-ciliate DRDC8 were neither actively growing nor dividing.

3.4.2 Fluorescence Microscopy of Phagosome-lysosome Fusion and Vacuolar Acidification within *Colpoda* RR

To provide evidence that phagosomal vacuoles containing *L. monocytogenes* DRDC8 fused with lysosomes as part of the process leading to killing and digestion of these bacteria, the cell permeant, acidotropic stain, LysoSensor$^\text{TM}$ Blue DND-167, was utilized as a probe to visualize lysosome trafficking, lysosome-phagosome fusion and phagolysosomal acidification. LysoSensor$^\text{TM}$ Blue DND-167 has a $pK_a$ value of 5.0 and exhibits very weak fluorescence at neutral pH when excited with UV light, but emits an intense blue fluorescence in acidic environments below pH = 5.0 (Lin *et al.* 2001). Figure 3.2 presents micrographs that show the lysosomes, phagosome-lysosome fusion and ultimately phagolysosomal acidification within *Colpoda* cells following phagocytosis of DRDC8 prior to feeding the ciliates with DRDC8, the ciliate cytoplasm contained numerous, small acidic vesicles that are presumed to be lysosomes (Figure 3.2A). However, at 0.5 h post feeding at 25°C, these structures fused with larger vacuoles,
presumably food vacuoles, that likely contained bacteria (Figure 3.2B). By 1 h post feeding, many large vacuoles within Colpoda cells were also stained with an intense blue fluorescence, indicating the vacuoles were acidified (Figure 3.2C). Overall, these micrographs indicate that fusion of small acidic vesicles (presumably lysosomes) fuse with larger vacuoles resulting in acidification of these larger structures.

These observations were confirmed by staining Colpoda cells with acridine orange, a vital stain for lysosomes. Figure 3.3A presents micrographs of Colpoda RR cells from co-cultures at 0.5 h post feeding with the GFP-expressing DRDC8 derivative ADGA, that show small acridine orange stained vesicles, juxtaposed with large vacuoles that acridine orange-stained lysosomes. Larger vacuoles stained with acridine orange were also observed, suggesting fusion of the smaller vesicles with larger vacuoles. This observation corresponded with data obtained for LysoSensor™ Blue DND-167 stained preparations. By 4 h post feeding, none of the Colpoda cell vesicles/vacuoles examined was stained by acridine orange.

3.4.3 Inhibition of Phagosome-Lysosome Fusion inhibits killing of DRDC8

Treatment of macrophages with a weak base such as NH₄Cl, results in alkalinisation of intracellular vesicles and inhibition of phagosome – lysosome fusion (Hart et al. 1991). This effect was used to determine the requirement for the phagosome-lysosome fusion step during processing of L. monocytogenes DRDC8 by Colpoda RR following co-culture at 25°C. Counts of viable, intra-ciliate DRDC8 cells within NH₄Cl-treated and control Colpoda RR preparations are presented as Figure 3.4A. At 0 h post feeding, counts of viable bacteria internalized within NH₄Cl-treated Colpoda RR (ca. 2.1 × 10³ cells mL⁻¹) was significantly less (p < 0.05) than the counts of viable bacteria internalized within untreated control Colpoda RR (ca. 6 × 10³ cells mL⁻¹). At 4 h post feeding, viable counts for untreated control co-cultures were undetectable, whereas counts for NH₄Cl-treated Colpoda RR remained at ca. 2 × 10³ cells mL⁻¹ for the entire 24 h of co-culture. The survival of DRDC8 within treated Colpoda cells was confirmed by TEM (Figure 3.4B). TEM showed the presence of apparently intact bacterial cells within NH₄Cl-treated Colpoda RR at 4 h post feeding. These bacterial cells were found to be either located singly within vacuoles of NH₄Cl-treated Colpoda RR or in groups within larger vacuoles. By 24 h post feeding (Figure 3.4C), apparently intact bacterial cells were present within
vacuoles of NH$_4$Cl-treated *Colpoda* RR. These bacterial cells had intact cell walls, and no morphological indication of degradation. None of the bacterial cells observed in sections of *Colpoda* showed septation indicative of replication. By comparison, TEM of control preparations at 4 and 24 h post feeding did not contain bacterial cells with intact cell walls (see Figure 3.1B and C).

### 3.4.4 Bafilomycin and Monensin inhibit Vacuole Acidification in *Colpoda* RR

As Lysosensor$^\text{TM}$ Blue DND-167 staining confirmed that *Colpoda* vacuoles containing *L. monocytogenes* DRDC8 cells are acidified following phagocytosis, the impact of inhibitors that prevent vacuole acidification on survival of phagocytosed bacterial cells was examined. Bafilomycin A1 specifically inhibits vacuolar type H$^+$-ATPases and prevents acidification of the phagosome (Yoshimori *et al.* 1991), whereas monensin intercalates into vacuole membranes and mediates exchange of monovalent cations through the membrane leading to a rise in vacuolar pH (Nakazato and Hatano 1991).

Counts of viable intra-ciliate DRDC8 cells following co-culture with bafilomycin treated *Colpoda* RR at 25°C are shown on Figure 3.5A. In comparison to counts of viable bacteria within untreated control *Colpoda* RR (*ca.* $6 \times 10^3$ cells mL$^{-1}$) at 0 h post feeding, counts of bacteria within bafilomycin-treated *Colpoda* RR were significantly lower (*ca.* $2.2 \times 10^3$ cells mL$^{-1}$) ($p < 0.05$). However counts of intra-ciliate bacteria within bafilomycin-treated *Colpoda* RR remained unchanged for up to 24 h post feeding ($p > 0.05$), whereas counts of bacteria within untreated control *Colpoda* decreased to undetectable levels by 4 h post feeding ($p < 0.05$). This data suggested that bafilomycin treatment of *Colpoda* reduced the efficiency of killing of *L. monocytogenes* cells trapped within vesicles. This contention was supported by TEM of bafilomycin treated *Colpoda* RR fed with DRDC8 (Figure 3.5). Sections of ciliates from co-cultures at 4 h and 24 h post feeding contained bacterial cells arranged within tight vacuoles (Figure 3.5B and C). These bacterial cells were apparently intact, had well-defined cell wall structures and the thick peptidoglycan layer typical of Gram positive bacteria was clearly visible. There were no signs of either bacterial degradation or bacterial replication within bafilomycin treated *Colpoda* RR either at 4 h or 24 h post feeding.
Treatment of *Colpoda* RR with monensin resulted in experimental outcomes that were similar to those obtained for cells treated with bafilomycin (Figure 3.6). Counts of intra-ciliate DRDC8 from co-cultures at 25°C, remained stable for 24 h post feeding, whereas counts for untreated ciliates reduced to undetectable levels within 4 h post feeding. Similarly, TEM of sections of monensin treated *Colpoda* cells co-cultured with DRDC8 revealed that the bacteria remained morphologically intact within tight vacuoles that apparently did not transition to typical food vacuoles.

### 3.4.5 Protease Inhibitors limit killing of DRDC8 within Phagolysosomes

To investigate whether proteolytic enzymes are involved in the killing of *L. monocytogenes* DRDC8 within vacuoles of *Colpoda* RR, a protease inhibitor cocktail was utilized to inhibit proteolytic activities of endogenous proteases within this ciliate. Co-cultures of protease inhibitor treated *Colpoda* RR cells with DRDC8 were incubated at 25°C. Untreated *Colpoda* cells were used as a control. Counts of intra-ciliate DRDC8 cells are presented in Figure 3.7A. As with *Colpoda* cells treated with chemical agents that inhibit phagosome-lysosome fusion and phagosome acidification, the count of intra-ciliate bacteria immediately after feeding to protease inhibitor-treated *Colpoda* RR (ca. $1.9 \times 10^3$ cells mL$^{-1}$) was ca. 3 fold lower ($p < 0.05$) than the count for control *Colpoda* RR (ca. $6 \times 10^3$ cells mL$^{-1}$). However, counts of viable intra-ciliate bacteria remained unchanged within protease inhibitor-treated *Colpoda* RR at 4 and 24 h post feeding (ca. $1.9 \times 10^3$ cells mL$^{-1}$) ($p > 0.05$). Conversely counts of bacteria within untreated control *Colpoda* RR decreased to undetectable levels within 4 h post feeding ($p < 0.05$).

TEM of sections of treated *Colpoda* cells confirmed the bacteriology data and showed that protease inhibitor treated ciliate cells taken from co-cultures at 4 and 24 h post feeding with DRDC8 contained intact bacterial cells located singly within vacuoles (Figure 3.7B and C). This observation contrasts that for untreated controls (see Figure 3.1). Some bacterial cells were also found within what is apparently a contractile vacuole (not shown). All sections of bacterial cells were characterized by morphologically intact cell wall complexes without any indication of degradation typical of untreated control co-cultures. None of the cell sections examined had developed septa typical of cell division.
3.4.6 L-NMMA-induced inhibition of Ciliate Nitric Oxide Production

To evaluate whether nitric oxide is involved in the killing of *L. monocytogenes* DRDC8 by *Colpoda* RR, the concentration of nitrite within ciliates from co-cultures at 25°C was measured by Griess reagent (Table 3.1). Concentrations of nitrite detected at 1 h, 4 h and 24 h post feeding were not significantly different to levels of nitrite of either *Colpoda* RR at 0 h post feeding (*p* > 0.05), or starved *Colpoda* RR (*p* > 0.05). Nitrite concentrations within co-cultures remained unchanged for up to 24 h post feeding (*p* > 0.05). Production of nitrite by *Colpoda* RR fed with *E. coli* DH5α was not significantly different to levels seen in *Colpoda* RR fed with DRDC8 (*p* > 0.05). As expected, nitric oxide synthase inhibitor L-NMMA-treated *Colpoda* RR did not produce any nitrite during co-cultures for any of the 24 h duration post feeding.

Interestingly, viable intra-ciliate bacterial counts on Figure 3.8A indicated that there was no significant difference in the counts of viable intra-ciliate DRDC8 within L-NMMA-treated *Colpoda* RR in comparison to untreated *Colpoda* RR for up to 24 h post feeding (*p* > 0.05). For both L-NMMA-treated and untreated samples, viable intra-ciliate DRDC8 counts at 0 h post feeding (ca. 6 × 10^3 cells mL\(^{-1}\)) decreased to ca. 7 × 10^2 cells mL\(^{-1}\) at 2 h post feeding (*p* < 0.05) and to undetectable levels by 4 h post feeding.

The reduction in counts of intra-ciliate bacterial cells, irrespective of treatment with L-NMMA was mirrored by TEM of sections of treated *Colpoda* cells. The morphological appearance of sections of bacterial cells within L-NMMA-treated *Colpoda* cells (Figure 3.8B and C) was identical to that of bacteria within untreated *Colpoda* cells (see Figure 3.1). Phagocytosed DRDC8 cells were degraded by L-NMMA-treated *Colpoda* RR within 4 h post feeding, similar to observations of TEM of untreated co-cultures (as shown on Figure 3.8B and C). The treated ciliates contained large food vacuoles with degraded particulates of bacterial cell size. No morphologically intact bacterial cells were observed within vacuoles of L-NMMA-treated *Colpoda* RR. Furthermore, no bacteria or large food vacuoles were detected within L-NMMA-treated *Colpoda* cells (Figure 3.8C).

3.4.7 Superoxide Production by *Colpoda* RR

Superoxide release by *Colpoda* RR in response to *L. monocytogenes* DRDC8 infection was measured by assay of the NADPH-dependent reduction of NBT in the presence or absence
of the NADPH oxidase inhibitor DPI (O'Donnell et al. 1993). By subtracting the amount of NBT reduced in the presence of inhibitor from the amount reduced in the absence of inhibitor, estimates of superoxide release caused strictly by the metabolic products of the respiratory burst within *Colpoda* RR were calculated. The data obtained are displayed as Figure 3.9.

The levels of DPI-inhibitable NBT reduction (µg mL⁻¹) after 1 h post feeding with DRDC8 (ca. 3.2 µg mL⁻¹) and 24 h post feeding (ca. 3.7 µg mL⁻¹) were not significantly different (*p* > 0.05). However, the levels of DPI-inhibitable NBT reduction of co-cultures with either live, or heat-killed *E. coli* DH5α, or heat-killed DRDC8 were significantly higher than that of co-cultures with live DRDC8 at the same time points tested (*p* < 0.05). Production of superoxide by bacteria only controls was negligible (significantly lower than other samples (*p* < 0.05)). These results demonstrated that the amount of superoxide produced by *Colpoda* RR co-cultured with live DRDC8 is less than that of *Colpoda* RR co-cultures with *E. coli* DH5α and heat-inactivated controls at similar time points.

### 3.5 Discussion

Since *Colpoda* RR is able to phagocytose and degrade DRDC8 (Chapter 2), this study aimed to identify the mechanisms of defense employed by *Colpoda* RR in killing DRDC8. There are no reported studies describing the mechanisms of defense employed by ciliated protozoans in response to intracellular bacterial pathogens, whereas there is a considerable body of literature that focuses on mechanisms used by mammalian cells to kill bacteria. Indeed an array of chemical inhibitors with well-described mechanisms of action has been identified for studies with mammalian cells (Conte et al. 1996). Some of these have been used to determine whether parallel bactericidal systems exist in *Acanthamoeba* spp. (Akya et al. 2009, Lamothe et al. 2004). In the present study, inhibitors known to affect phagosome-lysosome fusion, vacuole acidification, protease activity and nitric oxide production were utilized to study killing of internalized DRDC8 within *Colpoda* RR.

#### 3.5.1 Phagosome-lysosome Fusion mediates killing of *L. monocytogenes*

The ability of *L. monocytogenes* to survive within intracellular compartments within mammalian cells has been clearly demonstrated (Dramsi et al. 1993, Wood et al. 1993, Francis and Thomas 1996). However, this study has demonstrated that *Colpoda* RR
efficiently inactivates *L. monocytogenes* within 2 h of feeding on this bacterial pathogen. Indeed, *Colpoda* RR are able to feed on planktonic DRDC8 cells as well as graze efficiently on biofilms of DRDC8 (Chapter 2). Furthermore, the fact that DRDC8 cells are unable to invade *Colpoda* RR cells when phagocytosis is inhibited by the K⁺ efflux inhibitor TEA chloride, (see Chapter 2) indicated that *Colpoda* RR internalised bacterial cells within vacuoles. This conclusion contrasts with the well established InlA and InlB mediated invasion of mammalian cells by DRDC8.

Phagosome-lysosome fusion and vacuole acidification are important in degradation of phagocytosed bacterial cells by mammalian cells (Styrt and Klempner 1988, Huynh and Grinstein 2007). Bacterial cells are phagocytosed into an intracellular vacuole where microbicidal agents are deployed by the maturation process. During maturation, acidification of the vacuoles occurs due to presence of V-ATPases, following phagosome-lysosome fusion (Huynh and Grinstein 2007). Acidification of vacuoles results in the lytic activity of a variety of degradative enzymes such as proteases, as well as promoting the generation of hydrogen peroxide (Huynh and Grinstein 2007). In this study, acridine orange as well as the fluid phase probe Lysosensor™ Blue DND-167 were used to characterize the DRDC8-containing vacuoles within *Colpoda* RR. Acridine orange¹ has been shown to concentrate within lysosomes, becoming protonated and sequestered, causing them to appear as bright orange granules in fluorescence microscopy, whereas Lysosensor™ Blue DND-167 is a non-fluorescent probe compound that freely diffuses through the membrane and becomes highly fluorescent at an acidic pH (ca. < 5.0). Consequently this probe is useful for identifying trafficking of acidic vacuoles. In the context of this study, this probe was used to show that DRDC8-containing vacuoles within *Colpoda* RR fused with small acidic vesicles (presumably lysosomes), an outcome mirrored in *Colpoda* RR stained with acridine orange. As expected, the content of the larger vesicles became acidic shortly after phagosome-lysosome fusion. These results support the idea that for *Colpoda* at least, lysosome mediated acidification of vacuoles containing bacteria is a likely precursor to killing of bacteria and digestion. In this respect, the process mimics well-described cellular processes used by mammalian phagocytic cells. The fact that killing of intra-ciliate DRDC8 cells was impaired by inhibitors that target

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¹ 3, 6-bis dimethylaminoacridine.
phagosome–lysosome fusion (NH$_4$Cl) and vacuole acidification (bafilomycin A1 and monensin), lends strong support for the role of these cellular processes in *Colpoda*.

NH$_4$Cl is a weak base that interferes with the maturation of the phagosome and as a consequence leads to inhibition of phagosome-lysosome fusion (Hart and Young 1991). However, there are instances of contradictory outcomes reported that concern survival of bacteria within NH$_4$Cl-treated host cells. For example, Harley and Drasar (1989) reported that *L. pneumophila* within NH$_4$Cl-treated HeLa cells were able to survive and replicate within intracellular compartments, but Byrd and Horwitz (1991) later showed that *L. pneumophila* survived without replication within treated human monocytes. Nevertheless, in the current study, this weak base clearly blocked maturation of the phagolysosome to the point where DRDC8 was able to survive within *Colpoda* vacuoles, although no evidence for growth and cell division was detected.

The involvement of acidification of the phagosome following fusion with lysosomes, in killing of intra-ciliate DRDC8 was confirmed by use of neutralizing inhibitors such as bafilomycin A1 and monensin. Bafilomycin A has a membrane-permeant activity and specifically inhibits vacuolar-type proton translocating ATPases (V-ATPases) that are involved in vacuole acidification within treated cells (Conte *et al.*, 1996), whereas monensin intercalates into vacuole membranes and mediates the exchange of monovalent cations through the membrane resulting in the increase of the vacuolar pH (Nakazato and Hatano 1991). The effect of these inhibitors on survival of DRDC8 within *Colpoda* cells is directly comparable with studies that report impaired killing of bacteria within macrophage cell lines. For example, killing of intra-amoebic *L. monocytogenes* by *A. polyphaga* was impaired following treatments with both bafilomycin A1 and monensin (Akya *et al.*, 2009). Furthermore, bafilomycin A1 treatment of murine RAW 264.7 macrophages inhibited killing and degradation of phagocytosed *E. coli* (Frankenberg *et al.*, 2008). Similarly, inhibition of acidification of *Staphylococcus aureus*-containing vacuoles also resulted in impaired bacterial killing by alveolar macrophages (Bidani *et al.*, 2000). Di *et al.* (2006) has demonstrated the use of alveolar macrophages that carried cystic fibrosis transmembrane conductance regulator chloride channel mutations to directly implicate the role of vacuole acidification in the killing of bacteria.
Exactly why DRDC8 is able to survive within inhibitor-treated Colpoda RR, but is unable to replicate, is unclear. Several reports have suggested that survival without replication of M. avium or L. pneumophila within activated macrophages is due to replication restriction mediated by enhanced phagosome-lysosome fusion (Schaible et al. 1998, Santic et al. 2005). However this is an unlikely explanation for Colpoda cells treated with inhibitors designed to prevent fusion of lysosomes with the phagosome. An alternative, and more likely reason to explain this observation is that inhibition by NH₄Cl, bafilomycin and monensin was sufficient to allow survival, but insufficiently complete to allow growth of survivor L. monocytogenes cells within the phagosome. Other intact antibacterial mechanisms may also have been sufficient to repress growth without affecting survival. Furthermore, as Listeriolysin O expression is reduced at temperatures used for co-culture (Datta and Kothary 1993), L. monocytogenes would not be able to mediate destruction of the ciliate vacuole membrane to escape and replicate within the cytosol of Colpoda RR.

3.5.2 Proteases assist killing of L. monocytogenes

In addition to the role of lysosome fusion with phagocytic vacuoles and vacuolar acidification, a variety of endopeptidases (cysteine and aspartate proteases), exopeptidases (cysteine and serine proteases) and hydrolases located within phagosomes are also reported to degrade bacterial components within the phagosomes (Pillay et al. 2002). Substrates for exopeptidases, are generated by the C1 family of cysteine proteases of endopeptidases (Pillay et al. 2002).

Since the experimental data presented confirmed that impairment of proteases of Colpoda RR by protease inhibitors allowed the survival of DRDC8 within the ciliates, this outcome indicates that endo- and exo-peptidase of Colpoda RR are likely to be a factor involved in the killing and degradation of internalized DRDC8. Since the serine and cysteine protease inhibitors in the cocktail used in this study would have likely impaired the endopeptidases, the activity of exopeptidases would have also been limited as the endopeptidase activity is required to generate substrates for the exopeptidases. Although the effect of protease inhibitors on survival of DRDC8 within Colpoda cells is novel for work with bacteriophagous protozoans, previous studies have highlighted the antibacterial role of serine proteases within mammalian cells. For example, Rosenberger et al. (2004)
showed that protease regulation of macrophage cathelicidin-related antimicrobial peptide (CRAMP) expression and activity impaired the replication of intracellular *S*. *Typhimurium*. Similarly, Standish and Weiser (2009) demonstrated that inhibition of neutrophil granule proteases by serine protease inhibitors resulted in survival of *Streptococcus pneumoniae* within neutrophils. Whether serine proteases (or other proteases) in particular are involved in limiting the bacteriocidal response to *L. monocytogenes* within *Colpoda* cells is unknown. This question could easily be resolved by treatment of *Colpoda* cells used in co-cultures with specific protease inhibitors.

### 3.5.3 *L. monocytogenes* is insensitive to Nitric Oxide

Nitric oxide is a toxic radical synthesised from L-arginine by inducible nitric oxide synthase (iNOS). It is a central component of the mammalian innate immunity and an important bactericidal agent (Nathan and Shiloh 2000) central to killing ingested pathogens by activated macrophages (Nathan 1997). Intracellular pathogens such as *Mycobacterium tuberculosis* and *Leishmania major* are killed by a nitric oxide-dependent mechanism (Vouldoukis *et al*. 1995, Bekker *et al*. 2001). Many other published studies implicate nitric oxide produced by mammalian host cells in the killing of internalized bacteria. One of these studies has for example, showed that L-NMMA inhibition of nitric oxide synthesis within activated macrophages correlates with loss of control of intracellular replication of *Rhodococcus equi* (Darrah *et al*. 2000). This type of study implicates nitric oxide as a mediator of the killing of intracellular *R. equi* by activated macrophages. Furthermore, an increased *S*. *Typhimurium* burden was detected within reticulo-endothelial organs of iNOS mutant mice (Mastroeni *et al*. 2000). Increased levels of nitric oxide production were detected within peritoneal macrophages in response to interferon gamma in a separate study (Vazquez-Torres *et al*. 2000). In that study, the elevated levels of NO appeared to increase antibacterial activity against *S*. *Typhimurium*.

However, *L. monocytogenes* is generally regarded as insensitive to killing by NO (Bogdan 2012). Indeed, Bogdan (2012) cited a series of previous findings obtained with IFN-γ, IFN-γ, plus LPS, or IFN-γ + TNF stimulated macrophages from wild type, NOS-2−/−, or NOS-2 inhibitor treated macrophages, all of which failed to find a correlation between NO synthesis and killing of *Listeria* cells (Bogdan 1997, Edelson and Unanue
2002, Shiloh et al. 1999). The NO specific data in this present study confirm the insensitivity of *L. monocytogenes* and extend the finding to protozoan cells. Although NO is produced by *Colpoda RR* during co-culture with DRDC8, it apparently is not a factor involved in the killing of bacteria. Two lines of data support this contention:

1. Bacteriology and TEM data for *Colpoda* treated with the NO inhibitor L-NMMA demonstrated that there was no difference in the efficiency of bacterial killing of *L. monocytogenes* compared to untreated controls.

2. Levels of nitrite within *Colpoda RR* fed with DRDC8 were similar to that of co-cultures with the non-pathogenic *E. coli* DH5α, heat-killed DRDC8 and starved *Colpoda RR* controls.

Whether the level of nitric oxide produced by *Colpoda* following feeding with DRDC8 is too low (ca. 0.42 µM nitrite at 1 h post feeding, ca. 0.43 µM nitrite at 4 h post feeding and ca. 0.36 µM nitrite at 24 h post feeding) to have any significant bactericidal effect is unknown, although the cited studies involving mammalian cells suggests this is not the case. Nevertheless reported levels of NO synthesized by host cells are varied. A recent report by von Bargen et al. (2011) indicated that following infection with *R. equi*, macrophages produced ca. 10 µM NO at 24 h post infection. Summersgill et al. (1992) showed *L. pneumophila* were killed by nitric oxide released by murine macrophages, and the level of nitrite measured at 24 h post infection was ca. 58.6 µM. However, the data presented in this present study mirrors the outcome of a separate study by Beckerman et al. (1993) that showed killing of *L. monocytogenes* occurred even after treatment of activated macrophages with L-NMMA. The outcome of this present study could be an indication that though *Colpoda RR* does produce low levels of NO, the ciliate does not employ it as a mechanism of defence against phagocytosed pathogens.

### 3.5.4 Superoxides

NADPH oxidase within hosts is activated upon phagocytosis, resulting in the generation of intracellular superoxide (Takeya and Sumimoto 2003). The superoxide plays an important role in the oxygen-dependent antibacterial mechanisms of phagocytic cells (Fang 2004). Phagocytic cells are known to use a combination of oxidative mechanisms, including superoxide in addition to non-oxidative mechanisms such as pH, to defend against the
wide range of phagocytosed pathogenic bacteria (Roos and Winterbourn 2002). It has been previously established that the NADPH oxidase is important for eliminating *L. monocytogenes* within host cells (Shiloh *et al.* 1999). The outcomes of the present study that showed less superoxide production by *Colpoda* RR following feeding with DRDC8 in comparison to control co-cultures with *E. coli* DH5α and heat-killed DRDC8 controls mirrored the outcome of a separate study that demonstrated macrophages produced less superoxide following *Burkholderia cenocepacia* infection as compared to heat-inactivated *B. cenocepacia* and *E. coli* controls (Keith *et al.* 2009). Interestingly, a study provided evidence of Listeriolysin O inhibiting host NADPH oxidase by preventing its localization to phagosomes hence allowing the bacterium to escape the phagosome while avoiding the microbicidal respiratory burst (Lam *et al.* 2011). Whether the evidence provided by Lam *et al.* (2011) explains the outcome obtained in this present study is unclear. It is possible that Listeriolysin O expressed by DRDC8 within *Colpoda* RR managed to inhibit NADPH oxidase to a certain extent, resulting in less superoxide production in co-cultures with live DRDC8 in comparison to control co-cultures with *E. coli* DH5α and heat-killed DRDC8, but the level of Listeriolysin O expressed is likely not enough to allow the escape of the bacterium from the vacuole.

### 3.5.5 Inhibitor mediated uptake of *L. monocytogenes* by *Colpoda*

Phagocytosis is the primary mechanism used by ciliates for the uptake of bacteria. An invagination pinches off to form a large endocytic vesicle (phagosome) containing the ingested material (Alberts *et al.* 1994). This process is called endocytosis. The phagosome travels within the cell and ultimately fuses with a lysosome. The ingested substance is then digested (Alberts *et al.* 1994). A significantly reduced uptake of bacteria by all inhibitor-treated ciliates (except L-NMMA) compared to untreated ciliates seemed to indicate that phagocytosis of DRDC8 by treated *Colpoda* RR was affected by the inhibitory effects of the chemicals. This phenomenon has been a feature of a number of previous studies. One of the studies showed that besides inhibiting vacuole acidification, bafilomycin A1 also conferred inhibitory effects on the uptake of *E. coli* by alveolar macrophages (Bidani and Heming 1995). Treatment of *A. polyphaga* with monensin also resulted in a reduced uptake of *L. monocytogenes* by the amoeba compared to untreated control amoeba cells (Akya *et al.* 2009). One reason for this effect may be related to reduced Ca^{2+} levels within *Colpoda*.
Calcium (Ca$^{2+}$) is regarded as a requirement for phagocytosis (Peck and Duborgel 1985) and vacuole acidification (Downey et al. 1999), although a few other studies have reported conflicting outcomes concerning the importance of Ca$^{2+}$ for phagosome-lysosome fusion (Jaconi et al. 1990, Zimmerli et al. 1996, Dewitt and Hallett 2002). Importantly, increases in vacuole pH within macrophages, resulting from treatment by either NH$_4$Cl or bafilomycin A1 result in several fold decreases in Ca$^{2+}$ concentrations within the macrophage cells (Christensen et al. 2002). Thus, it seems likely that reduced levels of endogenous Ca$^{2+}$ brought about by NH$_4$Cl, bafilomycin and monensin treatment, are responsible for reduced uptake of *L. monocytogenes* by treated *Colpoda* cells. The AS buffer used to suspend *Colpoda* cells, has sufficient concentrations of Ca$^{2+}$ to promote active phagocytosis and phagosome-lysosome fusion within *Colpoda* RR in co-cultures with DRDC8. This suggests that the effects of the inhibitors used is localized within cells, is likely to be transitory, and dependent on continued presence of those inhibitors. However, it is not known why treatment of the ciliates with protease inhibitor cocktail also resulted in a reduced bacteria uptake, although it is interesting to speculate that this effect may be caused by the effect of proteases on signaling cascades necessary for phagocytosis.

### 3.5.6 Conclusions

When phagocytic cells “feed” on bacterial cells, the host phagosome undergoes a maturation process in which actin undergoes depolymerisation (Bengtsson et al. 1993), enabling fusion of the phagosomes with lysosomes (Flannagan et al. 2009). The phagosome-lysosome structure formed is an acidic, hydrolytic compartment in which the bacterial cell may be killed and digested. Defensins, cathelicidins, lysozymes, and a variety of proteases are recruited by the phagosome to kill the pathogen (Flannagan et al. 2009). It was interesting to note that inhibition of a single mechanism within *Colpoda* RR resulted in survival of DRDC8 within the ciliate. This observation indicated that killing of *L. monocytogenes* by *Colpoda* RR cannot be attributed to any one single mechanism. Phagocytosis and killing of bacteria by mammalian cells is a pathway that consists of a cascade of defined processes. Survival of bacteria within mammalian cells following inhibition of either one of these processes has been well established for mammalian cells. Interference with the uptake of bacteria such as *Pseudomonas aeruginosa* and *Yersinia enterocolitica* by macrophages and neutrophils by direct impairment of the phagocytic
machinery of the host has been previously demonstrated (Garrity-Ryan et al. 2000, Grosdent et al. 2002). Other types of bacteria such as *Mycobacterium tuberculosis* and *S. Typhimurium* have strategies to either counteract the acid accumulation within phagolysosomes, or have acquired genes that encode proteins that assist the bacteria to withstand the low pH within phagolysosomes (Park et al. 1996, Vandal et al. 2008). *P. aeruginosa, Enterococcus faecalis, Proteus mirabilis* and *Streptococcus pyogenes* have been shown to protect themselves by actively degrading antimicrobial peptides such as proteases that are recruited by the phagosome (Schmidtchen et al. 2002), whereas *Staphylococcus aureus* are able to resist the effects of phagosome-recruited antimicrobial peptides (Peschel et al. 2001). Hence it is not unusual to note the intra-ciliate survival of DRDC8 when one of the ciliate defence mechanisms was inhibited in this present study. Furthermore, it also shows that the *Colpoda* RR defence mechanism machinery is similar to that seen in mammalian cells.

Overall, the inhibitory effects of NH₄Cl, bafilomycin A1, monensin and protease inhibitor cocktail clearly demonstrated the important function of phagosome-lysosome fusion, vacuolar acidification and endogenous protease activity in regulating the killing and degradation of DRDC8 within *Colpoda* RR. It is also possible that the low pH within vacuoles of *Colpoda* RR plays a role in activation of enzymes and proteases involved in degradation of *L. monocytogenes* DRDC8 within these vacuoles. This corresponds with TEM that showed partial or complete degradation of cell wall material of vacuole-confined DRDC8 cells (Chapter 2). Ultimately however, the reduction in the ability of inhibitor-treated *Colpoda* RR to kill phagocytosed DRDC8 cells indicated that the fate of intra-ciliate DRDC8 is primarily determined by the ciliate; the bacterium on the other hand is unable to control its own survival and replicate within *Colpoda* RR.

In conclusion, the experimental results presented in this study indicated that phagosome-lysosome fusion and vacuole acidification are involved in both phagocytosis and degradation of *L. monocytogenes* DRDC8 by *Colpoda* RR. These data reveal that similar molecular principles are used by mammalian and *Colpoda* cells to phagocytose and degrade pathogenic bacteria. Further, ciliate proteolytic activities are required for killing and degradation of DRDC8 by *Colpoda* RR. However, the production of low levels of NO
by *Colpoda* RR combined with the insensitivity of *L. monocytogenes* to NO, suggests NO
does not play a role in the killing and degradation of DRDC8 by *Colpoda* RR.

### 3.6 References


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Figure 3.1: TEM of *Colpoda* RR co-cultured with *L. monocytogenes* DRDC8 at 25°C.

(A) Counts of intra-ciliate DRDC8 during co-culture with *Colpoda* RR. Results are the means of CFU ± standard deviations for triplicate samples.

(B) Food vacuoles (FV) of *Colpoda* RR at 4 h post feeding, containing degraded bacterial cells. Bar = 1.0 µm. Inset shows a high magnification micrograph of a food vacuole of *Colpoda* RR, containing degraded bacterial cells at 4 h post feeding. Note the loss of intact bacterial cell walls. Bar = 0.25 µm.

(C) As for (A) except co-culture after 24 h post feeding. Note the absence of any intact bacterial cells and food vacuoles. Note the mitochondria (mt) and cilia (c). Bar = 2.5 µm.
Figure 3.2: Phagosome-lysosome fusion and vacuolar acidification within *Colpoda* RR.

*Colpoda* RR were pre-treated with LysoSensor Blue DND-167 for 1 h at 25°C, followed by pulse feeding with *L. monocytogenes* DRDC8 for 1 h at 25°C, while still retaining LysoSensor™ Blue DND-167 in the suspension. Extra-ciliate bacteria were then removed by washing, and ciliates were incubated for a further 0.5 and 1 h at 25°C.

(A) Note the lysosomes (bright blue) scattered within *Colpoda* RR prior to co-culture with DRDC8. Bar = 5 µm.

(B) Note the lysosomes (bright blue) fused around the vacuoles containing bacterial cells (white arrows) at 0.5 h post feeding. Bar = 5 µm. Inset shows a high magnification micrograph of lysosomes (bright blue) fused around the vacuoles containing bacterial cells. Bar = 0.5 µm.

(C) Vacuole acidification containing bacteria (white arrows) at 1 h post feeding. Bar = 5 µm.
Figure 3.3: Phagosome-lysosome fusion within *Colpoda* RR.

*Colpoda* RR were pre-treated with acridine orange for 1 h at 25°C, followed by pulse feeding with GFP-expressing *L. monocytogenes* ADGA for 1 h at 25°C, while still retaining acridine orange in the suspension. Extra-ciliate bacteria were then removed by washing, and ciliates were incubated for a further 0.5 and 4 h at 25°C.

(A) Note the lysosomes (orange) fused around the vacuoles containing GFP-expressing bacterial cells (white arrows) at 0.5 h post-feeding. Bar = 5 µm.

(B) Note the absence of any lysosomal staining within *Colpoda* RR at 4 h post feeding. Bar = 5 µm.
Figure 3.4: NH₄Cl inhibits phagosome-lysosome fusion of *Colpoda* RR.

*Colpoda* RR pre-treated with NH₄Cl for 1 h at 25°C, were pulse fed with *L. monocytogenes* DRDC8 for 1 h at 25°C, without removing NH₄Cl. Extra-ciliate bacteria were then removed by washing, and ciliates were further incubated for a further 0.5, 1, 2, 4 and 24 h at 25°C. Counts of intra-ciliate bacteria were determined by plating ciliate lysates. Extra-ciliate bacteria were eliminated by washing prior to lysis of the ciliates. Co-cultures not treated with NH₄Cl were used as a control.

(A) Counts of intra-ciliate DRDC8 within treated and untreated *Colpoda* RR. Results are the means of CFU ± standard deviations for triplicate samples.

(B) TEM of NH₄Cl-treated *Colpoda* RR co-cultured with DRDC8 at 4 h post feeding. Note the intact bacterial cells (black arrows) present within vacuoles of *Colpoda* RR. Bar = 1.5 µm.

(C) As for (A) except co-culture after 24 h. Note the intact bacterial cells within vacuoles of *Colpoda* RR. Bar = 0.25 µm.
Figure 3.5: Bafilomycin A1 inhibits vacuolar acidification in *Colpoda* RR.

*Colpoda* RR pre-treated with bafilomycin A1 for 1 h at 25°C, were pulse fed with *L. monocytogenes* DRDC8 for 1 h at 25°C, without removing bafilomycin A1. Extra-ciliate bacteria were then removed by washing, and ciliates were further incubated for a further 0.5, 1, 2, 4 and 24 h at 25°C. Counts of intra-ciliate bacteria were determined by plating ciliate lysates. Extra-ciliate bacteria were eliminated by washing prior to lysis of the ciliates. Co-cultures not treated with bafilomycin A1 were used as a control.

(A) Counts of intra-ciliate DRDC8 within treated and untreated *Colpoda* RR. Results are the means of CFU ± standard deviations for triplicate samples.

(B) TEM of bafilomycin A1-treated *Colpoda* RR co-cultured with DRDC8 at 4 h post-feeding. Note the intact bacterial cells within vacuoles of *Colpoda* RR (black arrows). Bar = 1.5 µm.

(C) As for (A) except co-culture after 24 h. Bar = 0.25 µm.
Figure 3.6: Monensin inhibits vacuolar acidification of *Colpoda RR*.

*Colpoda RR* pre-treated with monensin for 1 h at 25°C, were pulse fed with *L. monocytogenes* DRDC8 for 1 h at 25°C, without removing monensin. Extra-ciliate bacteria were then removed by washing, and ciliates incubated for a further 0.5, 1, 2, 4 and 24 h at 25°C. Counts of intra-ciliate bacteria were determined by plating ciliate lysates. Extra-ciliate bacteria were eliminated by washing prior to lysis of the ciliates. Co-cultures not treated with monensin were used as a control.

(A) Counts of intra-ciliate DRDC8 within treated and untreated *Colpoda RR*. Results are the means of CFU ± standard deviations for triplicate samples.

(B) TEM of monensin-treated *Colpoda RR* co-cultured with DRDC8 at 4 h post feeding. Note the apparently intact bacterial cells (black arrows) within vacuoles of *Colpoda RR*. Bar = 0.75 µm.

(C) As for (A) except co-culture after 24 h. Note the presence of apparently intact bacterial cells within vacuoles of *Colpoda RR*. Bar = 1.5 µm.
Figure 3.7: Protease inhibitors affect survival of *L. monocytogenes* DRDC8 within *Colpoda* RR.

*Colpoda* RR pre-treated with protease inhibitor cocktail for 1 h at 25°C, were pulse fed with DRDC8 for 1 h at 25°C, without removing the protease inhibitor cocktail. Extra-ciliate bacteria were removed by washing, and ciliates were incubated for a further 0.5, 1, 2, 4 and 24 h at 25°C. Counts of intra-ciliate bacteria were determined by plating ciliate lysates. Extra-ciliate bacteria were eliminated by washing prior to lysis of the ciliates. Co-cultures not treated with protease inhibitor cocktail were used as a control.

(A) Counts of intra-ciliate DRDC8 within treated and untreated *Colpoda* RR. Results are the mean CFU ± standard deviations for triplicate samples.

(B) TEM of protease inhibitor cocktail-treated *Colpoda* RR co-cultured with DRDC8 at 4 h post feeding. Note the bacterial cells (white arrows) within vacuoles of *Colpoda* RR. Note the micronucleus (mic). Bar = 1.5 µm.

(C) As for (A) except co-culture after 24 h. Note the intact bacterial cells (white arrows) within vacuoles of *Colpoda* RR. Bar = 0.75 µm.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Incubation time (h)</th>
<th>Nitrite production (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starved Colpoda RR</td>
<td>0</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.35</td>
</tr>
<tr>
<td>Colpoda RR + L. monocytogenes DRDC8</td>
<td>0</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.36</td>
</tr>
<tr>
<td>Colpoda RR + E. coli DH5α</td>
<td>0</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.33</td>
</tr>
<tr>
<td>Colpoda RR + heat-killed L. monocytogenes DRDC8</td>
<td>0</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.41</td>
</tr>
<tr>
<td>Colpoda RR + L. monocytogenes DRDC8 + L-NMMA</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
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<td>1</td>
<td>0.00</td>
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<td>0.00</td>
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<tr>
<td></td>
<td>24</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Production of nitric oxide was detected by quantitating the amount of nitrite released by Colpoda RR during co-cultures with DRDC8, using Griess reagent. As a negative control, Colpoda RR were pre-treated with the nitric oxide synthase inhibitor L-NMMA. All samples were reacted with Griess reagent for 10 min in the dark at 25°C. Absorbance was measured at 550 nm in a microplate reader. Absorbance of sodium nitrite (NaNO$_2$) was used as a standard. Each value represents the mean of three replicate experiments.
Figure 3.8: L-NMMA inhibition of NO production by Colpoda RR.

Colpoda RR were pre-treated with L-NMMA for 1 h at 25°C, followed by pulse feeding with L. monocytogenes DRDC8 for 1 h at 25°C, without removing L-NMMA in the suspension. Extra-ciliate bacteria were then removed by washing and ciliates incubated for a further 0.5, 1, 2, 4 and 24 h at 25°C. Counts of intra-ciliate bacteria were determined by plating ciliate lysates. Extra-ciliate bacteria were eliminated by washing prior to lysis of the ciliates. Co-cultures not treated with L-NMMA were used as a control.

(A) Counts of intra-ciliate DRDC8 within treated and untreated Colpoda RR. Results are the means of CFU ± standard deviations for triplicate samples.

(B) TEM of L-NMMA-treated Colpoda RR co-cultured with DRDC8 at 4 h post feeding. Note the food vacuoles (FV), possibly containing degraded bacteria. Bar = 0.5 µm.

(C) As for (A) except co-culture after 24 h. Note the mitochondria (mt) and macronucleus (mac). Bar = 1.5 µm.
Figure 3.9: Intracellular superoxide production by *Colpoda RR*.

*Colpoda RR* was pulse fed with *E. coli* DH5α or *L. monocytogenes* DRDC8 for 1 h at 25°C. As controls, *Colpoda RR* was fed heat-killed suspensions of *E. coli* DH5α and DRDC8. Pulse fed *Colpoda RR* was washed to remove any extra-ciliate bacteria. Washed *Colpoda RR* cells were treated with NBT (1.25 mg.mL⁻¹) ± DPI chloride (NADPH oxidase inhibitor) for 1, 4 and 24 h post feeding at 25°C. The reduced NBT deposits were solubilized by adding 240 µL of 2 M KOH and 280 µL DMSO. Absorbance was measured at 570 nm. Amount of NADPH oxidase-dependent NBT reduction was calculated by subtracting the absorbance values for samples minus the DPI chloride from the absorbance values obtained in the presence of inhibitor. Each value represents the mean of three replicate experiments.
Chapter 4: Secretion of *L. monocytogenes* within Ciliate-derived Faecal Pellets

4.1 Abstract

There is limited knowledge on the encapsulation of bacteria in faecal pellets secreted by protozoans, even though the potential for spread of pathogenic bacteria within these structures is significant. For example, inhalation or ingestion of faecal pellets containing live, pathogenic bacteria secreted by protozoans may lead to infectious disease. However, it is not known whether virulent *Listeria monocytogenes* can survive ingestion by the ciliated protozoan *Colpoda* by secretion within faecal pellet structures. Two isolates of *Colpoda* sp.: *Colpoda* sp. strain RR from a natural environment, and *Colpoda* sp. strain MLS-5 from a food processing environment, were used in this study to investigate whether these protozoan isolates were able to secrete faecal pellets containing bacteria. TEM was used to show that co-cultures of *Colpoda* RR and *Colpoda* MLS-5 with *L. monocytogenes* DRDC8 resulted in secretion of faecal pellets containing intact bacterial cells. Plate counts, assay of microbial respiratory activity, and fluorescence microscopy proved DRDC8 cells present within faecal pellets were viable and metabolically active. To confirm that this observation also applied to other bacteria, co-cultures of *Colpoda* RR with the pathogenic *Salmonella enterica* serovar Typhimurium C5 and the non-pathogen, *Bacillus subtilis* were used to demonstrate release of faecal pellets containing these bacteria. Thus faecal pellet production by *Colpoda* is not influenced by pathogen type of the bacteria used in this study. The potential for pellet location of *L. monocytogenes* to enhance survival in the presence of bactericidal agents was investigated using modifications of standard gentamycin protection assays in which faecal pellets secreted by *Colpoda* RR and MLS-5 were treated with gentamycin and NaOCl for up to 24 h at 25°C. Viable counts of pellet-located *L. monocytogenes* DRDC8 and assays of microbial respiratory activity of the pellet-located DRDC8 cells were similar to that of the biocide-untreated DRDC8 controls, indicating that faecal pellet encapsulated DRDC8 cells are resistant to biocidal agents.
This work suggests that *Colpoda*-derived faecal pellets may provide a mechanism for transmission of *L. monocytogenes* and other pathogenic bacteria. Furthermore, bacteria encapsulated by faecal pellets may be resistant to disinfectants and cleaning agents used in food manufacturing and preparation facilities.

### 4.2 Introduction

Host-pathogen interactions that occur between *Legionella pneumophila* and amoeba (Rowbotham 1983 and 1986, Molofsky and Swanson 2004) have confirmed the importance of protozoans as a possible niche for a number of intracellular bacterial pathogens to evolve in order to infect mammalian cells (Molmeret *et al.* 2005). That premise is now well established for a range of other bacteria/protozoa interactions, including *Mycobacterium avium - Acanthamoeba* spp. (Steinert *et al.* 1998, Miltner and Bermudez 2000), *Salmonella enterica - Acanthamoeba rhysodes* (Tezcan-Merdol *et al.* 2004), *Escherichia coli O157 - Acanthamoeba* spp. (Barker and Brown 1994) and *Burkholderia cepacia - Acanthamoeba polyphaga* (Landers *et al.* 2000).

As a pathogen of both humans and animals, *Listeria monocytogenes* is one of the most virulent and ubiquitous pathogens as this facultative intracellular bacterium is well adapted to survive in the environment. As the etiological agent for listeriosis, *L. monocytogenes* causes infection when contaminated food is ingested (Cossart and Bierne 2001). By comparison with the large number of published reports concerning *L. monocytogenes*, there have been few studies dedicated to the ecology of *L. monocytogenes* in natural environments. In particular, the role of protozoans as a potential reservoir for *L. monocytogenes* has not been well studied, even though this bacterium has evolved an array of virulence factors that are important for invasion of cells and establishment of an intracellular lifestyle within mammalian cells (Cossart *et al.* 2003). *L. monocytogenes* has shown to have parasitizing effects on mammalian cells (e.g. HeLa and Caco-2 cell lines) and is pathogenic in mice (Francis and Thomas 1996). By contrast, previously demonstrated outcomes of co-cultures of *L. monocytogenes* with *Acanthamoeba* spp. have shown otherwise. Akya *et al.* (2010) showed that amoebae are able to actively phagocytose and kill bacteria located in phagolysosomal compartments within 2 h post ingestion. Interestingly, several groups have reported secretion of pathogenic prey bacteria from...
protozoan cells within membrane bound faecal pellets. For example, Rowbotham (1980 and 1986) described release of vesicle-like structures (faecal pellets) that contained an infectious dose of *L. pneumophila*. Following predation and phagocytosis by amoebae, the bacteria were released within pellets. These pellet associated bacterial cells were in addition to those released from lysed amoeba cells. Encapsulation of viable *L. pneumophila* cells within faecal pellets has also been confirmed for other protozoan hosts, such as *Tetrahymena* spp. (McNealy 2001) as well as *A. polyphaga* and *A. castellanii* (Berk et al. 1998). A separate study went further to show that *L. pneumophila* located within faecal pellets of *A. castellanii* were able to survive to up to 6 months within medium that had a poor nutrient availability (Bouyer et al. 2007). Furthermore, co-cultures of *T. pyriformis* with strains of *Escherichia coli* resulted in secretion of faecal pellet-like structures that contained viable, culturable bacteria (Schlimme et al. 1997). Brandl et al. (2005) also showed that up to 50 viable *S. enterica* cells are present per faecal pellet secreted by *Tetrahymena* sp. However, Gourabathini et al. (2008) demonstrated that while *Tetrahymena* sp. released faecal pellets containing *E. coli O157:H7* and *S. enterica* following grazing on these bacteria, this process did not occur when the ciliates grazed on *L. monocytogenes*.

Encapsulation of bacterial cells within membrane bound pellet-like structures presents these cells with a significant advantage in terms of survival in the presence of antibacterial agents. Berk et al. (1998) showed that faecal pellet encapsulated *L. pneumophila* cells retained viability following exposure to biocides (e.g. free chlorine, and which others) as well as cycles of freeze-thaw treatment. Similarly, *S. enterica* cells contained within *Tetrahymena* sp. faecal pellets exhibit higher resistance to low concentrations of chlorine in comparison to free-swimming bacteria (Brandl et al. 2005). However, to date there is no unequivocal evidence to support the contention that *L. monocytogenes* is either secreted within faecal pellet structures by predatory protozoans, or faecal pellet-associated cells are more resistant to bactericidal agents than individual cells.

In this study, data from co-culture experiments with two isolates of the ciliate *Colpoda* (RR and MLS-5) are presented that show that *L. monocytogenes* cells predated by these protozoans are secreted within faecal pellets. Furthermore, pellet associated bacteria, but not washed cell suspensions of *L. monocytogenes*, are able to survive exposure to
gentamycin and NaOCl. As a comparator, secretion of *Salmonella enterica* serovar Typhimurium C5 within *Colpoda* faecal pellets was also investigated.

4.3 Experimental Procedures

4.3.1 Organisms and Culture Conditions

The *Colpoda* sp. isolate RR (GenBank accession number GQ_475427) was previously isolated from Lotus pond at the Adelaide Botanical Gardens (Raghu Nadhanan 2008), whereas *Colpoda* sp. isolate MLS-5 (see Appendix A) was isolated from a South Australian food processing environment. Clonal populations were established and maintained in aerated water containing washed suspensions of *E. coli* DH5α as a food source. These cultures were incubated at 25°C and routinely monitored for ciliate growth.

*L. monocytogenes* strain DRDC8, an Australian dairy isolate known to invade and multiply within mammalian cells (Francis and Thomas 1996) was routinely cultivated in Listeria Enrichment Medium broth (LEM) or on LEM agar plates with incubation at 37°C overnight with aeration. GFP-expressing *L. monocytogenes* ADGA (Laboratory collection, University of Adelaide), a derivative of DRDC8, was grown in LEM broth supplemented with 10 μg mL⁻¹ erythromycin. *B. subtilis*, *E. coli* DH5α and *S. Typhimurium* C5 (Laboratory collection, University of Adelaide) were grown in LB broth (Lennox) with incubation at 37°C.

Where required, washed cell suspensions of DRDC8 in 1.5 mL microcentrifuge tubes were killed by heating for 45 min at 100°C.

4.3.2 Co-culture Assays

The ciliates from active clonal cultures fed with *E. coli* DH5α were harvested by gentle centrifugation (106 × g, 2 min). The cell pellet was washed once in modified Neff’s amoeba saline (AS) buffer (pH 7.0) (Smirnov and Brown 2004) and resuspended in fresh AS buffer at room temperature. Overnight broth cultures of bacteria incubated at 37°C were harvested by centrifugation (4000 × g, 20 min) and the cell pellet washed once in AS buffer followed by re-suspension in fresh AS buffer to a final concentration of 10⁹ cells mL⁻¹.
Washed suspensions of ciliates (200 μL) at concentrations of ca. 10^4 cells mL⁻¹ were then pulse fed with 200 μL of washed bacterial suspensions for 1 h at 25°C. Post feeding, the co-culture suspensions were washed free of any extra-ciliate bacteria, and the ciliates are incubated as required to determine survival of ingested bacteria.

To obtain faecal pellet suspensions free of *Colpoda* and extra-faecal pellet bacteria, the co-culture suspensions were centrifuged and the washed with AS buffer (106 × g, 2 min) till the suspensions were free of *Colpoda* ciliates. The washed suspensions were then washed with AS buffer to kill any remaining extracellular bacteria. Samples (100 μL) of washed suspensions were then serially diluted and plated out on agar plates to confirm the absence of any extracellular bacteria. Counts of free faecal pellets present after 0.5, 1, 4 and 24 h post feeding, were determined by direct microscopy with the aid of a haemocytometer. All counts were done in triplicates and mean concentrations of free faecal pellets were determined.

To estimate counts of viable bacteria within faecal pellets, faecal pellets were lysed by addition of 0.3% (w/v) Triton X-100 solution. Serial dilutions of lysates were then plated out on LEM agar (for *L. monocytogenes* DRDC8), LEM agar with 10 μg mL⁻¹ erythromycin (for GFP-expressing *L. monocytogenes* ADGA) or on LB agar (for *B. subtilis* and *S. Typhimurium* C5) and incubated overnight at 37°C. Following incubation, the counts of viable bacteria (CFU mL⁻¹) were determined.

The ability of viable bacteria to reduce XTT to a soluble, orange-coloured formazan product was used to confirm that bacteria within faecal pellets were both viable and actively respiring (Bensaid *et al*. 2000, Roslev and King 1993). Secreted faecal pellets from *Colpoda* that were fed with *L. monocytogenes* DRDC8 cells were lysed, and these DRDC8 cells were treated for 12 h with the XTT at a final concentration of 0.3 mg mL⁻¹ in AS buffer. Assays were assessed visually for formation of reduced XTT. Faecal pellets secreted by *Colpoda* fed with heat-killed DRDC8 cells were used as a negative control for the method. Gentamycin (10 μg mL⁻¹) killed treated suspensions of DRDC8 was also used as a negative control for the XTT assay.
4.3.3 Biocide Treatment

Co-culture suspensions of 4 h post feeding were washed free of both ciliates and any remaining extracellular *L. monocytogenes* DRDC8 as described in Schipper et al. (1994). Free faecal pellets collected and resuspended in AS buffer as described above. Samples (ca. 10⁴ faecal pellet mL⁻¹) of faecal pellet preparations were separately treated with different concentrations of gentamycin and NaOCl (supplied as a 25% solution). Each biocide was diluted in AS buffer to the following final concentrations: gentamycin (0.5, 5.0, 10, 20, 50 and 100 µg mL⁻¹) and NaOCl (0.25, 0.5, 1, 2, 4, 6, 8 and 10%). Faecal pellet suspensions were incubated with the biocides for 4 and 24 h, after which the suspensions were washed free of the biocides. The faecal pellets were then lysed by addition of 0.3% (v/v) Triton X-100 solution. Serial dilutions of lysates containing pellet-located bacteria were plated out on LEM agar and incubated overnight at 37°C. Following incubation, the numbers of CFU mL⁻¹ were determined. Counts of free DRDC8 suspensions in AS buffer that were treated with the biocides were used as a control.

4.3.4 Fluorescence Microscopy

Preparations of faecal pellets were harvested from co-cultures and these faecal pellets were lysed by 0.3% (v/v) Triton X-100 solution. Lysates were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit (catalog no. L-7012) (Molecular Probes). Briefly, 1µL each of SYTO 9 green-fluorescent nucleic acid stain and propidium iodide red-fluorescent nucleic acid stain were combined in 1 mL of AS buffer and thoroughly mixed. 3 µL of the dye mixture was then added into 1 mL of the solution containing lysates of faecal pellets. This stained lysates solution was incubated at 25°C in the dark for 15 min. Stained samples were then washed 2 times with AS buffer, and the washed samples were added between a slide and an 18 mm square coverslip. All stained samples were visualized with Olympus 1X70 microscope. The images were taken using Metamorph software version 6.3r7 (Molecular Devices).

4.3.5 Transmission Electron Microscopy (TEM)

Preparations of faecal pellets harvested from co-cultures, were fixed in PBS containing 4% paraformaldehyde, 1.25% (v/v) gluteraldehyde with overnight at incubation at 4°C, then embedded in 1% (w/v) agarose and post-fixed in 2% (w/v) OsO₄ in distilled water. After
post fixation, specimens were dehydrated in an ethanol series. The ethanol was replaced by propylene oxide and the specimens embedded in Pro Scitech Epoxy resin. Ultra-thin sections of resin-embedded specimens were stained with uranyl acetate and lead citrate and examined in Philips CM 100 transmission electron microscope.

### 4.3.6 Statistical Analysis

Counts of viable *Colpoda* cells, faecal pellets and viable pellet-located *L. monocytogenes* DRDC8 were calculated after co-culture of DRDC8 with *Colpoda* RR and MLS-5. All data reported are the means obtained from three independent experiments. Each experimental data point was the mean of three replicate samples. Tukey’s Multiple Comparison Test was used to compare the counts at different time-points. *p*-values of < 0.05 were considered statistically significant. All statistical tests were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, California).

### 4.4 Results

#### 4.4.1 Production of Faecal Pellets by *Colpoda* ciliates

Co-culture of *L. monocytogenes* DRDC8 with *Colpoda* RR or MLS-5 resulted in release of faecal pellet-like structures containing bacterial cells within 2 h post feeding at 25°C. Figure 4.1 (*Colpoda* RR) and Figure 4.2 (*Colpoda* MLS-5) are TEM micrographs that illustrate typical characteristics of faecal pellets secreted by these isolates of ciliates. These faecal pellets had diameters of 1.5 – 2.5 µm and comprised multiple, layered membranes structures. Since these secreted structures are made up of multiple pieces of membrane that are not simply cytoplasmic contents enclosed by a continuous membrane (which is what defines a vesicle), they are more correctly termed a faecal pellet (S. Berk, personal communication). Faecal pellet-associated bacterial cells were typically segregated within different compartments formed by the membrane layers in a manner similar to that described for faecal pellets secreted by *Acanthamoeba* spp. (Berk *et al*. 1998). Bacterial cells within all faecal pellets examined appeared to have intact cell wall structures. None of these cells exhibited any sign of either partial or complete loss of bacterial cell wall integrity.
In separate co-culture experiments using *Colpoda* RR, release of faecal pellets containing either *S. Typhimurium* C5 (Figure 4.3A) or *Bacillus subtilis* (data not shown) was demonstrated. These multi-layered membraneous faecal pellets were similar in size and structure to those containing DRDC8 cells. In some sections of C5-fed *Colpoda* RR, multilayered membraneous structures resembling faecal pellets, were located within intact ciliate cells (Figure 4.3B). These structures, which also contained bacterial cells, were located at the periphery of intact *Colpoda* ciliates are presumed to be precursor faecal pellets prior to release to the cell exterior.

To confirm that the bacterial cells located within faecal pellets secreted by each *Colpoda* isolate was the strain of *L. monocytogenes* fed to ciliates and not contaminating bacteria, *Colpoda* RR cells were co-cultured with the GFP-expressing, DRDC8-derived *L. monocytogenes* strain ADGA and the faecal pellets secreted were examined for presence of GFP. As expected, secreted faecal pellets appeared green when examined by fluorescence microscopy (Figure 4.4A and B), whereas control co-cultures fed with the non-GFP expressing *L. monocytogenes* DRDC8 (Figure 4.4C and D) did not secrete faecal pellets that contained GFP.

### 4.4.2 *Colpoda* secrete Faecal Pellets that contain *L. monocytogenes* DRDC8

Counts of viable ciliates, faecal pellets and pellet-located bacteria following co-culture of *L. monocytogenes* DRDC8 with *Colpoda* RR (Figure 4.5) and MLS-5 (Figure 4.6) were determined over 24 h post feeding at 25°C. Overall, the counts of viable ciliates, faecal pellets and pellet-located bacteria following co-culture with DRDC8 were similar for *Colpoda* RR and MLS-5. Based on the data presented on Figure 4.5, viable counts of *Colpoda* RR remained unchanged at ca. $1.3 \times 10^5$ cells mL$^{-1}$ from 0 to 12 h post feeding ($p > 0.05$), then increased to ca. $5 \times 10^5$ cells mL$^{-1}$ at 24 h post feeding ($p < 0.05$). Counts of faecal pellets within 4 h post feeding were ca. $1.2 \times 10^5$ faecal pellet mL$^{-1}$ ($p < 0.05$), and remained unchanged till 24 h post feeding ($p > 0.05$). Viable, pellet-located DRDC8 counts from lysates of faecal pellets were ca. $1.5 \times 10^4$ CFU mL$^{-1}$ at 4 h post feeding ($p < 0.05$), and only increased slightly to ca. $1.8 \times 10^4$ CFU mL$^{-1}$ at 24 h post feeding ($p > 0.05$). Similar results were obtained for co-cultures of DRDC8 with *Colpoda* MLS-5 (Figure 4.6). To confirm if the secretion of faecal pellets by *Colpoda* ciliates is a result of feeding on DRDC8, *Colpoda* RR and MLS-5 were incubated for up to 24 h within AS
buffer without the addition of bacteria (no bacteria control) (Figure 4.5 and Figure 4.6). There was no production of faecal pellets by the starved ciliates throughout the period of incubation.

In general, an increase in viable ciliates did not result in a simultaneous increase in counts of faecal pellets and pellet-located bacteria. Regardless of the *Colpoda* strain used for co-culture, counts of faecal pellets and pellet-located bacteria increased to maximum levels within 4 h post feeding and thereafter remained almost unchanged for up to 24 h post feeding.

Since the mean total number of faecal pellets in the suspension at 24 h post feeding was ca. $1.2 \times 10^5$ faecal pellet mL$^{-1}$ for *Colpoda* RR co-culture and $1.3 \times 10^5$ faecal pellets mL$^{-1}$ for *Colpoda* MLS-5 co-culture, a single *Colpoda* ciliate was estimated to have secreted an average of 4.00 - 4.08 faecal pellets. However it is possible that a proportion of *Colpoda* ciliates may secrete far more than 4.00 - 4.08 faecal pellets, whereas other *Colpoda* ciliates may secrete fewer faecal pellets, and some may not even secrete any faecal pellets.

Based on the counts of faecal pellets and pellet-located bacteria at 24 h post feeding (Figure 4.5 and Figure 4.6), faecal pellets from *Colpoda* RR and MLS-5 co-cultures were estimated to contain an average of ca. 8 bacterial cells per faecal pellet (Figure 4.7). These estimates do not preclude the possibility that many faecal pellets contained fewer or no bacteria, as TEM micrographs provided evidence that empty faecal pellets were secreted by *Colpoda* RR following co-cultures with DRDC8 (data not shown). It was not possible to directly enumerate the numbers of DRDC8 within each faecal pellet by direct microscopy, as the outer enclosing membrane of faecal pellets made observation the bacteria within difficult.

### 4.4.3 Viability of *L. monocytogenes* within Faecal Pellets

The viability of all *L. monocytogenes* DRDC8 cells located within secreted faecal pellets prepared from co-cultures with *Colpoda* RR and MLS-5 was assessed by using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Figure 4.8). Faecal pellets were lysed prior to staining. Bacterial cells that exhibited only green fluorescence, but not red fluorescence, were considered to be viable. Stained DRDC8 cells from lysed faecal pellets of *Colpoda* RR emitted green fluorescence characteristic of viable bacterial cells (Figure
No stained cells isolated from faecal pellet preparations exhibited red fluorescence (Figure 4.8B). As expected, faecal pellet preparations from *Colpoda* RR fed with heat-killed DRDC8 exhibited only red fluorescence (Figure 4.8C and Figure 4.8D). Similar results were obtained for DRDC8 cells within faecal pellets secreted by *Colpoda* MLS-5 (data not shown).

The ability of DRDC8 cells to reduce the tetrazolium salt XTT was used to confirm that pellet-located bacterial cells were viable and respiring (Figure 4.9 and Figure 4.10). Free faecal pellets containing viable and respiring DRDC8 cells secreted by *Colpoda* RR (Figure 4.9) and *Colpoda* MLS-5 (Figure 4.10) were lysed, and the pellet-located DRDC8 were treated with 0.3 mg mL\(^{-1}\) XTT, a microbial respiratory indicator, for 12 h. A positive XTT reaction was demonstrated for these samples (Well A). These samples that showed a positive XTT outcome were dark orange in colour, indicating production of orange formazan crystals through the reduction of XTT by viable and respiring pellet-located *L. monocytogenes* DRDC8. As expected, the negative controls for the XTT assay, comprising heat-killed pellet-located DRDC8 (Well B) as well as DRDC8 suspended in AS buffer (Well C), did not produce the orange colour, indicative of negative XTT reaction.

### 4.4.1 *L. monocytogenes* within Faecal Pellets are protected from killing by Gentamycin and Chlorine

Following co-cultures with *L. monocytogenes* DRDC8, secreted faecal pellets of both *Colpoda* RR and *Colpoda* MLS-5 were incubated separately in solutions of either gentamycin (0.5, 5, 10, 20, 50 and 100 µg mL\(^{-1}\)) or NaOCl (0.25, 0.5, 1, 2, 4, 6, 8 and 10%) at 25°C. Control preparations of DRDC8 cells suspended in AS buffer were treated with identical concentrations of each biocide. Survivor bacterial cells were estimated by plating on LEM medium (Table 4.1 (*Colpoda* RR) and Table 4.2 (*Colpoda* MLS-5). Treatment with both biocides for 4 and 24 h resulted in no loss of viability for bacteria located within faecal pellets for any of the biocide concentrations used. Counts of pellet-located DRDC8 within biocide-treated faecal pellets of both strains of *Colpoda* ciliates were the same as counts of pellet-located DRDC8 prior to biocide treatment (0 h post treatment) \((p > 0.05)\). However DRDC8 cells suspended in AS buffer were killed by either
biocide within a 4 h exposure, with the exception of cell suspensions treated with the lowest concentration (0.5 µg mL⁻¹) of gentamycin.

These results were confirmed by a positive XTT reaction (orange-coloured solution) for pellet-located DRDC8 (data not shown). XTT assay results for biocide treated suspensions of DRDC8 cells in AS buffer, were identical to those of treated faecal pellets prepared from *Colpoda* strains fed heat-killed DRDC8.

### 4.5 Discussion

Rowbotham (1980) was the first to provide evidence of amoebae as a vector of *Legionella* spp. More recently, Berk *et al.* (1998) demonstrated *Acanthamoeba* spp. expelled faecal pellets that contained bacterial cells into the extracellular environment. Since then, only a few published studies have demonstrated faecal pellet production by other protozoans following co-culture with different species of bacterial pathogens.

The genus *Colpoda* belongs to the class Colpodea, and is usually 20 - 50 µm in length with a reniform shape. *Colpoda* ciliates feed on bacteria as the major food source. Like *L. monocytogenes*, *Colpoda* can be found in a variety of environments, especially in soil and vegetation. For this reason, *Colpoda* may be a good model organism to analyse interactions between *L. monocytogenes* and protozoans. The choice of *L. monocytogenes* strain DRDC8 as the primary test bacterium for use in co-culture experiments was based on prior observation by Raghu Nadhanan (2008) that showed *Colpoda* RR predated pathogenic strains (eg. DRDC8 or EGD Kaufmann) and non-pathogenic strains (eg. Listeriolysin O insertion mutant, LLO17) equally well. All strains of *L. monocytogenes* were actively phagocytosed and degraded by the host ciliate.

In this study, we have demonstrated that *Colpoda* ciliates secrete faecal pellets containing live *L. monocytogenes* DRDC8 cells following co-culture. This outcome is unique, as previous studies have shown the inability of other protozoa species to secrete faecal pellets containing bacteria following co-cultures with *L. monocytogenes*. The amoeba *A. palestinensis* and the ciliate *T. pyriformis* did not produce any faecal pellets following feeding with *L. monocytogenes* (Brandl *et al.* 2005, Gourabathini *et al.* 2008). The ciliate *Glaucoma* sp. produced only a few faecal pellets following co-culture with *L. monocytogenes* (Gourabathini *et al.* 2008). Brandl *et al.* (2005) showed that
Tetrahymena sp. strain MB125 expelled only few faecal pellets following co-culture with L. monocytogenes, and these faecal pellets contained only few cells of L. monocytogenes. It is interesting that although the present study provided clear evidence to show Colpoda RR and MLS-5 secreted faecal pellets containing bacteria following co-cultures with L. monocytogenes and S. Typhimurium, Gourabathini et al. 2008 reported that Colpoda steinii did not secrete any faecal pellets containing bacteria following co-culture with L. monocytogenes and S. enterica. The reason for this difference is unclear. It is likely that different species of protozoa (even of the same genus) may differ in their ability to secrete bacteria within faecal pellets. Besides that, protozoans may also differ in terms of ability to secrete different isolates of the same bacterium within faecal pellets during co-culture. For example, studies by Gourabathini et al. (2008) and Brandl et al. (2005) utilized a different isolate of L. monocytogenes compared with the present study. This suggested that the ability of protozoans to produce L. monocytogenes-containing faecal pellets may be dependent on the isolate of L. monocytogenes used. Given that Colpoda RR is also able to secrete faecal pellets following co-cultures with either S. Typhimurium C5 or B. subtilis, this showed that the process of faecal pellet production by Colpoda RR is likely to be independent of either the bacteria or the bacteria’s pathogenicity.

Why most DRDC8 cells internalised by Colpoda RR and MLS-5 are unable to survive digestion (Chapter 2), and others are secreted within secreted faecal pellets is unknown. The simplest explanation may be that the intact bacteria become enmeshed in cellular debris targeted by the ciliate for release as faecal pellets. Another explanation is that these strains of Colpoda may have a unique mechanism in place for processing L. monocytogenes. Whether differential processing of DRDC8 by the ciliates is an attempt to expel food that it does not need, or is not able to digest, has yet to be determined. Alternatively, location of bacteria within faecal pellets may be a method utilized by bacteria to escape digestion by the host ciliates. These questions highlight the poor understanding about egestion methods employed by bacterial pathogens to escape from protozoan hosts.

Interestingly, the eventual fate of the bacteria-containing faecal pellets is not known. It is possible that these faecal pellets remain in the environment, or are taken up by the ciliates when food sources are scarce. Nevertheless, pellet-located bacteria are likely to
enjoy a privileged environment protected from external threat. The observation that viable, actively respiring DRDC8 cells are present within faecal pellets even after 24 h post feeding is a strong indication that there is no host bactericidal machinery remaining within the faecal pellets. Typically secreted faecal pellets are generally around 2.5 µm in diameter or less, and each faecal pellet can contain about 8 viable and actively respiring \(L.\) monocytogenes cells. As a consequence, these faecal pellets are potential infectious particles that may cause a severe infection when either faecal pellet-contaminated food or water is ingested. Though there have been no published studies on \(L.\) monocytogenes infections acquired by inhalation, these bacteria-containing faecal pellets should still be considered as a possible clinically important vector of disease transmission.

It has been well documented that host protozoans may enhance survival of bacterial pathogens within protozoans by protecting bacteria from exposure to disinfectants and sanitizers. Pathogens such as \(S.\) Typhimurium and \(Campylobacter\) jejuni have demonstrated resistance to free chlorine following ingestion by \(T.\) pyriformis (King et al. 1988). \(C.\) jejuni present within \(T.\) pyriformis and \(A.\) castellanii also exhibit increased resistance to Virudine, a disinfectant commonly used in the poultry industry (Snelling et al. 2005). Furthermore, Adekambi et al. (2006) reported that as many as 26 species of water-associated mycobacteria cells that were encapsulated within cysts of \(A.\) polyphaga were able to survive up to 24 h exposure to free chlorine. Bacteria encapsulated within faecal pellets secreted by protozoans have also been demonstrated to be resistant to biocidal agents. Brandl et al. (2005) for example, reported that \(S.\) Thompson cells located within faecal pellets of \(Tetrahymena\) sp. were protected during treatment with calcium hypochlorite (\(\text{Ca(ClO)}_2\)) concentrations of 0.13 and 0.42%. Similarly, Berk et al. (1998) showed that \(L.\) pneumophila cells within faecal pellets of \(Acanthamoeba\) spp. are resistant to external stresses such as cooling tower biocides, freeze-thawing and sonication processes. Consequently it is not surprising that DRDC8 cells encapsulated within faecal pellets of \(Colpoda\) RR and MLS-5 are resistant to concentrations of gentamycin (0 – 100 µg mL\(^{-1}\)) and NaOCl (0 – 10%) that are normally lethal to washed cell suspensions of this pathogen. The membranes that make up the faecal pellets may either act as a barrier to prevent the permeation of the biocides into the pellets, and/or organic materials present within the pellets may inactivate or prevent the biocide molecules damaging the bacterial
cells. The remarkable resistance of these pellet-located DRDC8 cells to these otherwise biocidal concentrations of disinfectants indicated that the faecal pellets are likely to be an excellent protective shield for the bacteria in the presence of disinfectants and cleaning agents used in food processing environments.

Food safety and hygiene and the eradication of food-borne pathogens have become a major concern for the food industry. Chlorine is one of the most commonly used and cost-effective sanitizer in food processing and handling applications. Chlorine-based sanitizers are used in a range of food processing environments and food contact articles for the purpose of killing, inactivating or reducing a wide number of food-borne pathogens. Sanitizers in food processing facilities are an example of measure taken to disinfect these commonly used environments. Commercially available chlorine-based solutions are generally used as sanitizing agents for equipments and product contact surfaces in food-processing environments to reduce levels of bacteria. Examples include Clorox Commercial Solutions™ Ultra Clorox Disinfecting Bleach (The Clorox Company of Canada, Ltd.), Ostro Brite (Ostrem Chemical Co. Ltd.) and GRUNT Chlorinated Detergent (Agrade Cleaning Supplies), which contain 5 - 8, 3.15 and 4% available chlorine respectively. Typically, for the purpose of disinfecting food processing equipment, recommended chlorine content within chlorine-based sanitizers is not more than 0.02% available chlorine (McGlynn 2004). In households, commercially available bleaches such as Clorox™ and Javex are solutions of NaOCl at 5.25%. Although chlorine-based sanitizers are effective in inactivating free bacterial cells, they may not be effective against bacteria encapsulated within faecal pellets. Since this study and others have shown that faecal pellet encapsulated bacteria are resistant to free chlorine concentration to up to 10%, it is unlikely that these sanitizers are able to effectively kill faecal pellet encapsulated bacteria when used at the recommended concentrations. For these reasons, faecal pellet associated bacterial pathogens represent an important concern in terms of health risk management for food processing facilities.

Although bacteria located in faecal pellets are protected from the action of chemicals and antibiotics, the survival of these bacteria may well depend on the ability of the faecal pellet membrane to remain intact. Brandl et al. (2005) has reported that faecal pellets produced by *T. pyriformis* are ‘difficult to lyse’, whereas the faecal pellets secreted by
either strain of *Colpoda* used in the present study were readily lysed by exposure to a 0.3% Triton X-100 solution. The ease with which *Colpoda* faecal pellets could be lysed, together with the resistance of *L. monocytogenes* DRDC8 enclosed within faecal pellets to biocide treatment demonstrate that chlorine based biocides combined with surfactants will be required to adequately destroy faecal pellet located *L. monocytogenes* and other pathogenic bacteria such as *S. Typhimurium*. Though radiation treatment has been shown to decontaminate pathogens such as *L. monocytogenes* and *Salmonella* spp. within foods (Farkas 1998), it is unsure if it is effective against bacterial cells that are encapsulated within faecal pellets.

Whether pellet-located *L. monocytogenes* cells are more or less virulent than free *L. monocytogenes* cells are unknown. It is possible that the intra-pellet environment carrying these bacteria may improve survival of *L. monocytogenes* during passage through the stomach. Future studies to investigate this could include inoculating animal models separately with different moi of pellet-located and free *L. monocytogenes* and tracking the rate at which these two types of *L. monocytogenes* can cause disease. Molecular studies can also be carried to study if there are any differences in the levels of expression of virulence factors between intra-faecal pellet and free *L. monocytogenes* cells.

Overall, the results of the present work indicate that *Colpoda* RR and *Colpoda* MLS-5 can possibly facilitate the dissemination of pathogenic bacteria inside faecal pellets that have impermeable barriers that prevent penetration of biocides such as gentamycin and NaOCl, and are found free in solutions. The fact that DRDC8 does not survive within vegetative cells of either *Colpoda* RR and *Colpoda* MLS-5 (Chapter 2), but is present within secreted faecal pellets, underscores the importance of this study in terms of risk of transmission of pathogens to humans. It is especially important to note that the food processing environment isolate *Colpoda* MLS-5 is not only able to secrete faecal pellets that harbours pathogenic bacteria, these faecal pellets also protect the pellet-located pathogenic bacteria within it from killing by concentrations of NaOCl that are usually used to disinfect the areas in food processing environments. This will have significant implications on both the food as well as health industries. Future studies need to investigate practical ways to eliminate pellet-located pathogens while still present within the faecal pellets. For example, targeting the faecal pellet membrane with disinfectants that
contains surfactants may help to lyse the faecal pellet membrane and assist the disinfectant to permeate the pellet contents. It would also be useful to determine if the faecal pellets are protected against dehydration or UV exposure. Furthermore, it would be interesting to investigate the survival time of faecal pellet associated bacteria within pellets suspended in water.

This study highlights the role of *Colpoda* RR and *Colpoda* MLS-5 as possible vectors in the transmission of *L. monocytogenes* through the production of faecal pellets under the laboratory conditions tested.

### 4.6 Acknowledgments

We thank Ruth Williams and Lynette Waterhouse of Adelaide Microscopy for their invaluable assistance with the TEM. We also thank Assoc. Prof. Renato Morona for his assistance with fluorescence microscopy. RRN was supported by The University of Adelaide Graduate Research Scholarship.

### 4.7 References


Figure 4.1: TEM of a faecal pellet secreted by Colpoda RR co-cultured with L. monocytogenes DRDC8.

Note the bacterial cells located within the multilayered membranes structure of the faecal pellet. Bar = 0.5 μm.
Figure 4.2: TEM of a faecal pellet secreted by *Colpoda* MLS-5 co-cultured with *L. monocytogenes* DRDC8.

Note the bacterial cells located within the multilayered membranes structure of the faecal pellet. Bar: =1 μm.
Figure 4.3: Faecal pellets from co-culture of *Colpoda* RR with *S. Typhimurium* C5.

(A) *Colpoda* RR secreted faecal pellets containing bacteria sized cells (arrows). Note the presence of extra-faecal pellet bacteria. Bar = 0.5 μm.

(B) A multi-layered, membranous faecal pellet-like structure located within an intact *Colpoda* RR cell. Note the bacteria sized cell within the faecal pellet (arrow). Bar = 1 μm.
Figure 4.4: Microscopy of GFP-expressing *L. monocytogenes* ADGA cells within faecal pellets secreted by *Colpoda* RR

GFP-expressing *L. monocytogenes* ADGA within a faecal pellet viewed under:

(A) Normal light. Bar: 1.5 μm.
(B) FITC filter. Bar: 1.5 μm.

Non GFP-expressing *L. monocytogenes* DRDC8 within a faecal pellet viewed under:

(C) Normal light. Bar: 0.7 μm.
(D) FITC filter. Bar: 0.7 μm.
Co-cultures of *Colpoda* RR and *L. monocytogenes* DRDC8 were incubated at 25°C for up to 24 h. Counts of ciliates and faecal pellets were determined by direct microscopy. Counts of pellet-located bacteria were determined following lysis of preparations of isolated faecal pellets. Extra-faecal pellet bacteria were eliminated by gentamycin treatment prior to lysis. Results presented are the means of three counts ± standard deviations.
Figure 4.6: Counts of Colpoda MLS-5, faecal pellets and pellet-located bacteria.

Co-cultures of Colpoda MLS-5 and L. monocytogenes DRDC8 were incubated at 25°C for up to 24 h. Counts of ciliates and faecal pellets were determined by direct microscopy. Counts of pellet-located bacteria were determined following lysis of preparations of isolated faecal pellets. Extra-faecal pellet bacteria were eliminated by gentamycin treatment prior to lysis. Results presented are the means of three counts ± standard deviations.
Figure 4.7: Estimates of bacterial cells per faecal pellet secreted by *Colpoda* RR and MLS-5.

Co-cultures of *Colpoda* RR and MLS-5 with *L. monocytogenes* DRDC8 were incubated at 25°C for up to 24 h. Counts of faecal pellets were determined by direct microscopy. Counts of pellet-located bacteria were determined following lysis of preparations of isolated faecal pellets. Extra-faecal pellet bacteria were eliminated by gentamycin treatment prior to lysis. For each time-point, counts of secreted faecal pellets and pellet-located DRDC8 were used estimate the mean number of bacteria per faecal pellet. Results presented are the means of three counts ± standard deviations.
Preparations of faecal pellets were lysed and the pellet-located *L. monocytogenes* DRDC8 were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit to determine the viability status of bacterial cells within faecal pellets. Stained preparations were examined by Phase contrast fluorescence microscopy. Live DRDC8 appear green following staining with SYTO® 9 green-fluorescent nucleic acid stain and heat-killed DRDC8 appear red following staining with propidium iodide.

(A) Live DRDC8 (FITC filter). Bar: 10 μm.
(B) Live DRDC8 (Texas Red filter). Bar: 10 μm.
(C) Heat-killed DRDC8 (FITC filter). Bar: 10 μm.
(D) Heat-killed DRDC8 (Texas Red filter). Bar: 10 μm.
Figure 4.9: XTT assay of faecal pellets expelled from *Colpoda* RR.

Co-cultures of *Colpoda* RR and *L. monocytogenes* DRDC8 were used to isolate preparations of faecal pellets. Faecal pellets were lysed, treated with XTT and incubated at 25°C for 12 h prior to assessment of formation of soluble, orange-coloured formazan product.

(A) Faecal pellets lysates from *Colpoda* RR fed viable DRDC8.

(B) Faecal pellets lysates from *Colpoda* RR fed heat-killed DRDC8.

(C) Suspension of gentamycin-treated DRDC8.
Figure 4.10: XTT assay of faecal pellets expelled from *Colpoda* MLS-5.

Co-cultures of *Colpoda* MLS-5 and *L. monocytogenes* DRDC8 were used to isolate preparations of faecal pellets. Faecal pellets were lysed, treated with XTT and incubated at 25°C for 12 h prior to assessment of formation of soluble, orange-coloured formazan product.

(A) Faecal pellets lysates from *Colpoda* MLS-5 fed viable DRDC8.
(B) Faecal pellets lysates from *Colpoda* MLS-5 fed heat-killed DRDC8.
(C) Suspension of gentamycin-treated DRDC8.
Table 4.1: Resistance of *Colpoda* RR pellet-located *L. monocytogenes* DRDC8 to treatment with gentamycin and NaOCl.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Incubation period (h)</th>
<th>Gentamycin concentration (µg mL⁻¹)</th>
<th>NaOCl concentration (%) †</th>
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<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Pellet-located</td>
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<td></td>
</tr>
<tr>
<td>DRDC8</td>
<td></td>
<td>1.3×10⁴</td>
<td>2.0×10⁴</td>
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<td>4.1×10⁵</td>
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<td>DRDC8 control</td>
<td></td>
<td>2.9×10⁵</td>
<td>4.3×10⁴</td>
</tr>
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</table>

Bacterial counts (CFU mL⁻¹) of pellet-located *L. monocytogenes* DRDC8 at concentrations of gentamycin at 50 and 100 µg mL⁻¹ and NaOCl 4, 6, 8 and 10% were also tested, and all counts were consistently 10⁴ CFU mL⁻¹.

Results are the means of CFU ± standard deviations for triplicate samples.

†: (%) denotes percentage of w/v available chlorine.
Table 4.2: Resistance of *Colpoda* MLS-5 pellet-located *L. monocytogenes* DRDC8 to treatment with gentamycin and NaOCl.

<table>
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<tr>
<th>Sample Type</th>
<th>Incubation period (h)</th>
<th>Gentamycin concentration (µg mL⁻¹)</th>
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<th>0.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
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<tbody>
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<td>Pellet-located</td>
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<td>1.6×10⁴</td>
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<td>1.7×10⁴</td>
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<tr>
<td>DRDC8</td>
<td></td>
<td></td>
<td>(± 0.284)</td>
<td>(± 0.194)</td>
<td>(± 0.138)</td>
<td>(± 0.065)</td>
<td>(± 0.129)</td>
</tr>
<tr>
<td>DRDC8 control</td>
<td></td>
<td></td>
<td>3.8×10⁴</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(± 0.201)</td>
<td>(± 0.106)</td>
<td>(± 0.000)</td>
<td>(± 0.000)</td>
<td>(± 0.000)</td>
</tr>
<tr>
<td>Pellet-located</td>
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</tr>
<tr>
<td>DRDC8</td>
<td></td>
<td></td>
<td>(± 0.098)</td>
<td>(± 0.134)</td>
<td>(± 0.174)</td>
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Results are the means of CFU ± standard deviations for triplicate samples.

† : (%) denotes percentage of w/v available chlorine.

Bacterial counts (CFU mL⁻¹) of pellet located *L. monocytogenes* DRDC8 at concentrations of gentamycin at 50 and 100 µg mL⁻¹ and NaOCl 4, 6, 8 and 10% were also tested, and all counts showed were consistently 10⁴ CFU mL⁻¹.
Chapter 5: General Discussion

*L. monocytogenes* has no recognized animal reservoir. While *Acanthamoeba* spp. is known to harbor a number of intracellular bacterial pathogens (Rowbotham 1980, Winiecka-Krusnell *et al.* 2002, Abd *et al.* 2005, Miltner and Bermuda 2000), the consensus of studies indicate *L. monocytogenes* is phagocytosed and rapidly degraded by the host amoeba (Huws *et al.* 2008, Akya *et al.* 2010). Whether the latter observation applies to all phagotrophic protozoans is a matter of conjecture. It was against this background that the present study evaluated the interaction between *L. monocytogenes* and the common ciliate, *Colpoda*.

To achieve this aim, *Colpoda* suspensions were pulse fed *L. monocytogenes* cells for a period of 1 h, and the ciliates washed to remove remaining bacterial cells followed by incubation for up to 24 h post feeding. This approach was selected over an *ad libitum* feeding protocol, to allow observation of sequential stages of processing of phagocytosed bacteria by *Colpoda*. *Ad libitum* feeding was considered inappropriate because individual *Colpoda* cells were likely to continuously feed, would contain phagocytosed bacteria at all stages of degradation and therefore key stages of development would be difficult to identify. A disadvantage of the pulse feeding protocol is that ciliates incubated for long periods of time are likely to be nutritionally stressed, suffer sub-lethal injury and undergo encystment. Any of these outcomes could compromise the ability of *Colpoda* to normally process and digest phagocytosed bacteria. However for short-term studies of a few hours duration, this method clearly allows observation of the process of phagocytosis and digestion of bacteria within vacuolar compartments. Indeed the basic protocol developed was used to examine interactions between *L. monocytogenes* strains, *B. subtilis*, *S. Typhimurium* C5 and *E. coli* DH5α, as well as to examine the histology and cell biology of phagocytosis and digestion of internalized bacteria. Furthermore, the protocol proved useful in evaluating secretion of bacteria within faecal pellets and cysts.

Importantly, the co-culture protocol was used together with microscopic techniques to unequivocally establish that *Colpoda* cells are phagotrophic for *L. monocytogenes* and other Gram positive and Gram negative bacteria. Judicious use of GFP-expressing strain of
"L. monocytogenes" together with fluorescence microscopy, established that this facultatively intracellular mammalian pathogen, was rapidly phagocytosed by host Colpoda cells. By employing a K⁺ channel inhibitor to block ciliate phagocytosis, L. monocytogenes was shown to be unable to induce uptake as has been described for mammalian cells. Similarly, the co-culture method was also valuable in evaluating aspects of the cell biology of phagosome docking with lysosomes, phagosome acidification, proteolysis, NO and superoxide production in the killing of L. monocytogenes by Colpoda cells. Indeed, this work confirmed published data that indicated L. monocytogenes is insensitive to NO expressed by host cells.

A novel outcome of the work that has important public health implications was the observation that Colpoda that were with fed L. monocytogenes cells had secreted faecal pellets containing these bacterial cells. Confirmation of the bacteria within these pellets was established through the use of GFP tagged bacterial cells. Furthermore, these bacteria were shown to be viable and metabolically active. While this finding is novel for L. monocytogenes, the principal that bacteria are secreted in faecal pellets by protozoans such as Acanthamoeba sp. has been previously established (Berk et al. 1998, Bouyer et al. 2007) and Tetrahymena sp. (Brandl et al. 2005). From a public health perspective, these pellet-located bacterial cells were demonstrated to be resistant to concentrations of gentamycin and sodium hypochlorite (NaOCl) well above concentrations needed to kill planktonic forms of L. monocytogenes. The implications for public health are not immediately obvious, but if these encapsulated forms of bacterial cells were to become established in food processing environments, then this may explain why it is difficult to decontaminate these premises. There is ample opportunity for ciliates like Colpoda to colonize floors and drains, especially in wet areas. Given that one Colpoda isolate used in this study (MLS-5) was isolated from a food processing environment, this scenario is an interesting one worthy of further evaluation.

An interesting challenge faced during this study was the maintenance of the ciliate cultures. Viable ciliate suspensions were an absolute requirement for co-culture studies. Regular sub-culturing of Colpoda RR was required to ensure an adequate supply of ciliates, but also to ensure that the cultures did not encounter conditions that promoted encystment. Encystment is related to shortages of nutrients, changes in temperature, and
adverse environmental conditions. Despite best efforts, throughout the duration of this study, Colpoda cultures regularly encysted. This occurred despite the presence of high bacterial prey populations as food source (E. coli DH5α, ca. 10⁹ cells mL⁻¹).

Another challenge of this work was provision of ciliate suspensions free of extracellular bacteria. Typically gentamycin treatment of mammalian cells infected with suspensions of intracellular pathogens is the most common means used to eliminate extra-cellular bacteria without destroying intra-cellular bacteria (Schipper et al. 1994). However, preliminary optimization experiments showed that gentamycin adversely affected the viability of Colpoda (Raghu Nadhanan 2008). Exposure of Colpoda to gentamycin at concentrations as low as 2 μg mL⁻¹ caused a 10 fold decrease in numbers of viable ciliates within 15 min following treatment. Hence this study relied on washing co-culture suspensions with AS buffer as means of removing any extra-ciliate bacteria. Preliminary experiments established that this reduced the counts of extracellular bacteria in ciliate suspensions to undetectable levels.

Colpoda ciliates also lost viability if subjected to centrifugation that exceeded ca. 200 x g. This was a particular challenge given ciliate suspensions had to be washed multiple times to properly remove any extra-ciliate bacteria within the suspensions. Colpoda ciliates have a maximum growth temperature of about 28 - 30°C. This upper growth temperature limit restricts the use of these ciliates in experiments that are clinically relevant for L. monocytogenes, for example to determine the effect of Listeriolysin O expression by L. monocytogenes at 37°C (the temperature at which Listeriolysin O is optimally expressed) on the fate of intra-ciliate L. monocytogenes.

The requirement for use of washed cell suspensions of bacteria for co-cultures with Colpoda cells was also a mandatory requirement. Culture media (e.g. LEM) used to culture L. monocytogenes caused immediate and dramatic loss of Colpoda viability. These cells typically lyse within minutes of exposure to sterile, fresh media (Raghu Nadhanan 2008) and indicated that LEM medium contained components that were lethal to Colpoda RR. To the best of the author's knowledge, this phenomenon has not previously been reported. To the contrary, yeast cultures have been shown to stimulate growth of rumen ciliates (Jouany et al. 1998). Peterson (1942) showed that yeast particles were essential for the continued growth of the ciliate Colpidium campylum, as the yeast stimulated the digestive mechanism.
of the ciliate. These anecdotal reports suggest that the yeast extract component of LEM is unlikely to have caused this loss of viability of Colpoda cells. Whether the Colpoda are sensitive to tryptic soy extract, the only additional component of LEM, remains to be established. Nevertheless, the technological issues described above highlight the need for careful assessment of culture and cell handling practices when working with ciliates. It is simply not sufficient to transfer methods used to study interactions of human pathogens with mammalian systems to a protozoan system without careful validation.

Although the advantages of pulse feeding of Colpoda have been discussed, there are circumstances where an ad libitum feeding protocol may also be advantageous. In particular, co-cultures to up to 48, 96 h or even longer co-cultures would require this feeding method. Indeed it would be interesting to determine whether the constant presence of L. monocytogenes cells during extended co-cultures would provide the bacteria with opportunities to parasitize Colpoda ciliates. Another application would be to produce large numbers of faecal pellet-located L. monocytogenes for studies of biocide resistance, the long term stability of the pellet membrane complex, and to assess whether the pellet environment is sufficient to maintain the viability of the entrapped bacterial cells.

In addition, there are possibilities that in this study, not all forms of Colpoda-pathogen interactions have been identified. It is possible that different species of Colpoda ciliates may behave differently in different environment niches, in which other outcomes may likely be obtained. Hence, employing models of Colpoda-pathogenic bacteria co-cultures that mimic a variety of environmental conditions such as composition of nutrients, levels of salt concentration, as well as presence of other competitors such as other protozoans, is likely to reflect the real outcome of bacteria/protozoa interaction in the environments. Besides that, co-culture of Colpoda with mixed populations of pathogenic bacteria may give L. monocytogenes cells an advantage that enables them to parasitize the ciliate cytoplasmic compartment. For example, a combination of L. monocytogenes, S. Typhimurium and Shigella flexneri may be co-cultured with Colpoda to determine if there is any difference in the outcome of the co-culture in comparison to co-culture of Colpoda with a single type of bacteria at one time. Since different types of bacteria co-exist in the real environment, this experiment may be of particular public health relevance.
5.1 Significance and Future Directions

A particularly intriguing aspect of the presented work is that it is unclear why on one hand, *L. monocytogenes* strains (and indeed also *S. Typhimurium C5*) are able to effectively parasitize mammalian cells, but are unable to parasitize *Colpoda* ciliates. There are several reasons that may explain this conundrum. It is possible that Listeriolysin O expression is insufficient to allow escape of the bacteria from phagocytic vacuoles. Construction of a *L. monocytogenes* strain that constitutively expresses Listeriolysin O could be carried out and used for co-culture with *Colpoda* ciliates to determine whether constitutive expression of Listeriolysin O has any virulent effects on *Colpoda* ciliates. It is also possible that the PrfA regulation of virulence genes of *L. monocytogenes* is either inhibited or not induced in the *Colpoda* cellular environment. Under these circumstances, all of the PrfA regulated genes, including *plcA* (encoding a phospholipase) and *hly* (encoding Listeriolysin O), would not be expressed and the bacterium would not be able to escape the phagosome before destruction by the host. It would therefore be interesting to examine whether extracts of *Colpoda* ciliates were able to inhibit/induce PrfA regulation of expression of genes within the Listeria pathogenicity island, for example *hly, actA* etc.

The ability of *Colpoda* ciliates to naturally feed on bacteria as food source, including those that are highly pathogenic to mammalian cells such as *L. monocytogenes* and *S. Typhimurium* suggests that the ciliates may have experienced evolutionary changes over the years to acquire the ability to phagocytose and degrade even professional intracellular bacterial pathogens. Increased knowledge regarding the genes of *Colpoda* ciliates involved in the intracellular killing of pathogens such as *L. monocytogenes* and *S. Typhimurium* should be required. Hence, a further detailed study should be done to provide a greater understanding of the mechanisms employed by *Colpoda* ciliates to kill intracellular bacteria pathogens such as *L. monocytogenes* and *S. Typhimurium* that will be crucial for the designing of new therapies to treat infections caused by intracellular pathogens.

The formation of slow-growing subpopulations of bacterial cells known as Small Colony Variants (SCVs) are increasingly recognized as important contributors to clinical disease and resistance to therapeutic intervention. SCVs are better able to persist in mammalian cells and are less susceptible to antibiotics than wild-type variants. Besides that, SCVs can cause latent or recurrent infections on emergence from the protective
environment of the host cell. SCVs are able to survive within both professional and non-professional phagocytic cells (Alexander and Hudson 2001, Sinha and Herrmann 2005). However there are no studies that show the role SCVs of *L. monocytogenes* in interaction with non-mammalian cells. Given the fact that SCVs are capable of modulating mammalian host immune responses, studies could be done to determine the relevance of *L. monocytogenes* SCVs interactions with protozoans such as *Colpoda* ciliates. The outcomes of this interaction in comparison to wild type variants of the *L. monocytogenes* can be investigated.

Whether pathogenic bacteria within faecal pellets secreted by *Colpoda* ciliates are infectious to mammalian cells and capable of causing disease needs to be further clarified. Furthermore it would be interesting to determine whether *L. monocytogenes* cells present within the outer walls of encysted *Colpoda* cysts are able to survive *Colpoda* encystment for long periods, and in particular, if these bacteria are resistant to disinfections. If the bacteria survive encystment, then this would provide evidence to suggest that these ciliates could under certain circumstances, act as a reservoir for *L. monocytogenes*.

The fact that *Dictyostelium discoideum* kills ingested *S. Typhimurium*, but allows intracellular survival of *Legionella* sp. (Skriwan *et al.* 2002) suggests that it is possible that other isolates of *Colpoda* ciliates that were not used in this study may interact differently with *L. monocytogenes*. Further *in vitro* experiments of co-cultures of *L. monocytogenes* with other isolates of *Colpoda* ciliates as well as other ciliate types can be carried out to determine whether or not they can act as a natural reservoir for *L. monocytogenes* in the environments.

**5.2 Conclusions**

This study has described some important features of the interaction between pathogenic *L. monocytogenes* DRDC8 cells and the ciliates *Colpoda* RR (an isolate of natural environment) and *Colpoda* MLS-5 (an isolate of food processing environment). The uptake and fate of *L. monocytogenes* cells internalized by these *Colpoda* ciliates were also assessed by this study. The main conclusions are as follows:

- *Colpoda* RR and MLS-5 rapidly phagocytose and degrade *L. monocytogenes* DRDC8 as food source in a manner that is independent of the level of expression of
Listeriolysin O normally required for escape of these pathogenic bacteria into the cytoplasm of mammalian cells.

- *Colpoda* RR is also able to feed on the pathogenic *S. Typhimurium* C5 as well as *E. coli* DH5α and *B. subtilis*.

- Immediately after feeding of *Colpoda* cells, phagocytosed *L. monocytogenes* DRDC8 are present within the ciliate cytoplasm in either tight vacuoles or as groups of cells within large food vacuoles. The cells within food vacuoles are degraded within 4 h post feeding. A similar outcome was observed for *S. Typhimurium* C5, *E. coli* DH5α and *B. subtilis*.

- Killing and degradation of Gram positive bacteria such as *L. monocytogenes* DRDC8 and *B. subtilis* by *Colpoda* RR is a slower process compared to degradation of Gram negative bacteria such as *S. Typhimurium* C5 and *E. coli* DH5α by *Colpoda* RR.

- Different isolates of *Colpoda* digest *L. monocytogenes* at different rates.

- Phagosome-lysosome fusion and vacuole acidification occur when *Colpoda* RR phagocytose *L. monocytogenes* DRDC8. Inhibition of phagosome-lysosome fusion, vacuole acidification and proteases activity allows survival without replication of intra-ciliate DRDC8, suggesting that these cellular processes are required for killing of *L. monocytogenes*.

- Nitric oxide is not involved in the killing and degradation of *L. monocytogenes* DRDC8 by *Colpoda* RR.

- *Colpoda* RR and MLS-5 are able to graze on biofilms of pathogenic *L. monocytogenes* strains DRDC8, EGD Kaufmann and KE925 as well as biofilms of *S. Typhimurium* C5, *B. subtilis* and *E. coli* DH5α as food source. However, supernatants of *L. monocytogenes* biofilms do not contain inhibitory compounds that affect the viability and grazing activity of *Colpoda* RR and *Colpoda* MLS-5.

- *L. monocytogenes* DRDC8 can escape killing by *Colpoda* if these cells are secreted within faecal pellets or they become located within *Colpoda* RR cysts. The faecal pellets contain live, respiring DRDC8. Furthermore, production of faecal pellets containing bacteria by *Colpoda* RR is independent of the type of bacteria it feed on,
as faecal pellets containing *L. monocytogenes* DRDC8, *S. Typhimurium* C5 and the non-pathogenic *B. subtilis* are produced following independent co-cultures with these bacteria.

- *L. monocytogenes* DRDC8 cells encapsulated within faecal pellets of *Colpoda* RR and *Colpoda* MLS-5 are resistant to gentamycin and NaOCl.

5.3 References


Appendix A: Sequence data for 18S rRNA of *Colpoda* sp. MLS-5

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