Chapter 5: Co-cultivation of *L. monocytogenes* with other *Acanthamoeba* spp.

### 5.1 Introduction

On the basis of data presented in Chapter 4, *A. polyphaga* is clearly able to phagocytose and kill *L. monocytogenes* within a few hours post co-cultivation. *L. monocytogenes* is unable to evade amoeba killing mechanisms and establish an intra-cellular lifestyle within amoeba trophozoites. In fact, microscopic evidence presented confirmed that bacteria were confined to, and degraded within, amoeba cytoplasmic vacuoles. It is therefore likely that these bacteria have little chance to express virulence genes normally required to escape from the phagosome of mammalian cells. This outcome was the same for several different *L. monocytogenes* strains including environmental and clinical isolates as well as an avirulent variant of strain DRDC8.

It is possible that these observations are peculiar to certain *Acanthamoeba* species or strains. There are published studies that indicate not all *Acanthamoeba* isolates can harbour intra-cellular bacteria (Lamothe *et al.*, 2004). Therefore, to determine whether the ability of *A. polyphaga* to kill *L. monocytogenes* is (Tezcan-Merdol *et al.*, 2004) shared by other *Acanthamoeba* species, co-culture experiments and microscopic studies identical to those described for *A. polyphaga* were conducted using isolates from water and soil, namely *A. castellanii*, *A. lenticulata* and another *Acanthamoeba* sp. These all are free-living amoebae that feed on bacteria and other microorganisms in natural environments. In the case of *A. castellanii*, several bacterial pathogens are known to be able to survive and/or replicate within this type of amoeba (Abd *et al.*, 2003; Abd *et al.*, 2005; Essig *et al.*, 1997).

### 5.2 Results

#### 5.2.1 Isolation and Identification of *Acanthamoeba* spp.

**5.2.1.1 Isolation of Axenic Cultures from Environmental Samples**

*Acanthamoeba* species used in this study were isolated from soil and water samples. These natural, free-living amoebae were initially identified on the basis of cyst morphology e.g. size...
and wall layers (ectocyst and endocyst). Accordingly, they can be divided into three groups I, II and III. Group I has larger cysts (18 µm or more) with stellate endocyst and smooth or wrinkled ectocyst. Group II has smaller cysts (<18 µm) with polyhedric, globular, ovoid, or stellate endocysts and wavy ectocysts, which is the most abundant amoebae in environment and contains species such as *Acanthamoeba castellanii* and *Acanthamoeba polyphaga*. Finally group III has relatively large cysts (<19 µm) with globular or ovoid endocysts and smooth or wavy ectocysts (Schuster and Visvesvara, 2004).

The morphology of amoebae, and in particular *Acanthamoeba* cells, varies according to prevailing environmental conditions, and consequently it is difficult to distinguish *Acanthamoeba* spp. to species level using morphological characteristics alone (Smirnov and Brown, 2004). Nevertheless, most amoebae isolated displayed morphological characteristics typical of *Acanthamoeba* Group II. Moreover, the environmental samples were contaminated with a wide range of environmental micro-organisms, including protozoa, fungi and nematodes. Thus, in order to isolate axenic *Acanthamoeba* spp., they were initially cultured on lawns of *E. coli* cells plated on NNA plates in an X configuration pattern. Over a period of 48 to 72 h, amoeba trophozoites, multiplied and grew outward to the marginal zones of NNA plates that contained no bacteria. In this area of plates, the absence of prey bacterial cells resulted in rapid encystment of amoeba trophozoites.

To isolate and clone particular amoeba cells, a small slice of agar that contained a few isolated amoeba cysts was excised and placed on a fresh NNA spread with a lawn of heat-killed *E. coli* cells. This process was repeated as required to obtain pure cultures of amoebae. Pure cultures (or clones) were finally cultured in PYG medium supplemented with antibiotics and incubation at 22ºC. These procedures resulted in isolation of nine axenic *Acanthamoeba* clones, namely A1, A3, A4, A13, A17, AE, AS2, AC012 and AC238 (see Table 2.3).

### 5.2.1.2 18S rDNA analysis of Axenic Cultures of *Acanthamoeba* spp.

The identity of cloned cultures of *Acanthamoeba* spp. were confirmed by sequence analysis of PCR amplified DNA encoding a variable part of 18S rRNA (400 to 500 bp) of amoeba genomic DNA. *A. polyphaga* AC012 was included as a control. The PCR products were sequenced and resultant sequences were subjected to BLAST nucleotide comparison analysis to find the best matches in GenBank Nucleotide Database (Table 2.3).
BLAST analysis of *Acanthamoeba* ACO12 18S rDNA sequence revealed that it was highly similar to 18s ribosomal RNA sequences reported for a number of *A. polyphaga* strains that have previously been lodged in the GenBank Nucleotide Database. The three most similar matches were: *A. polyphaga* strain OX-1 (GenBank Accession AF019051), *A. polyphaga* strain ATCC30872 (GenBank Accession AY026244) and *Acanthamoeba* sp. S7 (GenBank Accession DQ087289) with similarity of 334/340 (98%), 329/340 (96%) and 331/343 (96%) respectively. Given these highly sequence similarities, AC012 was classified as an *A. polyphaga* isolate. This free-living amoeba is a common resident of soil and water and naturally feeds on bacteria or other small single-celled organisms (Schuster, 2002). A number of bacterial pathogens are capable of surviving or multiplying within *A. polyphaga* cells *in vitro* (Gaze *et al.*, 2003; Lamothe *et al.*, 2004; Landers *et al.*, 2000).

BLAST analysis of sequence data obtained from isolate AC328 showed a high similarity with a number of known *Acanthamoeba* strains. The three of most significant matches (100% similarity over 399 bases) for AC328 were *A. castellanii* ATCC 50374 (GenBank Accession U07413), *Acanthamoeba* sp. S9 (GenBank Accession DQ087304) and *Acanthamoeba* sp. ATCC 30868 (GenBank Accession AY549558). Given the identity of sequence data, AC328 was confirmed as *Acanthamoeba* and is likely to be *A. castellanii*.

BLAST analysis of sequence data for isolate AE revealed that it also shared a high sequence similarity with *A. castellanii* (GenBank Accession AF114438), *A. castellanii* Neff ATCC 50373 (GenBank Accession ACU07416), and *A. castellanii* strain ATCC 50373 (GenBank Accession AF534137.1) with 406/416 bp (97%), 406/416 bp (97%) and 403/413 bp (97%) nucleotide matches respectively. *A. castellanii* is a common water and soil amoeba and widely spread in environment. It naturally feeds on bacteria and other micro-organisms. This amoeba has been shown to be capable of harbouring bacterial pathogens (La Scola and Raoult, 2001).

Nucleotide sequence alignments indicated that A1, A4, A17 and AS2 sequences were either identical or shared high sequence similarities. Sequence data for isolate A1 was 99% similar to A4 (shared 372/373 bp), and identical to that obtained for isolate A17; 98% similar to AS2 (369/375 bp). Sequence data for isolate AS2 was 98% similar to that of A17 (372/378 bp), AS2 sequence was 98% similar to A4 (371/378 bp), and A17 sequence 99% similar with that of A4 (375/376 bp). Nucleotide BLAST analysis demonstrated that these isolates are shared high sequence similarity with known ribosomal RNA gene sequences for a number of *A. lenticulata*
strains. The three most similar sequences were for: PD2S (GenBank Accession U94741) *A. lenticulata* strain 25/1 (U94740) and *A. lenticulata* strain NJSP-3-2 (U94738) all with (99%; 373/374 nucleotide matches) sequence similarity. These high sequence similarities indicated these isolates were likely to be *A. lenticulata*. *A. lenticulata* is also widely spread in soil and water.

For A13 and A2 isolates no exact matches to specific *Acanthamoeba* species were found to entries in the GenBank Nucleotide Database using BLASTn comparisons. However, the 18S rDNA sequences shared high sequence similarity with sequences for a number of *Acanthamoeba* spp. The three most similar sequence matches for A2 isolate were: *Acanthamoeba* sp. CDC V390 (GenBank Accession AY703004), *Acanthamoeba* sp. CDC V388 (GenBank Accession AY703003) and *Acanthamoeba* sp. CDC V382 (GenBank Accession AY703002) all with 415/416 (99%) nucleotide matches. A13 shared high sequence similarity with sequences for *Acanthamoeba* sp. MA47 (GenBank Accession EF050504), *Acanthamoeba* sp. MA43 (GenBank Accession EF050502) and *Acanthamoeba* sp. EGM5 (GenBank Accession EF050492) with 332/342 (97%), 332/342 (97%) and 324/333 (97%) nucleotide matches respectively.

To provide additional visual confirmation of the identity of 18S rDNA sequences with that of known cultures (*A. polyphaga* ATCC30872, *A. lenticulata* PD2S and *A. castellanii* ATCC50374), the nucleotide sequence data was aligned using ClustalX (Thompson *et al*., 1997) (Appendix B) and the aligned data used as input for the molecular evolutionary genetics analysis package, Mega V3.1 (Kumar *et al*., 2004). Nucleotide sequences for *A. polyphaga* ATCC30872, *A. lenticulata* PD2S and *A. castellanii* ATCC50374 were included in this analysis as control sequence data. The phylogenetic affiliation of all sequences was assessed by examination of a dendrogram constructed from a pairwise distance matrix prepared using a UPGMA method and a Kimura 2-parameter nucleotide analysis model (Figure 5.1). Bootstrap values were derived from 500 sampling events of the aligned sequence data. Notably, with the exception of A13, all sequence data clustered into three separate groups that corresponded with the proposed identities (Figure 5.1). Examination of the dendrogram clearly shows that the 18S rDNA sequence of isolates A1, A4, A17 and AS2 are very similar to that of *A. lenticulata* PD2S (GenBank Accession U94741). Similarly, the 18S rDNA sequence of isolates A2, AA2 and AE are very similar to that of *A. castellanii* strain ATCC50374 (GenBank Accession U07413) and the sequence data for *A. polyphaga* AC012 is similar to that of *A. polyphaga* strain ATCC30872.
(GenBank Accession AY026244). The sequence data for A13 was different from each of the three primary groups of isolates described, but most similar to *A. castellanii*.

### 5.2.2 Growth of *A. castellanii* and *A. lenticulata* on Lawns of *L. monocytogenes*

*A. castellanii* strain AC328 and *A. lenticulata* strain AS2 were used individually for co-cultures on plates of NNA with *L. monocytogenes* strain DRDC8 and non-virulent bacterium *E. coli* DH5α. Inoculated plates were incubated for up to 3 d at 22, 30, and 37°C. At daily intervals, each co-culture plate was examined and the diameter of amoeba growth zones measured as previously described for *A. polyphaga*.

Figure 5.2 presents the growth zone data obtained for co-cultures with *A. castellanii* AC328. *A. castellanii* did not grow significantly when co-cultured at 37°C, but did grow during co-culture at 22 and 30°C. Compared with *A. polyphaga* (see Chapter 4.3.2.1), this strain of amoeba grew slowly when co-cultured with either *E. coli* or *L. monocytogenes* at 22 and 30°C. Even after 3 d of co-culture *A. castellanii* cells only covered 1/3 to 1/2 of the NNA plate surface, whereas the growth zone of *A. polyphaga* AC012 covered 100% of the available plate area. However, this amoeba grew marginally faster when co-cultured on *E. coli* when compared co-culture on *L. monocytogenes*. The morphological characteristics of *A. castellanii* trophozoites and cysts when co-cultured with *L. monocytogenes* and *E. coli* were similar. No evidence for bacterial growth on plates, or the presence of amoeba cell debris, was obtained and this was taken to indicate that the bacterial cells did not kill amoebae or grow on materials released from amoeba trophozoites.

By comparison with *A. castellanii* AC328, *A. lenticulata* AS2 grew well when co-cultured with either *L. monocytogenes* DRDC8 or *E. coli* DH5α at 22, 30 and 37°C (Figure 5.3). After 3 d of co-cultivation, *A. lenticulata* cells covered almost the entire surface of the co-culture plates. However, unlike *A. castellanii* and *A. polyphaga* AC012, these amoeba cells grew more rapidly when co-cultured at 37°C than at 30 or 22°C. As was the case for *A. polyphaga* and *A. castellanii*, *A. lenticulata* trophozoites grew marginally faster when co-cultured on *E. coli* cells compared with growth on *L. monocytogenes* DRDC8. Furthermore, like *A. polyphaga*, *A. lenticulata* trophozoites tended to encyst earlier at 37°C than when co-cultured at 22°C and 30°C. In addition, no evidence for killing of *A. lenticulata* by bacterial cells was obtained. The morphological characteristics of amoeba trophozoites grown on *E. coli* and *L. monocytogenes* at
each of the three incubation temperatures seemed similar. Neither enlargement of amoeba cytoplasmic vacuoles nor lysis of amoeba trophozoites on plates was observed.

5.2.3 Co-cultivation of *L. monocytogenes* with Monolayers of *A. lenticulata* and *A. castellanii*

Co-culture of *A. lenticulata* and *A. castellanii* on lawns of bacteria provided good preliminary data to indicate that these amoebae were capable of grazing on bacterial cells and used them as a source of nutrients. To provide quantitative information on the interaction, monolayers of *A. lenticulata* and *A. castellanii* in 24 well trays were infected with *L. monocytogenes* DRDC8. The trays were incubated at 22°C. To prevent early encystation and starvation stress of amoeba trophozoites, heat-killed *E. coli* cells were added daily as described (see Chapter 2.7.2). At regular intervals during co-culture, counts of bacteria (total and intra-amoebic), amoebae and percent cysts were determined as described for *A. polyphaga*.

5.2.3.1 Co-culture of *A. lenticulata* AS2 and *L. monocytogenes*

Data obtained for *A. lenticulata* AS2 is presented in Figure 5.4a, b. Counts of total and intra-amoebic bacteria declined during co-culture. However, the reduction in counts of intra-amoebic bacteria was more significant, and counts of bacteria changed from ca. 10^5 CFU.mL^-1 to ca. 10^3 CFU.mL^-1 over 96 h of co-culture. By contrast, estimates of the total numbers of bacteria (intra-amoebic plus extra attached bacteria) reduced by only 10 fold. These findings suggested that *A. lenticulata* AS2, like *A. polyphaga* AC012, phagocytosed *L. monocytogenes* cells and killed these internalised bacteria. Counts of amoeba cells fluctuated only slightly over the duration of the experiment (Figure 5.4b). As expected, a proportion of amoeba trophozoites gradually encysted, and by the end of the experiment, about 45% of all amoeba cells were present as cysts.

5.2.3.2 Co-culture of *A. castellanii* AC328 and *L. monocytogenes*

When *A. castellanii* AC328 was co-cultured with *L. monocytogenes* over a period of 72 h, the progression of changes in counts of bacteria and amoebae was similar to that reported for *A. lenticulata* (Figure 5.5a, b compared with Figure 5.4b). However, counts of intra-amoebic bacteria decreased from ca. 10^4 CFU.mL^-1 by only 10 to 15 fold compared with the 100 fold decrease obtained with *A. lenticulata* (Figure 5.5a). Furthermore, counts of total bacteria increased by 10 fold over the period of co-culture. This result indicated extra-amoebic bacteria
grew quite well on nutrients released from amoeba cells or grew on heat killed *E. coli* cells routinely added to the co-culture as a food source for amoebae to prevent early encystation. Further, the lower rate of reduction of intra-amoebic bacteria compared with that obtained for *A. lenticulata* and *A. polyphaga* AC012 corresponded with the slower rate of growth observed when this amoeba was co-cultured with *L. monocytogenes* on the surface of NNA plates. Counts of amoeba cells declined by *ca.* 40% over the entire period of co-culture (Figure 5.5b). Only about 7% of all amoeba cells were present as cysts at the end of co-culture.

### 5.2.4 Prolonged Co-cultures with *Acanthamoeba* spp.

Co-culture experiments with *A. lenticulata* AS2 and *A. castellanii* AC328 described in Section 5.2.3, indicated that *L. monocytogenes* was not capable of growth or survival within either type of amoebae during short term co-culture (up to 96 h). To determine the outcome of co-cultures over prolonged periods (up to 20 d), *A. lenticulata* AS2, *A. castellanii* AC328 and *Acanthamoeba* sp. strain A2 were co-cultured with *L. monocytogenes* DRDC8 and the avirulent, *hly* variant, LLO17, in 75 cm² flasks for 20 d at 30°C, as previously described for *A. polyphaga*. As controls, *L. monocytogenes* cells were also cultured in AS and/or AS plus ACM in the absence of amoebae.

#### 5.2.4.1 Co-culture of *A. lenticulata* AS2 and *L. monocytogenes*

Counts of bacteria, amoebae and percent of amoebae present as cysts for co-culture experiments with *A. lenticulata* are shown in Figure 5.6a, b. Counts of both DRDC8 and LLO17 decreased from *ca.* $10^7$ CFU.mL⁻¹ to *ca.* $10^3$ CFU.mL⁻¹ over the first 5 to 6 d of co-cultivation, after which, only marginal decreases in cell counts were observed (Figure 5.6a). Counts obtained for DRDC8 and LLO17 were similar at all stages of co-culture. There was no obvious difference between counts of bacteria obtained for control cultures and co-cultures with amoeba cells, an outcome different to that observed for *A. polyphaga* (see Chapter 4.3.2.3). Counts of amoeba cells and the percent of amoeba present as cysts increased slightly over the first 2 d of co-culture, but then declined from *ca.* $2.4 \times 10^3$ cells.mL⁻¹ to less than $6 \times 10^2$ cells.mL⁻¹ after 20 d (Figure 5.6b). Furthermore, counts of amoeba cells in AS buffer alone were similar to those in co-culture. Amoeba trophozoites formed cysts at all stages of co-culture. After 8 d of co-culture, 30 to 40 percent of amoebae were present as cysts, and by day 10, this had increased to more than 80%.
This data indicated that *A. lenticulata* AS2 trophozoites did not actively reduce the counts of viable bacteria used for co-culture. One explanation for this unexpected observation was that the viability of bacterial cells was sustained by nutrients released from amoeba cells which compensated the bacterial elimination. The contention that *A. lenticulata* did not actively feed on *L. monocytogenes* is supported by the fact that trophozoites numbers during co-culture with bacteria were similar to those observed for identical suspensions of control preparations of amoeba cells (in the non-nutrient containing AS buffer). Furthermore, trophozoites underwent early encystation (Figure 5.6b) during co-culture and this fact suggested that trophozoites were under nutrient/physiological stress.

5.2.4.2 Co-culture of *A. castellanii* AC328 and *L. monocytogenes*

Counts of bacteria, amoeba and percent cysts during co-culture and when bacteria and amoeba were separately suspended in AS buffer are presented in Figure 5.7a, b. In general counts of bacteria in co-culture with amoeba declined less rapidly than when the bacteria were suspended in ACM alone and no differences between counts of DRDC8 and LLO17 were observed. Furthermore, the rate of change for bacterial counts in co-culture was similar to that observed for co-culture with *A. lenticulata* AS2. After 20 d of co-culture, counts of bacteria declined from ca. $10^7$ CFU.mL$^{-1}$ to between $10^2$ and $10^3$ CFU.mL$^{-1}$ (Figure 5.7a).

Counts of amoeba cells suspended in AS buffer or in co-culture with bacterial cells were almost identical throughout the 20 d duration of the experiment (Figure 5.7b). The proportion of amoeba present as cysts increased from ca. 10% after 8 d to ca. 100% after 20 d. This result supported the above result for the less reduction in the number of bacteria, which indicated amoeba trophozoites did not effectively phagocytose and kill bacteria.

Identical experiments conducted for the interaction of *A. castellanii* during co-culture with a clinical isolate of *L. monocytogenes* (KE504) (Figure 5.8a, b) produced identical results to that obtained for *L. monocytogenes* strain DRDC8. Counts of amoebae and the percent of amoeba present as cysts in co-cultures with DRDC8 and KE504 were similar (Figure 5.8b).

Consequently, *A. castellanii* AC328, like *A. lenticulata* AS2, was unable to reduce the number of bacteria during co-culture at a rate different to the observed decline in counts of bacteria suspended in ACM alone. This conclusion is supported by the fact that numbers of
amoeba did not increase during co-culture but instead declined to low levels over the first 10 to 12 d of co-culture at a rate similar to that observed for amoeba suspended in AS buffer alone.

5.2.4.3 Co-culture of *Acanthamoeba* sp. A2 and *L. monocytogenes*

Trends of counts of bacteria, amoeba and percent of amoeba present as cysts during co-culture of *Acanthamoeba* sp. strain A2 were similar to those obtained for *A. lenticulata* AS2. Counts of DRDC8 and LLO17 decreased from ca. $10^7$ CFU.mL$^{-1}$ to $<10^3$ CFU.mL$^{-1}$ during the first 6 to 8 d of co-culture (Figure 5.9a). No significant differences in counts of DRDC8 and LLO17 were observed over the entire period of co-culture.

Counts of amoeba declined from ca. $1.8 \times 10^3$ to ca. $1.2 \times 10^3$ cells.mL$^{-1}$ over the 20 d co-culture (Figure 5.9b). The observed reduction in counts of amoebae over the entire co-culture period was smaller compared to that observed for other co-cultures with different amoebae. Interestingly, almost 80% of all amoebae were present as cysts by the first 4 d of co-culture.

5.2.5 Microscopy of *Acanthamoeba* Infected with *L. monocytogenes*

Co-culture experiments described above, provided information that implied both *A. castellanii* AC328 and *A. lenticulata* AA2 were capable of feeding on *L. monocytogenes* cells, albeit slowly by comparison with *A. polyphaga* AC012 (see Chapter 4.3.2). To examine the cell – cell interactions between *L. monocytogenes* and *A. lenticulata* and *A. castellanii* during co-culture, monolayers of infected amoebae were examined by immuno-fluorescent microscopy and TEM. In particular, the aim of this work was to provide more information about the cellular interactions over the first few hours of co-culture.

5.2.5.1 Fluorescence Microscopy of *A. lenticulata* and *A. castellanii*

Micrographs of *A. lenticulata* cells as monolayers after infection with *L. monocytogenes* DRDC8 cells are shown in Figure 5.10. Immediately after infection of the amoeba, few immuno-labelled bacteria were observed within the amoeba cells (Figure 5.10a, b). This outcome differed significantly from that obtained for *A. polyphaga* AC012. After 1 h and 3 h of co-culture, only a small proportion of amoeba contained immuno-labelled bacteria. With longer co-culture, no intact immuno-labelled intra-amoebic bacteria were observed (Figure 5.10e, f) and the majority
of amoeba cells were present as cysts. During extended co-culture, immuno-labelled bacteria were only found associated with the surface of amoeba cells.

Similar results were obtained for *A. castellanii*. Trophozoites contained labelled intra-amoebic bacteria 1 to 2 h post infection (Figure 5.11). However, no labelled bacterial cells were observed within amoeba cells after 3 to 4 h of co-culture. With extended co-culture, the majority of amoeba cells were present as cysts.

### 5.2.5.2 TEM of *A. lenticulata* co-cultured with *L. monocytogenes* DRDC8

Although immuno-fluorescence microscopy of infected amoeba monolayers showed that *L. monocytogenes* cells were internalised by *A. castellanii* and *A. lenticulata* trophozoites and degraded after 3 to 5 h co-culture, detailed information about the interaction at the sub-cellular was not obtained. To fill this data gap, sections of infected amoeba cells were examined by TEM. Micrographs of thin sections of amoeba cells infected with *A. lenticulata* are shown in Figure 5.12.

Bacterial cells were not found in any section of uninfected control *A. lenticulata* cells (Figure 5.12a). However, in infected amoeba cells, bacterial cells were found located within tight vacuoles in thin sections of amoeba after 2 h of co-culture (Figure 5.12b, c). Bacterial cells within vacuoles had identifiable cell walls and were apparently intact. After 4 to 5 h co-culture, almost all sections of bacteria located within vacuoles displayed loss of cell wall structures. Sections of vacuoles that contained bacteria were surrounded by mitochondria and lysosome-like vesicles, some of which appeared to have merged with adjacent phagosomal vacuoles. No evidence of long term bacterial survival or multiplication within phagosomal vacuoles or within the cytoplasm amoeba trophozoites was obtained. The sub-cellular features of infected amoeba cells were similar to those described for *A. polyphaga* (see Chapter 4.3.2.5.2).

### 5.3 Discussion

All *A. castellanii*, *Acanthamoeba* sp. and *A. lenticulata* are recognized as free-living amoebae found in soil and water. As these amoebae rely on phagocytosis of other cellular organisms to provide the necessary nutrients for growth and reproduction, they could potentially act as reservoirs for facultative intra-cellular bacterial pathogens. Interestingly, various Gram-negative bacterial pathogens e.g. *Vibrio cholerae*, *Legionella pneumophila*, acid fast bacterium...
Mycobacterium avium, and Chlamydia pneumoniae, (Abd et al., 2005; Bozue and Johnson, 1996; Cirillo et al., 1997; Essig et al., 1997; La Scola and Raoult, 2001; Moffat and Tompkins, 1992) are known to be able to co-exist within various types of amoebae. Chlamydia and several Gram-negative bacteria have also been isolated from naturally occurring Acanthamoeba sp. (Fritsche et al., 1993). However, in general, information describing the interaction of Gram-positive bacteria with amoebae has not been widely reported, although (Weekers et al., 1995) has shown that Gram-positive bacteria are more sensitive to the bacteriolytic activity of A. polyphaga and A. castellani than Gram-negative bacteria. Nevertheless, not all species of amoeba are able to harbour bacteria (Marolda et al., 1999). For this reason, work described in this thesis chapter was designed to study the interaction of Acanthamoeba spp. other than A. polyphaga with L. monocytogenes during co-culture.

As was reported for co-cultures using A. polyphaga AC012 in Chapter 4, co-culture experiments with A. lenticulata and A. castellani as well as Acanthamoeba sp. AA2, showed that L. monocytogenes cells could not survive more than a few hours within phagosomes of these amoebae. That conclusion is supported by light and transmission electron microscopic examination of infected amoebae at different stages of co-culture. In fact, TEM of thin sections of amoeba cells containing L. monocytogenes clearly showed that the bacterial cells were rapidly degraded within phagosomes and apparently never had an opportunity to grow and multiply either within the phagocytic vacuoles or the host amoeba cytoplasm.

Although the three types of Acanthamoeba used in co-cultures described in this chapter, were able to kill L. monocytogenes, the rates of killing were different to that observed for A. polyphaga AC012. For example A. polyphaga AC012 and A. lenticulata AS2 actively phagocytosed and killed L. monocytogenes cells, but A. castellani AC328 in particular, differed in that it did not grow well on lawns of either L. monocytogenes or E. coli cells at either 30 or 37°C. This observation suggested that A. castellani AC328 killed L. monocytogenes cells more slowly than either A. lenticulata AS2 or A. polyphaga AC012. Whether this is the case for other types of bacteria remains to be determined. A. lenticulata AS2 and Acanthamoeba AA2 also did not predate and kill L. monocytogenes as actively as A. polyphaga AC012. Furthermore, prolonged co-culture of each of the three Acanthamoeba strains used did not result in growth of amoeba populations – in fact the trophozoites used for those experiments rapidly converted to the cyst form. Hence it is likely that none of the three strains described was as predatory for
L. monocytogenes cells as A. polyphaga AC012. On the basis of the different results obtained for co-cultures, it is reasonable to conclude that different Acanthamoeba spp. respond differently during co-culture with L. monocytogenes – a result that is in agreement with observations published by (Marolda et al., 1999).

It is significant that the observed rate of decline in counts of L. monocytogenes cells during co-culture was not significantly different from that observed for identical populations of L. monocytogenes suspended in ACM. Thus although the amoeba may predate and kill bacterial cells at a low level, survival of extra-amoebic L. monocytogenes cells may be more significant. Data shown indicated that L. monocytogenes cells may survive saprophytically by utilizing nutrients released by amoeba cells during co-culture. Indeed this is a result that may have implications for the persistence of this pathogen in adverse environmental conditions.

As reported in Chapter 4 of this thesis, the outcomes of co-cultures of L. monocytogenes and the three Acanthamoeba spp. used are in stark contrast with results reported by Ly and Müller (1990). The contradictory conclusions may reflect differences in strains of amoeba and the approaches used to evaluate the outcomes of co-cultivation experiments. Ly and Müller (1990) used an Acanthamoeba sp. isolated from tap water, whereas work described in this thesis used Acanthamoeba isolated from pond waters as well as a laboratory strain of A. polyphaga. Further they used only one strain of L. monocytogenes which was isolated from tap water, whereas the present study used several environmental and clinical isolates of L. monocytogenes in co-culture with several isolates of Acanthamoeba spp. Bacterial and amoeba cells were co-cultured in tap water as opposed to the AS buffer used in this study. Clearly from the results of work described in Chapters 4 and 5 of this thesis, the viability of L. monocytogenes varies significantly depending on the suspending medium used. For example, important differences in viability were noted when these bacterial cells were suspended in AS buffer and ACM (see Chapter 4.3.2.3). In fact the bacterial cells were able to survive in ACM. Furthermore, although the Ly and Müller (1990) study reported the use of fluorescence microscopy of infected amoebae to demonstrate presence of intra-cellular bacteria, the method used was not described nor were micrographs published. Consequently a direct comparison with results shown in this thesis is not possible. Another important difference between the two studies involves the different co-culture methods used. Ly and Müller (1990) used batch co-culture methods only whereas the present work deployed quantitative assays based on infection of monolayers of amoeba that allowed
simultaneous microbiological and microscopic analysis. Furthermore, although they isolated both *Acanthamoeba* sp. and *L. monocytogenes* strain form tap water, they incubated co-culture medium at 36°C which would not commonly represent the environmental temperature for water.

**5.4 Conclusions**

This study has described the important features of *L. monocytogenes* interaction with several free-living *Acanthamoeba* spp. The results indicate that, at least under *in vitro* conditions used, amoebae effectively kill *L. monocytogenes*, although with different rates, irrespective of virulence genes and large-plasmid associated genes. However, *L. monocytogenes* cells clearly grow on materials released from amoeba cells during encystations, lysis and growth. This may render bacteria more able to survive in different environmental conditions.
Figure 5.1: Phylogenetic affiliation of 18S rDNA sequence data from *Acanthamoeba* isolates.

Sequence data was aligned with ClustalX and the aligned sequence used as input for the molecular evolutionary genetics analysis package, Mega V3.1. The dendrogram was constructed from a pairwise distance matrix by using a UPGMA method and a Kimura 2-parameter nucleotide model. The scale for the tree indicates inferred evolutionary distance. Bootstrap values were derived from 500 sampling events of the aligned sequence data. Identities of isolates are inferred by similarity to sequences of reference isolates (shown in blue type).
Figure 5.2: Growth of A. castellanii AC328 on lawns of L. monocytogenes and E. coli DH5α at 22, 30 or 37°C.

Growth of A. castellanii is reported as the diameter of amoeba growth zones on co-culture plates. Data are the mean of three independent observations.

Panel A: Incubation at 22°C, Panel B: Incubation at 30°C, Panel C: Incubation at 37°C.
Figure 5.3: Growth of *A. lenticulata* AS2 on lawns of *L. monocytogenes* DRDC8 and *E. coli* DH5α at 22, 30 or 37°C.

Growth of *A. lenticulata* is reported as the diameter of amoeba growth zones on co-culture plates. Data shown are the mean of three observations.

Panel A: Incubation at 22°C, Panel B: Incubation at 30°C, Panel C: Incubation at 37°C.
Figure 5.4:  Counts of *L. monocytogenes* and *A. lenticulata* during co-culture at 22°C.

Monolayers of *A. lenticulata* AS2 cells in wells of tissue culture trays were infected with *L. monocytogenes* DRDC8 (MOI = 100 bacterial cells per amoeba cell) followed by incubation at 22°C for 1 to 2 h. After removal of extra-amoebic bacteria by washing with AS buffer, the infected amoebae were incubated at 22°C for up to 92 h. Each monolayer preparation was processed to determine the total viable count of bacteria, numbers of amoebae and percentage of amoeba cysts. Counts of amoeba cells and cysts were carried out using hemocytometer to determine numbers of amoebae per mL culture and percent cysts respectively. Counts of bacteria were determined by plating on BHI medium. Results shown are the mean of 3 replicates. Error bars represent the standard deviation about the mean counts obtained.

Panel A:  Counts of intra- and extra-amoebic bacteria.

Panel B:  Counts of amoebae and percent cysts.
Intra-amoebic bacteria

Total number of bacteria

Duration of co-culture (h)

Amoebae mL⁻¹

Percent cysts

Number of amoebae

Percent cysts

CFU mL⁻¹
Figure 5.5: Counts of *L. monocytogenes* and *A. castellanii* during co-culture at 22°C.

Monolayers of *A. castellanii* AC328 cells in wells of tissue culture trays were infected with *L. monocytogenes* DRDC8 (MOI = 100) followed by incubation at 22°C for 1 to 2 h. After removal of extra-amoebic bacteria by washing with AS buffer, the infected amoebae were incubated at 22°C for up to 72 h. Each monolayer preparation was processed to determine the Total viable count of bacteria, numbers of amoebae and percentage of amoeba cysts. Counts of amoeba cells and cysts were carried out using hemocytometer to determine numbers of amoebae per mL culture and percent cysts respectively. Counts of bacteria were determined by plating on BHI medium. Results shown are the mean of 3 replicates. Error bars represent the standard deviation about the mean counts and mean percent cysts obtained.

Panel A: Counts of intra-amoebic bacteria and total number (intra- and extra-amoebic) of bacteria.

Panel B: Counts of amoebae and percent cysts.
A
Intra-amoebic bacteria
Total number of bacteria

B
Number of amoebae
Percent cysts

Duration of co-culture (h)
Figure 5.6: Counts of *L. monocytogenes* and *A. lenticulata* during co-culture at 30 °C.

*L. monocytogenes* DRDC8 and the avirulent variant LLO17 were cultured in flasks containing 25 mL AS buffer and *A. lenticulata* AS2 trophozoites at MOI of 1000 bacteria per amoeba cell. Identical suspensions of bacteria were also cultured in AS buffer and AS buffer plus ACM as controls. Counts of viable bacteria were determined by plating on BHI agar. Amoebae were also cultured in AS buffer as a control. Counts of amoeba cells and cysts in 10 to 20 microscopic fields (100 times magnification) was used to determine numbers of amoebae per mL culture and percent cysts respectively.

Panel A: Counts of viable *L. monocytogenes* DRDC8 and LLO17 during co-culture. The viability of suspensions of each strain of bacterium in AS buffer alone was used as a control. Counts shown are the mean of 3 replicates. Error bars represent the standard deviation about the mean counts of bacteria.

Panel B: Mean counts of amoeba cells and percent cysts during co-culture with *L. monocytogenes* strains DRDC8 and LLO17. Suspensions of amoebae in AS buffer were used as a control. Error bars represent the standard deviation about the mean counts of trophozoites and percent cysts. Closed symbols represent the number of amoeba cells and open symbols represents the percent of encysted amoebae.
Figure 5.7: Counts of *L. monocytogenes* and *A. castellanii* during co-cultivation at 22ºC.

*L. monocytogenes* DRDC8 and the avirulent variant LLO17 were added to tissue culture flasks containing 25 mL AS buffer and *A. castellanii* AC328 trophozoites at a MOI of 1000 bacteria per amoeba cell. Identical suspensions of bacteria were also cultured in AS buffer plus ACM as a control. Amoebae were also cultured in AS buffer as control. Counts of bacteria were determined by plating samples on BHI agar. Counts of amoeba cells and cysts in 10 to 20 microscopic fields (100 times magnification) was used to determine numbers of amoebae per mL culture and percent cysts respectively.

Panel A: Counts of viable *L. monocytogenes* DRDC8 and LLO17 during co-culture. The viability of suspensions of each strain of bacterium in AS buffer alone was used as a control. Counts shown are the mean of 3 replicates. Error bars represent the standard deviation about the mean counts of bacteria.

Panel B: Mean counts of amoeba cells and percent cysts during co-culture with *L. monocytogenes* strains DRDC8 and LLO17. Suspensions of amoebae in AS buffer were used as a control. Error bars represent the standard deviation about the mean counts of trophozoites and percent cysts. Closed symbols represent the number of amoeba cells and open symbols represents the percent of encysted amoebae.
Figure 5.8: Counts of *L. monocytogenes* KE504 and *A. castellanii* during co-cultivation at 22°C.

*L. monocytogenes* strains DRDC8 and KE504 were added to flasks containing 25 mL AS buffer and *A. castellanii* AC328 trophozoites at a MOI of 1000 bacteria per amoeba cell. Counts of viable bacteria were determined by plating on BHI agar. Counts of amoeba cells and cysts in 10 microscopic fields (100 times magnification) was used to determine numbers of amoebae per mL culture and percent cysts respectively.

**Panel A:** Counts of viable *L. monocytogenes* during co-culture. Counts shown are the mean of 3 replicates. Error bars represent the standard deviation about the mean counts of bacteria.

**Panel B:** Mean counts of amoeba cells and percent cysts during co-culture with *L. monocytogenes* strains. Error bars represent the standard deviation about the mean counts of trophozoites and percent cysts. Closed symbols represent the number of amoeba cells and open symbols represents the percent of encysted amoebae.
Figure 5.9: Counts of L. monocytogenes AAC1 and Acanthamoeba A2 during co-cultivation at 22°C.

L. monocytogenes DRDC8 and plasmid cured AAC1 were added to flasks containing 25 mL AS buffer and Acanthamoeba sp. strain A2 trophozoites at a MOI of 1000 bacteria per amoeba cell. Counts of viable bacteria were determined by plating on BHI agar. Counts of amoeba cells and cysts in 10 – 20 microscopic fields (100 times magnification) was used to determine numbers of amoebae per mL culture and percent cysts respectively.

Panel A: Counts of viable L. monocytogenes during co-culture. Counts shown are the mean of 3 replicates. Error bars represent the standard deviation about the mean counts of bacteria.

Panel B: Mean counts of amoeba cells and percent cysts during co-culture with L. monocytogenes strains. Error bars represent the standard deviation about the mean counts of trophozoites and percent cysts. Closed symbols represent the number of amoeba cells and open symbols represents the percent of encysted amoebae.
Figure 5.10: Localisation and survival of *L. monocytogenes* within *A. lenticulata* AA2 at 22°C.

Monolayers of *A. lenticulata* strain AA2 were infected with *L. monocytogenes* DRDC8 at an MOI of 100. After washing to remove extra-amoebic bacteria, the preparations were incubated at 22°C for 0, 1, 4, 19 and 28 h. Monolayers were fixed and FITC immuno-labelled using antibodies specific for bacterial cells. Immuno-labelled preparations were examined with a fluorescent microscope. Bar marker=20 µm.

Panel A: Time = 0 h. Labelled bacterial cells are attached to the cell surface of amoebae and some have been phagocytosed by amoeba cells.

Panel B: As for Panel A except at higher magnification. The amoeba cell shown contains a large number of immuno-labelled bacterial cells.

Panel C: Time = 1 h. Note the lower density of immuno-labelled bacterial cells within amoeba compared with that shown in Panel B.

Panel D: Time = 4 h. Amoeba cells typically contain only a few immuno-labelled bacterial cells.

Panel E: Time = 19 h. Majority of amoeba cells are free of immuno-labelled bacterial cells. By 19 h incubation, a significant number of amoeba cells had commenced encystation.

Panel F: Time = 28 h. Most amoeba cells have commenced encystation.
Monolayers of *A. castellanii* strain AC328 on coverslips in were infected with *L. monocytogenes* DRDC8 at an MOI of 100. After removal of extra-amoebic bacteria (Time = 0 h), the monolayer preparations were incubated at 22°C for 0, 1, 3, 19 and 20 h post washing. Monolayers were fixed and FITC immuno-labelled using antibodies specific for bacterial cells. Immuno-labelled preparations were examined with a fluorescent microscope. Bar = 20 µm:

**Panel A**: Time = 0 h. Note the stained bacteria attached to amoebae surfaces as well as those phagocytosed by amoeba cells.

**Panel B**: Time = 0 h. High magnification view of stained bacteria attached to amoeba cells. Less bacteria were internalised by this amoeba in comparison to other amoebae.

**Panel C**: Time = 1 h. Most labelled bacteria have been phagocytosed by amoeba cells.

**Panel D**: Time = 3 h. Surface associated and phagocytosed bacteria.

**Panel E**: Time = 19 h. Labelled bacteria attached to the surface of amoeba cells undergoing encystations. Usually amoeba undergoing encystation contained few if any labelled internalised bacterial cells.

**Panel F**: Time = 22 h. Labelled bacterial cells associated with the surface of amoebae undergoing encystation.
Figure 5.12: TEM of *L. monocytogenes* within vacuoles of *A. lenticulata* AA2.

Monolayers of *A. lenticulata* strain AA2 were infected with *L. monocytogenes* DRDC8 cells at an MOI of 50 bacteria per amoeba cell and incubated at 22°C for 1 h. Extra-amoebic bacteria were removed by washing, followed by incubation at 22°C for 0, 1, 2, and 4 h. Infected amoeba cells were prepared for examination by TEM.

Panel A: Control, uninfected amoeba cell at t = 2h. Note the large number of vacuolar structures within the cytoplasm of the amoeba cell shown.

Panel B: An infected amoeba cell after 2 h incubation. The micrograph of the cell shows several bacterial cells (B) located within vacuoles.

Panel C: A bacterial cell (B) located within a vacuole in an amoeba cell after 2h incubation. Note the intact cell wall (CW) and the vacuolar membrane (M).

Panel D: An infected amoeba cell after 4 h incubation. Note the bacterial cells (B) located within vacuole structures and the loss of bacterial cell wall structure compared with that shown in Panel C.

Panel E: A bacterial cell (B) within a vacuole. Note the distinct loss of bacterial cell wall integrity. Also note the cluster of lysosome-like structures clustered around the vacuole shown and in various stages of fusion with the vacuolar membrane.

Panel F: As for Panel E.