Chapter 6: Mechanism of Phagocytosis and Killing of *L. monocytogenes* by *A. polyphaga*

6.1 Introduction

Intra-cellular bacterial pathogens have evolved various strategies to evade killing mechanism(s) in order to survive within mammalian host cells. As an intra-cellular pathogen, *L. monocytogenes* is able to survive and replicate within different types of mammalian cells, including macrophages, enterocysts and hepatocytes (Cossart and Sansonetti, 2004). As is the case for other intercellular pathogens, invasion of mammalian cells by *L. monocytogenes* consists of several well defined steps, i.e. adhesion, phagocytosis, evasion of the killing mechanism of host cells and finally survival and multiplication within the host cell cytosol. This bacterium attaches to host cell surface receptors using surface proteins (InlA and InlB). Attached cells are subsequently internalised by the host either via a passive or an induced phagocytosis (or zipper mechanism). Once confined within phagosomes, *L. monocytogenes* cells express LLO, phospholipases and other virulence associated gene products that allow it to escape from the phagosome and gain access to the cell cytoplasm. When the bacteria have accessed the cytoplasm, ActA recruits monomeric actin to one end of bacterial cells where it is polymerised to form actin tails. Thus addition of actin monomer to actin polymer tails by bacterial cells, allows movement within the cytosol and spread to adjacent uninfected cells via listeriopods. Phagocytosis of bacteria within listeriopods leads to a new invasion cycle in adjacent cells.

Although *L. monocytogenes* is able to escape killing in the phagosome of mammalian cells, this is clearly not the case when this bacterium is phagocytosed by *Acanthamoeba* cells (see Chapters 4 and 5). Indeed, *A. polyphaga* cells are able to phagocytose *L. monocytogenes* cells and rapidly reduce the population of viable bacterial cells. Bacterial cells within the phagosome are rapidly destroyed and eliminated within a short period of time. *L. monocytogenes* cells are degraded within tight cytoplasmic vacuoles (the phagosome) usually within 2 to 5 h after phagocytosis by the amoeba trophozoites. Thus in contrast to mammalian cells, *L. monocytogenes* is not able to invade the cytosol of *A. polyphaga* cells and other *Acanthamoeba* spp. to establish an intra-cellular lifestyle (see Chapters 4 and 5). Apparently these bacteria are either not able to express key virulence
genes necessary for escape from phagosome, or they do not have sufficient defenses to overcome the oxidative burst and degradative enzymes characteristic of the Acanthamoeba phagolysosome.

Phagocytosis is an actin-based process that involves polymerisation of monomeric G-actin to polymeric F-actin. This allows eukaryotic cells to internalise small particles such as prokaryotic cells. Acanthamoeba spp. are free-living amoebae which have evolved an efficient mechanism to phagocytose and kill bacteria and other cells that they use as sources of nutrients. For example, A. castellanii phagocytoses heat-killed E. coli by an actin-dependent mechanism that can be diminished in the presence of phosphatidylinositol 3-kinase (PI 3-kinase) inhibitors (Alsam et al., 2005).

Eukaryotic cells generally employ two main mechanisms by which they kill internalised microorganisms. One mechanism involves production of toxic oxygen radicals such as super oxide and hydrogen peroxide and/or nitrite oxide. The second is degradation of internalized organisms as a result of delivery of lysosomal hydrolases by lysosomes following lysosome-phagosome fusion. These two killing mechanisms are not necessarily independent of each other; rather they overlap and together, facilitate killing of a wide variety of microorganisms (Gildea et al., 2005).

However, the mechanism involved in phagocytosis and killing of L. monocytogenes by A. polyphaga is unclear. To provide a better understanding of these processes, different types of inhibitors were used to inhibit pathways that seemed to be implicated in the phagocytosis and killing of bacteria by amoeba trophozoites.

6.2 Experimental Approach

Amoeba monolayers were pre-treated with different target specific inhibitors prior to co-culture with L. monocytogenes strain DRDC8 at 22ºC. Since results presented in Chapters 4 and 5 showed bacteria were killed by amoeba trophozoites irrespective of the incubation temperatures used, these experiments were uniformly carried out at 22ºC; a temperature representative of environmental conditions. The inhibitors used were: ammonium chloride, Suramin, Monensin, Bafilomycin A, Cytochalasin D and Wortmannin. Phagocytosis and killing of bacterial cells by amoeba trophozoites was assessed by plate counting methods and microscopy.
6.3 Results

6.3.1 Phagocytosis of Viable and Heat-killed *L. monocytogenes* by *A. polyphaga* on Plates

Results presented in Chapters 4 and 5 indicated that *A. polyphaga* can effectively phagocytose *L. monocytogenes* cells and kill bacteria within a few hours of co-culture. In order to determine whether the phagocytosis of *L. monocytogenes* by *A. polyphaga* was an induced or inactive process(s), co-cultures of amoeba trophozoites on lawns of live and heat-killed *L. monocytogenes* were prepared and the size of amoeba growth zones measured and used to compare the growth of amoeba trophozoites on live and killed bacteria at 30ºC. The growth rate of amoebae on bacteria used to estimate the rate of bacterial phagocytosis by amoeba trophozoites (Figure 6.1).

This experiment showed that amoeba trophozoites could grow on both live and killed bacterial cells at similar rates. After 1 or 2 d of co-culture, the size of the growth zone of amoeba trophozoites on lawns of either live or killed bacterial cells was almost the same. However, after 3 to 4 d of co-culture, the growth zone of amoeba trophozoites on lawns of live bacteria was slightly greater than that observed for amoeba grown on lawns of heat-killed bacteria. This result indicated that amoeba trophozoites may grow marginally more rapidly on live bacterial cells compared to the heat killed bacteria. Given the fact that *A. polyphaga* is able to phagocytose and digest bacterial cells as food, their growth on both live and killed *L. monocytogenes* reflected their ability to uptake bacterial cells. Consequently, *A. polyphaga* could uptake live and killed *L. monocytogenes* with almost similar rate irrespective of the bacterial induced phagocytosis activity.

6.3.2 Mannose-binding Protein and Phagocytosis of *L. monocytogenes*

Alsam *et al.* (2005) have reported that the mannose binding protein (MBP) expressed by *A. castellanii* cells plays a role in phagocytosis of killed *E. coli* cells. To assess the potential impact of MBP on phagocytosis of live *L. monocytogenes* DRDC8 by *A. polyphaga* AC012, monolayers of these amoeba cells were pre-treated with 100 mM mannose for 1 h at 22ºC. Untreated amoebae and amoebae pre-treated with the same concentration of galactose and glucose were used as controls (as described by Alsam *et al.*, 2005). The monolayers were then washed with AS buffer and incubated with *L. monocytogenes* cells (MOI=100 bacteria per amoeba cell) for 1 h. Extra-amoebic
bacteria were removed by washing in AS buffer and the counts of viable intra-amoebic bacteria were determined by plating amoeba lysates on BHI plates.

The Log10 counts of bacteria recovered from mannose, glucose and galactose pre-treated amoebae were not significantly different from that recovered from the control co-culture (Figure 6.2). This data indicated that none of these sugars affected uptake of bacteria by amoeba trophozoites.

6.3.3 Impact of Actin Polymerisation Inhibitors on Phagocytosis

The role of microfilament and cytoskeleton rearrangement in phagocytosis by mammalian cells and other eukaryotes, such as amoebae, has been documented in a number of published reports (Alsam et al., 2005; Cossart and Sansonetti, 2004; Elliott and Winn, 1986). These cellular processes are normally dependent on actin polymerisation and are sensitive to the actin polymerisation inhibitors, Cytochalasin D and Wortmannin. Cytochalasin D is a specific actin polymerization inhibitor (Weihing, 1978) that has been extensively used to study actin polymerization dependent processes in mammalian cells (Elliott and Winn, 1986) and amoebae (Alsam et al., 2005; King et al., 1991). Wortmannin is a phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor that inhibits actin polymerisation in mammalian cells. PI 3-kinases are versatile signaling molecules that play crucial roles in receptor-mediated signal transduction, actin remodeling and membrane trafficking (Downes et al., 2005). Thus, Wortmannin can be used to explore the convergence of two major cell signaling systems, those using G-protein-coupled receptors and those using receptor tyrosine kinases (Ui et al., 1995). Wortmannin has been successfully used as phagocytosis inhibitor in mammalian cells since it can enter intact cells. To determine if actin polymerization plays a role in phagocytosis of *L. monocytogenes* by *A. polyphaga*, these inhibitors were used to pre-treat the monolayers of amoeba trophozoites prior to co-culture.

6.3.3.1 Effect of Cytochalasin D on Phagocytosis

Monolayers of *A. polyphaga* AC012 were pre-treated with either of two concentrations (50 and 100 µM) of Cytochalasin D for 1 h. Treated amoeba cells were then infected with *L. monocytogenes* DRDC8 (MOI = 100 bacteria per amoeba cell) and the co-cultured cells incubated for 24 h at 22 ºC. Monolayers of amoebae that had received no pre-treatment or had been pre-treated with dimethyl sulfoxide (DMSO) (a solvent for the Cytochalasin D)
were included as control cultures. Total viable counts of total bacteria (extra-amoebic and intra-amoebic bacteria) were determined at 0, 5 and 24 h post co-culture to determine the proportion of bacteria that had been predated by amoeba trophozoites.

At the commencement of the co-culture experiment (t = 0 h), total counts of bacteria in four co-culture media were essentially the same (Figure 6.3). After 5 h of co-culture, the counts of bacteria recovered from treated and untreated amoebae declined by ca. 8 fold. However, after 24 h of co-culture, statistically significant differences in the counts of bacteria recovered from the control co-cultures and the pre-treated amoeba co-cultures were apparent. While counts of bacteria recovered from co-cultures with Cytochalasin-treated amoebae remained almost unchanged, counts of bacteria in the control co-cultures declined by almost 100 fold relative to counts at the commencement of the experiment. These results showed that pre-treatment of amoeba trophozoites with Cytochalasin D resulted in a significant change in the ability of amoebae to phagocytose *L. monocytogenes* cells compared with control co-cultures.

As DMSO was used as a solvent for Cytochalasin D, control co-cultures were set up using monolayers pre-treated with only DMSO (diluted 1 in 100 in Milli-Q water). These control co-cultures were used to rule out any possible impact of this solvent on phagocytosis of *A. polyphaga*. Since counts of bacteria recovered from these control co-cultures were similar to those obtained for co-cultures that used untreated amoebae (Figure 6.3), the concentration of DMSO used was assumed to not have an adverse effect on phagocytosis of *L. monocytogenes* by amoeba cells.

Identically treated amoebae that had been infected with FITC-labelled *L. monocytogenes* cells were also examined by fluorescence microscopy at different times during co-culture. Control (untreated) amoebae were readily able to phagocytose *L. monocytogenes* (Figure 6.4a, b). After 3 h incubation, the number of viable bacteria within vacuoles of host amoebae declined (Figure 6.4b). However, the number of bacteria phagocytosed by Cytochalasin-treated amoebae appeared to be lower than that observed for untreated amoebae at the same incubation times (Figure 6.4c, d).

Counts of clusters/clumps of bacterial cells within vacuoles inside amoebae were used as a means of quantitating this microscopy data. Typically individual FITC-labelled bacterial cells within amoebae could not be distinguished. These clusters/clumps, when viewed in a single plane appeared to contain more than one cell. Nevertheless, each
amoeba contained a number of clusters and counts of these clusters/clumps were used as an estimate of the number of bacteria within each amoeba cell. The data was plotted as Box and Whisker plots (Figure 6.5) and Student t-tests used to compare counts of bacterial clumps obtained for control and experimental preparations. Although the mean (and median) count of clumps of bacteria within amoebae was less than that obtained for the control preparations, the difference was not statistically significant.

Overall, the experimental results indicated that Cytochalsin D treatment lead to a reduction in the uptake of *L. monocytogenes* cells by *A. polyphaga*, presumably by interfering with actin-mediated phagocytosis.

### 6.3.3.2 Effect of Wortmannin on Phagocytosis

Amoeba that had been pre-treated with Wortmannin at concentrations of 500 nM and 1 µM for 1 h were used to co-culture with *L. monocytogenes* as described for Cytochalasin D. This allowed an evaluation of the effect of Wortmannin on phagocytosis of *L. monocytogenes* cells by *A. polyphaga* trophozoites. Two control co-cultures were set up using untreated and DMSO-pre-treated amoebae (diluted 1:100).

Figure 6.6 presents the mean total viable counts of bacteria recovered from Wortmannin pre-treated amoeba during co-culture for 0, 5 and 24 h. The total viable counts of bacteria obtained were essentially identical (*ca. 10^9* CFU.mL^-1) for the control and experimental co-cultures at t = 0 h. After 5 h of co-culture, counts of bacteria declined for all co-cultures by 8 to 10 fold (*ca. 10^8* CFU.mL^-1). However, after 24 h of co-culture, the counts of bacteria in co-cultures with Wortmannin-treated and untreated amoebae were significantly different (p<0.001). Counts of bacteria recovered from co-cultures with untreated amoeba were more than 100 fold less (*ca. 10^7* cells.mL^-1) than counts obtained at the commencement of co-culture. However, the number of bacteria in co-culture with Wortmannin-treated amoebae remained almost unchanged. Counts of bacteria recovered from co-cultures with Wortmannin pre-treated amoeba varied in a dose dependent manner. Counts of bacteria recovered from co-cultures with amoebae pre-treated with 500 nM of Wortmannin were lower than counts obtained for amoebae pre-treated with 1 µM Wortmannin. These experimental results suggested that pre-treatment of amoeba trophozoites with Wortmannin reduced the uptake of *L. monocytogenes* compared to untreated trophozoites; a result that was similar to results obtained for co-cultures with Cytochalasin-treated amoebae.
Microscopic examination of Wortmannin-treated amoeba trophozoites during coculture with *L. monocytogenes*, supported the results described above. The number of FITC-labelled bacteria presented within vacuoles of Wortmannin pre-treated amoebae (Figure 6.4e, f) was less than that observed for control (untreated amoeba) co-cultures (Figure 6.4a, b). As described for Cytochalasin D, the mean counts of clumps/clusters of intra-amoebic bacterial cells obtained for pre-treated and control amoeba were not statistically different (Figure 6.5). This was expected, as Wortmannin pre-treatment made the bacterial clumps within amoeba cells more diffuse and smaller. Consequently, the count of clumps per amoeba cells was increased relative to the control preparations. Furthermore, many Wortmannin treated amoebae could not be counted as they detached from the glass (see Section 6.3.3.3). Overall, these results indicated Wortmannin pre-treatment reduced the uptake of *L. monocytogenes* cells by *A. polyphaga* trophozoites.

6.3.3.3 Impact of Wortmannin and Cytochalasin D on Trophozoite Morphology

To determine the effect of pre-treatment with Cytochalasin D, Wortmannin (and the solvent, DMSO) on the morphology of *A. polyphaga* trophozoites, monolayers of amoebae were examined by phase contrast microscopy. Untreated amoebae were used as a control. Figure 6.7a – d presents micrographs of representative amoeba cells following treatment. Control (untreated amoebae) maintained their shape and ability to adhere to glass surfaces throughout the period of the experiment (24 h). However, Wortmannin and Cytochalasin D pre-treated trophozoites displayed a rounded shape and most of these cells lost the ability to attach to glass surfaces. DMSO pre-treated cells also adopted a rounded shape, but these cells did not lose ability to attach to surfaces. These observations demonstrated that these inhibitors had an impact on actin filament formation in pseudopodia of amoebae and affected the ability of the amoebae to attach to surfaces.

6.3.4 Suramin induced Inhibition of Phagosome - Lysosome Fusion and Intra-amoebic Killing

Suramin is a polybasic anion that binds strongly to plasma proteins and enters cells by endocytosis. It becomes concentrated in their lysosomes where it can inhibit many enzymes, including some of the lysosomal proteases (Gildea et al., 2005). Suramin has been used previously in several studies to interfere with phagosome-lysosome fusion in macrophages. For example research showed that Suramin prominently diminished the
phagosome-lysosome fusion in mouse macrophage in uptake of mycobacterium but its effect was temporary (Draper et al., 1979).

The fusion of phagosomes with lysosomes plays an important role in degradation of phagocytosed particles by eukaryotic cells. In order to assess the impact of phagosome lysosome fusion on intra-amoebic killing of *L. monocytogenes*, the bacteria were co-cultured with amoeba trophozoites pre-treated with Suramin and also with untreated amoebae as a control. Amoeba monolayers were pre-treated with Suramin (300 µg.mL\(^{-1}\)) for 1 h, then washed in AS buffer followed by inoculation *L. monocytogenes* cells (MOI = 100 bacteria per amoeba cell). After incubation at 22°C for 1 h, remaining extra-amoebic bacteria were removed by gentle washing with AS and the monolayers were incubated at 22°C. The counts of viable intra-amoebic bacteria were determined 6 h post incubation by lysis of the amoeba cells followed by plating lysates on BHI. Untreated amoebae were used in co-culture with bacteria as a control.

Figure 6.8 shows the counts of viable bacteria recovered from monolayers of trophozoites. The counts of bacteria recovered from co-cultures with pre-treated amoebae (*ca. 6.1 \times 10^7\) CFU.mL\(^{-1}\)) were *ca. 2* fold higher than counts obtained for the control co-culture with untreated amoebae (*ca. 3 \times 10^7\) CFU.mL\(^{-1}\)). This difference was statistically significant (p<0.05). Since the number of bacterial cells and trophozoites were the same at the beginning for both the pre-treated and control co-cultures, the higher counts of bacteria recovered from treated trophozoites indicated that Suramin significantly reduced the rate of killing of bacterial cells by trophozoites.

In order to find out whether Suramin also affected the ability of trophozoites to phagocytose *L. monocytogenes*, monolayers of amoebae were pre-treated with Suramin (200 µg.mL\(^{-1}\)) as previously described. The counts of intra-amoebic bacteria in co-cultures were determined at two different time points, after a short (1.5 h) co-culture period and following an extended period of co-culture (6 h). The rationale for this approach was that after 1.5 h of co-culture, bacterial cells will have been phagocytosed, but the majority of them will not have been degraded by amoeba trophozoites. However, by 6 h of co-culture, the majority of intra-amoebic bacteria will have been killed. Thus counts of viable bacteria after 1.5 h of co-culture were used to estimate the inhibitory effect of Suramin on phagocytosis by trophozoites, whereas counts of bacteria recovered after 6 h were used to assess the effect of the inhibitor on the killing of bacterial cells by amoebae relative to the control cultures.
The counts of bacteria recovered from pre-treated and control co-cultures after 1.5 h of co-culture differed significantly (Figure 6.9). Indeed, the mean number of viable bacteria recovered from co-cultures with Suramin pre-treated amoebae was about half \((8.5 \times 10^5 \text{ CFU.mL}^{-1})\) that recovered from the control co-culture \((1.5 \times 10^6 \text{ CFU.mL}^{-1})\). Although this difference was not statistically significant \((p>0.05)\), this data suggested that the uptake of bacterial cells by trophozoites could be reduced by Suramin pre-treatment of host amoebae. Similarly, after 6 h of co-culture; counts of viable bacteria recovered from co-cultures with pre-treated amoebae were slightly higher \((6.5 \times 10^5 \text{ CFU.mL}^{-1})\) than the counts obtained for the control co-culture \((5 \times 10^5 \text{ CFU.mL}^{-1})\), although this difference was not statistically significant. Two way analysis of variance of the data indicated that although the mean counts at any one sampling time point were not significantly different, the counts at 1.5 h and at 6 h were significantly different \((P<0.01)\). Overall, the reduction in intra-amoebic killing of bacteria in co-cultures with pre-treated amoebae \((1 \times 10^6 \text{ CFU.mL}^{-1})\) compared to control co-cultures \((2 \times 10^5 \text{ CFU.mL}^{-1})\) is consistent with results for co-cultures that used amoebae pre-treated with 300 µg.mL\(^{-1}\) of Suramin. Collectively this data suggested the Suramin had a significant impact on uptake and intra-amoebic killing of \(L.\ monocytogenes\) by \(A.\ polyphaga\) trophozoites.

### 6.3.5 Phagosome Acidification and Intra-amoebic Killing

Phagosomal acidification has a key role in degradation of phagocytosed bacterial cells by mammalian macrophages (Downey et al., 1999; Styrt and Klempner, 1988). This process can be effectively inhibited by treatment of eukaryotic cells with ammonium chloride, Bafilomycin A and Monensin.

Ammonium chloride is a weak base that can permeate intact cell membrane. It can accumulate in acidic compartments within the cell cytoplasm and neutralize the pH. In addition, ammonium chloride can also interfere with maturation of the phagosome and inhibit phagosome-lysosome fusion (Hart and Young, 1991). Bafilomycin A is a macrolide antibiotic with a membrane-permeant activity and is a relatively specific inhibitor for vacuolar-type proton translocating ATPases (v-ATPases). This group of ATPases is involved in phagosome acidification (Bowman et al., 1988). Monensin acts as a cationic ionophore and carrier, which is involved in equilibrating extra-cellular and intra-cellular pH (Prabhananda and Kombrabail, 1992). In view of these effects, these
substances were used to pre-treat *A. polyphaga* trophozoites in order to evaluate the impact of phagosomal acidification on intra-amoebic killing of *L. monocytogenes*.

6.3.5.1 Ammonium chloride Pre-treatment

Amoeba monolayers were pre-treated with ammonium chloride (final concentration of 20 and 30 mM for 1 h). Pre-treated and untreated (control) monolayers were then inoculated with *L. monocytogenes* DRDC8 (MOI = 100 bacteria per amoeba cell) and incubated at 22°C for 1 h. After a washing step to remove extra-amoebic bacteria, the monolayers were incubated at 22°C. Counts of viable intra-amoebic bacteria were determined at 1, 4 and 6 h after washing, by plating amoeba lysates on BHI.

Changes in the counts of intra-amoebic bacteria over the 6 h of the co-culture experiment are shown in Figure 6.10. Statistically significant differences (*p*<0.001) in the counts of bacteria recovered from pre-treated amoebae and control amoebae were obtained, especially after 4 and 6 h of co-culture. The results clearly showed ammonium chloride reduced the intra-amoebic killing of *L. monocytogenes*. After 1 h of co-culture, the mean counts of intra-amoebic bacteria recovered from co-culture with pre-treated amoebae were not statistically different from counts for the control co-culture (*p*>0.05). However, after 4 and 6 h of incubation, the mean counts of bacteria recovered from pre-treated co-cultures were significantly greater than the mean count obtained for the control co-culture. In fact, the mean count of bacteria for pre-treated co-cultures was about 4× greater than the control co-culture. These results clearly showed that ammonium chloride pre-treatment significantly reduced the intra-amoebic killing of *L. monocytogenes* compared to control preparations. Furthermore, the results of this experiment also indicated that pre-treated amoeba trophozoites are still able degrade and kill bacteria albeit at a much reduced rate.

Additional evidence to support the microbiological data described above, was obtained by microscopic examination of amoeba cells from these co-culture experiments. This was achieved by infecting pre-treated and control amoebae with FITC-labelled *L. monocytogenes* DRDC8 cells. Presence of labelled bacteria within amoebae was then monitored by fluorescence microscopy. Figure 6.4a presents micrographs that show labelled bacterial cells within untreated control amoeba cells and Figure 6.4g, h shows the results obtained for pre-treated amoeba cells after 1 and 3 h post co-cultivation. At 1 h post co-culture (Figure 6.4g), the mean count of bacteria internalized by amoeba trophozoites was almost the same as that of untreated amoebae (Figure 6.4a). After 3 h co-
cultivation, however, the mean number of intra-amoebic bacteria recovered from pre-treated amoebae (Figure 6.4h) was greater than for the untreated amoebae (Figure 6.4b). Furthermore, the intra-amoebic bacterial cells in pre-treated amoebae were spread within amoeba trophozoites as separate clusters, whereas the number of bacterial clusters in untreated amoebae was in general less and of a smaller size. When counts of clumps/clusters of intra-amoebic bacteria for control and pre-treated amoebae were compared, a highly significant difference (p<0.001) in the mean counts of these clusters/clumps was observed for co-cultures after 3 h, but not after 1 h. As expected, the mean count of clusters/clumps of bacterial cells in pre-treated amoebae was greater than that obtained for control co-cultures (Figure 6.11). Together, these results clearly showed ammonium chloride significantly slowed down the mechanism utilized by \textit{A. polyphaga} trophozoites to kill \textit{L. monocytogenes} cells.

6.3.5.2 Bafilomycin A Pre-treatment

To determine the impact of Bafilomycin A pre-treatment on ability of amoeba trophozoites to phagocytose and destroy \textit{L. monocytogenes} DRDC8 cells, monolayers of amoebae were pre-treated with a solution containing 80 nM Bafilomycin A for 1 h at 22ºC. Pre-treated and control monolayer preparations were infected with \textit{L. monocytogenes} DRDC8 cells at an MOI of 100 bacteria per amoeba cell. Extra-amoebic bacteria were removed by washing in AS buffer and the monolayers were incubated at 22ºC. Counts of intra-amoebic bacteria in co-cultures were determined at 1.5 and 6 h post incubation by plating amoeba lysates on BHI plates. Counts of bacteria recovered from amoebae are presented in Figure 6.12.

After 1.5 h of co-culture, the mean count of bacteria recovered from pre-treated amoeba was not significantly different (p>0.05) from the mean counts obtained for control co-cultures. Thus Bafilomycin A treatment apparently did not affect the ability of \textit{A. polyphaga} trophozoites to phagocytose \textit{L. monocytogenes}. However, after 6 h of co-culture, the mean count of viable bacteria (\textit{ca.} $9 \times 10^5$ CFU.mL$^{-1}$) recovered from co-culture with pre-treated amoebae was higher (P<0.05) than the mean count obtained for control co-cultures (\textit{ca.} $5 \times 10^5$ CFU.mL$^{-1}$), although the difference in counts was not significantly different (p>0.05). Nevertheless, this data indicated that Bafilomycin treatments may have weakly inhibited the killing of \textit{L. monocytogenes} cells phagocytosed by amoeba trophozoites.
When taken together, this data showed that Bafilomycin A did not significantly affect the uptake of *L. monocytogenes* by *A. polyphaga* trophozoites, although this inhibitor may have weakly inhibited intra-amoebic killing of *L. monocytogenes*. Moreover, the results of these experiments tend to provide additional support for the results obtained from pre-treated amoebae with ammonium chloride.

### 6.3.5.3 Monensin Pre-treatment

The impact of Monensin on intra-amoebic killing of *L. monocytogenes* was assessed by pre-treatment of amoeba monolayers with 5 µM Monensin for 1 h followed by washing the monolayers with AS and inoculation with bacteria (MOI=100 bacteria per amoeba cell). After 1 h incubation at 22ºC followed by washing to remove the extra-amoebic bacteria, the counts of viable intra-amoebic bacteria in co-cultures with treated and untreated amoebae (a control) were determined after further 1.5 and 6 h of incubation.

The results obtained are presented as Figure 6.13. After 1.5 h of co-culture, the mean counts of viable bacterial cells recovered from co-cultures with treated amoebae (*ca.* 10⁶ CFU.mL⁻¹) was lower than the mean count obtained for the control co-culture (1.5 × 10⁶ CFU.mL⁻¹), although a statistical analysis of Log₁₀ transformed data indicated that difference was not significant (i.e. *p*>0.05). Since the viable count of intra-amoebic bacteria after 1.5 h of co-culture represented uptake rather than killing of bacteria, the above result indicated that Monensin may have inhibited phagocytosis of bacterial cells by amoeba trophozoites.

After 6 h of co-culture, the mean counts of bacteria recovered from pre-treated (6 × 10⁵ CFU.mL⁻¹) and control (5 × 10⁵ CFU.mL⁻¹) co-cultures were not significantly different. However, the mean counts of bacteria recovered after 6 h of co-culture from both control and experimental amoebae was less than those obtained after 1.5 h of co-culture. These observations were taken to indicate that Monensin only partially inhibited intra-amoebic killing of *L. monocytogenes* over the entire 6 h period of the experiment. However, given the significant reduction in the numbers of intra-amoebic bacteria recovered from pre-treated amoeba after only 1.5 h of co-culture compared with the control, Monensin essentially inhibited the uptake of *L. monocytogenes* cells by the amoeba trophozoites.

To assess the dose-dependence of Monensin on intra-amoebic killing of bacteria, amoeba cells were pre-treated with either 5 or 20 µM concentrations of Monensin prior to infection with *L. monocytogenes* as previously described. Untreated amoebae were used as
a control. Mean counts of viable intra-amoebic bacteria after 4 h of incubation are presented as Figure 6.14. The mean count (3 × 10^5 CFU.mL^-1) of bacteria recovered from amoeba pre-treated with 5 µM Monensin was not significantly different (i.e. p>0.05) from the mean count (3.2 × 10^5 CFU.mL^-1) obtained for the control co-culture. However, the mean count of viable bacteria recovered from amoeba pre-treated with 20 µM Monensin was significantly less (1.9 × 10^5 CFU.mL^-1) than the mean count obtained for the control co-culture (p<0.05). This data indicated pre-treatment of amoebae with Monensin has a dose dependent effect on ability of amoeba trophozoites to phagocytose *L. monocytogenes* cells.

### 6.4 Discussion

In view of the fact that *A. polyphaga* and other species of *Acanthamoeba* are able to phagocytose and rapidly kill *L. monocytogenes* cells, the work presented in this chapter sought to characterise aspects of the molecular and cellular mechanisms involved. This work relied on use of inhibitory agents for which a well described mechanism of action has been identified for mammalian cells and in some cases, amoeba cells. In particular the work focused on use of inhibitors that are known to inhibit the actin dependent phagocytic process, those that interfere with lysosome fusion with phagocytic vacuoles and processes that occur in the phagolysosomal vacuole.

The fact that amoeba trophozoites can grow on lawns of live and killed *L. monocytogenes*, indicated that amoeba cell can effectively phagocytose these bacteria and digest them as a food source. More importantly, the fact that heat killed bacterial cells can be phagocytosed by trophozoites, indicated that this process is not mediated by *L. monocytogenes*. Rather, uptake of bacterial cells is mediated by amoeba trophozoites. *A. polyphaga* trophozoites are professional phagocytes capable of ingesting different types of bacteria as food nutrients (Bottone *et al.*, 1994; Weekers *et al.*, 1993). Thus the InlA/InlB based mechanism deployed by *L. monocytogenes* to initiate uptake by mammalian non-professional phagocytic cells (as reported by Pizarro-Cerda and Cossart, 2006) is apparently either not used, or is not active under the co-culture conditions used with trophozoites. Nevertheless, on the basis of the experiments described, a role for the InlA/InlB system in bacterial mediated uptake by amoeba cells cannot be excluded. Co-cultures with *L. monocytogenes* InlA/B mutants will be required to eliminate this bacterial cell dependent mechanism of phagocytosis. If InlA and InlB do not play a role in uptake
of bacteria by amoebae, then data obtained for appropriate co-culture experiments should not be different from that obtained for control experiments using the parental strains of bacteria.

Both Cytochalasin D and Wortmannin reduced the ability of *A. polyphaga* to phagocytose *L. monocytogenes* cells. This effect was most evident from an analysis of counts of viable bacteria recovered from control and pre-treated amoebae and supported by microscopy and to a lesser extent an analysis of numbers of clumps/clusters of bacterial cells within amoebae. It is likely that data variability contributed to the absence of any statistically significant difference between the mean counts of these clusters/clumps observed for the control and experimental groups. Furthermore, the estimates of number of clusters/clumps are likely to be under estimates of the number of vacuoles containing bacteria as the micrographs used to count clusters/clumps represent a single planar view. Thus clumps of cells may be hidden from view by clumps above. Another source of variance may lie in the fact that Cytochalasin D and Wortmannin treatments reduced the ability of amoebae to remain attached to the surface of glass slides used in these assays. Thus many affected amoebae may not have been included in quantitative estimates of counts of clusters/clumps of bacterial cells. Overall however, the conclusion drawn from all data is that these inhibitors affected actin polymerisation within trophozoites to an extent that cytoskeletal rearrangement necessary for phagocytosis of *L. monocytogenes* cells was decreased. This outcome is consistent with other reported research. For example, Cytochalasin D has been extensively used as an inhibitor of actin polymerisation that affects phagocytosis in mammalian cells (Elliott and Winn, 1986; Finlay and Falkow, 1988). Further, it has been reported that Cytochalasin D reduced uptake of *L. pneumophila* by *A. castellanii* (Moffat and Tompkins, 1992). It has also been shown that Cytochalasin D can inhibit actin filament formation and significantly reduce amoeba locomotion and phagocytic activity (Ravdin *et al*., 1985; Stockem *et al*., 1983).

Wortmannin is a phosphoinositide 3-kinase (PI 3-kinase) inhibitor in mammalian cells. PI 3-kinase is a heterodimers of an 85 kDa regulatory subunit (p85α, β) and a 110 kDa catalytic subunit (p110α, β). The p85 subunit activates catalytic function of the p110 subunit. It binds irreversibly to the p110 catalytic subunit of the PI 3-knase (Ui *et al*., 1995). Research has shown that infection of mammalian cells with *L. monocytogenes* results in increased amounts of cellular phosphoinositide derivatives, indicating PI 3-kinase is implicated in cellular invasion by *L. monocytogenes* (Ireton *et al*., 1996).
*L. monocytogenes* uptake by non-macrophage cells is induced by host tyrosine phosphorylation, PI 3-kinase activity, and rearrangements in the actin cytoskeleton mediated by InlB gene of these bacteria.

Entry of *L. monocytogenes* into cell lines, including Vero cells, is impaired by treatment of cells with Wortmannin or LY294002 (Ireton *et al.*, 1996), both of which are specific inhibitors of PI 3-kinase (Ui *et al.*, 1995; Vlahos *et al.*, 1994). This data correlated with the result presented in this Chapter that indicated pre-treatment of *A. polyphaga* trophozoites with Wortmannin reduced the ability of amoebae to phagocytose *L. monocytogenes* cells. In view of the published data and that presented in this thesis, it seems that phagocytosis of *L. monocytogenes* by *A. polyphaga* is an actin-polymerization dependent process and that phosphatidilinocytol 3-kinase plays a role in this process. This implies that the mechanism of phagocytosis in *A. polyphaga* is similar to that of mammalian cells. Thus actin-mediated cytoskeletal rearrangement is required for phagocytosis and this plays a key role in bacterial uptake by eukaryotic cells. This process has been preserved across different types of eukaryotic cells including *Acanthamoeba*. However, the detailed molecular basis of actin polymerisation and the role of PI 3-kinase in phagocytosis of *Acanthamoeba* species remain to be investigated.

Interestingly, high concentrations of mannose, and other six carbon sugars, had no observable effect on uptake of live *L. monocytogenes* cells by *A. polyphaga* trophozoites. This result contrasts with published reports which indicated mannose binding protein was implicated in uptake of killed *E. coli* by *A. castellanii* (Alsam *et al.*, 2005) and attachment of amoeba trophozoites to human cell receptors (Garate *et al.*, 2004). However, this unexpected result could indicate one or more of the following:

(a) The MBP (or similar surface associated molecules) of *A. polyphaga* is not involved in receptor mediated uptake of *L. monocytogenes*.

(b) *A. polyphaga* trophozoites may not express MBP under experimental conditions tested. Expression of MBP prior to co-culture experiments could in future work be tested by either by immuno-fluorescent labelling of cells using anti MBP serum, or by Western blot analysis of amoeba membrane fractions. Furthermore, any future work should include co-culture experiments that use a control bacterium known to be phagocytosed by this receptor e.g. *E. coli*.
(c) An alternative receptor is involved in phagocytosis of *L. monocytogenes*. Presence of an alternative receptor could be determined by attempts to inhibit the receptor by addition of high concentrations of a variety of sugars previously implicated as inhibitors of receptor mediated attachment/phagocytosis.

In fact *Acanthamoeba* cells may use different surface receptors to facilitate uptake of Gram-positive and negative bacterial cells. For example, Allen and Dawidowicz (1990) have shown that exogenous mannose has no effect on latex beads phagocytosis by *Acanthamoeba castellanii* but blocked the uptake of yeast particles by the amoeba cells. As a supporting evidence, it has been reported that *Legionella pneumophila* cells used different surface receptors to enable them to attach *A. polyphaga* cells but, they are not involved in attachment to mammalian cells (Gao *et al.*, 1997). However, the molecular determinants responsible to characterize and determine these receptors and uptake mechanisms used by *Acanthamoeba* spp. to internalize *L. monocytogenes* cells have yet to be determined.

Phagosome acidification mediates the activation of lysosomal enzymes involved in digestion of phagocytosed particles by mammalian cells. This process can be inhibited by neutralizing agents such as ammonium chloride, as well as inhibitors such as Bafilomycin A and Monensin. Indeed, the microbiology and microscopy data obtained for this section of work showed that pre-treatment of *A. polyphaga* trophozoites with ammonium chloride and Bafilomycin A resulted in a significant reduction in the ability of *A. polyphaga* trophozoites to kill intra-amoebic *L. monocytogenes*, *i.e.* pre-treatment with ammonium chloride resulted in improved survival of intra-amoebic *L. monocytogenes* cells. Interestingly, Monensin only slightly affected the intra-amoebic killing of *L. monocytogenes* cells, but instead inhibited the uptake of bacteria by the amoeba cells. Since Monensin has a general impact on cell organelles and intra-cellular transportation systems, it is perhaps not surprising that this inhibitor resulted in a small but measurable reduction in the uptake of *L. monocytogenes* by *A. polyphaga*.

Collectively the impact of Bafilomycin A and ammonium chloride clearly indicated the significant role of acidification of the phagosome on intra-amoebic killing of bacteria. These results could imply the role of phagosomal pH in activation of enzymes involved in degradation of bacterial cells within phagolysosomes. This is consistent with TEM evidence (see Chapters 4 and 5), that indicated *L. monocytogenes* cells are killed within tight vacuoles of *A. polyphaga* trophozoites.
It is of interest that although *L. monocytogenes* cells can readily survive within mammalian cells, they cannot escape from the phagosomes of cells treated with Bafilomycin A; presumably because LLO is not activated and therefore cannot mediate destruction of the phagosomal membrane. Consequently the intra-cellular growth of bacteria in these treated cells is impaired (Conte *et al.*, 1996). Clearly, the situation for *L. monocytogenes* cells following phagocytosis by Bafilomycin A pre-treated amoebae is quite different. This inhibitor caused a reduction in capacity of *A. polyphaga* to kill *L. monocytogenes* cells and thus the bacterial cells survived longer in pre-treated amoebae. Together with the microbiological data obtained for co-cultivation experiments described in Chapters 4 and 5, this data provides strong evidence that *L. monocytogenes* is unable to survive or replicate within *A. polyphaga* cells.

Suramin has been used by several researchers to study the killing mechanism of bacteria by mammalian cells. For instance, PMNs pre-treated with Suramin for 30 min showed significant reduction in bactericidal activity (Roilides *et al.*, 1993). It has been shown that GTPase is critical for phagosome-lysosome fusion and intra-cellular killing of *L. monocytogenes* by mammalian macrophages (Alvarez-Dominguez and Stahl, 1999; de Chastellier and Berche, 1994). The results of these investigations indicated that Suramin reduced the ability of mammalian cells to kill intra-cellular bacteria. This is in agreement with the results presented in this thesis chapter. In this case, Suramin treatment resulted in a small, but significant reduction in the rate of intra-cellular killing of *L. monocytogenes* cells by *A. polyphaga* trophozoites compared to the control co-culture that used untreated amoeba cells. Given that co-culture data as well as TEM of sections of bacteria phagocytosed by amoebae clearly showed that *L. monocytogenes* cells are rapidly killed within tight vacuoles in *A. polyphaga* trophozoites surrounded by several small lysosome-like vesicles, this implicates phagosome-lysosome fusion as an important process by which *A. polyphaga* mediates killing of *L. monocytogenes* cells.

Overall, it is important that the effects of inhibitors used on phagocytosis and intra-amoebic killing not be overstated. Differences between treated and untreated amoeba cells, with respect to phagocytosis or survival of *L. monocytogenes* are very modest and never more than one log. Inhibition of phagocytic killing would potentially cause a dramatic effect, which would allow a higher survival rate of the infecting bacteria. Consequently, a re-evaluation of the experimental method used may be required. In particular, optimization of the experimental conditions used for these assays, including
inhibitor concentration, should be determined. The use of bacterial sized particles as an alternative to *L. monocytogenes* should be employed as controls for study of inhibition of phagocytosis.

### 6.5 Conclusion

The data presented indicates that actin polymerisation and cytoskeletal rearrangement are involved in phagocytosis of *L. monocytogenes* cells by *A. polyphaga* trophozoites. This data reveals the similar principles of molecular mechanisms used by different types of eukaryotes in uptake of bacteria. Further, both phagosomal acidification and phagosome-lysosome fusion are involved in killing and degradation of *L. monocytogenes* cells by *A. polyphaga*. However, the MBP receptor and *Listeria* induced phagocytosis determinants do not play important role in uptake of bacteria by amoeba trophozoites.
Figure 6.1: Growth *A. polyphaga* on lawns of *L. monocytogenes*.

Suspensions of live and heat-killed *L. monocytogenes* DRDC8 cells in AS, were spread on NNA plates and after drying, 10 µL of an *A. polyphaga* AC012 suspension (containing $5 \times 10^3$ cells) was placed on the centre of plates, followed by incubation at 30°C. The amoeba growth zones on plates were measured (mm) each day up to 4 d from the commencement of co-culture.

An average of 3 measures was used to estimate the mean size of each growth zone. Error bars represent the standard deviation about the mean size of the growth zone.
Monolayers of *A. polyphaga* AC012 amoebae in 24-well trays were pre-treated with glucose, galactose and mannose (all with 100 mM) for 1 h at 22°C. After washing with AS, the monolayers were inoculated with *L. monocytogenes* DRDC8 cells at an MOI of 100 bacteria per amoeba cell and incubated at 22°C for 1 h. Extra-amoebic bacteria were removed by 3 washes in AS buffer followed by counting of intra-amoebic bacteria by lysing of amoeba cells with Triton X-100 and plating on BHI. A control was set up with the same condition except amoebae were not pre-treated with the sugars.

The bacterial numbers presented are the means of 3 independent counts. Error bars represent the standard error about the mean counts of bacteria. Experimental and control data were compared using a Student t-test. ns = not significant.
Figure 6.3: Survival of *L. monocytogenes* within Cytochalasin D pre-treated *A. polyphaga* cells.

Monolayers of *A. polyphaga* AC012 trophozoites in 24-well trays were pre-treated with Cytochalasin D (50 and 100 µM) for 1h at 22°C. After washing the monolayer with AS, *L. monocytogenes* DRDC8 cells were added at an MOI of 100 bacteria per amoeba cell, followed by incubation at 22°C. At 6 and 24 h post inoculation, total viable counts of bacteria were performed by Triton X-100 lysis of amoeba cells and plating on BHI with incubation at 37°C.

The bacterial numbers presented are the means of 3 independent counts. Error bars represent the standard deviation about the mean counts of bacteria. Asterisks indicate that the mean counts for treated amoeba co-cultures are significantly different from counts obtained for the control co-cultures. *** = p<0.001. ns = not significant. Data was compared using a two way analysis of variance of Log<sub>10</sub> transformed data.
Figure 6.4: Fluorescence microscopy of *L. monocytogenes* in *A. polyphaga* pretreated with Cytochalasin D, Wortmannin and NH₄Cl.

The bar markers represent 20 µm.

Panel A: Control (untreated) amoeba cells after 1 h of co-culture with FITC-labelled *L. monocytogenes* cells.

Panel B: Control (untreated) amoeba cells after 3 h of co-culture with FITC-labelled *L. monocytogenes* cells.

Panel C: Cytochalasin D treated amoeba cells after 1 h of co-culture with FITC-labelled *L. monocytogenes* cells.

Panel D: Cytochalasin D treated amoeba cells after 3 h of co-culture with FITC-labelled *L. monocytogenes* cells.

Panel E: Wortmannin (500 nM) treated amoeba cells after 1 h of co-culture with FITC-labelled *L. monocytogenes* cells.

Panel F: Wortmannin (500 nM) treated amoeba cells after 3 h of co-culture with FITC-labelled *L. monocytogenes* cells.

Panel G: NH₄Cl treated amoeba cells after 1 h of co-culture with FITC-labelled *L. monocytogenes* cells.

Panel H: NH₄Cl treated amoeba cells after 3 h of co-culture with FITC-labelled *L. monocytogenes* cells.
Figure 6.5: Effect of Cytochalasin D and Wortmannin pre-treatment on phagocytosis of *L. monocytogenes*.

Pre-treated monolayers of *A. polyphaga* AC012 trophozoites were infected with FITC-labeled *L. monocytogenes* DRDC8 cells at an MOI of 100. Infected amoebae were incubated at 22°C. Monolayers were examined by fluorescence microscopy. Counts of clumps of intra-amoebic bacterial cells were determined after incubation for 1h at 22°C. Data was presented as a Box and Whisker plot. The solid line within each box is the median and the box, which represents the first and third quartiles indicates the spread of the data. The plus sign within the boxes represents the mean of the data. The bars show the spread of data within the 10 to 90 percentile range. Solid dots show data points that fall outside of that range. Number of observations: control amoebae, 81; Cytochalasin D pre-treated amoebae, 27; Wortmannin pre-treated amoebae, 39.

A Student t-test was used to determine whether experimental data sets were statistically different from control data sets. *ns* = not significant.
Monolayers of *A. polyphaga* AC012 cells in 24-well trays were pre-treated with Wortmannin (500 nM and 1 µM) for 1 h at 22°C. After washing the monolayer with AS, *L. monocytogenes* DRDC8 cells were added at an MOI of 100 bacteria per amoeba followed by incubation at 22°C. At 6 and 24 h post inoculation the total viable counts of bacteria were performed by lysing amoebae with Triton X-100 and plating on BHI with incubation at 37°C.

The bacterial numbers shown are the mean of 3 independent counts. Error bars represent the standard error about the mean counts of bacteria. Asterisks indicate that the mean counts for treated amoeba co-cultures are significantly different from counts obtained for the control co-cultures. *** = p<0.001. ns = not significant. Data was compared using a two way analysis of variance of Log_{10} transformed data.
Figure 6.7: Morphology of Cytochalasin D, Wortmannin and DMSO pre-treated *A. polyphaga*.

Monolayers of *A. polyphaga* AC012 trophozoites were examined by phase contrast microscopy. The bar marker represents 20 µm. The panels are labelled as follows:

**Panel A:** control (untreated) amoeba preparations.
**Panel B:** pre-treated with DMSO (1/100 diluted in AS buffer).
**Panel C:** amoebae pre-treated with Cytochalasin D.
**Panel D:** amoebae pre-treated with Wortmannin.
Figure 6.8: Survival of *L. monocytogenes* in Suramin pre-treated *A. polyphaga* cells.

Monolayers of *A. polyphaga* AC012 trophozoites in 24-well trays were pre-treated with Suramin (300 µg.mL⁻¹) for 1 h at 22°C. After washing with AS, the monolayers were inoculated with *L. monocytogenes* DRDC8 cells at an MOI of 100 bacteria per amoeba cell and incubated at 22°C for 1 h followed by washing out the extra-amoebic bacteria with AS buffer. The monolayers were then incubated at 22°C for 6 h and the counts of intra-amoebic bacteria were determined by plating of amoeba lysates on BHI plates. Untreated amoeba monolayers were used as controls.

The numbers of bacteria presented are the mean of 3 independent counts. Error bars represent the standard deviation about the mean counts of bacteria. An asterisk (*) indicates that the mean is significantly different (p<0.05) from that of the control data. Data obtained was compared using a Student’s t-test.
Monolayers of *A. polyphaga* AC012 trophozoites in 24-well trays were pre-treated with Suramin (200 µg.mL⁻¹) for 1 h at 22°C. After washing with AS, the monolayers were inoculated with *L. monocytogenes* DRDC8 cells at an MOI of 100 bacteria per amoeba cell and incubated at 22°C for 1 h followed by washing out the extra-amoebic bacteria with AS buffer. The monolayers were then incubated at 22°C. After 1.5 and 6 h co-culture, the counts of viable intra-amoebic bacteria were determined by lysing amoebae with Triton X-100 and plating the lysate on BHI. A control was set up with the same condition except amoebae were not pre-treated with Suramin.

The mean numbers of bacteria presented are the results of 4 independent replicates. Error bars represent the standard deviation about the mean counts of bacteria. *ns* indicates that the mean counts for treated amoeba co-cultures are not significantly different from counts obtained for the control co-cultures at each time point. Data was compared using a two way analysis of variance of Log_{10} transformed data.
Monolayers of *A. polyphaga* AC012 trophozoites in 24-well trays were pre-treated with NH₄Cl (20 mM) for 1 h at 22°C. After washing the monolayers, *L. monocytogenes* DRDC8 cells were added to an MOI of 100 bacteria per amoeba cell followed by incubation at 22°C for 1 h. The extra-amoebic bacteria were washed out with 3× washing in AS. At 1, 4 and 6 h post inoculation the counts of viable intra-amoebic bacteria were performed by lysis of amoeba cells with Triton X-100 (final concentration of 0.3%) and plating the lysate on BHI.

The mean counts of bacteria are based on 3 independent replicates. Error bars represent the standard deviation about the mean counts of bacteria. Asterisks indicate that the mean counts for treated amoeba co-cultures are significantly different from counts obtained for the control co-cultures. ⋆⋆⋆ = p<0.001 and ⋆⋆ = p<0.01. ns = not significant. Data was compared using a two way analysis of variance of Log₁₀ transformed data.
Figure 6.11: Number of clumps of intra-amoebic *L. monocytogenes* in NH$_4$Cl pre-treatment *A. polyphaga*.

Ammonium chloride pre-treated monolayers of *A. polyphaga* trophozoites were infected with FITC-labeled *L. monocytogenes* DRDC8 cells at an MOI of 100. Infected amoebae were incubated at 22°C for 1 and 3 h. Monolayers were examined by fluorescence microscopy. Counts of clumps of intra-amoebic bacterial cells were determined. Data was presented as a Box and Whisker plot. The solid line within each box is the median and the box, which represents the first and third quartiles, indicates the spread of the data. The plus sign within the boxes represents the mean of the data. The bars show the spread of data within the 10 to 90 percentile range. Solid dots show data points that fall outside of that range. Number of observations: Control amoebae at 1 h, 81; Control amoebae at 3 h, 54; Ammonium chloride pre-treated amoebae at 1 h, 78; Ammonium chloride pre-treated amoebae at 3 h, 70.

A Student t-test was used to determine whether experimental data sets were statistically different from control data sets. *** = p<0.001, ns = not significant.
Figure 6.12: Survival of *L. monocytogenes* DRDC8 following phagocytosis by Bafilomycin A pre-treated *A. polyphaga* AC012 cells.

Monolayers of amoebae in 24-well trays were pre-treated with Bafilomycin A (80 nM) for 1 h at 22°C. After washing with AS, the monolayers were inoculated with bacteria at MOI of 100 bacteria per amoeba cell and incubated at 22°C for 1 h followed by washing out the extra-amoebic bacteria with AS buffer. The monolayers were then incubated at 22°C for 6 h and the counts of viable intra-amoebic bacteria were determined by lysis of amoebae with Triton X-100 and plating the lysate on BHI. A control was set up with the same condition except amoebae were not pre-treated with Bafilomycin A.

The counts of bacteria are the mean of 3 replicates of independent counts. Error bars represent the standard deviation about the mean counts of bacteria. ns indicates that the mean counts for treated amoeba co-cultures are not significantly different from counts obtained for the control co-cultures at each time point. Data was compared using a two way analysis of variance of Log_{10} transformed data.
Monolayers of *A. polyphaga* AC012 trophozoites in 24-well trays were pre-treated with Monensin (5 and 20 µM) for 1 h at 22°C. After washing with AS, the monolayers were inoculated with *L. monocytogenes* DRDC8 cells at an MOI of 100 bacteria per amoeba cell and incubated at 22°C for 1 h followed by washing out the extra-amoebic bacteria with AS buffer. The monolayers were then incubated at 22°C. After 1.5 and 4 h of co-culture, the counts of viable intra-amoebic bacteria were determined by lysis of amoebae with Triton X-100 and plating the lysate on BHI. A control was set up with the same condition except amoebae were not pre-treated with Monensin.

The numbers of bacteria presented are the means of 3 replicates of independent counts. Error bars represent the standard deviation about the mean counts of bacteria. ns indicates that the mean counts for treated amoeba co-cultures are not significantly different from counts obtained for the control co-cultures at each time point. Data was compared using a two way analysis of variance of Log_{10} transformed data.
Figure 6.14: Survival of *L. monocytogenes* in *A. polyphaga* cells pre-treated with different concentrations of Monensin.

Monolayers of *A. polyphaga* AC012 trophozoites in 24-well trays were pre-treated with Monensin (5 and 20 µM) for 1 h at 22°C. After washing with AS, the monolayers were inoculated with *L. monocytogenes* DRDC8 cells at an MOI of 100 bacteria per amoeba cell and incubated at 22°C for 1 h followed by washing out the extra-amoebic bacteria with AS buffer. The monolayers were then incubated at 22°C for 4 h and the counts of viable intra-amoebic bacteria were determined by lysis of amoebae with Triton 100 and plating the lysate on BHI. A control was set up with the same condition except amoebae were not pre-treated with Monensin.

The numbers of bacteria presented are the mean of 3 replicates of independent counts. Error bars represent the standard deviation about the mean counts of bacteria. Experimental and control data sets were compared using a Student’s t-test of Log_{10} transformed data. An asterisk (*) indicates that the mean is significantly different (p<0.05) from that of the control data. ns indicates no significant difference between the control and experimental data sets.
Chapter 7: General Discussion

7.1 Introduction

*Listeria monocytogenes* is an important human pathogen which produces severe disease in pregnant women, neonates and immuno-compromised individuals including elders and people with cancer. Humans usually become infected as a result of consumption of contaminated foods, particular “ready-to-eat” foods. Given the increase in the proportion of aged persons in populations over recent decades, the longer survival of cancer patients and also the popularity of ready to eat food e.g. refrigerated food, listeriosis remains a major food-associated public health concern in developed countries. In fact a number of reports have indicated that the prevalence of listeriosis in developed countries has increased during recent years (Garcia-Alvarez *et al.*, 2006; Koch and Stark, 2006). One of the main reasons for this trend is the fact that *L. monocytogenes* can grow in refrigerated foods, even in the presence of commonly used inhibitory agents used as preservatives.

Typically, this bacterium contaminates foods via raw materials or food processing environments. However, the current knowledge of the ecology of this important bacterium and in particular, the mode of transmission and survival in the environment, remains very limited. Filling this information gap may assist the food industry to minimise the potential for contamination of food materials by this opportunistic pathogen. Recent studies of the microbial ecology of other intra-cellular pathogens has highlighted the potential role of protozoan hosts to act as reservoirs of these bacteria and indeed, act as vectors for dissemination to mammalian hosts. In particular, *Acanthamoeba* spp. have been studied as an alternative host for these pathogens (Greub and Raoult, 2004). Since *Acanthamoeba* spp. are wide spread and naturally feed on free-living bacteria, it is reasonable to assume that this group of protozoa could interact with *Listeria* spp. in the environment.

*Acanthamoeba* spp. have been successfully used as a model host system to study the interaction of bacteria with free-living protozoa *in vitro*. Moreover, the isolation, growth, maintenance and co-culture methods for *Acanthamoeba* spp. have been well established. However, although the interaction of several Gram-negative bacterial pathogens with *Acanthamoeba* spp. has been well studied, there are few published reports of related
research dedicated to Gram-positive bacterial pathogens. It is against this background that the work described in this thesis was undertaken.

To achieve this aim, *Acanthamoeba* co-culture assays were developed in order to examine the interaction of *L. monocytogenes* cells with *Acanthamoeba* trophozoites to assess the potential impact of this group of amoebae on environmental survival and ecology of *L. monocytogenes*. A further aspect of this work was to assess the role of plasmids carried by *L. monocytogenes* on ability of these bacteria to interact with *Acanthamoeba* cells. That work was assisted by sequence analysis of a part of the large plasmid carried by *L. monocytogenes* strain DRDC8. Plasmid DNA specific PCR primers were used to detect two plasmid encoded marker genes (*cadA* and *ctpA*) as well as confirm plasmid-cured variants of *L. monocytogenes* DRDC8. Moreover, the mechanism associated with uptake and intra-amoebic killing of *L. monocytogenes* DRDC8 by *A. polyphaga* was studied.

### 7.2 Plasmid Associated Genes in *L. monocytogenes* DRDC8

DNA sequence data obtained for a part of the large plasmid carried by *L. monocytogenes* DRDC8 (as a collaborative laboratory project), provided important information about the potential function of proteins encoded by genes carried on plasmid DNA. That analysis indicated that the types of genes and the genetic organisation of plasmid genes was similar to that reported by whole genome sequencing projects for plasmids carried by other *Listeria* species. Furthermore, the nucleotide and amino acid sequence data for those genes shared extraordinary similarity to those identified in other Gram-positive bacteria. For example, cadmium and copper transport associated genes encoded on this plasmid DNA were similar to genes found in *Enterococcus hirae* in terms of organisation and sequence conservation (Solioz and Stoyanov, 2003). In addition, two adjacent ORFs that encoded replication proteins were similar to counterparts encoded on a large plasmid in *Enterococcus fecalis* (Schwarz *et al.*, 2001), and an ORF that encoded a restriction modification gene also was essentially identical to one carried on the *L. innocua* large plasmid pLI100. The latter ORF was located on the 3’ end of the replication initiation protein (RepB) gene and this organisation was also conserved in the *L. innocua* large plasmid (pLI100). A potential reason for these similarities may lie in the observation that
within a 30 Kbp region of the large plasmid of DRDC8, five open reading frames encode polypeptides that resemble transposase-like proteins described in *L. innocua* and *Lactococcus lactis*. Furthermore a two gene operon with similarity to genes encoding a known cadmium efflux system is flanked by transposase genes and associated inverted repeats. The significance of this observation will be discussed later.

Given the high similarity of genes encoded by *L. monocytogenes* plasmid DNA with other Gram-positive bacteria, it is reasonable to assume the large plasmid is implicated in exchange of genetic traits among *Listeria* species and other Gram-positive bacteria. For example, it has been reported that a 37 Kb plasmid of *L. monocytogenes* was self transferable to other Gram-positive bacteria including *Enterococcus* and *Staphylococcus* (Poyart-Salmeron *et al.*, 1990). Moreover, several of the ORFs encoded by the large plasmid of *L. monocytogenes* DRDC8 have high sequence similarity to ORFs encoded on plasmids found in *Lactococcus lactis, Lactobacillus* spp. and *Streptococcus* spp.. Since strain DRDC8 was originally isolated from a dairy processing environment, it is possible that these genes were acquired from other bacteria associated with dairy products such as *L. lactis*. The fact that the copper haemostasis genes encoded on the DRDC8 large plasmid have not previously been reported for other *Listeria* genomic sequences, suggested that strain DRDC8 may have acquired these genes to allow it to effectively acquire Cu$^{2+}$, and to control levels of this critical micronutrient, from the environment in response to copper limited/toxic conditions. Furthermore, this suggested that interspecies plasmid exchange may play an important role in evolution of plasmid-associated genes carried by *Listeria* spp. However, the ability of the large plasmid from DRDC8 to be mobilised by conjugation has yet to be established.

The number of ORFs that encoded transposases suggested that transposition events may have played a role in evolution of the DRDC8 large plasmid. Transposition events involving the host bacterial chromosome, or other mobile genetic elements such as conjugative plasmids and bacteriophage genomes, may also have been critical to that process. This notion is supported by reports that most heavy metal ion transporters and regulators are carried on plasmids in bacteria (Dabbs and Sole, 1988; Lemaitre *et al.*, 1998; Liu *et al.*, 2002; Silver, 1996). The cation transport regulator genes protect bacteria against high concentrations of heavy metals, which are highly toxic for bacterial cells (Lebrun *et
al., 1994; Solioz and Stoyanov, 2003). Moreover, the multiple copies of transposase genes carried by the DRDC8 plasmid implied that plasmids in Listeria are likely to be highly variable and subject to frequent DNA insertion and deletion events. This process may contribute to variation in the size and genetic variety of plasmids isolated from different strains of L. monocytogenes. That assertion is supported by the fact that the deduced polypeptide sequences of the transposases are most similar to those encoded by plasmids found in other Gram-positive bacteria (Polzin and Shimizu-Kadota, 1987). Thus transposition events may have been responsible for introduction of genes associated with cation transport and homeostasis in this group of bacteria. This phenomenon may have occurred via the proximity of bacterial cells in environmental niches, or during growth, colonisation and infection of animal such as ruminants, where bacteria from different genera could exchange plasmids, phage or DNA fragments.

7.3 Interaction of L. monocytogenes and Acanthamoeba spp.

Remarkably, the results of co-culture experiments with L. monocytogenes strain DRDC8 and Acanthamoeba polyphaga (as well as other Acanthamoeba spp.), showed L. monocytogenes is unable to establish an intra-cellular lifestyle within Acanthamoeba spp. under the experimental conditions tested, even though strain DRDC8 is capable to effectively parasitise mammalian cells (e.g. HeLa, CaCo2 and J774 cell lines) and is pathogenic for mice (Francis and Thomas, 1997). Indeed L. monocytogenes is phagocytosed by amoeba trophozoites and effectively killed within 2 – 5 h after phagocytosis. The experimental data to support this contrary conclusion was derived from simple plate cultures of amoebae on lawns of bacteria, quantitative microbiological data obtained from co-cultures with monolayers of amoebae, prolonged co-cultures of amoebae with bacteria in AS buffer and microscopic examination of bacteria within the phagosomes of amoeba trophozoites.

The results of plate co-cultures indicated amoebae could grow equally well on L. monocytogenes and E. coli. Further, those simple experiments provided strong preliminary evidence that the trophozoites could feed on and kill the bacteria on these plates. This observation was consistent for co-cultures carried out within the biokinetic temperature range of the amoeba cultures used (i.e. <37°C). However, our observations of
plate co-cultures were different from other research reports for Gram-negative bacteria. Marolda et al. (1999), for example, reported that co-culture of Burkholderia cepacia and A. polyphaga resulted in a delay of amoeba encystation and amoeba trophozoites subsequently developed large cytoplasmic vacuoles that contained live motile bacteria. Further, Gaze et al. (2003), reported that Salmonella Typhimurium co-cultured with A. polyphaga on plates, formed colonies. That observation indicated these bacteria could grow in the presence of amoeba trophozoites. Indeed, both of the mentioned Gram-negative bacteria were able to survive within amoeba hosts. Thus the preliminary plate culture data indicated that the way L. monocytogenes interact with Acanthamoeba cells is quite different than that reported for Gram-negative bacterial pathogens. Consequently Gram-negative bacterial pathogens may have evolved effective mechanisms that allow interaction and survival within host amoeba cells.

Data obtained from co-cultures of monolayers of amoeba with L. monocytogenes cells provided the most convincing evidence that Acanthamoeba trophozoites can effectively kill internalised bacteria shortly after phagocytosis. Since intra-amoebic L. monocytogenes cells are killed within 2 – 5 h post co-cultivation, that observation suggested that amoeba trophozoites may be able to kill L. monocytogenes before these bacteria can escape from phagosomes and gain access to the cytoplasm of amoeba trophozoites. As was observed for plate co-cultures, the outcome of bacterial interaction with monolayers of amoebae was independent of incubation temperature. Surprisingly this was the case even when incubation temperatures of 37ºC were used; that temperature has been demonstrated to be optimal for the expression of key L. monocytogenes virulence genes necessary for intracellular invasion and parasitism of the cytoplasmic compartment of mammalian cells. Given the fact that the number of amoebae remained almost unchanged for the duration of short-term co-culture experiments (5 h), amoeba trophozoites are unlikely to be lysed by phagocytosed L. monocytogenes cells. In fact, those observations indicated that intra-amoebic L. monocytogenes cells are not able to evade the killing mechanism(s) employed by Acanthamoeba cells. At co-culture incubation temperatures optimal for expression of L. monocytogenes virulence factors, these factors are either not expressed in the amoeba host, or are ineffective in enabling the bacteria to escape the phagosome before phagosome-phagolysosome derived factors cause inactivation/killing of bacterial cells.
Failure of intra-amoebic *L. monocytogenes* cells to escape the phagocytic vacuoles was confirmed by examination of amoebae monolayers infected with *L. monocytogenes* using both Fluorescent microscopy and TEM techniques. Fluorescent microscopy studies complemented microbiological data and confirmed that the bacterial cells are phagocytosed by amoeba trophozoites shortly after co-culture. Importantly, the TEM micrographs provided a clear demonstration that the bacterial cells are degraded once confined within amoeba vacuoles 2 - 4 h after phagocytosis. No evidence of bacterial replication within amoeba trophozoites was obtained, nor was any evidence obtained for trophozoite destruction as a result of infection by *L. monocytogenes*. In addition, this data also showed *L. monocytogenes* cells are killed within amoeba phagosomes before bacteria able to escape from phagosomes and get access to the amoeba cytoplasm. Presence of the concentrated lysosome-like vesicles and mitochondria in the amoeba cytoplasm surrounding the phagocytic vacuoles suggested lysosomal enzymes were probably involved in degradation of bacterial cells.

The fact that both the LLO (*hly*) mutant and plasmid-cured strains of *L. monocytogenes* were killed by amoebae as quickly as the wild type parent provided strong direct evidence that *Listeria* virulence genes and plasmid associated genes have no obvious impact on bacterial interaction with amoeba cells. These results agree with previously reported research that indicated *L. monocytogenes* did not replicate within trophozoites of *A. castellanii* nor did the major virulence gene of *L. monocytogenes* (*hly*), change the outcome of bacterial interaction with this amoeba (Zhou et al., 2007). However, this outcome is contrary to events that describe the interaction of *Listeria* with mammalian cells. For example, it has been shown that *hly* mutants are completely avirulent for mammalian cells (Michel et al., 1990; Portnoy et al., 1988) and are not able to invade the cytoplasm of infected cells. In addition, plasmid encoded factors do not affect the outcome of bacterial interaction with amoebae. Indeed the exact role of *L. monocytogenes* virulence genes and plasmid associated genes in interaction with other single celled protozoa needs to be thoroughly investigated.

In order to more carefully evaluate whether *L. monocytogenes* is able to express virulence genes on entry to *Acanthamoeba* cells, several promoter-GFP transcriptional fusions were constructed and used in co-culture experiments. Results obtained showed
that *prfA* (and hence *hly*, *plcA*, *plcB*, *actA* and *mpl*) was not expressed when bacteria are phagocytosed by amoebae, even though the same construct expressed GFP when used to infect HeLa cells. PrfA is normally a key regulator of virulence genes; and expression of *prfA* is required for *L. monocytogenes* to escape from early phagosome in mammalian cells to the cytoplasmic compartment of those host cells (Freitag *et al.*, 1993). Conversely, PrfA mutants are avirulent for mammalian cells (Chakraborty *et al.*, 1992; Freitag *et al.*, 1993).

This evidence, together with the fact that *hly* mutants behave identically to wild-type parent strains in the *Acanthamoeba* co-culture model, indicated that *L. monocytogenes* cells unable to express the major virulence regulator gene, *prfA*, and consequently other virulence determinants controlled by PrfA such as listeriolysin O (LLO), PlcA and PlcB within trophozoite phagosomes.

The results of co-culture assays and microscopic examination presented in Chapters 4 and 5 are also contrary to the results of the only other published study of co-culture of *L. monocytogenes* with amoeba cells (Ly and Müller, 1990). That work indicated *L. monocytogenes* cells could survive and multiply within amoeba trophozoites. One explanation for this contradiction may be the use of different co-culture methodologies and amoeba strains. For example, Ly and Müller (1990) did not use ACM as a control medium. This may be important as it has been shown independently, that during co-culture with amoeba trophozoites, bacterial cells could grow on the materials released and secreted by actively growing amoeba cells, amoeba undergoing encystation and lysed amoeba cells (Marolda *et al.*, 1999; Steinert *et al.*, 1998). Moreover, work described in this thesis showed that *L. monocytogenes* can grow in ACM. Therefore, molecules released from amoeba cells may provide the necessary nutrients for saprophytic growth of *L. monocytogenes* during co-culture in tap water and consequently, provide an alternative explanation for the bacterial growth in co-culture with amoeba cells noted by Ly and Müller (1990).

It is also of interest that Ly and Müller (1990) used only one strain of *Acanthamoeba* sp. (unknown species) for their experiments. Their results may not be generally applicable to other *Acanthamoeba* spp., since it has been shown that different *Acanthamoeba* spp. produce different degradative enzymes (Weekers *et al.*, 1995) and consequently not all
types of these amoeba may necessarily support intra-cellular survival of a particular bacterium.

Importantly, the work described in this thesis established, for the first time, through the use of microscopy that *L. monocytogenes* strains were unable to survive within the intra-cellular compartments of the several *Acanathamoeba* spp. tested. Although Ly and Müller (1990) indicated that the strain of *L. monocytogenes* used by them was able to survive within amoeba cells, unequivocal evidence was not published to show that these bacteria were maintained in a viable state inside amoeba trophozoites for extended periods of time. It is possible that their experiments simply reflected the ability of *L. monocytogenes* to grow saprophytically on molecules released by amoeba cells. Given the significantly longer survival time of *L. monocytogenes* in co-culture with amoebae compared with cultures in AS buffer (control), this indicated the bacterial cells benefited from the materials normally secreted/released from amoeba cells during encystation or lysis as previously reported for co-cultures with *B. cepacia* (Marolda *et al.*, 1999) and *Mycobacterium avium* (Steinert *et al.*, 1998). This notion is further supported by the similarity of survival data for *L. monocytogenes* cells in amoeba-conditioned medium compared with that of co-cultures with amoeba cells. Collectively, this information indicated bacteria can survive in the extra-amoebic environment. Thus although *L. monocytogenes* cells may not survive within intra-cellular compartments of amoeba trophozoites, proximity of bacteria to amoebae (and nutrients released from amoebae) in the natural environment may actually facilitate survival of bacterial cells.

Co-culture of *Acanthamoeba* with several Gram-negative and/or acid fast bacteria has indicated these groups of bacteria are able to grow, or in some cases multiply, within amoeba hosts. For example, *Acanthamoeba* spp. harbour *Legionella pneumophila, Salmonella Typhimurium, Simkania negevensis, Burkholderia cepacia, Mycobacterium avium* and *Chlamydia pneumoniae* (Essig *et al.*, 1997; Gao *et al.*, 1997; Gaze *et al.*, 2003; Kahane *et al.*, 2001; Marolda *et al.*, 1999; Steinert *et al.*, 1998). However, with the exception of the work published by Ly and Müller (1990), interactions between Gram-positive bacteria and *Acanthamoeba* spp. have received scant attention. There is therefore, insufficient evidence to unequivocally say whether various Gram-positive bacterial pathogens can survive or replicate within amoeba hosts.
Apparently, Gram-negative bacteria are much more resistant to the bactericidal mechanisms of *Acanthamoeba* spp. than are Gram-positive bacteria (Weekers *et al.*, 1995). This implies that there may be fundamental differences between Gram-positive and Gram-negative bacteria in terms of their ability to interact with *Acanthamoeba* spp. An obvious structural difference between Gram-positive and Gram-negative bacteria that may be important is the accessibility of the peptidoglycan layer of the Gram-positive bacterial cell wall to various amoeba-derived enzymes. Furthermore, differences in bacteriolytic sensitivities noted for various Gram-negative bacteria may be linked to the presence of specific proteins, lipoproteins or lipopolysaccharides in the Gram-negative bacterial cell wall complex. For example, anionic polyelectrolytes located in the outer membrane of Gram-negative bacteria can hamper bacteriolysis (Ginsburg, 1988).

The fact that *S. Typhimurium* (see Chapter 4.3.3) is able to survive within *A. polyphaga*, whereas *L. monocytogenes* strains do not, exemplifies the fundamental difference between Gram-positive and Gram-negative bacteria in terms of adaptations necessary for survival within amoebae. Microscopic examination of co-cultures showed that *S. Typhimurium* is able to survive and grow within phagocytic vacuoles of amoeba trophozoites for extended periods of time, whereas *L. monocytogenes* cells are rapidly eliminated within a few hours. This might reflect different mechanisms employed by Gram negative bacterial pathogens to avoid killing mechanisms utilized by professional eukaryotic phagocytes e.g. Type III and IV secretions systems.

Bacteria that are natural endosymbionts of amoeba cells have been isolated from naturally occurring amoebae. Interestingly, these bacteria are typically Gram-negative bacteria or *Chlamydia*, but not Gram-positive bacteria. For example, *Bacteriodes* and *Chlamydia* have been isolated from *Acanthamoeba* spp. and *Hartmanella* (Collingro *et al.*, 2005; Fritsche *et al.*, 1993; Horn and Wagner, 2004). Thus it is possible that Gram-positive bacteria may not able to survive the killing mechanisms utilised by *Acanthamoeba* spp. However, as so little data describing the interaction between Gram-positive bacteria and amoebae is presently available, additional work is required to better characterise the potential for other Gram-positive bacteria to survive within the intra-amoebic environment.

It is recognized that the present study included only a restricted set of isolates of *L. monocytogenes* and *Acanthamoeba* spp. Consequently it is possible that not all forms of
interactions have been identified. Different free-living amoebae may function differently in environmental niches in which different outcomes are possible. For example, bacterial growth conditions can have a significant impact on the efficiency of entry into amoebae (Tezcan-Merdol et al., 2004). Therefore, the use of experimental co-culture models which mimic different environmental conditions such as salt concentration, osmotic pressure, nutrient composition and presence of other microbial competitors, may provide a better insight into the real environmental interaction of bacteria with amoeba cells.

7.4 Interactions are Independent of Plasmid Genes

Sequence data obtained from the DRDC8 large plasmid provided a means to select and confirm plasmid-cured variants of *L. monocytogenes*. In particular, PCR amplification of two cation transporting genes (*cadA* and *ctpA*) was used to select and confirm the loss of the large plasmid from cured variants of *L. monocytogenes* DRDC8 (see Chapter 3.3.4.2). Isolation of plasmid cured variants allowed an assessment of the potential impact of plasmid associated genes on bacterial interaction with both mammalian and *Acanthamoeba* cells.

Significantly, results presented in this thesis, also indicated that the large plasmid carried by *L. monocytogenes* DRDC8 (and other isolates) was not maintained during invasion and intra-cellular growth within HeLa cells. This indicated that the plasmid is essentially unstable in the absence of selective pressures which are apparently not relevant to *in vitro* cell culture. Importantly, this result may be one explanation for the absence of large plasmids in clinical isolates of *L. monocytogenes*. This concept is supported by unpublished reports that the plasmid encoded *ctpA* gene is restricted to environmental isolates of *L. monocytogenes*. This hypothesis could be tested by comparison of the plasmid content using plasmid isolation and PCR amplification of plasmid encoded genes from freshly isolated clinical and environmental specimens of *L. monocytogenes*.

The observation that plasmid DNA was lost in serial passage through HeLa cell cultures, indicated that the large plasmid may have no significance for intra-cellular growth
of *L. monocytogenes* within mammalian cells. Rather it is likely that the plasmid encoded genes are aid survival and persistence of these bacteria in environmental niches (Lebrun et al., 1992). Given the currently accepted dogma that plasmid-borne genes provide no selective advantage to bacteria grown in enriched media, it is not surprising that the increased metabolic load imposed by maintenance of plasmid DNA leads to the success of populations of plasmidless isolates over those that continue to maintain plasmid DNA. Thus it seems the large plasmid encoded genes carried by *L. monocytogenes* DRDC8 are not absolutely required for growth within cell cytoplasm of *in vitro* cultured HeLa cells.

Whether this outcome also applies to *in vivo* models of infection has yet to be tested. However, there is published data that indicates that the results of *in vitro* infections of cell culture systems may not simply apply to *in vivo* models of infection. Francis and Thomas (1996) for example, showed that a *L. monocytogenes* strain DRDC8 *ctpA* mutant (DSE201) was less able to persist in the liver and spleen of mice and in particular, were more readily cleared from the liver. By contrast, no difference in growth of the mutant and the wild type parent in cultured HeLa and J774 cells was observed. In that study, the contradiction between experimental outcomes for *in vitro* versus *in vivo* models of infection was thought to be related to a reduction in levels of available copper ions in liver and spleen tissue as a response to inflammation caused by infection. That phenomenon has previously been reported as a response to infection of mammalian animal model systems by different infectious agents (Beisel, 1977; Crocker et al., 1992; Matousek de Abel de la Cruz et al., 1993). Under condition of reduced copper concentrations in inflamed liver, *ctpA* mutants would be expected to be growth limited as copper is an essential growth factor and *ctpA* mutants are partially defective in transporting this important trace nutrient. So the copper limiting condition in the liver, for example, might be expected to select for *L. monocytogenes* carrying plasmid encoded *ctpA*. This phenomenon is unlikely to occur in cell culture models where the unrestricted availability of nutrients has no selective advantage for the large plasmid.

The presence of plasmid-encoded cation transport systems may play roles in survival of bacteria by enhancing their ability to keep intra-cellular homeostasis of metal elements. These elements may present with various concentrations in environmental habitats. Given capability of *L. monocytogenes* to grow and survive in a wide range of environmental
conditions, auxiliary genes carried on plasmids may be critical for the persistence of bacteria in environmental habitats. In these environments, bacterial cells may be exposed to many chemical compounds at concentrations that require careful regulation in order to maintain homeostasis within the bacterial cell cytosol. The presence of large plasmid in ca. 55% of environmental isolates of *L. monocytogenes* screened is unlikely to be a coincidence and that data provides indirect support for a role in assisting *L. monocytogenes* to survive in the environment (see Chapter 3.3.4). This view is consistent with other published research which indicates bacterial genes involved in resistance/tolerance to toxic metal ions are usually carried on plasmids (Dabbs and Sole, 1988; Lemaitre *et al*., 1998; Liu *et al*., 2002; Silver, 1996). For example, cadmium efflux genes found on plasmids in *L. monocytogenes* may protect these bacteria against toxic concentrations of cadmium in the environment (Lebrun *et al*., 1994). Similarly copper is an essential element for all live organisms including bacteria, but if present in high concentration, is toxic for viability of bacterial cells. Consequently, plasmid-associated copper transport genes might play an important role in environmental survival and ecology of *L. monocytogenes*. However, the exact role of plasmid-encoded genes in environmental survival of *L. monocytogenes* and their potential contribution are yet to be investigated. The function of these genes could be tested by mutation in each genes and cultivation of the mutated bacteria in a minimum culture medium supplemented with different compounds such as metal ions which resemble environmental habitats.

### 7.5 Mechanisms of Phagocytosis and Killing

The contrasting outcomes of *L. monocytogenes* infection of amoeba trophozoites and mammalian cells provided an opportunity to examine the mechanisms used by *A. polyphaga* to phagocytose and kill *L. monocytogenes*. This information may support speculation that phagocytic eukaryotic cells from different evolutionary lines exploit similar mechanisms to phagocytose and digest bacterial cells. The basic conservation in biochemical and structural features of physiological processes in eukaryotic cells is likely to limit the variety of mechanisms exploited by eukaryotic cells to phagocytose and degrade prokaryotic cells. It seems that actin polymerization, phagosome lysosome fusion
and phagosome acidification are among those common mechanisms used by different eukaryotic cells.

The rapid killing of \textit{L. monocytogenes} by \textit{Acanthamoeba} spp. suggests that these amoebae may employ antimicrobial peptides as a means of destroying bacterial prey. \textit{Entamoeba histolytica}, and indeed other \textit{Entamoeba} spp., are well known for an ability to produce antimicrobial peptides that are particularly active against Gram positive bacteria and wall-less forms of bacteria, but not Gram negative bacteria (Leippe, 1999). Accumulated evidence indicates that pore forming peptides, collectively known as amoebapores, are expressed by \textit{Entamoeba histolytica} and form an integral part of the cytolytic machinery of pathogenic amoebae. These peptides are discharged into phagosomes containing engulfed bacteria where they interact with bacterial cytoplasmic membranes and ultimately cause the inactivation of the bacterial cell (Andrã \textit{et al.}, 2003; Bracha \textit{et al.}, 1999; Bruhn and Leippe, 2001). Since \textit{Acanthamoeba} spp., like \textit{Entamoeba} spp. are free-living bacteriovorous amoebae, it is likely that the former also express amoebapore-like molecules as part of an armory of molecules that are recruited to destroy bacterial cells. The potential for amoebapore-like molecules to play a role in killing \textit{L. monocytogenes} by \textit{Acanthamoeba} spp however, has not been specifically addressed by studies described in this thesis. Furthermore, no published reports of cytolytic peptides expressed by \textit{Acanthamoeba} spp. are available. Nevertheless, the role of these molecules in intra-amoebic killing of Gram positive bacterial cells could form the basis of future studies designed to specifically address this issue.

Inhibition of actin polymerisation by Cytochalasin D and Wortmannin effectively reduced the ability of \textit{A. polyphaga} to phagocytose \textit{L. monocytogenes} DRDC8 in a manner similar to that reported for mammalian cells. This suggested that the underlying involvement of actin in the mechanism of phagocytosis is similar for both mammalian cells and \textit{A. polyphaga}. In fact, the actin-mediated cytoskeletal rearrangements play a key role in bacterial entry into the eukaryotic cells and have been preserved across different types of eukaryotic cells including \textit{Acanthamoebae}. Further, given the significant impact of Wortmannin on reduction of phagocytosis of \textit{L. monocytogenes} by amoeba trophozoites, suggested that phosphatidilinositotol 3-kinase probably plays a role in this process. This data is consistent with the research reports for mammalian cells which indicated the role of
actin polymerisation and PI 3-kinase in phagocytosis of bacterial cells by mammalian macrophages (Elliott and Winn, 1986; Finlay and Falkow, 1988; Ui et al., 1995; Vlahos et al., 1994). However, the exact molecular mechanism of actin polymerization and the role of PI 3-kinase in the phagocytosis process of bacteria by Acanthamoeba species remain to be investigated.

Alsam et al. (2005) have reported that the mannose binding protein play a role in uptake of killed E. coli by A. castellanii. Further, it seems that mannose-binding protein (MBP) of Acanthamoeba is also involved in the pathogenesis of the Acanthamoeba infection by mediating the adhesion of amoeba cells to the human cells (Garate et al., 2004). However, the result presented in Chapter 6 indicated phagocytosis of L. monocytogenes by A. polyphaga cannot be inhibited by mannose. One explanation for this observation is that Acanthamoeba cells may use different receptor mediated mechanisms for uptake different bacterial cells and non-cellular particles. For example, Allen and Dawidowicz (1990) reported that although exogenous mannose had no effect on phagocytosis of latex beads by A. castellanii, uptake of yeast particles by the amoeba cells was inhibited by mannose. Further, it has been reported that Legionella pneumophila uses different surface receptors to attach to A. polyphaga cells but not to mammalian cells (Gao et al., 1997). However, much remains to be uncovered about the molecular determinants implicated in attachment and uptake of different bacterial pathogens interacted with Acanthamoeba spp.

Bacterial induced phagocytosis is not a significant factor in uptake of bacterial cells by amoeba trophozoites. Indeed, the L. monocytogenes induced mechanism plays a key role in uptake of bacterial cells by non-phagocytic cells not by professional macrophages (Pizarro-Cerda and Cossart, 2006). This is consistent with the fact that A. polyphaga trophozoites have a professional phagocytic capability that is responsible for uptake of different types of bacteria as food nutrients (Bottone et al., 1994; Weekers et al., 1993). However, the role of L. monocytogenes surface proteins, InlA/B, and other surface associated proteins in the uptake of L. monocytogenes by amoeba trophozoites need to be investigated. One method for testing the role of each gene could be using a mutation strain for each of these genes and compare their uptake to the wild type by experimental models using bacteriological methods and microscopy.
The results presented also indicated that phagosome acidification plays a role in intra-amoebic killing of *L. monocytogenes* by *A. polyphaga*. Pre-treatment amoebae with BafilomycinA and ammonium chloride slowed down intra-amoebic killing of *L. monocytogenes* by *A. polyphaga*. These results could imply the role of phagosomal pH in activation of enzymes involved in the degradation of bacterial cells within phagolysosomes. This data is supported by the TEM results (see Chapters 4 and 5), that indicated *L. monocytogenes* cells are killed within tight vacuoles in *A. polyphaga* trophozoites. This is also consistent with the report that acidification of phagosome is necessary to kill *Aspergillus fumigatus* by macrophages (Ibrahim-Granet *et al.*, 2003). This may be an interesting result since it is generally accepted that acidification of the phagosome is necessary for activation of LLO required for rupture of the phagosome membrane and movement of *L. monocytogenes* into the host cell cytoplasm. Clearly intra-amoebic *L. monocytogenes* cells are unable to escape into the trophozoites cytoplasm. Consequently, these bacterial cells are unable to express LLO, cannot express sufficient LLO to allow rapid escape from the phagolysosome, or the LLO is unable to interact with the membrane lipids of amoebae in the same way as it does for mammalian cell vacuolar membranes.

Research showed that phagosome - lysosome fusion was involved in intra-cellular killing of *L. monocytogenes* by mammalian macrophages (Alvarez-Dominguez and Stahl, 1999; de Chastellier and Berche, 1994). This is consistent with results presented in Chapter 6 which indicated fusion of lysosomes with the phagosome is involved in the intra-amoebic killing of *L. monocytogenes* by *A. polyphaga* trophozoites. Further, this correlates with the co-culture results presented in Chapters 4 and 5. For example, TEM clearly showed that *L. monocytogenes* cells are killed within tight vacuoles in *A. polyphaga* trophozoites surrounded by small lysosome-like vesicles, some of which appeared to have fused with the phagosome.

### 7.6 Future Directions

Biofilms, including those found on some surfaces and drains of food processing environments are likely to be niches for a variety of protozoa capable of grazing on bacteria. Consequently, there may be opportunity for a variety of types of interactions
between *L. monocytogenes* and protozoan cells that may result in formation of reservoirs of bacteria. *In vitro* co-culture studies involving *L. monocytogenes* with other protozoa, e.g. other amoebae, flagellates and ciliates need to be done to determine whether or not they could act as environmental reservoir for *L. monocytogenes*. For example, research has shown that one type of amoeba is not able to harbour all types of bacteria; rather a distinct type of amoeba may harbour only a few certain types of bacteria. For example *S. Typhimurium* can survive within *A. polyphaga*, but is eliminated by the soil-borne amoeba, *Dictyostelium discoideum*. Conversely *Legionella* can survive within this amoeba (Skriwan et al., 2002). Therefore, more investigation is required to determine whether other types of amoebae or protozoa can harbour *L. monocytogenes* or act as environmental reservoirs for this intra-cellular pathogen.

As described previously, environmental conditions may also change the outcome of interaction between *L. monocytogenes* with free-living *Acanthamoebae*. For example, plasmid genes could provide bacteria with auxiliary genes, which make them more versatile in physiological capability and as a result become resistant to amoeba killing mechanisms. In such environmental conditions bacteria may play differently once they are phagocytosed by amoeba cells. This hypothesis could be tested using the co-culture of *L. monocytogenes* with different amoebae in media which are mimic real environmental conditions. Co-culture conditions could be manipulated to examine the effects of changes in salt, nutrient compositions, temperature, humidity and presence of other microbial competitors. This may allow a determination of environmental selective pressure for intra-amoebic survival of both amoebae and bacteria.

If other protozoan hosts support the intra-cellular growth/survival of *L. monocytogenes* cells, it would be of interest to investigate the genetic and phenotypic basis that underpin survival in one host vs killing in another such as *A. polyphaga*. Such genetic traits supported the intra-cellular survival of bacteria could be sought in different types of free-living protozoa which can then be co-cultured with bacteria to find out whether they are able to harbour *L. monocytogenes*. This investigation may provide a better insight into the outcome of the environmental interaction between *L. monocytogenes* and free-living protozoa.
One interesting field may be the investigation of bacterial killing mechanisms used by free-living amoebae. Given the fact that the amoeba phagocytosis and killing of bacterial pathogens are shared similar features with mammalian cells, they can be used as an alternative cell culture model to study the mechanisms and regulatory cascades involved in the uptake and killing of bacteria by eukaryotic cells. Further, the fact that free-living amoebae naturally feed on bacteria, they have been evolved over the long period of evolution to acquire maximum capability to uptake and digest different types of bacteria as food nutrients. Thus, the mechanisms employed by amoebae to kill different bacterial cells may provide important information which can be used in designing new drugs to fight the increasing bacterial drug-resistance. In particular, the potential for *Acanthamoeba* spp to elicit antimicrobial peptides capable of lysing bacteria may represent an interesting aspect for investigation. That work could as mentioned previously in this chapter, identify new classes of antimicrobial peptides that may be useful alternatives to existing antibiotics used to combat disease caused by bacterial pathogens.

### 7.7 Conclusions

In conclusion, this study has described some important features of interaction between *L. monocytogenes* cells with free-living *Acanthamoeba* spp. and the role of plasmid-borne genes in intra-cellular survival of this opportunistic pathogen. The uptake and killing mechanisms of *L. monocytogenes* by amoeba trophozoites were also assessed by this work. The main conclusions are as follows:

1. Sequence analysis of *L. monocytogenes* DRDC8 plasmid DNA demonstrated the significant homology of gene organisations and sequence similarities with plasmid genes found in other *Listeria* spp. and Gram-positive bacteria.

2. The plasmid-encoded determinants are not required for intra-cellular survival in mammalian cell culture. Plasmid cured variants grow as well as the wild type parent. Furthermore, the large plasmid is unstable and is lost during serial passage of DRDC8 in HeLa cell culture.

3. *Acanthamoeba* spp. used for co-culture experiments are able to phagocytose and effectively kill pathogenic and non-pathogenic (*hly* mutants) strains of *L. monocytogenes* cells.
4. *L. monocytogenes* cells are not able to survive or replicate within the amoeba trophozoites or cysts.

5. *L. monocytogenes* DRDC8 large plasmid associated genes have no impact on susceptibility to intra-amoebic killing of bacteria.

6. *L. monocytogenes* cells are not able to express virulence genes once confined within phagosome in *Acanthamoeba* trophozoites.

7. The mannose binding protein expressed by some strains of amoebae is not implicated in phagocytosis of *L. monocytogenes* cells by *A. polyphaga* trophozoites.

8. *L. monocytogenes* cells are killed within phagosomes in amoeba trophozoites and both phagosome acidification and phagosome lysosome fusion are involved in the killing process of bacteria.

9. *Acanthamoeba* spp. are not able to harbour *L. monocytogenes* within their cells and consequently are unlikely to act as an environmental reservoir for *L. monocytogenes*, at least under the conditions tested.

10. However, *L. monocytogenes* cells can grow saprophytically on materials released form amoeba trophozoites during encystations or lysis. The potential for saprophytic growth of *L. monocytogenes* in complex multicellular communities may contribute to persistence and survival of the bacteria.