

ROLE OF *ACANTHAMOEBA* SPP. IN THE  
ENVIRONMENTAL SURVIVAL OF  
*LISTERIA MONOCYTOGENES*

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## **ABSTRACT**

*Listeria monocytogenes* causes a potentially deadly disease of man and is a major source of contamination in food industry. The mechanism of survival and persistence of *L. monocytogenes* in the environment is not fully known. The present study investigates the possible role of *Acanthamoebae* in the survival and persistence of *L. monocytogenes* in the environment. This was achieved through experiments that brings together the two organisms in a co-culture and then examined ability of bacteria to survive in the presence of amoeba, inside amoeba trophozoites and in their cysts. The effects of intracellular survival on *L. monocytogenes*' morphology, ability to form biofilms and respond to biocides inside and outside the cysts were also examined. In summary, *L. monocytogenes* Scott A was found to survive and grow in *Acanthamoeba* over 72 h. In addition, exposure of bacteria to manganese enhanced intracellular growth and survival of *L. monocytogenes* within *Acanthamoeba*. While *L. monocytogenes* Scott A survived and replicated in *A. castellanii*, it barely survived in *A. polyphaga* and never survived in *A. culbertsoni*. None of the other strains of *L. monocytogenes* tested were able to survive in *Acanthamoeba*. Autophagy, which was previously shown to aid survival of *L. monocytogenes* in macrophages, was also found contribute to survival within *Acanthamoeba*. In addition to surviving within *A. castellanii* trophozoites, *L. monocytogenes* Scott A also survived encystment of the host amoeba. *L. monocytogenes* sequestered in cysts were protected from high level of chlorine that is lethal to free bacteria. In addition, *L. monocytogenes* recovered from cysts were predominantly filamentous and demonstrated enhanced ability to form biofilm and also exhibited increased resistance to a disinfectant and some antibiotics that are normally used in treatment of listerial infections. The observations suggest that *A. castellanii* could potentially contribute to the survival, dissemination, and persistence of bacteria in the environment.

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## **ABBREVIATIONS**

**AGE-** Amoebic granulomatous encephalitis

**ANOVA-** Analysis of variance

**BSA-** Bovine serum albumin

**BHI-**Brain heart infusion

**Caco-2-** Human colonic carcinoma cell line

**CCV-** Clathrin-coated vesicle

**CFU-** colony forming unit

**CM-** conditioned medium

**CNS-** central nervous system

**DMSO-** Dimethyl sulphoxide

**DNA-** Deoxyribonucleic acid

**DPD-** N-N-Diethyl-P-phenylenediamine

**DPI-** Diphenyleneiodonium

**ER-** endoplasmic reticulum

**EV-** endocytic vesicle

**FBS-** Foetal bovine serum

**FLA-** free-living amoeba

**hly-** haemolysin

**H<sub>2</sub>O<sub>2</sub>-** Hydrogen peroxide

**Hpt-** Hexose phosphate transporter

**IFN- $\gamma$ -** Gamma interferon

**IL-** Interleukins

**InIA-** Internalins A

**InIB-** Internalins B

**kDa**- Kilodalton

**LE**- Late endosome

**LLO** – Listeriolysin O

**LPS**- Lipopolysaccharides

**K<sub>2</sub>HPO<sub>4</sub>**- Dipotassium hydrogen phosphate

**MAC**- Minimum amoebicidal concentration

**3-MA**- 3-Methyladenine

**MIC**- Minimum inhibitory concentration

**MM**- maintenance medium

**MnCl<sub>2</sub>·4H<sub>2</sub>O**- Manganese II chloride tetrahydrate

**MnSOD**- Manganese superoxide dismutase

**MOI**- Multiplicity of infection

**NADPH**- Nicotinamide adenine dinucleotide hydrogen phosphate

**Na<sub>2</sub>HPO<sub>4</sub>**- Disodium hydrogen phosphate

**Nf-κB**- Nuclear factor kappa

**NOD**- Nuclear oligodimerization domain

**O<sub>2</sub><sup>•-</sup>**-Superoxide

**OD**- optical density

**OH<sup>•</sup>** - Hydroxyl radical

**P60**- 60-Kilodalton protein

**PBS**- Phosphate buffer saline

**PC-PLC**- Phosphatidylcholine-specific phospholipase C

**PI-PLC**- Phosphatidylinositol-specific phospholipase C

**PLC**- Phospholipase C

**PrfA**- Positive regulatory factor A

**RER**- Rough endoplasmic reticulum

**RM**- Repeated measures

**RNA**- Ribonucleic acid

**RNS**- Reactive nitrogen species

**ROS**- Reactive oxygen species

**rpm**- Revolutions per minute

**SDS**- Sodium dodecyl sulphate

**SE**- sorting endosome

**SOD**- Superoxide dismutase

**RS**-  $\frac{1}{4}$  strength Ringer's solution

**TEM**- Transmission electron microscope

**TLR**- Toll-like receptors

**TNF**- Tumor necrotic factor

**TSA**- Tryptone soya agar

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# Chapter 1. General Introduction

## 1.1 Free-living amoebae

Free-living amoebae (FLA) are unicellular protozoa commonly found in soils and aquatic environments. FLA are the major predators of bacteria in the soil (Clarholm, 1981, Rodriguez-Zaragoza, 1994, Huws *et al.*, 2005). Their feeding activities help recycle minerals and increase soil fertility (Sinclair *et al.*, 1981). All free-living amoebae are capable of living freely in the environment but some are also opportunistic pathogens of man (Marciano-Cabral and Cabral, 2003, Schuster and Visvesvara, 2004). The pathogenic strains mainly belong to three genera, *Acanthamoeba*, *Naegleria* and *Balamuthia* (Visvesvara *et al.*, 2007, Marciano-Cabral, 2009).

### 1.1.1 *Acanthamoeba*

The genus *Acanthamoeba* is the most common amoeba if not the most common protozoa in soil and water samples (Schuster and Visvesvara, 2004). They feed mainly on bacteria and detritus in the environment and reproduces by binary fusion (Visvesvara *et al.*, 2007). *Acanthamoeba* is an aerobic organism. It can easily be cultivated in the laboratory on non-nutrient agar plates coated with bacteria such *Escherichia coli* (Visvesvara *et al.*, 2007). They can also grow axenically (in the absence of living organism) in culture media containing peptone, yeast extract and glucose (Schuster, 2002, Khan, 2006).

The genus *Acanthamoeba* can be classified based on their on the morphological characteristics of their cysts in three groups (Khan, 2006). They can also be classified based their rRNA sequences into 15 different genotypes (T1-T15) (Khan, 2006, Visvesvara *et al.*, 2007).

### 1.1.2 Biology and life-cycle of *Acanthamoeba*

*Acanthamoeba* undergo at least two developmental stages during their life cycle, the trophozoite which is the vegetative feeding form and the resistant cyst (Figure 1.1). A typical *Acanthamoeba* trophozoite is 12- 35µm in diameter and has one nucleus that is centrally located (Khan, 2006). The cytoplasm is granulated and contains numerous mitochondria, ribosomes, food vacuoles, and a contractile vacuole (Visvesvara *et al.*, 2007). *Acanthamoeba* trophozoites exhibit spine-like structures on their surface called pseudopodia (acanthopodia) with which they adhere to surfaces, move from place to place and capture their prey (Khan, 2006). Trophozoites feed voraciously on bacteria, algae, yeasts and detritus present in the environment and multiply by binary fission (Greub *et al.*, 2004, Visvesvara *et al.*, 2007).

Under harsh conditions such as prolonged starvation, desiccation, extremes of pH, hyperosmosis, cold and heat, trophozoite differentiates into a non-dividing, double-walled resistant cyst by a process of encystment (Murti and Shukla, 1984, Khunkitti *et al.*, 1998, Cordingley and Trzyna, 2008)( Figure 1.2). During the process of encystment, amoeba expels contents of cytoplasmic vacuole and decreases in cell volume, weight and diameter by reducing the amount of water, excess cytoplasmic organelles, RNA, glycogen, triacylglycerides and protein. It then rounds up to form a pre-cyst containing a single-wall. The removal of particulate material from its cytoplasm continues and the pre-cyst eventually develops into mature cyst by forming another wall called ectocyst (Stewart and Weisman, 1972).

The mature cyst wall consist of two layers, the outer (exocyst) and the inner (endocyst) which are separated by an electron-lucent intercyst space with an average thickness of 840 nm. (Weisman, 1976, Lemgruber *et al.*, 2010). The cell wall is composed of 36-45 % protein and 20-34 % carbohydrate. The bulk of the carbohydrate is cellulose (Barrett

and Alexander, 1977). The major constituents of the endocyst is cellulose while exocyst is composed of protein and polysaccharides (Lemgruber *et al.*, 2010).

Encystment is associated with increased levels of cyclic adenosine monophosphate (Weisman, 1976, Murti and Shukla, 1984).

Cysts are smaller than trophozoites and measure 5 -20  $\mu\text{m}$  in diameter (Khan, 2006). They are immotile but can be carried by air currents and can remain metabolically inert as long as conditions are unfavourable for hatching (Byers, 1979, Murti and Shukla, 1984).

*Acanthamoeba* cysts are highly resistant to disinfection, desiccation and extreme temperatures and can remain viable more than 20 years (Kilvington and Price, 1990, Marciano-Cabral and Cabral, 2003, Sriram *et al.*, 2008, Coulon *et al.*, 2010). Trophozoites emerge from cysts under favourable conditions leaving behind the outer shell by a process called excystation ( Figure 1.2). Excystation is mediated by glutamic acid and certain other amino acids. During this process, there is breaking of dormancy and depolymerisation of proteases and cellulase (Murti and Shukla, 1984).

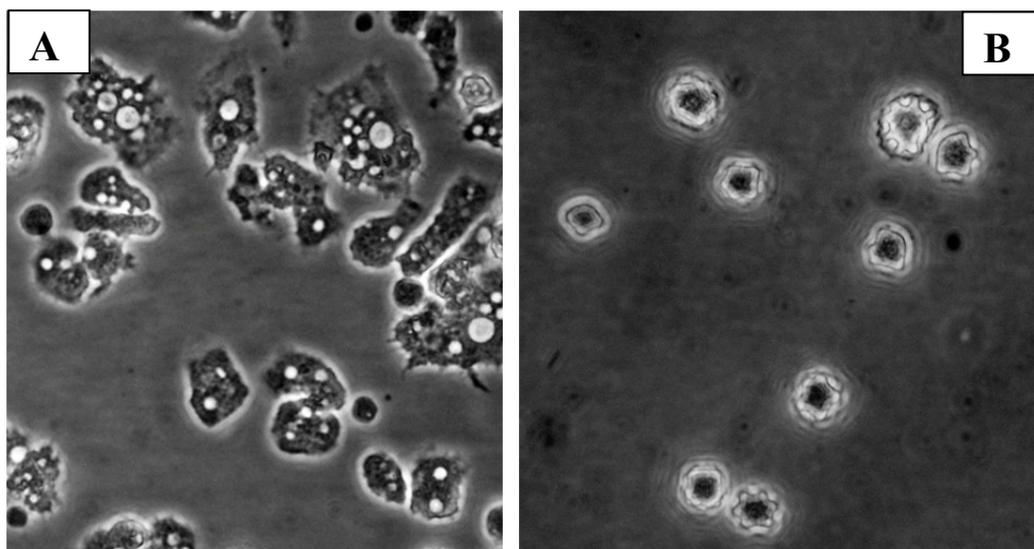


Figure 1.1 Phase microscopy images of the developmental forms of *Acanthamoeba* (A) Trophozoites and (B) Resistant cysts. Adapted from this study.  $\times 400$ .

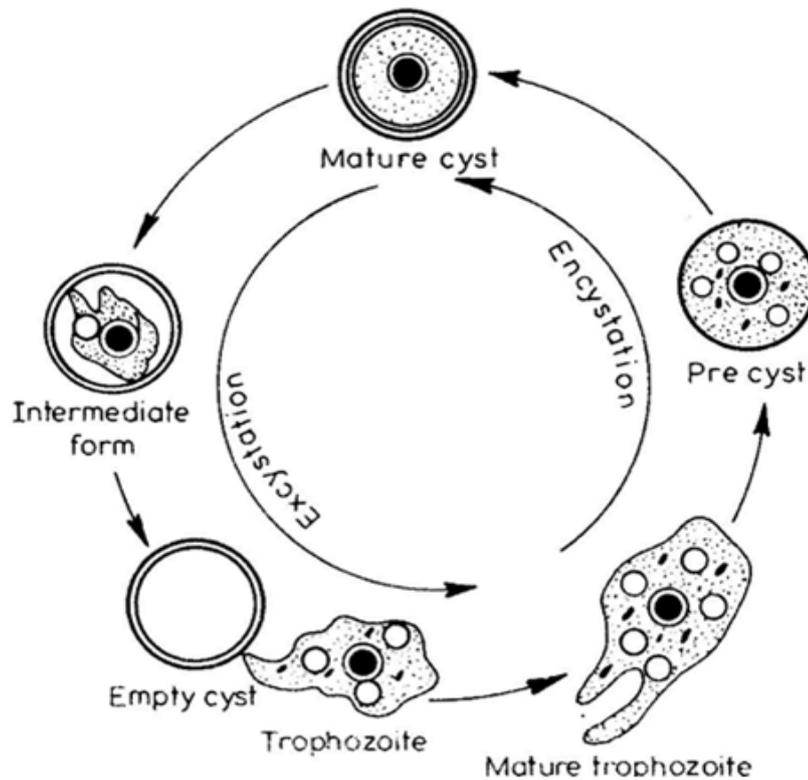


Figure 1.2 Life cycle of free-living amoeba.

During normal growth conditions amoeba exist as a trophozoite but when conditions becomes unfavourable amoeba begins to encyst forming first the pre-cyst and then mature cyst. When favourable conditions return, the trophozoites hatch from the cyst. Adapted from (Murti and Shukla, 1984).

### 1.1.3 Ecology

Free-living amoebae are cosmopolitan in distribution. They constitute about 50 % of the total number of protozoa in soil (Rodriguez-Zaragoza, 1994). Being the main predators of bacteria in the soil, FLA play an important role in ecosystem by increasing nutrient recycling and linking lower trophic levels with higher ones (Clarholm, 1981, Sinclair *et al.*, 1981). Their relative abundance in nature is dependent on the season, bacterial numbers, temperature, pH and salinity (Rodriguez-Zaragoza, 1994).

The genus *Acanthamoeba* is the most common FLA and indeed, the most common protozoa found in freshwater, soil and atmosphere (Weisman, 1976, Schuster and Visvesvara, 2004). *Acanthamoeba* species are present in all types of environment all over the world including public water supplies, swimming pools, bottled water, seawater, pond water, stagnant water, freshwater lakes, river water, distilled water and from atmosphere. In addition, *Acanthamoeba* has been recovered from hospitals, dialysis units, eye wash stations, human nasal cavities, pharyngeal swabs, lungs tissues, skin lesions, corneal biopsies, cerebrospinal fluid (CSF) and brain necropsies (Marciano-Cabral and Cabral, 2003, Visvesvara *et al.*, 2007).

#### **1.1.4 Feeding**

*Acanthamoeba* feeds mainly on bacteria (Visvesvara *et al.*, 2007). However, not all bacteria are equally suitable as food source. *Acanthamoeba* show preference for bacteria that are not pigmented or encapsulated as the mucoid capsules impedes phagocytosis by the amoebae while the bacterial toxins are often toxic (Weekers *et al.*, 1993, Visvesvara *et al.*, 2007). In addition, they also prefer Gram negative to Gram positive bacteria because of the thickness of Gram positive cell wall which make their digestion difficult (Alexander, 1981, Gonzalez *et al.*, 1990, Weekers *et al.*, 1993). Food uptake occurs by both phagocytosis and pinocytosis (Chambers and Thompson, 1976). Phagocytosis is a receptor-dependent ingestion of a large particle greater than 0.5  $\mu\text{m}$  in diameter, into vacuoles, whereas pinocytosis is a non-specific uptake of liquid food into vacuoles (Cardelli, 2001, Duhon and Cardelli, 2002). Although both processes in *Acanthamoeba* are independent of each other, they both are suppressed by inhibitors of oxidative metabolism and low temperature but less affected by inhibitors of glycolysis (Chambers and Thompson, 1976, Bowers, 1977). Bowers demonstrated that increasing the rate of phagocytosis suppressed pinocytosis and suggested that phagocytosis has greater

preponderance over pinocytosis as a mean of feeding in *Acanthamoeba* (Bowers, 1977). *Acanthamoeba* also ingest bacteria, yeast or cells by use of temporary structures formed on their surface, called 'food-cups' or amebostomes (Khan, 2001, Marciano-Cabral and Cabral, 2003).

### **1.1.5 Phagocytosis**

Phagocytosis is a word derived from the Greek word meaning 'cell eating'. It is a term used to describe the ingestion of a particle by a biological cell. Multiple steps are involved in phagocytosis of particles by professional phagocytes such as macrophages and amoeba, (Cardelli, 2001, Haas, 2007). Ultimately, the ingested particle is delivered to the cell interior contained within a membrane-bound vacuole known as phagosome, formed when a phagocyte wraps a portion of its plasma membrane around the particle, followed by plasma membrane fusion at the tip of the particle (Touret *et al.*, 2005, Yeung *et al.*, 2006). Phagocytosis of food particles by amoeba was enhanced when cultures were incubated with mechanical agitation (Avery *et al.*, 1995).

The processes of phagocytosis in amoeba are in many respects similar to that of mammalian immune phagocytes (Lock *et al.*, 1987). Firstly, both begin with recognition and binding of a particle to a receptor found on their surfaces (Vogel *et al.*, 1980, Lock *et al.*, 1987, Cardelli, 2001). Secondly, both receptors can be blocked by mannose to prevent binding (Brown *et al.*, 1975, Lock *et al.*, 1987, Allen and Dawidowicz, 1990). Thirdly, binding of particles to the receptors in both cells stimulates polymerisation of monomeric G-actin into filamentous F-actin that aids in internalisation of the particle (Allen and Aderem, 1996, Alsam *et al.*, 2005). In addition, the process in both can be inhibited by cytochalasin D and genistein (Alsam *et al.*, 2005). Finally, both processes can be stimulated by nutrient deprivation (Martinet *et al.*, 2009).

The main difference between phagocytosis by *Acanthamoeba* and phagocytosis by immune phagocytes is that, whereas *Acanthamoeba* uses the process primarily for nutritional purpose, the immune phagocytes on the other hand use phagocytosis mainly for defence purposes (Neuhaus *et al.*, 2002).

### **1.1.6 Phagosome maturation**

The limiting membrane of a nascent phagosome is derived from plasmalemma and its fluid contents are a sample of the extracellular medium. Consequently, its lumen is a benign environment incapable of killing invading microorganisms. These capabilities are acquired subsequently as a result of maturation (Ulsamer *et al.*, 1971, Vieira *et al.*, 2002, Haas, 2007). Phagosome maturation is a progressive sequence of fusion and fission reactions and interactions with components of endocytic pathway that modifies the composition of phagosome limiting membrane and of its contents to give rise to a hybrid vacuole called phagolysosome (Figure 1.3). Maturation therefore bestows on the phagosome its microbicidal function (Allen and Aderem, 1996, Duhon and Cardelli, 2002, Vieira *et al.*, 2002, Haas, 2007). Shortly after sealing, the nascent phagosome merges with the early endosome also called sorting endosome (SE), followed by late endosomes (LE) and eventually lysosomes that provides most of the microbicidal components. The fusion of phagosome and lysosome which marks the climax of the maturation process has been demonstrated *in vitro* in *Acanthamoeba* (Oates and Touster, 1980). A key event in the maturation of phagosome is the progressive acidification of the lumen, from near-neutral to pH 5 (Bouvier *et al.*, 1994). Phagosomal acidification is mediated through a vacuolar-type H<sup>+</sup>-ATPase located in phagosomal membrane and this translocates protons from cytoplasm into intra-phagosomal space (Lukacs *et al.*, 1990). The acidic pH play 4 major roles: 1) it is lethal to some pathogens (Horwitz and Maxfield, 1984, Schneider *et al.*, 2000), 2) it favours dismutation of

superoxide (Harvey, 2000, Jankowski *et al.*, 2002), 3) it provides optimal condition for the activity of hydrolytic enzymes(Amaral *et al.*, 2007) and 4) it is a pre-requisite for phagosome-lysosome fusion (Yates *et al.*, 2005).

Calcium is another important messenger that regulates maturation of phagosome (Zimmerli *et al.*, 1996). It mediates fusion with late endosomes and lysosomes and also helps to maintain the dynamic equilibrium of organelles in the late endocytic pathway (Peters and Mayer, 1998, Pryor *et al.*, 2000).

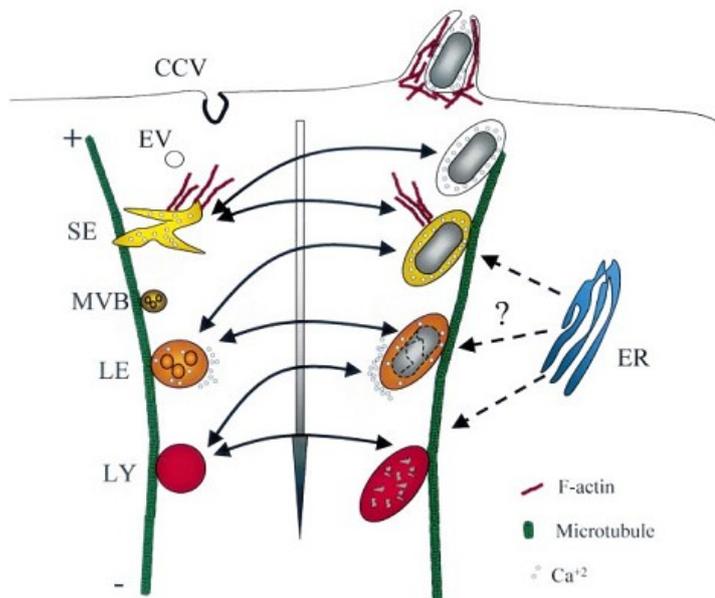


Figure 1.3 Schematic representation of phagosome maturation.

Following uptake of a particle, the phagosome matures as it undergoes fusion (arrows) with vesicles of the endocytic pathway (left). CCV, clathrin-coated vesicle; EV, endocytic vesicle; SE, sorting endosome; MVB, multi-vesicular bodies LE, late endosome; LY, lysosome; ER, endoplasmic reticulum. Adapted from (Vieira *et al.*, 2002)

### 1.1.7 Respiratory burst of phagocytosis

*Acanthamoeba castellanii* has a branched mitochondrial electron –transport chain that terminates in three distinct oxidases. The cytochrome oxidase of the main phosphorylating transport chain that is resistant to azide but sensitive to potassium cyanide (KCN), the alternative oxidase that is insensitive to cyanide but inhibited by salicylhydroxamic acid and the main oxidase that is insensitive to azide and salicylhydroxamic acid but inhibited by cyanide (Edwards and Lloyd, 1978, Lloyd *et al.*, 1979). The alternative oxidase pathway which is also called oxidative metabolism is a phagocytosis-dependent but non-phosphoylating process that is independent of the mitochondrial cytochrome oxidase although it consumes the latter's reducing power (Davies *et al.*, 1991, Jarmuszkiewicz *et al.*, 2008). Part of the cyanide-insensitive respiration in amoeba occurs in the phagolysosomal granules and resembles the NADPH oxidase of neutrophils and macrophages (Drath and Karnovsky, 1975, Brooks and Schneider, 1985, Davies *et al.*, 1991, Hampton *et al.*, 1998).

Although the alternative oxidase is a constitutive activity at a basal level in amoeba, activation of the pathway results to abrupt two-fold increase in oxygen uptake together with the onset of the production of a series of compounds formed from oxygen including superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and a number of additional oxygen-containing compounds, all of which are highly reactive (Brooks and Schneider, 1985). These changes in oxidative metabolism are collectively known as the respiratory burst (Babior, 1984, Borregaard, 1985) (Figure 1.4).

The NADPH oxidase of phagocytes that is responsible for respiratory burst consists of one membrane-bound and four cytosolic components (Dahlgren and Karlsson, 1999). When activated, electrons are transported from this NADPH oxidase in the cytosol by *cytochrome b* to oxygen present in phagosome to generate superoxide anions ( $O_2^-$ ). The

$O_2^-$  ions are unstable and immediately dismutate to  $O_2$  and  $H_2O_2$  in a reaction catalysed by the enzyme superoxide dismutase (Fridovich, 1978, Borregaard, 1985, Babior, 1999). The  $O_2^-$  and  $H_2O_2$  generated as primary products during respiratory burst are not sufficiently bactericidal but can undergo secondary reactions to generate other reactive oxygen species (ROS) such as hydroxyl radicals ( $\bullet OH$ ) that are strongly anti-microbial (Babior, 1984, Dahlgren and Karlsson, 1999, Kehrer, 2000). Hydroxyl radical is extremely reactive with biological molecules as such, can damage cell proteins, DNA and lipids (Hampton *et al.*, 1998).

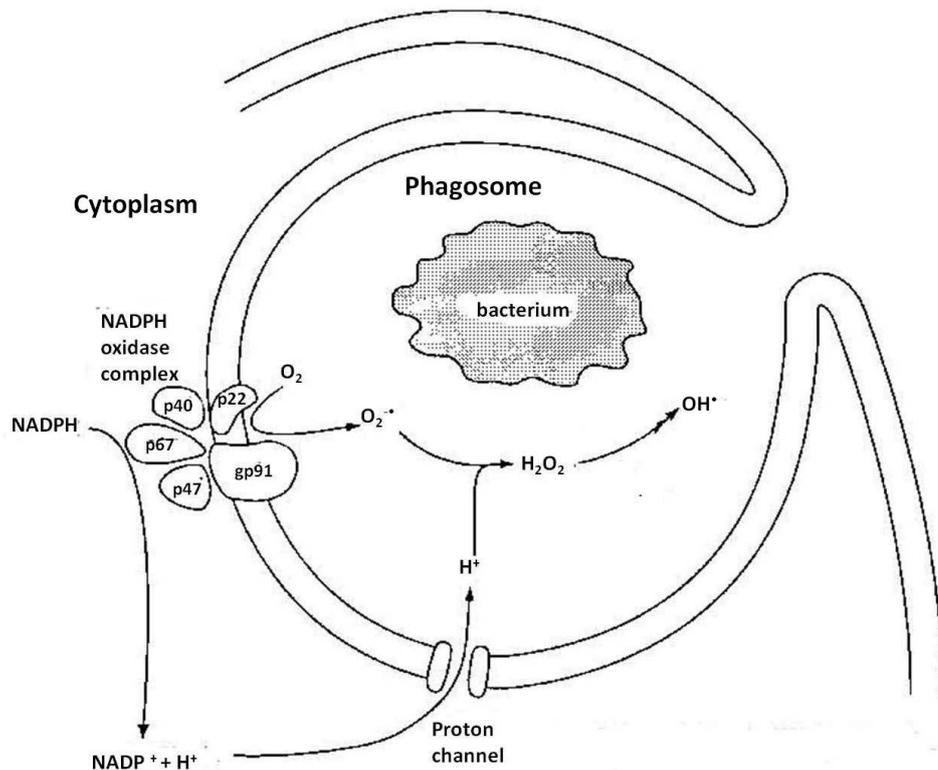


Figure 1.4 Schematic representation of respiratory burst in *Acanthamoeba*.

Electrons are transported to  $O_2$  in the lumen of phagosome via the NADPH oxidase in the membrane of phagosome leading to formation of superoxide ( $O_2^\bullet$ ) and secondary oxidants such as hydroxyl radical ( $OH^\bullet$ ) which is extremely reactive and can cause damage to DNA, proteins and lipids.

### **1.1.8 Diseases caused by *Acanthamoeba***

Several species of *Acanthamoeba* including *A. castellanii*, *A. culbertsoni*, *A. hatchetti*, *A. healyi*, *A. polyphaga*, *A. rhyodes*, *A. astronyxis*, and *A. divionensis* are known to cause granulomatous amoebic encephalitis (GAE), an insidious, chronic and mostly fatal disease of man, particularly in the immunocompromised or debilitated people (Marciano-Cabral, 2009). In addition to causing GAE, *Acanthamoeba* also causes *Acanthamoeba* keratitis (AK) in immunocompetent people (Schuster *et al.*, 2003). AK is a potentially blinding infection of the cornea that is most commonly associated with contact lens wearers (Alizadeh *et al.*, 2005, Khan, 2006). More than 95 % of *Acanthamoeba* isolates that produce keratitis belong to T4 genotype (Khan, 2001)

## **1.2 *Listeria monocytogenes***

The genus *Listeria* comprise of a group of Gram positive, facultative anaerobic rods that do not form spores. They are widespread in the environment in which they live as saprophytes and are motile at 10°C to 25°C. There are currently 6 species of the genus *Listeria*: *L. monocytogenes*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri*. Of this six species only *L. monocytogenes* and *L. ivanovii* are pathogenic to humans and/or animals causing a disease known as listeriosis (Mainou-Fowler *et al.*, 1988, Rocourt, 1988, Vazquez-Boland *et al.*, 2001). While *L. monocytogenes* is known to infect both humans and animals, *L. ivanovii* is considered to infect ruminants only (Snapir *et al.*, 2006) although there are reported cases of human infections with *L. ivanovii* which occurred mainly in immunocompromised patients (Cummins *et al.*, 1994, Lessing *et al.*, 1994, Snapir *et al.*, 2006, Guillet *et al.*, 2010).

*L. monocytogenes* is a Gram-positive, non- spore forming, motile, aerobic or facultative anaerobic coccobacillus bacterium measuring approximately 0.4- 0.5 µm in diameter

and 1- 2 µm in length and exist either as single bacterial cells or chains of multiple cells of similar size (Farber and Peterkin, 1991, Romick *et al.*, 1996, Rowan *et al.*, 2000a). It is catalase positive, oxidase negative and expresses beta-haemolysin (Jemmi and Stephan, 2006). The bacterium is actively motile by means of peritrichous flagella and exhibits characteristic tumbling motility at 20-25°C but does not synthesize flagella at body temperatures (Farber and Peterkin, 1991).

*L. monocytogenes* is ubiquitous in the environment and has the ability grow at refrigeration temperatures. The bacterium generally grows and reproduces at temperatures from 1°C to 45°C with the optimum between 30°C and 37°C (Rowan and Anderson, 1998, Jemmi and Stephan, 2006). *L. monocytogenes* is further subdivided into 13 serotypes (serovars) based on the somatic (O) and flagella (H) antigen (Nadon *et al.*, 2001).

### **1.2.1 Historical developments**

*L. monocytogenes* was first discovered in 1926 by E.G.D Murray and others and was named *Bacterium monocytogenes* because it caused characteristic monocytosis in infected rabbits and guinea pigs in their laboratory in Cambridge England. It was renamed *L. monocytogenes* by Pirie in 1940 in honour of Joseph Lister the English surgeon who introduced principles of antiseptis to standard surgical procedures (Farber and Peterkin, 1991, Vazquez-Boland *et al.*, 2001, Hof, 2003).

The first case of human disease caused by *L. monocytogenes* was reported in Denmark in 1929 (Vazquez-Boland *et al.*, 2001). However, it was not until it caused a large outbreak of invasive disease with a high case-fatality rate in the Maritime Provinces in Canada that it was recognized as a serious public health problem.(Swaminathan and Gerner-Smidt, 2007). Because of its high case fatality rate *L. monocytogenes* infections

now ranked among the topmost frequent causes of death due to foodborne illnesses in industrialized countries (Mead *et al.*, 1999, de Valk *et al.*, 2005).

## **1.2.2 Listeriosis**

### **1.2.2.1 Pathogenesis**

Infection is acquired by consumption of contaminated foods (Lunden *et al.*, 2004, Mead *et al.*, 2006). After ingestion, *L monocytogenes* crosses the mucosal barrier of the intestine and enters the blood stream. Once in the blood stream, *L monocytogenes* disseminates to the mesenteric lymph nodes, spleen and liver. The resident macrophages in the liver (Kupffer cells) engulf and kill most of bacteria causing a decrease in size of viable bacteria population during the first 6 h after infection. The surviving bacteria that escaped destruction by Kupffer cells then invade liver hepatocytes and grow in numbers for 2-5 days. After sufficient multiplication in the liver bacteria re-enter the blood stream and spread to various organs including central nervous system (CNS) and placenta of pregnant women (Vazquez-Boland *et al.*, 2001, Drevets and Bronze, 2008).

### **1.2.2.2 Clinical features**

Human listeriosis can be classified as invasive and non-invasive listeriosis and usually presents as one of three clinical syndromes namely febrile gastroenteritis, maternal-foetal neonatal listeriosis, or bacteremia with or without CNS involvement (Schuchat *et al.*, 1991, Drevets and Bronze, 2008). The main clinical symptoms of non-invasive listeriosis are febrile gastroenteritis which is characterised by diarrhoea, fever, abdominal pain, chills, headache and myalgias. In pregnant women, the onset of listeriosis usually presents as a non-specific mild flu-like symptoms. The disease at this stage is often self-limiting and most patients recover without antimicrobial treatment (Farber and Peterkin, 1991, Drevets and Bronze, 2008). Invasive listeriosis in non-

pregnant adults usually occurs in persons with underlying health conditions (Vazquez-Boland *et al.*, 2001). It typically presents as bacteraemia or as CNS infection including meningitis, meningoencephalitis, and abscesses in the brain (Schuchat *et al.*, 1991, Drevets and Bronze, 2008). Spontaneous abortion and stillbirths occurs as complications in pregnant mother (Delgado, 2008). Vertical transmission from mother to foetus can result to neonatal disease (Vazquez-Boland *et al.*, 2001).

### **1.2.2.3 Epidemiology**

Listeriosis is a serious foodborne disease with an average case-fatality rate of 20-30 % in vulnerable people (Mead *et al.*, 1999, Swaminathan and Gerner-Smidt, 2007).

Because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to food-borne illness in developed countries and ranks as the third or fourth most common cause of bacterial meningitis in North America and Western Europe (Drevets and Bronze, 2008). The incidence of the disease worldwide is, however, low and varies between 0.1-11.3 per million in different countries (Gellin *et al.*, 1991, Swaminathan and Gerner-Smidt, 2007). In England and Wales, however, the incidence of listeriosis has been on a rise due to socio-economic deprivation, increase cases of malignancies, kidney diseases, liver diseases, diabetes and alcoholism (Gillespie *et al.*, 2010, Mook *et al.*, 2011). Most cases of human listeriosis are sporadic although cluster cases (Gillespie *et al.*, 2006) and large outbreaks have been reported in various parts of the world (Table 1.1). A majority of the outbreaks are caused by strains of serotype 4b (McLauchlin, 1990, Jemmi and Stephan, 2006, Swaminathan and Gerner-Smidt, 2007)

Table 1.1 Examples of outbreaks of human food-borne listeriosis .

<b>Country</b>	<b>Year</b>	<b>Food</b>	<b>Cases</b>	<b>Deaths</b>	<b>Serotype</b>
United States of America	1976	Raw salad	20	5	4b
New Zealand	1980	Fish	22	7	1/2a
Canada	1981	Coleslaw	41	18	4b
United States of America	1983	Milk	49	14	4b
United States of America	1985	Soft cheese	142	30	4b
Switzerland	1983-1987	Soft cheese	122	34	4b
United Kingdom	1987-1989	Pate	355	94	4b
France	1993	Pork tongue in aspic	279	NK	4b
France	1993	Pork rillettes	38	10	4b
United States of America	1994	Milk	45	0	1/2b
Sweden	1994-1995	Fish	9	2	4b
France	1995	Soft cheese	17	4	4b
Canada	1996	Crab meat	2	0	1/2b
Italy	1997	Salad	1566	0	4b
United States of America	1998-1999	Hot dogs	50	>8	4b
Finland	1998-1999	Butter	25	6	3b
Finland	1999	Fish	5	NK	1/2a
France	1999-2000	Pork rillettes	10	2	4b
France	1999-2000	Pork tongue in jelly	32	10	4b
United States of America	2000	Turkey meat	29	7	NK
Switzerland	2005	Soft cheese	3	1	NK

NK: not known      Adapted from (Jemmi and Stephan, 2006)

### **1.2.3 Infection of mammalian cells**

*L. monocytogenes* is a facultative intracellular pathogen which has the ability to multiply in the cytoplasm of wide variety of human and animal cell types. The cells in which *L. monocytogenes* grow and survive can be broadly classified into two, professional and non-professional phagocytes (Mengaud *et al.*, 1996, Cossart and Lecuit, 1998). The professional phagocytes actively take in *L. monocytogenes* through the process of phagocytosis and include, macrophages (Makino *et al.*, 2005, Ohya *et al.*, 2005), polymorphonuclear leucocytes (Drevets, 1999) and monocytes (Peck, 1989, Drevets, 1999). The non-professional phagocytes on the other hand take in *L. monocytogenes* by passive phagocytosis and they include, intestinal epithelial cells (Karunasagar *et al.*, 1994), endothelial cells (Greiffenberg *et al.*, 1998), hepatocytes (Wood *et al.*, 1993), fibroblasts (Gasnov *et al.*, 2006) and transformed cells (Farber and Speirs, 1987).

#### **1.2.3.1 Intracellular life cycle in mammalian cells**

Cell infection is characterized by several distinct steps (Figure 1.5). The first step is adhesion of bacteria to cell surface mediated by a number of *Listeria* adhesins including autolysin amidase (Ami), fibronectin binding protein (Fbp), proteins (p60, p104), ActA and insulin-like growth factor II (IGFIIR) (Dramsi *et al.*, 2004, Dussurget *et al.*, 2004, Gasnov *et al.*, 2006). Binding to cell surface is immediately followed by internalization through active phagocytosis as in the case of professional phagocytes or induced phagocytosis (invasion) as in non-professional phagocytes (Ireton and Cossart, 1997, Cossart and Lecuit, 1998, Pizarro-Cerda and Cossart, 2006). Invasion of non-professional phagocytes is by ‘zipper’ mechanism in which a bacterium gradually sinks into a dip-like structure of the host cell surface until it is finally enveloped in a vacuole (Cossart *et al.*, 2003). Two listerial surface proteins, internalins A (InlA) and internalins

B (InlB) mediate entry of *L. monocytogenes* into non-phagocytes (Braun *et al.*, 1998, Cossart and Toledo-Arana, 2008).

Upon entry, *L. monocytogenes* becomes trapped within a single-membrane vacuole and after 30-45 min, produce a pore-forming protein called listeriolysin O (LLO), which lyses phagosome membrane to allow escape of *L. monocytogenes* to cytoplasm (Henry *et al.*, 2006). In addition, *L. monocytogenes* also secretes phospholipases C (PLC) that facilitate escape from phagosomes (Poussin *et al.*, 2009).

Soon after escape, *L. monocytogenes* quickly multiply in the cytoplasm with generation time close to those observed in rich medium (O'Riordan and Portnoy, 2002, Joseph *et al.*, 2006). Following replication, *L. monocytogenes* secretes ActA which recruits host actin and polymerize them into a network of filaments. The actin filaments then rearrange to form a long actin 'comet' tail on one end of the bacterium which propels *Listeria* into rapid random motion (Dabiri *et al.*, 1990, Tilney *et al.*, 1990).

Once the moving bacteria come in contact with plasma membrane, they induce formation of protrusions, called listeriopods which penetrate into uninfected neighbouring cells. The bacteria are again engulfed resulting in the formation of two membrane secondary vacuoles from which bacteria escape to initiate a new infection cycle (Gedde *et al.*, 2000, Cossart and Toledo-Arana, 2008).

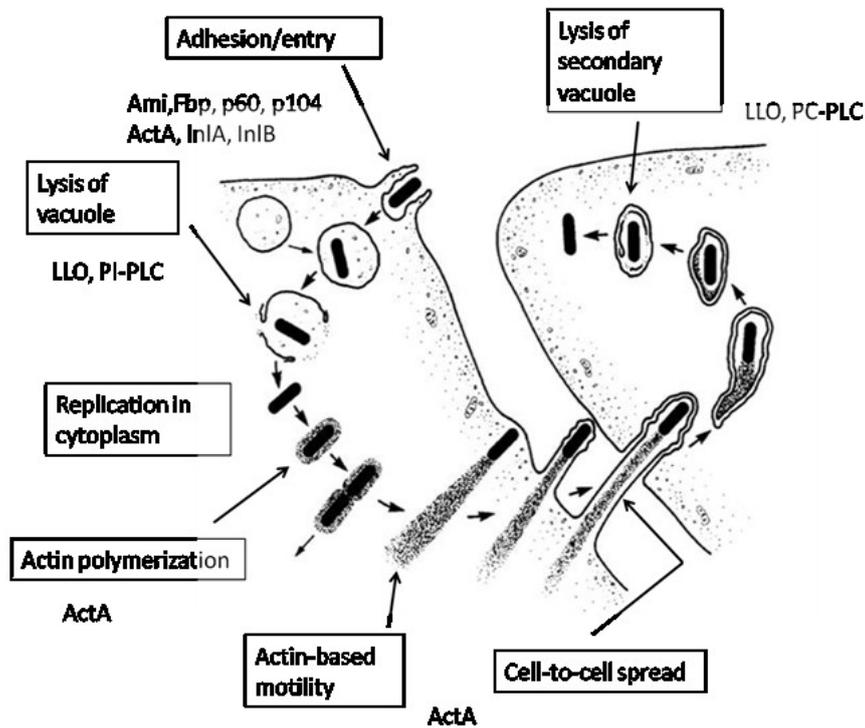


Figure 1.5 The infection cycle of *L. monocytogenes* in mammalian host cell. Each step of the infection is shown together with bacterial factors involved in the intracellular survival of *L. monocytogenes*. Adapted from Tinley and Portnoy (Tinley and Portnoy, 1989)

### 1.2.3.2 Virulence factors involved in infection of mammalian cells

#### 1.2.3.2.1 Listeriolysin O

Listeriolysin O (LLO) is a member of the pore-forming, cholesterol-dependent Cytolysin family encoded by *hly* gene. This 60-kDa secreted protein is the primary determinant for *Listeria* escape from primary and secondary vacuoles (Gedde *et al.*, 2000, Dussurget *et al.*, 2004). LLO monomers binds to 3 $\beta$ -hydroxy group of cholesterol molecule on the phagosomal membrane of mammalian cells, insert and oligomerize to a pore-forming unit which bore pores of varying sizes (Dramsai and Cossart, 2002, Bavdek

*et al.*, 2007). LLO contains a conserved undecapeptide Tryp residue that is intimately involved in membrane insertion (Rossjohn *et al.*, 1997, Billington *et al.*, 2000). Perforation of phagosome membrane increases pH and decreases Ca<sup>2+</sup> levels within the vacuole and consequently delays maturation of phagosome to facilitate escape of *L. monocytogenes* into cytoplasm (Henry *et al.*, 2006, Shaughnessy *et al.*, 2006, Burrack *et al.*, 2009). The pore-forming activity of LLO is pH-dependent. It is active at acidic pH range of 4.9-6.7 and optimal at pH 5 but is rapidly inactivated at neutral or alkaline pH (Beauregard *et al.*, 1997, Schuerch *et al.*, 2005). In addition, the pore forming activity of LLO is also dependent on the concentration of cholesterol where high concentration in membrane was shown to restore low activity of LLO at high pH values (Bavdek *et al.*, 2007).

A PEST-like sequence has been identified at the N-terminus of LLO which rapidly degrade the toxin when present in cytosol in order to prevent lysis of host cells(Lety *et al.*, 2001).

There is accumulating evidence to suggest that LLO is a multifunctional virulence factor with many important roles in the host-parasite interaction other than phagosomal membrane disruption (Kayal and Charbit, 2006). In addition to intracellular expression LLO is also expressed extracellularly in growth medium (Moors *et al.*, 1999). Exogenous and endogenous exposure to LLO induce a number of host cell responses, including cell proliferation and focus formation in transfected fibroblasts, activation of the Raf–Mek–mitogen-activated protein, activation of NF-κB (Vasconcelos and Deneer, 1994, Kayal and Charbit, 2006), activation of endothelial cells leading to expression of cell adhesion molecule (Drevets, 1998) and protective immunity(Hara *et al.*, 2007).

#### **1.2.3.2.2 ActA**

ActA are a 639-amino protein coded by *ActA* gene. The mature form of the protein is divided into three distinct regions: (i) carboxyl-terminal (C-terminal) which is highly hydrophobic and anchors the protein to bacteria cell wall. (ii) A central region of proline-rich repeats that stimulates *Listeria* actin-based motility and, (iii) amino-terminal (N-terminal) region which is rich in cationic residue (Vazquez-Boland *et al.*, 2001). The N-terminal initiates F-actin assembly by interacting with host cells proteins such as Arp2/3 complex, profiling, vasodilator-stimulated protein, cofilin and G-actin which results in polymerization of the monomeric actin into a network of filaments that rearranged to form actin comet tails (Dabiri *et al.*, 1990, Tilney *et al.*, 1990, Pizarro-Cerda and Cossart, 2006). Actin polymerization provides the propelling force that moves bacteria through cytoplasm and into adjacent cells (Dabiri *et al.*, 1990, Robbins *et al.*, 1999).

Beside its role in actin nucleation and actin-based motility, ActA is also actively involved in permeabilization of phagosome and escape of *Listeria* into cytoplasm (Poussin and Goldfine, 2010). In addition, the complete coating of listerial surface by ActA has been shown to camouflage the organism from autophagic recognition and provide platform for initiating intracellular bacterial motility (Birmingham *et al.*, 2007, Yoshikawa *et al.*, 2009).

#### **1.2.3.2.3 Phospholipases**

*L. monocytogenes* produces two distinct phospholipases C, phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad range phosphatidylcholine-specific phospholipase C (PC-PLC) (Smith *et al.*, 1995). While PI-PLC is synthesized in active form and only requires host cell protein kinase C beta (PKC $\beta$ ) for translocation, PC-PLC on the other hand is produced as an inactive precursor and requires bacterial zinc-

dependent metalloprotease and a host cysteine protease to activate it by cleaving part of the precursor (Vazquez-Boland *et al.*, 2001, Poussin *et al.*, 2009).

PI-PLC helps in escape of bacteria from primary phagosome while PC-PLC, which is only active during cell-to-cell spread of bacteria, helps in the dissolution of inner membrane of vacuoles of the spreading bacteria (Alberti-Segui *et al.*, 2007, Yeung *et al.*, 2007). Phospholipases have also been shown to be essential for evasion of cellular autophagy (Py *et al.*, 2007).

#### **1.2.3.2.4 Superoxide dismutase**

*L. monocytogenes* constitutively express a manganese-containing superoxide dismutase (MnSOD) encoded by *lmsod* in response to environmental factors such as growth temperature, anaerobic condition and salts (Brehm *et al.*, 1992, Vasconcelos and Deneer, 1994, Myers *et al.*, 2003). MnSOD secreted by SecA2 pathway has been identified as a novel virulence factor that contributes to escape of *L. monocytogenes* from vacuole and was critical for virulence in mice (Welch *et al.*, 1979, Archambaud *et al.*, 2006). The activity of enzyme was however down regulated by phosphorylation on serine and threonine residues that operates outside of PrfA network (Vasconcelos and Deneer, 1994, Archambaud *et al.*, 2006).

#### **1.2.3.3 Regulation of virulence genes expression**

The most important *Listeria* virulence genes (i.e., *prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*, *inlA*, *inlB*, *inlC*, and *hpt*) are regulated by a 27 kDa protein called positive regulatory factor A (PrfA), the only regulator that is directly involved in regulation of virulence gene expression within infected host cells (Dussurget *et al.*, 2004). Regulation of virulence genes occur through binding of PrfA to a 14 bp palindromic sequence (PrfA box) in the -41 region of target promoters (Freitag *et al.*, 1993, Dussurget *et al.*, 2004).

Other genetic factors that regulate gene expression are sigmaB, Hfq, VirR and small RNAs (Cossart and Toledo-Arana, 2008).

Environmental factors such as growth temperature, acidity and growth medium have also been shown to modulate expression of virulence genes (Coffey *et al.*, 1996). For example, PrfA –dependent transcription is weak below 30°C but induced at 37°C (Vazquez-Boland *et al.*, 2001). Similarly, wild-type strains expressed PrfA-regulated genes very weakly in rich medium such as BHI but expression was strong when BHI was supplemented with charcoal (Ripio *et al.*, 1996). On the other hand, fermentable carbohydrates caused strong repression of virulence genes (Datta and Kothary, 1993).

It has been shown that adaptation to mildly acidic conditions and growth in an iron-rich medium enhanced the invasiveness for Caco-2 cells and macrophage-like cells (Conte *et al.*, 2000, Garner *et al.*, 2006). Other environmental conditions that are known to regulate expression of virulence genes are heat shock (van der Veen *et al.*, 2007), osmolarity (Gardan *et al.*, 2003), starvation, (Christiansen *et al.*, 2004), salts (Coffey *et al.*, 1996) and, reactive oxygen intermediates (Makino *et al.*, 2005).

#### **1.2.3.4 Host defence against infection**

*Listeria monocytogenes* is able to invade and multiply in a variety of cells by manipulating endocytic and many host-cell signalling cascades to its advantage (de Chastellier and Berche, 1994, Pizarro-Cerda and Cossart, 2006). However, the host cells are capable of detecting *Listeria* infection at different cellular compartments by expressing innate immune receptors that trigger antibacterial defence pathways. The receptors include the membrane-bound Toll-like receptors (TLRs) and the soluble, cytosolic nuclear oligodimerization domain (NOD)-like receptors (Eitel *et al.*, 2011). These initiate a signalling cascade that leads to activation of the transcription factors such as NF- $\kappa$ B and IFN regulatory factor 3 (IRF3), which culminates in immune

activation and upregulation of genes involved in host defence including cytokine production (Corr and O'Neill, 2009).

Since the intracellular life cycle of *L. monocytogenes* allows it to avoid humoral defences such as antibodies and complement, the innate immune responses are therefore the first and important lines of defence against infection (Delgado *et al.*, 2009). Macrophages are the key mediators in eliciting both innate and adaptive immune responses. They perform multiple functions, including the phagocytosis and digestion of invading pathogens, presentation of antigen to T- lymphocytes, and the production of cytokines that activate various other cell types. Activation of macrophages with soluble stimuli such as cytokines enhances all three of these activities (Shaughnessy and Swanson, 2007). The most potent activators of macrophages in *Listeria* infections are cytokines particularly interferon gamma (IFN- $\gamma$ ), bacterial lipopolysaccharides (LPS), and interleukins (IL-6 and  $\alpha$ -IL-10) (Peck, 1989, Higginbotham *et al.*, 1992, Brombacher *et al.*, 1999).

Macrophages activation plays a significant role in the elimination of *L. monocytogenes* infections (Shaughnessy and Swanson, 2007). Activation of macrophages have been shown to rapidly clear *L. monocytogenes* infection through production of reactive oxygen intermediates and reactive nitrogen intermediates which prevent escape of *Listeria* from vacuoles to facilitate fusion of phagosome with lysosome (Ohya *et al.*, 1998, Myers *et al.*, 2003, Shaughnessy and Swanson, 2007). Production of ROI can also stimulate autophagy against the pathogen (Scherz-Shouval *et al.*, 2007, Rada *et al.*, 2008).

Autophagy also functions in innate immunity response against *L. monocytogenes* infection (Py *et al.*, 2007, Yano *et al.*, 2008, Corr and O'Neill, 2009). Autophagy can be

regulated by cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-13 and acts as an output of both innate and adaptive immunity responses (Delgado *et al.*, 2009).

#### **1.2.4 *Listeria* and biofilm formation**

Previous studies have documented the ability of *L. monocytogenes* to colonise and persist for long periods as biofilms on food-processing equipments (Lee Wong, 1998, Norwood, 1999) with subsequent contamination of raw and processed foods (Møretrø and Langsrud, 2004, Harvey *et al.*, 2007).

Reports have shown that flagella motility is essential for *Listeria* biofilm formation on abiotic surfaces (O'Neil and Marquis, 2006, Lemon *et al.*, 2007). In addition, the quantity of biofilm formed was found to correlate with phylogenetic division of *L. monocytogenes* (Borucki *et al.*, 2003) and amount of extracellular carbohydrate produced (Chae *et al.*, 2006). It has been suggested that cultivation of *L. monocytogenes* in medium containing NaCl, glucose or both influence its ability to adhere and form biofilm on abiotic surfaces (Briandet *et al.*, 1999a, Pan *et al.*, 2010).

Spontaneously occurring variant of *L. monocytogenes* with characteristic rough colonies and filamentous cell morphology have been isolated from food and clinical samples (Rowan *et al.*, 2000a, Rowan *et al.*, 2000b, Monk *et al.*, 2004). In addition, *Listeria* was induced to form filaments by exposing bacteria to a range of adverse growth conditions such as high concentration of NaCl in the presence (Bereksi *et al.*, 2002) or absence of acid (Isom *et al.*, 1995, Bereksi *et al.*, 2002), acid conditions (Isom *et al.*, 1995), sub-lethal alkaline (Giotis *et al.*, 2007), increased CO<sub>2</sub> environments (Jydegaard-Axelsen *et al.*, 2005)(Jydegaard-Axelsen *et al.*, 2005) in the presence of antimicrobial agents such as trimethoprim and co-trimoxazole (Minkowski *et al.*, 2001), and above-optimum growth temperature (Rowan and Anderson, 1998). Filamentous *Listeria* has

been shown to have enhanced ability to form biofilm on steel surfaces (Monk *et al.*, 2004).

Reports have shown that *L. monocytogenes* growing in biofilms are protected against cleaning and disinfection and are difficult to eradicate (Norwood and Gilmour, 2000, Pan *et al.*, 2006).

### **1.3 Autophagy**

The word autophagy is derived from the Greek words ‘auto’ (self) and ‘phagy’ (eat) meaning to “eat oneself”(Levine and Klionsky, 2004, Apel *et al.*, 2009). It is a bulk degradative mechanism that occurs constitutively at basal levels in all eukaryotic cells, and under normal growth conditions, primarily for the routine turnover of worn-out, dispensable or dysfunctional cytoplasmic organelles and soluble proteins (Dunn, 1994, Mizushima, 2005, Lee, 2009). Autophagy however, can be upregulated under stress conditions such as starvation, hypoxia, oxidative stress, overcrowding, high temperature, infection and accumulation of damaged or superfluous of organelles in order to maintain cellular homeostasis and to assure cell survival (Reggiori and Klionsky, 2002, Levine and Klionsky, 2004).

The main stages involved in autophagy pathway are induction, execution and maturation and this culminates in the formation of a double-membrane-bound vacuole called autophagosome that sequesters cytoplasmic material (mitochondria, portions of cytoplasm, bacteria, etc). The vacuole undergoes a progressive maturation and eventually, fuses with lysosome to degrade its contents (Kirkegaard *et al.*, 2004, Mizushima, 2005)(Figure 1.6).

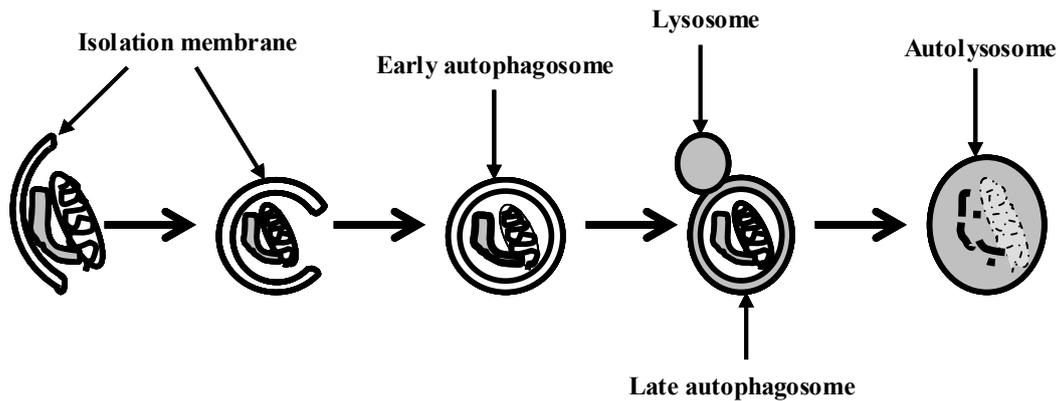


Figure 1.6 A typical models for the formation of autophagosome in eukaryotic cells.

Following induction isolation membrane buds off from rough endoplasmic reticulum (RER) and elongates enwrapping selected cytoplasmic components to form double-membrane vesicle ( early or immature autophagosome) which progressively matures by fusion with endocytic vesicles to form late autophagosome and autolysosome containing degradative enzymes to digest the sequestered materials. Adapted from (Mizushima, 2005).

### 1.3.1 Autophagy in mammalian cell infections

There is a growing body of evidence that autophagy functions in many aspects of innate and adaptive immunity, including immune activation, survival of infected cells, immune cell homeostasis, and degradation of pathogens (Mizushima *et al.*, 2008, Deretic and Levine, 2009, Orvedahl and Levine, 2009). However, some intracellular pathogens are able to resist autophagy digestion to survive in the host cell (Dorn *et al.*, 2002, Kirkegaard *et al.*, 2004, Levine *et al.*, 2011).

#### 1.3.1.1 Role of autophagy in control of infection

Pathogenic organisms that invade cells internalize in phagosome by phagocytosis and are usually delivered to lysosome for degrading (Vieira *et al.*, 2002). Some pathogens,

for example *L. pneumophila* and *L. monocytogenes* are able to subvert phagosome or escape to cytoplasm to avoid digestion (Segal and Shuman, 1998, Henry *et al.*, 2006). Often times, the cell will employ a second line of defence such as autophagy to destroy the invading pathogen (Sanjuan and Green, 2008).

Autophagy has been described as playing a pivotal surveillance role in recognition and eradication of pathogens that have evaded killing by the endosomal system (Dorn *et al.*, 2002, Orvedahl and Levine, 2009, Todde *et al.*, 2009) For example, *M. tuberculosis* and *L. pneumophila* are able to survive in phagosomes of macrophages by inhibiting fusion of the vacuoles with lysosomes. However, when the host cell autophagy is activated during infection, the phagosome is modified and rapidly fuses with lysosomes resulting in the death of intracellular bacteria (Amer and Swanson, 2005, Deretic and Levine, 2009).

Similarly, bacteria that escape from phagosome to cytoplasm such as group A Streptococcus, *S. typhimurium* and *L. monocytogenes* have also been shown to be targeted for destruction by autophagy (Nakagawa *et al.*, 2004, Birmingham *et al.*, 2006, Py *et al.*, 2007).

Other pathogens or their toxins that have been targeted and destroyed by autophagy include: *Burkholderia pseudomallei* (Cullinane *et al.*, 2008), *T. gondii* (Ling *et al.*, 2006), lethal toxin (LT) of *Bacillus anthracis* (Tan *et al.*, 2009a), cytotoxin of *Vibrio cholerae* (Saka *et al.*, 2007), VacA exotoxin of *Helicobacter pylori* (Terebiznik *et al.*, 2009), and HIV-1 (Kyei *et al.*, 2009).

### **1.3.1.2 Microbial adaptations to autophagy**

Intracellular organisms are often targeted for destruction by autophagy to prevent them from establishing a replicative niche within the host (Mizushima, 2005). However, many micro-organisms have developed multiple strategies to subvert autophagic death

and some even utilise the autophagic pathway to enhance their intracellular survival (Kirkegaard *et al.*, 2004, Shintani and Klionsky, 2004, Deretic and Levine, 2009). Herpes simplex virus 1 (HSV-1) for instance, counteracts the ability of neurons to induce autophagy through binding of its neurovirulence proteins to autophagy-promoting proteins (Alexander and Leib, 2008, Orvedahl and Levine, 2008) while *Coxiella burnettii* (Romano *et al.*, 2007), *Porphyromonas gingivalis* (Dorn *et al.*, 2001, 2002, Belanger *et al.*, 2006), *L. pneumophila* (Amer *et al.*, 2005), Hepatitis C virus (Sir *et al.*, 2008a, Sir *et al.*, 2008b), *Staphylococcus aureus* (Schnaith *et al.*, 2007) and *L. monocytogenes* (Birmingham *et al.*, 2008a, Birmingham *et al.*, 2008b) prevent maturation of autophagosomes to autolysosomes.

On the other hand, some pathogens avoid recognition by the autophagy machinery. A typical example is *Shigella flexneri* which secretes IcsB, a type III secretion system to help camouflage the bacterium from autophagic recognition (Ogawa *et al.*, 2005, Ogawa and Sasakawa, 2006). *L. monocytogenes* on the other hand coats its surface with ActA to escape recognition by autophagy (Yoshikawa *et al.*, 2009). In addition, *L. monocytogenes* actin-based motility mediated by ActA also helps the organism evade host cell autophagic killing in the later stages in infection of macrophages (Birmingham *et al.*, 2007, Py *et al.*, 2007, Birmingham *et al.*, 2008b).

Another way by which intracellular pathogens subvert their host's autophagy response is by utilising the functions or components of autophagy to enhance their intracellular survival and replication. For example, *L. pneumophila* (Ogawa and Sasakawa, 2006, Isberg *et al.*, 2009), *L. monocytogenes* (Birmingham *et al.*, 2008a), *Francisella tularensis* (Checroun *et al.*, 2006), *P. gingivalis* (Dorn *et al.*, 2001, 2002, Belanger *et al.*, 2006), *Brucella abortus* (Pizarro-Cerda *et al.*, 1998b) and *C. burnettii* (Gutierrez *et al.*, 2005, Romano *et al.*, 2007) modify the autophagosome and use it as a protective

niche for their survival whereas Coxsackievirus B3 (Wong *et al.*, 2008), *Chlamydia trachomatis* (Al-Younes *et al.*, 2004), *L. pneumophila* (Otto *et al.*, 2004, Dubuisson and Swanson, 2006, Isberg *et al.*, 2009), *Toxoplasma gondii* (Wang *et al.*, 2009), *V. parahaemolyticus* (Deretic and Levine, 2009) or *Leishmania amazonensis* (Pinheiro *et al.*, 2009) induce autophagy to foster their intracellular replication within the host cells.

#### **1.4 Free-living amoebae as host for intracellular pathogens**

*Acanthamoeba* play host to a number of intracellular microbial pathogens including bacteria, fungi, protozoa and viruses (Greub and Raoult, 2004). The most extensively studied intra-protozoan survival of bacteria is that of intracellular survival of *L. pneumophila* in free-living amoebae of the genus *Acanthamoeba* (Harb *et al.*, 2000, Greub and Raoult, 2004, Schuster and Visvesvara, 2004).

The lists of pathogens that are hosted by *Acanthamoeba* are ever growing and include species of bacteria, viruses, fungi and smaller protozoa found in the environment (Greub and Raoult, 2004, Thomas *et al.*, 2010). Some of important bacterial pathogens that have been shown to survive in *Acanthamoeba* are listed in Table 1.2.

Table 1.2 Some intracellular bacteria pathogens that can survive in *Acanthamoeba*

Bacteria spp	Host amoeba	Survival		Reference
		Trophozoites	Cysts	
<i>Acinetobacter baumannii</i>	<i>A. castellanii</i>	+	+	(Cateau <i>et al.</i> , 2011)
	<i>A. culbertsoni</i>	+	+	
<i>B. cepacia</i>	<i>A. castellanii</i>	+	ND	(Lamothe <i>et al.</i> , 2004),(Marolda <i>et al.</i> , 1999)
	<i>A. polyphaga</i>	+	ND	
<i>C. jejuni</i>	<i>A. polyphaga</i>	+	ND	(Axelsson-Olsson <i>et al.</i> , 2005, Axelsson-Olsson <i>et al.</i> , 2010)
<i>Chlamydia pneumoniae</i>	<i>A. castellanii</i>	+	ND	(Essig <i>et al.</i> , 1997)
<i>C. burnetti</i>	<i>A. castellanii</i>	+	ND	(La Scola and Raoult, 2001)
<i>E. coli</i> K-1 and K-12	<i>A. castellanii</i>	+	ND	(Alsam <i>et al.</i> , 2006, Jung <i>et al.</i> , 2008)
<i>F. tularensis</i>	<i>A. castellanii</i>	+	ND	(Abd <i>et al.</i> , 2003)
<i>H. pylori</i>	<i>A. castellanii</i>	+	ND	(Winiecka-Krusnell <i>et al.</i> , 2002)
<i>L.pneumophila</i>	<i>A. castellanii</i> <i>A. polyphaga</i>	+	+	(Kilvington and Price, 1990, Cirillo <i>et al.</i> , 1994, Cirillo <i>et al.</i> , 1999, Ohno <i>et al.</i> , 2008)
<i>M. avium</i>	<i>A. castellanii</i> <i>A. polyphaga</i>	+	+	(Cirillo <i>et al.</i> , 1997, Steinert <i>et al.</i> , 1998)
<i>S. typhimurium</i>	<i>A. rhyssodes</i>	+	-	(Tezcan-Merdol <i>et al.</i> , 2004)

<i>Shigella sonnei</i> <i>S. dysenteriae</i>	<i>A. castellanii</i> <i>A. astronyxis</i> <i>A. healyi</i>	+	ND	(Jeong <i>et al.</i> , 2007, Saeed <i>et al.</i> , 2009)
<i>V. cholera</i> <i>O139</i>	<i>A. polyphaga</i>	+	ND	(Sandstrom <i>et al.</i> , 2010)

+, bacteria able to survive; - bacteria not able to survive; ND, not done

#### 1.4.1 Interactions of *L. monocytogenes* with *Acanthamoeba*

*L. monocytogenes* is primarily a saprophytic bacterium that is well adapted to survival in soil and organic matter but it is also an opportunistic pathogen capable of surviving in human and animal cells and causing a serious disease condition (Gray *et al.*, 2006, Velge and Roche, 2010). Strangely, the virulence gene products used by *L. monocytogenes* to survive in these cells are maintained during their saprophytic existence suggesting that the gene products may also be required for survival in perhaps other eukaryotic organisms such as *Acanthamoebae* that co-exist with *L. monocytogenes* in the environment (Gray *et al.*, 2006). Studies have shown that the maintenance of virulence genes in environmental pathogens such as *Cryptococcus neoformans*, *Parachlamydia acanthamoebae*, *L. pneumophila* and *Mycobacterium avium* is a result of interaction of the microbes with soil borne free-living amoebae such as *Acanthamoeba* (Cirillo *et al.*, 1994, Cirillo *et al.*, 1997, Steenbergen *et al.*, 2001, Molmeret *et al.*, 2005).

Ly and Muller were first to suggest that *L. monocytogenes* can survive predation by the *Acanthamoeba* and even replicate inside the amoeba eventually killing their hosts (Ly and Muller, 1990a, Ly and Muller, 1990b). However, they did not describe the fate of internalised bacteria within protozoa either at cellular or sub-cellular level. In addition, the authors used non-axenic amoebae, which mean that there was high probability that

the cells were contaminated with other environmental bacteria. Moreover, Ly and Muller failed to show a clear distinction between extracellular bacterial growth and intracellular replication within the protozoa. This is necessary because later reports showed that *L. monocytogenes* can grow extracellularly in co-culture with *Acanthamoeba* (Zhou *et al.*, 2007, Huws *et al.*, 2008, Akya *et al.*, 2009b, 2010).

Previously, Zhou and colleagues suggested that some strains of *L. monocytogenes* were able to survive inside *A. castellanii* at 37°C (Zhou *et al.*, 2007). However, they did not find evidence of intracellular bacterial replication within the amoeba, rather they showed that intracellular *L. monocytogenes* numbers reduced over 72 h of infection (Zhou *et al.*, 2007).

Huws and co-workers on the other hand reported that *L. monocytogenes* were unable to survive or grow in *A. polyphaga* during co-cultures at 37°C (Huws *et al.*, 2008).

Recently, Akya and colleagues also found that *L. monocytogenes* did not survive in *A. polyphaga* when co-cultures were incubated at 15°C, 22°C or 37°C (Akya *et al.*, 2009a, 2009b, 2010).

In as much as the 37°C used in previous studies is the optimum temperature for growth and expression of *L. monocytogenes* virulence genes required for survival within mammalian cells (Leimeister-Wachter *et al.*, 1992, Yamada *et al.*, 2006), this temperature may not be suitable for survival of *L. monocytogenes* within *Acanthamoeba* since *Acanthamoeba* rapidly form cysts at 37°C (Marolda *et al.*, 1999, Greub *et al.*, 2004, Akya *et al.*, 2010). On the other hand, incubation of *L. monocytogenes*-*Acanthamoeba* cultures at 15°C and 22°C may affect expression of *L. monocytogenes* virulence genes *in vivo*. The optimum temperature for expression of LLO is between 30-37°C (Leimeister-Wachter *et al.*, 1992, Yamada *et al.*, 2006). The low temperature of incubation may also favour bacterial predation by *Acanthamoeba* (Bowers, 1977,

Leimeister-Wachter *et al.*, 1992, Greub *et al.*, 2004, Huws *et al.*, 2006). It was previously reported that *L. pneumophila* were able to grow in *Acanthamoeba* when cultures were incubated at 35°C but were eliminated by the same amoeba at 22°C or below (Nagington and Smith, 1980, Anand *et al.*, 1983, Ohno *et al.*, 2008).

In addition to incubation condition that could determine the outcome of interaction of *L. monocytogenes* with *Acanthamoeba*, the choice of the strain of *L. monocytogenes* or species of *Acanthamoeba* as host can also determine the fate of *L. monocytogenes* within *Acanthamoeba*. The ability of bacteria to survive in *Acanthamoeba* will obviously depend on whether the *Acanthamoeba* species is permissive for survival of the test strain or species of bacteria. Dey and colleagues recently found that the amoeba, *Willaertia magna* was able to inhibit the intracellular growth of *L. pneumophila*, Paris while permitting growth of others (*L. pneumophila*, Philadelphia and *L. pneumophila*, Lens) belonging to the same serotype (Dey *et al.*, 2009).

In view of the forgoing, it is very necessary to carry out further studies to ascertain if *L. monocytogenes* can survive in *Acanthamoeba*. This will require deliberate inclusion of new strains of *L. monocytogenes* strains and *Acanthamoeba* species that were not used in previous studies. In addition, it will require optimising conditions of incubations that could potentially promote survival of *L. monocytogenes* within *Acanthamoeba*.

## **1.5 The aims of this study.**

The aims of this study are to investigate the interactions of *L. monocytogenes* with *Acanthamoeba* spp, determine whether *L. monocytogenes* is able to survive and grow inside trophozoites of *Acanthamoeba* and also survive their encystment, be protected from disinfectant while inside the cyst and grow again after release. The study will also test if intracellular survival within *Acanthamoeba* can affect the morphology of *L. monocytogenes*, its ability to form biofilms and its response to biocides. The ability of

*L. monocytogenes* to survive in *Acanthamoeba* and the influence such survival could have on the ability of the organism to form biofilms and respond to biocides may explain the persistence of *L. monocytogenes* in food industry as biofilms (Carpentier and Cerf, 2011) and the resistance of such biofilms to disinfectants that are commonly used for routine cleaning in food environments (Norwood and Gilmour, 2000, Pan *et al.*, 2006). Each of the following chapters contains the detail for each investigation together with their hypotheses, aims and objectives.

## **Chapter 2. Materials and Methods**

### **2.1 Media and reagents**

All chemicals and reagents unless stated in the text were obtained from Sigma-Aldrich Chemical Company, Dorset, UK while media were obtained from Oxoid, Basingstoke, UK. Media, buffers and reagents were prepared in clean glassware using deionised water and were either sterilised by autoclaving at 121°C for 15 min or by filtration in the case of media or reagents that are heat labile.

### **2.2 Bacterial strains, assay and storage condition**

The bacteria species used in this study are shown on Table 2.1. They were stored at -80°C in growth medium. For routine day-to-day experiment bacteria were sub-cultured on tryptone soya agar (TSA) incubated at 37°C overnight and maintained at 4°C for 2 weeks, sealed with paraffin wax. A maximum of three subcultures were done before a fresh inoculation was made. For co-culture experiments, bacteria were sub-cultured onto fresh TSA plates and incubated at 32°C for 24 h. Bacterial colonies were harvested from plates using a moist sterile cotton bud to make a suspension in fresh quarter-strength Ringer's solution (RS)(Oxoid, Basingstoke, UK) in a 30 ml capacity sterile polystyrene universal bottle (Sterilin, Southport, UK). Bacterial suspension was vortexed vigorously to mix and concentrations were measured by their optical densities at 600 nm in a WPA CO8000 cell density meter (Biochrom, Cambridge, UK) and adjusted to the desired OD. To calibrate *Listeria* standard curve several concentrations were made from a stock suspension. CFU of viable bacteria in each dilution was

determined and graph of standard calibration curve was obtained by plotting CFU values of bacteria concentrations against their corresponding ODs (Figure 2.1).

For experiments that involved use of *L. monocytogenes* exposed to manganese ions, 1M stock solution of manganese chloride tetrahydrate previously prepared and filter-sterilised was incorporated into TSA (final concentration *c.* 2- 4 mM) while the medium was between 50°C- 55°C before dispensing into plates.

Table 2.1 Bacterial species

Organism	Description	Serotype	Source	Reference
<i>L.monocytogenes</i>				
strains				
Scott A	Wild type strain	4b	Clinical	(Jordan <i>et al.</i> , 2008)
EGDe	Wild type strain	1/2a	Clinical	(Archambaud <i>et al.</i> , 2006)
C52	Wild type strain	1/2a	Clinical	(Jordan <i>et al.</i> , 2008)
10403s	Wild type strain	1/2a	Clinical	(Jordan <i>et al.</i> , 2008)
10403s $\Delta$ hly	mutant	1/2a	-	-
10403s $\Delta$ prfA	mutant	1/2a	-	(Fadaee-Shohada <i>et al.</i> , 2010)
<i>Escherichia coli</i> JM101	Wild type strain	-	-	(Meyer <i>et al.</i> , 2005)

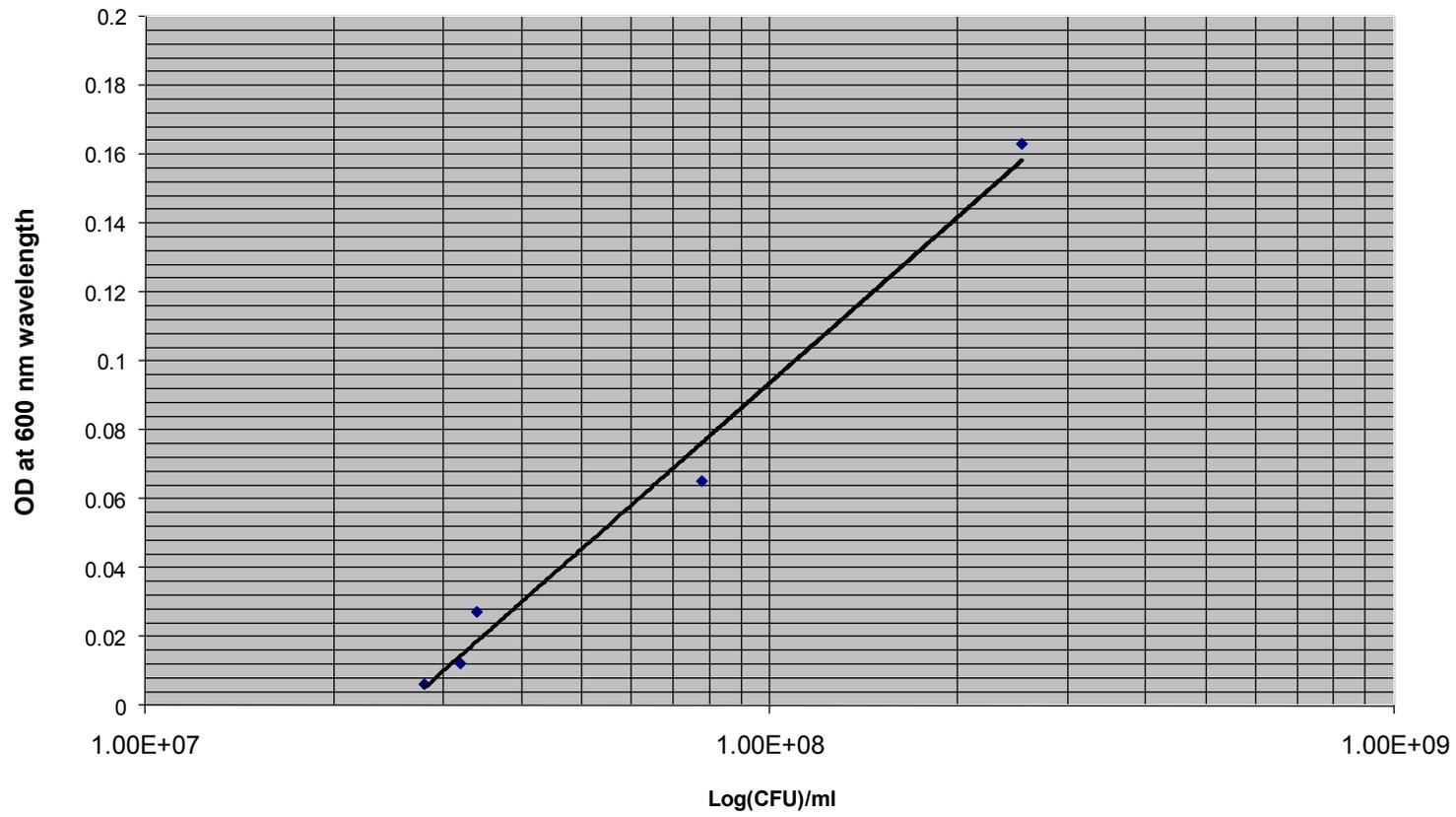


Figure 2.1 *Listeria monocytogenes* standard curve

## **2.2.1 Determination of bacterial viability**

### **2.2.1.1 Automated method**

This method used an automated spiral plater (WASP; Don Whitley Scientific, Shipley, UK) to inoculate bacteria onto TSA (Cheng and Portnoy, 2003). A sample was plated using the following settings: Deposition type: Log; fill mode: manual/syringe; deposition volume, 50 µl per plate in a continuously decreasing volume across 10 cm Petri dish (Figure 2.2). Prior to plating a sample stylus of the spiral plater was sanitised in 2400 ppm free chlorine disinfectant, prepared by dissolving two chlorine-releasing tablets (Presept; Johnson and Johnson Medical UK) in 1L deionised water. 500 ml of the disinfectant was placed in the machine and this was replaced with fresh disinfectant once weekly. Following disinfection, the stylus was rinsed in quick succession in sterile distilled water contained in two water pots. Fresh water was used after every 24 h time-point experiment. The stylus was sterilised before and after plating a sample. In addition, the sterility of the plater was determined before and after each batch of samples by plating sterile double-distilled water on TSA. Following 24-48 h incubation at 37°C, colony forming units (CFU) of viable bacteria were counted in a ProtoCOL system (Synbiosis, Nuffield Road Cambridge, UK) and estimated using Spearman–Karber computations.



Figure 2.2 Colonies of *L. monocytogenes* on TSA spread by means of a spiral plating device.

#### **2.2.1.2 Manual method**

The drop plate method described by Chen and co-workers (Chen *et al.*, 2003) for counting bacterial colonies was adopted with slight modifications. Here a multi-channel pipette (Biohit Oyj, Finland) was employed to carry out a 10-fold serial dilution of bacterial suspension across wells of a 96-well microtitre plate (Figure 2.3). 20  $\mu$ l of bacterial suspension was transferred to the first well containing 180  $\mu$ l D/E neutralising broth (Difco Laboratories Michigan, USA) and then mixed by pipetting 6 times. After mixing, 20  $\mu$ l was taken and serially diluted up to the sixth well containing 180  $\mu$ l RS in place of D/E neutralising broth and then discarding 20  $\mu$ l after the sixth dilution. Each sample dilution was done in triplicate wells. After mixing, 50  $\mu$ l from each dilution was

spotted on a corresponding segment marked on TSA plate. Plate was then left undisturbed on the bench to dry before they were incubated at 37°C for 24 h. The dilution with countable colonies of 20 or more was used to determine viable bacterial number which was expressed as colony forming units per ml (CFU/ml).

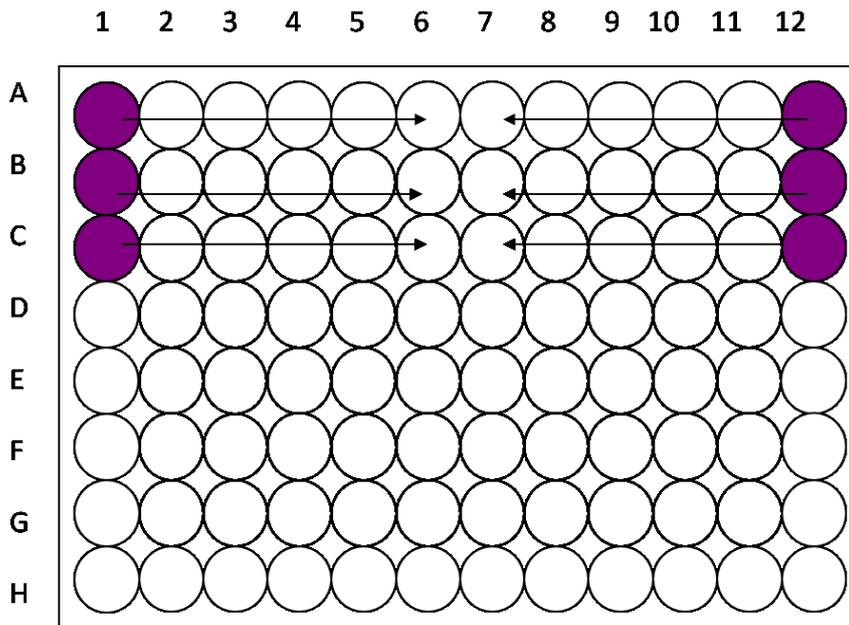


Figure 2.3 Outline of serial dilution of culture in 96-well microtitre plate.

Well with purple colour contained 180  $\mu$ l each DE/broth while the remaining 5 wells contained 180  $\mu$ l each RS. Arrows point at direction of serial dilution.

### 2.3 Amoebae culture and culture condition

The protozoa species used in the present study are listed in Table 2.2. The organisms were maintained as axenic cultures in a semi-defined *Acanthamoeba* growth medium (SK #6 medium) (Appendix 1E) supplemented with 40 U/ml penicillin and 40 mg/ml streptomycin (Hughes *et al.*, 2003). The monolayer of cells was incubated in a 175 cm<sup>2</sup> tissue culture flasks (Nunc Roskilde, Denmark) at 28°C until confluent growth was attained (3-4 days) before use in co-culture experiments. Growth medium was replaced

with fresh medium containing antibiotics every 4 d. Fresh cell culture was prepared from stock after 4-5 passages. For use in co-culture studies amoebae were suspended by tapping hard the bottom of the flask and pelleted in a centrifuge (Beckman coulter, High Wycombe, UK) at  $1000 \times g$  for 3 min. After three washes in RS, the pellet was re-suspended to 10 ml in the same solution. A tenfold dilution of amoeba cells in RS was done and their viability determined (see below).

Table 2.2 *Acanthamoeba* species

Protozoa	Description	Source	Reference
<i>Acanthamoeba castellanii</i>	ATCC strain	Human eye infection	(Pasricha <i>et al.</i> , 2003)
<i>A.culbertsoni</i>	ATCC strain	Monkey kidney tissue	(Rocha-Azevedo <i>et al.</i> , 2010)
<i>A. polyphaga</i> (Ros)	Wild type strain	<i>Acanthamoeba</i> keratitis	(Hughes and Kilvington, 2001)

### 2.3.1 Determination of amoebae viability

The viability of *Acanthamoeba* was determined by the trypan blue exclusion method (Strober, 2001). Here, 50  $\mu$ l of diluted cell suspension was mixed with equal amount 0.8 % (w/v) trypan blue (final concentration 0.4 %) for 5 min. 20  $\mu$ l cell suspension was removed and filled the chamber of a modified Fuchs-Rosenthal haemocytometer. Viable cells (unstained cells) were counted in five of the nine large squares of the chamber using 20 $\times$  objective of a CKX41 phase contrast microscope (Olympus, Essex, UK). Cell counts was multiplied by the dilution factor times a thousand and expressed as count/ml.

## **2.4 Cryopreservation of cultures**

For long time storage, protozoa and bacteria cultures were cryopreserved as previously described (Simione, 1998). To cryopreserve protozoa culture, 0.5 ml of cell suspension ( $10^6$  trophozoites/ ml) in the growth medium was placed in a cryotube containing equal amount of 10 % (v/v) foetal bovine serum (FBS) and 10 % (v/v) dimethyl sulfoxide (DMSO) to give a final concentration of 5 % FBS and 5 % (v/v) DMSO. The tube was labelled and placed in Nalgene freezing box designed to achieve a slow uniform cooling of 1°C per minute. The box was immediately placed in -80°C freezer overnight. Thereafter it was removed and stored directly at -80°C until use. The frozen stock was reconstituted by thawing in a water bath at 37°C and contents of the vial were immediately transferred to a tissue culture flask containing fresh growth medium with antibiotics and incubated at 28°C. Similarly, bacterial strains that were to be cryopreserved were first grown overnight in TSB at 37°C with vigorous shaking (200 r.p.m.). Cells were pelleted at  $2000 \times g$  for 20 min and re-suspended in fresh medium at a concentration of  $\approx 10^9$  CFU/ml. 0.5 ml bacterial suspension was added to equal amount of 100 % (v/v) sterile glycerol and stored directly at -80°C. For use, the frozen stock culture was removed, slightly thawed at room temperature and a loopful of bacteria was streaked on TSA and incubated at 37°C overnight.

## **2.5 Screening of detergents for cell lyses**

Ten percent (10 %) stock solutions of each of the following detergents: Nonidet-p40, saponin, sarcosine, SDS, taurocholic acid, tween-80 and triton-x100 were made and filter-sterilised. The Stock solutions were stored at room temperature for 1 month. For use, 1 % (v/v) detergent solution was made by diluting 1ml stock in 9 ml distilled water. Serial doubling dilution of detergent in 100 µl RS was carried out in triplicate across a

96-well microtitre plate leaving the last set of wells to serve as controls. An equal amount of *A. castellanii* ( $10^5$  cells/ml) was added to each well and the plate was left undisturbed on the bench for 10 min. Plates were examined under an inverted microscope for cell lysis. The lowest concentration of detergent that completely lysed trophozoites was considered the minimum amoebicidal concentration (MAC). The viability of *L. monocytogenes* was also tested at the MAC as follows: 1 ml of *Listeria* suspension (OD  $\approx$  0.15) was diluted in 9 ml of RS and 1 ml of this suspension was incubated with 1 ml detergent (2 times MAC) at room temperature. At 0, 10 and 30 min 0.2 ml of the bacteria-detergent suspension was removed and neutralised with 0.8 ml DE/ neutralising broth. Neutralisation was allowed for 10 min and thereafter a hundred-fold dilution of the bacterial suspension was done in RS and then plated onto TSA. Following incubation at 37°C for 24 h, CFU of viable bacterial cells were counted and recorded.

## **2.6 Co-culture experiments**

Co-culture of *L. monocytogenes* with protozoa was done as follows. Ten ml of cell suspension ( $5 \times 10^5$  trophozoites/ml) in RS was pipetted into a 25 cm<sup>2</sup> capacity tissue culture flask (NUNC) and mixed with 1 ml *L. monocytogenes* Scott suspension (OD  $\approx$  0.2) in RS. Co-culture was then incubated at 32°C with or without shaking. The shaking incubation was done in an orbital incubator (Sanyo Europe, Etten-Leur, Netherlands) at 100 r.p.m. At intervals up to 96 h, 100  $\mu$ l samples of co-culture was pipetted into a micro centrifuge tube and an equal volume of 0.13 % (v/v) sarcosine (final concentration = 0.06 %) was added and incubated at room temperature for 10 min. The tube was then vortexed at 2800 r.p.m on a minishaker (Camlab Cambridge, UK) for 30 s to ensure complete cell lysis. Cell lysate containing both extracellular and intracellular bacteria was diluted where necessary with RS and spread onto a TSA plate

in triplicate using a spiral-plater. After incubation at 37°C for 24 h the CFU were recorded. The viability of amoebae in co-culture was also determined using trypan blue exclusion method as described in section 2.3.1. *L. monocytogenes* incubated alone in RS and amoebae incubated alone in RS served as control samples.

## **2.7 Viability of *Listeria* in *Acanthamoeba*-conditioned medium**

Thirty millilitres of viable amoeba trophozoites ( $5 \times 10^5$ /ml) were incubated in fresh RS at 32°C without shaking for 24 h. After incubation, cells were pelleted at  $300 \times g$  for 3 min and the supernatant was filter sterilised by passing through a low protein binding membrane filter of pore size 0.2  $\mu\text{m}$  (Pall Corporation, Washington New York, USA). The filtrate, henceforth called conditioned medium (CM), was immediately used to cultivate *Listeria* in a similar fashion as in co-culture studies described above. Here, 1 ml of *L. monocytogenes* ( $\text{OD} \approx 0.2$ ) was inoculated into 9 ml CM in a small tissue culture flask and incubated at 32°C with or without shaking. At time points up to 96 h, samples of cultures were taken to determine viable bacteria number after serial dilution. *L. monocytogenes* grown in fresh RS in the same conditions served as the control experiment.

## **2.8 Invasion assays**

Gentamicin protection assay was used to determine uptake and/or intracellular survival of *L. monocytogenes* in amoeba (Van Langendonck *et al.*, 1998). In this method, co-culture of *L. monocytogenes* with amoeba were carried out as described above except that extracellular bacteria were removed from co-culture medium after a limited time of contact by washings and gentamicin treatment before intracellular bacteria were assessed. Prior to this experiment the sterility of amoeba was tested by lysing small

uninfected trophozoites with sarcosine and spreading the cell lysate on to TSA and incubated at 37°C for 24 h.

### **2.8.1 Uptake of *L. monocytogenes* by *Acanthamoeba***

To demonstrate uptake of *Listeria* into vacuoles of amoeba cells *A. castellanii* trophozoites were co-incubated with *L. monocytogenes* at 32°C for 1 h. Thereafter, cells were pelleted by centrifugation at  $300 \times g$  for 3 min and washed 5 times with RS to reduce the number of bacteria that were not associated with amoeba. For complete elimination of extracellular *Listeria*, infected cells were incubated with 500 µg/ml gentamicin for 1 h. After this, infected cells were pelleted and washed three times to remove gentamicin and lysed for 10 min with 0.06 % (v/v) sarcosine to liberate any intracellular bacteria which were then detected and counted after incubation in TSA.

In experiments to determine effect of multiplicity of infection (MOI) on uptake of *Listeria*, 10 ml of amoeba suspension ( $5 \times 10^5$ /ml) in RS was placed in each 25 cm<sup>2</sup> capacity tissue culture flask and infected with *L. monocytogenes* at varying ratios. After 1 h incubation at 32°C, cells were washed and treated with gentamicin and intracellular bacteria were determined as described above.

To determine effect of duration of incubation on uptake of *Listeria*, 30 ml cell suspension of *Acanthamoeba* was placed in a 75 cm<sup>2</sup> tissue culture flask and infected with *L. monocytogenes* in RS at a ratio of 1 amoeba to 100 *Listeria*. At 0.5 h, 1 h, 2 h, 3 h and 4 h, 5 ml sample of co-culture was taken, washed five times with RS and CFU of intracellular bacteria were counted after gentamicin treatment and cell lysis.

### **2.8.2 Light microscopy assay of bacteria uptake**

Internalisation of *L. monocytogenes* into vacuoles of amoeba was also demonstrated by light microscopy of samples stained with giemsa or toluidine. For the giemsa method *L. monocytogenes* were mixed together as described in section 2.8.1. Using a Pasteur

pipette, a drop of co-culture was delivered onto a microscope slide and incubated at 32°C for 1 h. After incubation, extracellular bacteria were removed by gentle washing with stream of distilled water for 1 min. The slide was air-dried and fixed in absolute methanol for 10 min followed by staining with giemsa solution in 0.5 % (w/v) Na<sub>2</sub>HPO<sub>4</sub>, 0.5% (w/v) K<sub>2</sub>HPO<sub>4</sub>, pH 6.8 for 1 h as described by Newsome *et al.*, 1998. Infected cells were examined under 100 × oil immersion objective of a light microscope (Carl Zeiss Welwyn Garden City, UK) and images were captured using an InfinityX-32 camera (Mazurek Optical Services, Southam, UK).

In the second method, infected cells were fixed with 2.5 % (v/v) glutaraldehyde buffered with 0.05M HEPES (pH 7.2) at 4°C overnight and then processed up to the resin embedding stage (see section 2.8.5). Semi- thin sections (0.5µm) were cut from the blocks using an ultracut microtome (Reichert-Leica, Marseille, France). The sections were then dried onto glass slides and stained with toluidine blue for 30 s. Excess dye was washed off with double distilled water and air-dried. Stained cells were examined under 100 × oil immersion objective of a Ceti Magnum- T positive phase contrast microscope fitted with a si-3000 digital camera (Medline Scientific Chalgrove, UK). Images were captured with the camera and viewed with XLi-Cap 17 software (XL imaging, Swansea, UK).

### **2.8.3 Intracellular growth kinetics of *Listeria* within *Acanthamoeba***

Thirty millilitres *Acanthamoeba* suspension consisting of  $5 \times 10^5$  *Acanthamoeba* trophozoites per ml of RS in 75 cm<sup>2</sup> tissue culture flask were infected with *L. monocytogenes* suspension at MOI of 1:150. Co-culture was incubated at 32°C with shaking for 1 h to allow ingestion of bacteria. Afterwards, infected cells were harvested into a 25 cm<sup>2</sup> centrifuge tube by gently scraping the wall of the flask with a cotton bud. The infected cells were then pelleted by centrifugation at 300 × g for 3 min followed by

washing 5 times with RS to remove non-engulfed bacteria. Afterwards, infected cells were incubated with 500 µg/ml gentamicin for 1 h to kill remaining extracellular bacteria that were not removed by washing. After three further washes with RS to remove gentamicin, infected cells were suspended in 20 % (v/v) sterile SK6 medium (maintenance medium) (Appendix 1F) without antibiotics. Infected cells were again pelleted at  $300 \times g$  for 3 min and the supernatant was tested for extracellular bacteria by spreading on TSA as described in section 2.2.1.1 and thereafter cells were re-suspended and incubated at 32°C. This time represented 0 h after infection. At time points 0 h, 4 h and subsequently 24 h from the start of infection, 1 ml sample of infected cells was removed into 15 ml centrifuge tube and spun at  $300 \times g$  for 3 min. The supernatant was tested for the presence of extracellular bacteria and thereafter tube was vortexed at 1800 rpm for 30 s to re-suspend cells in the pellet. 0.5 ml cell suspension was pipetted into a micro centrifuge tube, lysed with equal volume of approximately 0.13 % (v/v) sarcosine (final concentration 0.06 %). The lysate was then diluted in RS and spread on TSA as described in section 2.6. CFU of viable bacteria were counted after incubation for 24 h at 37°C. The controls included *L. monocytogenes* incubated alone or *Acanthamoeba* alone incubated alone in RS and treated the same way as control sample.

#### **2.8.4 Microplate assay of intracellular bacterial survival**

*Acanthamoeba castellanii* was allowed to grow to confluence in a 75 cm<sup>2</sup> tissue culture flask and then 2 ml of cell suspension in growth medium was added to each of 6 well microtitre plate and incubated at 28°C for overnight to form monolayer of cells. After incubation 2 growth medium was aspirated and approximately  $2 \times 10^7$  CFU *L. monocytogenes* in RS grown for overnight in brain heart infusion (BHI) broth (BD) at 37°C was used to inoculate cells in each well and incubated for 6 h at 37°C. Afterwards RS was aspirated and cell monolayer was washed once. The monolayer was then

incubated in 2 ml of growth medium containing 100 µg/ml gentamicin for 1 h at room temperature. Following incubation the medium was again aspirated and cells were washed two times with RS. After washing, 2 ml of growth medium containing 10µg/ml gentamicin was added and incubated at 37°C. At time points 0 h and subsequently 24 h intervals, growth medium was removed and the cell monolayer was washed once with RS. The cells were then removed by scrapping with a sterile cotton bud and lysed by passing through a 27-gauge needle 5-7 times. The cell lysate was spread onto TSA plate after dilution and CFU were counted after 24 h incubation at 37°C as described above (Zhou *et al.*, 2007).

### **2.8.5 Transmission electron microscopy to assess intracellular survival**

*Acanthamoeba castellanii* infected with *L. monocytogenes* was examined in transmission electron microscopy as described by (Hayat, 1970). In this experiment, *A. castellanii* trophozoites ( $10^6$  cells/ml) were infected with *L. monocytogenes* at a multiplicity of infection of 1:300. Infected cells were fixed at times up to 48 h post infection with 2.5 % (v/v) glutaraldehyde buffered with 0.05M HEPES (pH 7.2) at 4°C overnight. Samples were then taken to the Advanced Microscopy Centre of School of Biological Sciences where they were processed by the Technicians as follows: The cells were washed 3 times for 20 min in fresh 0.05 M HEPES (pH 7.2) and post-fixed in 1 % (w/v) osmium tetroxide in 0.05 M HEPES for a further 1.5 h. After subsequent washes in 0.05 M HEPES for 20 min and in double distilled water for another 20 min, samples were dehydrated in a series of increasing concentrations of ethanol. Finally, samples were embedded using LR white resin. Thin sections (approx. 80 nm thick) and semi-thin sections (0.5 µm) were cut from the blocks using an ultracut microtome (Reichert-Leica Marseille, France) and were collected on copper grids. The semi-thin sections were dried onto glass slides and stained with toluidine blue and examined in a light

microscope (see section 2.8.2.). The thin sections on the other hand were stained with 2 % (w/v) uranyl acetate in 30 % (v/v) methanol for 15 min followed by 3 min in Reynolds' lead citrate (30% v/v) and examined using a transmission electron microscope (JEOL 1220) using an accelerated voltage of 80 KV. Digital images were captured using a SIS Megaview III camera.

### **2.8.6 Detection of superoxide radicals in infected cells**

To show that *Acanthamoeba* generates toxic oxygen radicals during phagocytosis of *L. monocytogenes*, *A. castellanii* cells were pre-incubated with 10 µg/ml diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase (O'Donnell *et al.*, 1993) for 15-30 min before infection with *L. monocytogenes* and during incubation in maintenance medium. The number of intracellular bacteria was assessed at 0 h and 4 h of infection as described in section 2.8.3 (O'Donnell *et al.*, 1993)

### **2.8.7 Extraction of listerial soluble proteins**

A suspension of *L. monocytogenes* grown on TSA alone or TSA plus MnCl<sub>2</sub>.4H<sub>2</sub>O was prepared in phosphate buffer saline (PBS) and adjusted to OD<sub>600 nm</sub> = 1 (c. 10<sup>9</sup> CFU/ml). Four millilitres of this suspension were pipetted into a 7 ml capacity sterile polystyrene bijou bottle (Sterilin) and chilled for 30 min in ice. Bacterial proteins were extracted by sonication with a Sanyo Soniprep 150 (MSE, London, UK). Prior to sonication the probe was sanitised with 70 % (v/v) ethanol and it was then inserted into the bacterial sample to the depth of at least 1 cm. Sonication was performed on ice at an amplitude of 8 microns for 15 s per pulse, up to a maximum of 10 pulses with a 30 s interval between pulses. The probe was sanitised with 70 % ethanol after each sample and immersed in ice for 1 min to prevent overheating. Following completion of sonication (when the cloudy cell suspension becomes translucent), the cell debris were pelleted by

centrifuging at  $10,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The clear supernatant containing soluble proteins was collected and stored at  $-80^{\circ}\text{C}$  until use.

### **2.8.8 Determination of protein concentration in the cell extract**

The protein concentration of bacterial extracts was determined using the Bradford protein assay method in a 96-well microtitre plate (Bradford, 1976). In this method a 1 in 5 dilution of Bradford reagent (Sigma) was done by adding 1 ml of the reagent to 4 ml of phosphate buffer and filtering through a low-protein binding membrane filter (0.2  $\mu\text{m}$  pore size) to remove particles. A two-fold serial dilution of 1mg/ml bovine serum albumin (Europa Bioproducts Ltd, Cambridge, UK) (Appendix 1A) solution was carried out. A single 1 in 10 dilution of bacterial extract was also done. Ten microlitres of each concentration of BSA or extract was added into triplicate wells of a microtitre plate followed by addition of 200  $\mu\text{l}$  of the diluted Bradford reagent and mixed by gentle pipetting. The blank consisted of 200  $\mu\text{l}$  of the diluted Bradford reagent. The plate was then incubated at room temperature for 5 min and afterwards absorbance at 595 nm was read in a model 680 Bio-Rad microplate reader (Bio-Rad Laboratories Hercules California, USA). The average absorbance of each concentration of BSA standard and extract was calculated and subtracted from the average of the blank. Standard curve of BSA concentration was determined by plotting absorbance against concentration of BSA. A trend line was drawn. The linear regression ( $R^2$ ) value and equation of the line were recorded. Finally, total protein concentration of bacterial extract was determined by substituting the value of their absorbance in the standard curve equation.

### **2.8.9 Quantitative SOD activity in bacterial extract.**

The quantitative SOD activity of bacterial extract was done using the colorimetric method (Sigma Aldrich). Reaction cocktail was prepared as described in appendix 1G.7. Xanthine oxidase check was done by pipetting 2.8 ml of the reaction cocktail into each

of two cuvettes and use to equilibrate a spectrophotometer (Jenway Scientific Equipment, Stone, UK) at 550 nm. Afterwards 0.2 ml deionised water was added to one of the cuvette to serve as blank while 0.1 ml dH<sub>2</sub>O and 0.1 ml xanthine oxidase (Appendix 1G.5) were added to the second cuvette. Absorbances were measured at 550 nm for 5 min. The change in absorbance for the uninhibited (xanthine oxidase) and blank should be  $0.025 \pm 0.005$  if not, the concentration of xanthine oxidase (Appendix 1G.5b) was adjusted to meet this requirement. For test experiment the following reagents were added (reaction mix):

	Blank (ml)	Uninhibited (ml)	Test (ml)	Test 2 (ml)	Test (ml)
Reaction cocktail (Appendix 1G.7)	2.8	2.80	2.80	2.80	2.80
Deionised water	0.20	0.10	0.00	0.10	0.02
SOD (appendix 1G.6)	0.00	0.00	0.10	0.09	0.08
Equilibrate then add XOD	0.00	0.10	0.10	0.10	0.10

(Appendix 1G.5)

The reaction mix (3 ml) containing 50 mM K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 0.1 mM EDTA, 0.01 mM cytochrome c, 0.05 nM xanthine, 0.005 units xanthine oxidase and 1 unit SOD was mixed by inversion and absorbance was read at 550 nm for 5 min.

The percent inhibition was calculated as:

$$\frac{\Delta A_{550\text{nm}}/\text{min uninhibited} - \Delta A_{550\text{nm}}/\text{min inhibited}}{\Delta A_{550\text{nm}}/\text{min uninhibited}} \times 100$$

$$\Delta A_{550\text{nm}}/\text{min uninhibited} - \Delta A_{550\text{nm}}/\text{min Blank}$$

$$\text{Units/ml Enzyme: } \frac{\text{Percent inhibition} \times \text{DF}}{100}$$

$$50 \% \times 0.10$$

Where DF = Dilution factor

50 % = inhibition of the rate of cytochrome c reduction per the unit definition

0.10 = Volume (in ml) of enzyme used in each test.

## **2.9 Autophagy assay**

To investigate the role played by autophagy in intracellular survival of *L. monocytogenes*, *A. castellanii* was pre-incubated with 10 mM 3-methyladenine an inhibitor of autophagy for 20-30 min before infection with *L. monocytogenes* (Caro *et al.*, 1988). Intracellular bacteria survival was then assessed by the quantitative gentamicin protection and electron microscopy methods as described in sections 2.8.3 and 2.8.5.

## **2.10 Synchronous encystment/excystment of *Acanthamoeba***

Co-culture of *A. castellanii* with *L. monocytogenes* was carried out in a 75 cm<sup>2</sup> culture flask as described in section 2.8.3, except that a large number of amoeba cells (10<sup>6</sup> trophozoites/ml) were used and the infection ratio was respectively 1: 300. Co-culture flasks were incubated at 32°C with agitation at 100 r.p.m in an orbital incubator. After 1 h or 4 h of incubation, infected cells were respectively induced to form cysts while a flask was removed from the shaker and incubated without shaking for up to 24 h post-infection before cells were induced to form cyst.

For encystment, infected cells were harvested by gentle scrapping with a sterile cotton bud and then pelleted at 300 × g and washed 2 times with RS and once with Neff's encystment medium (Appendix 1) to reduce number of extracellular bacteria. Infected cells were then suspended in Neff's encystment medium and incubated at 32°C with shaking at 100 rpm for 4-6 days for trophozoites to form cysts. A control of amoeba alone and *L. monocytogenes* alone in RS were also incubated under the same conditions. After encystment, any cysts that had formed were harvested by scraping the surface of

the flask with a cotton bud. The cysts were then pelleted by centrifugation at  $1200 \times g$  for 10-15 min and washed two times with RS. The cysts were then incubated in 3 % (v/v) hydrochloric acid at room temperature for 24 h. Following incubation, cysts were again washed three times with sterile RS and the supernatant of the third wash was tested for presence of bacterial contamination by plating on triplicate TSA plates and incubation at 37°C. The washed cysts were then re-suspended in fresh SK6 medium without antibiotic and incubated at 32°C for hatching. The medium was inspected daily using an inverted microscope for hatching of amoeba and release of bacteria. Images were captured with a Camedia C-5050 zoom digital camera (Olympus, Essex, UK).

### **2.10.1 Susceptibility of amoebae cysts to chlorine**

Infected cysts were produced as described in section 2.10. Following acid treatment, cysts were washed, pooled and counted in a modified Fuchs-Rosenthal haemocytometer and adjusted. The ability of the *Listeria* trapped in cysts to survive chlorination was then tested as described by Kilvington and Price (Kilvington and Price, 1990). In this method, a 5 ml cyst suspension in RS ( $1 \times 10^6$  cysts/ ml) was placed into 15 ml centrifuged tubes. Meanwhile, 200 mg/l free chlorine stock was prepared from 10 % (v/v) sodium hypochlorite using phosphate buffer saline (pH 7.3). For this, a solution of 10 % (v/v) sodium hypochlorite was prepared in phosphate buffer saline and incubated at room temperature for 30 min. After incubation sodium hypochlorite was diluted and amount of free chlorine was measured using the N; N-Diethyl-p-Phenylenediamine (DPD) method in a Lovibond Comparator 2000+ (Tintometer Ltd, Lovibond House Amesbury, UK) in accordance with the manufacturer's instructions. Here, a chlorine colour disc (3/40B), which measures between 0.2 mg/l to 4mg/l free chlorine, was fitted into the comparator and two 10 ml cells were rinsed with the sample to be tested. One

cell was filled to the 10 ml mark with the sample and placed in left hand compartment of the comparator to act as blank, while a few drops of sample were placed into the second cell and one tablet of N; N-Diethyl-p-Phenylenediamine (DPD 1) was added, crushed with a clean stirring rod and topped up to the 10 ml mark with sample. This was mixed thoroughly by inversion and placed in the right hand compartment of the comparator. The colour disc was rotated against day light until a colour match was found and the corresponding value of chlorine on the disc was recorded. The sodium hypochlorite was then diluted in RS to give concentrations of 20-200 mg/l free chlorine. Five ml of each of the diluted free chlorine solutions was added to the cyst suspension and incubated at room temperature for 18 h. RS was added to control samples in place of chlorine.

Following incubation, disinfectant was removed by washing two times with RS containing 18 mg/ml sodium thiosulphate and once with RS alone (Kilvington and Price, 1990). The final pellet was re-suspended in 10 ml of SK6 medium and incubated in 25 cm<sup>2</sup> tissue culture flask at 32°C for hatching and release of intracellular bacteria which were monitored daily with an inverted phase contrast microscope for up to 14 days.

## **2.10.2 Identification of bacteria released from cysts**

### **2.10.2.1 Light microscopy**

Bacteria in hatching medium were examined with a 40× objective of a phase contrast microscopy for characteristic morphology and motility and images were captured using a Camedia C-5050 zoom digital camera (Olympus, Essex, UK)

### **2.10.2.2 Colonial morphology**

A loopful of bacteria in hatching medium was streaked on TSA plate and incubated for 24 h at 37°C. Thereafter bacterial colonies were examined for characteristic small, transparent, grey colonies (Harrison *et al.*, 2000).

### **2.10.2.3 Gram reaction**

Thin smear of bacteria was made on a clean glass slide. The smear was air-dried and heat-fixed by passing over a Bunsen flame. The smear was flooded with 0.5 % (w/v) crystal violet for 1 minute and excess stain was removed by gentle washing with water. After washing the slide was flooded with 0.4 % (w/v) Gram iodine for 1 minute and rinsed with water. After washing slide was decolourised by gentle streaming with acetone ( 3 parts of acetone and 1 part isopropyl alcohol) for 5 s. Smear was then counterstained with 0.5 % (w/v) safranin for another 1 min and rinsed with water (Coico, 2005). Slides were then air-dried and examined under 100 × oil immersion objective of a Ceti Magnum- T positive phase contrast microscope fitted with si-3000 digital camera (Medline Scientific Chalgrove, UK). Bacteria images were captured with the camera and viewed with XLi-Cap 17 software (XL imaging, Swansea, UK).

### **2.10.2.4 Acid production from mannose**

Bacteria isolates were tested for ability to produced acid from 1 % (w/v) mannose by inoculating a colony of an overnight growth on TSA into a tube of peptone water sugar medium (Appendix 1) incubated at 37°C for 18-24 h. After incubation, the tube was examined for growth and fermentation of mannose evident by acid production which changed the medium from purple to yellow (Barrow and Feltham, 1993).

### **2.10.2.5 Negative staining of bacteria**

Bacteria were washed twice with RS and re-suspended in 1 ml of same solution. 5  $\mu$ l bacteria suspension was applied onto the surface of a fresh glow discharged pioloform coated-grids. The grid was then suspended in the neck of a bottle containing 25 % (v/v) glutaraldehyde for 5 min to both fix the sample and adsorb it to the carbon film surface. Excess sample was removed by touching the right angles of the grid with filter paper to leave a thin film of sample. Grid was then rinsed twice with double distilled water and excess fluid was removed with filter paper. Sample was stained by adding 2 drops 1 % (v/v) uranyl acetate and excess stain was removed and air dried. Stained samples were viewed in a JEOL 1220 TEM with accelerating voltage of 80kV. Images were captured using Megaview III digital camera with analysis software (Harris, 1997).

### **2.10.2.6 API *Listeria* test**

Confirmation that intracellular bacteria recovered from amoeba cysts were *L. monocytogenes* was done using API *Listeria* test kits (BioMerieux, Basingstoke, UK) (Bille *et al.*, 1992). This consists of 10 cupules containing dehydrated substrates for enzymatic or sugar fermentations tests including test for the presence or the absence of arylamidase (DIM test), hydrolysis of esculin (ESC), presence of  $\alpha$ -mannosidase ( $\alpha$ -MAN), and acid production from D-arabitol (DARL), D-xylose (XYL), L-rhamnose (RHA),  $\alpha$ -methyl-D-glucoside (MDG), D-ribose (RIB), glucose-1- Phosphate (G1P), and D-tagatose (TAG).

Colonies of 18-24-h culture on TSA were picked with a sterile cotton bud and suspended in 2 ml of sterile deionised water (BioMerieux) at an OD<sub>600 nm</sub> of 0.4 ( $\approx 3 \times 10^8$  CFU/ml). About 3 ml of demineralised water was distributed into dimples of the incubation wells of incubation box to create a humid atmosphere. Bacterial suspension

was then distributed into the 10 cupules (100  $\mu$ l for the DIM test and 50  $\mu$ l for the other cupules) of the strip. Then the strip box was closed and incubated at 37°C for 18 to 24 h. Following incubation, one drop of ZYM B (supplied by the manufacturer) was added to the DIM test and allowed to react for 3 min and then all of the reactions were read. Reactions were determined according to colour changes and identification was obtained with the numerical profile of *L. monocytogenes* as indicated in the manufacturer's instructions.

### **2.10.3 Listerial susceptibility to biocides**

*Listeria*-amoebae suspension from hatching medium was first centrifuged at 300  $\times$  g for 2 min to pellet trophozoites. The supernatant containing only *L. monocytogenes* was then centrifuged at 3000  $\times$  g for 15 min. The pellet was washed 3 times with RS and re-suspended in same solution. The OD<sub>600 nm</sub> of the suspension was determined and adjusted to OD<sub>600nm</sub> = 0.005 ( $\approx 1 \times 10^6$  CFU/ml) with RS. The bacterial sample was then tested for susceptibility to biocides using a micro dilution assay in 96-well microtitre plate as described by Garcia and colleagues (Garcia *et al.*, 2000). In this method, 50  $\mu$ l TSB was dispensed into 11 of the 12 wells of a microtitre plate starting from the second well. 100  $\mu$ l of disinfectants (five quaternary ammonium compounds and sodium hypochlorite) or antibiotics stock solutions (2 mg/ml) was pipetted into the first well and it then was serially diluted (2-fold) across the wells while discarding same amount after the 11th well. The 12th well was without a biocide and served as the negative control. 50  $\mu$ l of the bacterial suspension was added into each well (starting from right to left) to give a final inoculum of about  $5 \times 10^5$  CFU/ml. Each test was done in triplicate and the plates were then incubated at 37°C for 24 h. The control samples consisted of *L. monocytogenes* incubated in TSB at 37°C overnight and *L. monocytogenes* recovered from amoebae cysts, sub-cultured into TSB and incubated in

the same conditions. They were processed and added to wells of microtitre plate as in test sample. The plates were then incubated at 37°C for 24 h and thereafter wells were visually assessed for turbidity. The last well with no visible growth gave the minimum inhibitory concentration.

#### **2.10.4 Assay for biofilms formation**

The assessment of biofilm formation was done using a quantitative microtitre plate assay (Djordjevic *et al.*, 2002). In this method, a colony of *L. monocytogenes* was inoculated into 10 ml of TSB and incubated at 37°C for overnight. Optical density of culture was determined and diluted with RS to  $OD_{600\text{ nm}} = 0.7$ . About 200  $\mu\text{l}$  of the diluted bacterial sample was added into the well of a 96-well polystyrene microtitre plate (Greiner Bio-One, Stonehouse, UK) in triplicate whereas fresh TSB or SK6 medium was added to 6 wells and served as control. The plate edges were sealed with paraffin wax to minimise evaporation and then the plate was incubated at 37°C for 2 h. Following incubation, culture medium was carefully aspirated from wells by tilting the plate slightly to avoid touching its bottom with the pipette tip. After washing for two times with 200  $\mu\text{l}$  RS, the plate was air-dried for 15 min next to a Bunsen flame and then heat-fixed in an oven at 80°C for 30 min without the plate lid. After fixing, biofilms were stained by adding 220  $\mu\text{l}$  0.1 % (w/v) crystal violet in deionised water into each well for 1 min. The stain was then removed and the well was rinsed 3-4 times with 220  $\mu\text{l}$  RS to remove unbound stain. The crystal violet was solubilised by adding 220  $\mu\text{l}$  80 % (v/v) ethanol in acetone and mixing by pipetting several times. One hundred microliters of the destaining solution was transferred from each well to a new microtitre plate and their absorbance was read at 595 nm in a model 680 Bio-Rad

microplate reader (Bio-Rad laboratories Hercules CA, USA) after 15 min of adding ethanol.

## **2.11 Statistical analyses**

Data were analysed using GraphPad Prism version 5.01 for Windows (Graphpad software, San Diego California, USA). Two- way ANOVA with Bonferroni multiple comparison post tests, One-way ANOVA with Tukey post test and Student t-test were used to analyse data as indicated in the Results section.

## Chapter 3. Co-culture studies

### 3.1 Introduction

#### 3.1.1 Protozoan predation of bacteria and ecological significance

Protozoa are heterotrophic organisms that derive their nourishment by feeding on bacteria, viruses and other eukaryotic organisms such as fungi and algae (Parry, 2004, Matz and Kjelleberg, 2005). Protozoa, and especially free-living amoebae, are considered to be the most important grazers of bacteria in the soil (Clarholm, 1981, Bamforth, 1985), biofilm communities (Huws *et al.*, 2005, Snelling *et al.*, 2006) and activated sludge (Ratsak *et al.*, 1996). Their grazing activity helps regulate the size of bacterial populations (Clarholm, 1981, Bamforth, 1985, Kuuppo-Leinikki, 1990) and enhances the turnover of nutrients, particularly nitrogen through mineralisation of organic compounds trapped in bacterial biomass when they decompose organic detritus (Sinclair *et al.*, 1981, Ratsak *et al.*, 1996, Ronn *et al.*, 2002, Huws *et al.*, 2005, Petropoulos and Gilbride, 2005, Pogue and Gilbride, 2007).

Studies have also shown that protozoan grazing can stimulate the activity of bacteria, particularly the fast growing bacteria, through excretion of nutrients and growth stimulating factors that promote growth or persistence of such in the presence of protozoa (Coleman *et al.*, 1977, Habte and Alexander, 1978, Alexander, 1981, Sambanis and Fredrickson, 1988, Sinclair and Alexander, 1989, Gurijala and Alexander, 1990, Levrat *et al.*, 1992).

Although bacteria are the main source of food for protozoa, not all bacteria are equally suitable as a food source (Singh, 1941, Weekers *et al.*, 1993, Wang and Ahearn, 1997). Some protozoa prefer Gram negative bacteria to Gram positive bacteria as a food source

because Gram positive bacteria have a thicker cell wall that makes them difficult to digest (Gonzalez *et al.*, 1990, Weekers *et al.*, 1993, Ronn *et al.*, 2002). Even among the Gram negative bacteria, those that produce toxic pigments such as *Serratia marcescens* were shown to be inedible to free-living amoebae such as *Acanthamoeba castellanii*, *A. polyphaga* and *Hartmannella vermiformis* (Weekers *et al.*, 1993, Wang and Ahearn, 1997).

Another factor that determines the suitability or otherwise of bacteria as food sources for protozoa is the state of bacteria. Singh found that the vegetative cells of spore forming bacteria were preferred to the spores as a food source for amoebae (Singh, 1941).

### **3.1.2 Bacterial adaptations to protozoan grazing**

Predation by protozoa is a major factor that is responsible for bacteria mortality in the soil, freshwater and marine ecosystem (Clarholm, 1981, Bamforth, 1985) and a principal determinant of the morphological structure, taxonomic composition and physiological status of bacterial communities (Jurgens and Matz, 2002, Ronn *et al.*, 2002, Huws *et al.*, 2005). However, bacteria have developed multiple adaptive strategies to overcome predation pressure by protozoa. The adaptations can act either before ingestion (pre-ingestional or extracellular) or after bacteria have been taken into phagosome (post-ingestional or intracellular) (Matz and Kjelleberg, 2005). One example of the pre-ingestional adaptation is the production of toxic substances that kills or wades off predators. *Bacillus licheniformis*, for instance, produces amoebicin, a lytic compound that antagonises *Naegleria fowleri* to prevent their phagocytosis (Cordovilla *et al.*, 1993), while *Chromatium vinosum* and *S. marcescens* produce pigments that makes them unsuitable for consumption by *A. castellanii*, *A. polyphaga* and *H. vermiformis* (Weekers *et al.*, 1993).

Secondly, some bacteria adapt to predation pressure by forming a bulky, oversized morphology that is not edible to protozoa (Jurgens and Matz, 2002). *Flectobacillus*, for example, responds to grazing pressure by *Ochromonas* by forming filamentous cells (Hahn *et al.*, 1999), while *Pseudomonas aeruginosa* responds by sticking together in clusters, called microcolonies, that reach a size beyond the feasible prey size for the predators (Jurgens and Matz, 2002, Matz and Kjelleberg, 2005). *Cyanobacteria oscillitoria* and *Aphanizomenon* on the other hand form tight threads and aggregates, respectively, to avoid ingestion by *Naegleria* (Xinyao *et al.*, 2006).

Another pre-ingestional adaptation of bacteria to protozoan grazing is increased bacterial motility. Matz and Jürgens found that a proportion of highly motile bacterial cells increased during grazing by flagellates and that the rate of ingestion of bacteria dropped at speeds of  $> 25 \mu\text{ms}^{-1}$  (Jurgens and Matz, 2002).

Once ingested, bacteria in protozoan vacuoles are faced with acidification and enzymatic degradation. Some bacteria have developed mechanisms to resist digestion and even survive and exit from protozoa (Harb *et al.*, 2000, Greub *et al.*, 2004, Snelling *et al.*, 2006). For example, *Synechrococcus* have been shown to resist digestion by *Tetrahymena* through the protective function of its S-layers (Matz and Kjelleberg, 2005) (Mart and Kjelleberg, 2005). *M. avium* and *L. pneumophila* on the other hand are able to survive and replicate in vacuoles of amoeba by inhibiting fusion of phagosomes with lysosomes and exit by lysing the amoebae (Bozue and Johnson, 1996, Cirillo *et al.*, 1997, Gao *et al.*, 2006) It has been suggested that adaptation of bacterial pathogens to intracellular life within protozoa may provide them with the means to infect more evolved, mammalian cells (Harb *et al.*, 2000, Molmeret *et al.*, 2005, Hilbi *et al.*, 2007). Indeed, growth of *L. pneumophila* and *M. avium* in *A. castellanii* has been shown to

increase their ability to invade macrophages and cause disease in mice (Cirillo *et al.*, 1994, Cirillo *et al.*, 1997).

### **3.2 Hypothesis**

*Listeria monocytogenes* can survive and grow in co-culture with protozoa given the right incubation condition. To test this hypothesis the following objectives were tackled:

- a. To determine the viability of *L. monocytogenes* in the presence and absence of amoeba
- b. To determine the viability of amoebae in co-culture with *Listeria*
- c. To assess the role of culture conditions on survival of both organisms in co-culture
- d. To evaluate survival of *L. monocytogenes* in amoeba- conditioned medium

### 3.3 Results

#### 3.3.1 Listerial survival in co-culture with *Acanthamoeba*

Results of co-culture of *L. monocytogenes* and three *Acanthamoebae* species are presented in Figure 3.1A-C. The results show that the mean CFU counts of *L. monocytogenes* (0h-96 h) in co-culture with either of the *Acanthamoeba* species was significantly ( $P < 0.0001$ ) higher than the mean counts of *L. monocytogenes* that were incubated alone. However, the numbers of CFU of *L. monocytogenes* fell significantly ( $P < 0.05$ ) from 0 h to 96 h incubation both in the presence of amoebae and in the amoeba-free medium. Nonetheless, the average rate of bacterial reduction in co-culture with amoeba cells was considerably lower than the rate of reduction recorded when *Listeria* was incubated alone ( $P < 0.05$ ). For example, the average fall in numbers of bacteria in the presence of amoebae was between 0.1 logs to 0.3 logs CFU/ml for every 24 h of incubation whereas numbers reduced on average by 0.7 logs CFU/ml when *Listeria* was incubated alone, without amoeba, over the same period.

Although the numbers of *L. monocytogenes* declined in co-culture with either of the amoebae species tested, the overall reduction in co-culture over 96 h of incubation varied with *Acanthamoeba* species. Whereas 1.2 logs CFU and 0.7 logs CFU reduction was obtained in the presence of *A. castellanii* (Figure 3.1A) and *A. culbertsoni* (Figure 3.1B) respectively after 96 h incubation, about 0.5 logs reduction was recorded in the presence of *A. polyphaga* over the same incubation period (Figure 3.1C). These differences were statistically significant ( $P < 0.05$ ). This suggests that *Listeria* thrives relatively better in co-culture with *A. polyphaga* than in co-culture with either *A. castellanii* or *A. culbertsoni*.

The results of survival of amoebae trophozoites in co-culture, on the other hand, showed no significant differences ( $P > 0.05$ ) in the mean counts (0 h-96 h) of trophozoites of either of the amoeba species in co-culture with *L. monocytogenes* and

the mean counts of their corresponding cultures that were incubated in medium without bacteria (Figure 3.1A-C). In addition, the number of *Acanthamoebae* trophozoites declined significantly ( $P < 0.05$ ) in the presence, as well as in the absence, of *L. monocytogenes* (Figure 3.1A-C). As with counts of *L. monocytogenes* in co-culture, the average numbers of amoebae trophozoites in co-culture varied with the species of *Acanthamoeba*. For instance, the average numbers of *A. polyphaga* trophozoites in co-culture (Figure 3.1C) for the 96 h duration of incubation was significantly higher ( $P < 0.001$ ) than the average numbers of either *A. castellanii* (Figure 3.1A) or *A. culbertsoni* (Figure 3.1B) over the same incubation period. A similar pattern was evident when *Acanthamoebae* species were incubated alone (Figure 3.1A-C).

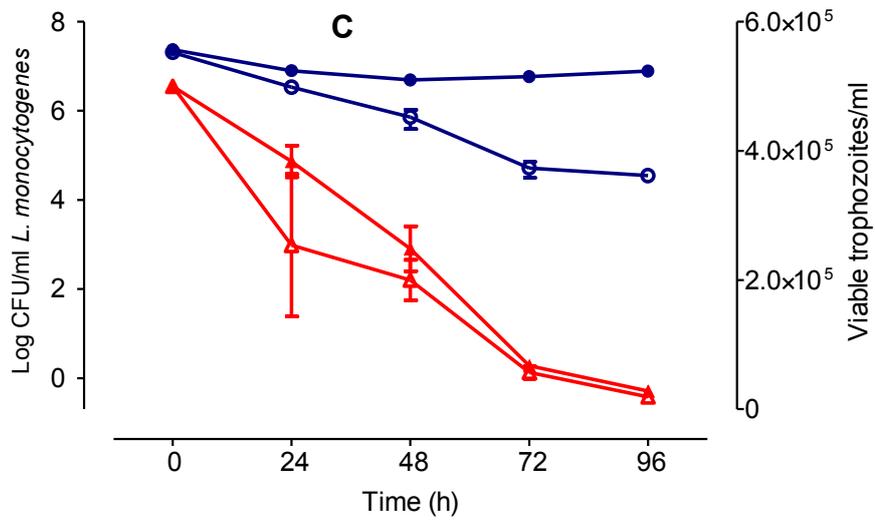
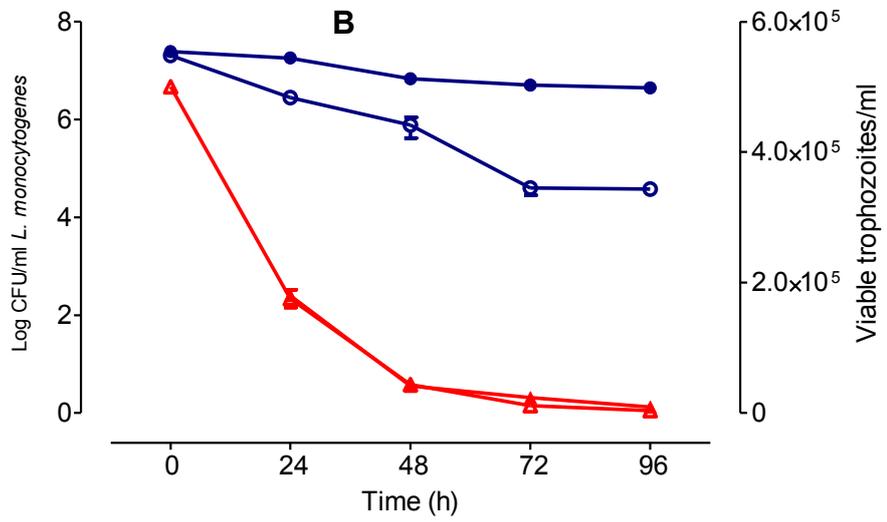
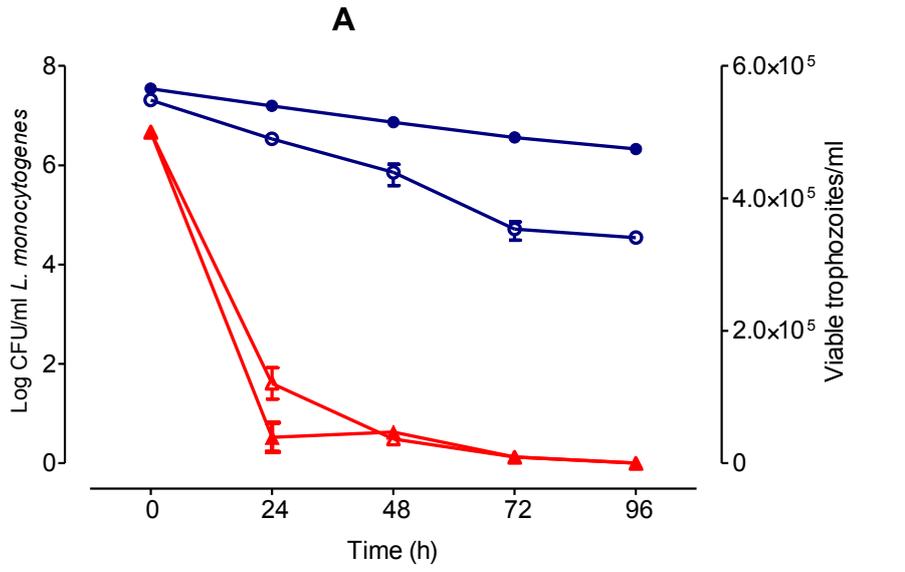


Figure 3.1 Survival of *L. monocytogenes* Scott A in co-culture with *Acanthamoeba*

(A) *A. castellanii*, (B) *A. culbertsoni* and (C) *A. polyphaga* incubated at stationary position. Mean CFU counts of *L. monocytogenes* in co-culture with *Acanthamoeba* (●) and in their absence (○). *Acanthamoeba* trophozoite counts in co-culture with *Listeria* (★) were compared with number of trophozoites incubated alone (▲). Error bars represent standard error of mean counts of triplicate experiments.

### 3.3.2 Effect of culture condition on listerial survival in co-culture

To test whether changing the culture condition will affect survival of *L. monocytogenes*, co-cultures were incubated with mechanical agitation in a rotary shaker at 100 rpm. The purpose was to increase the concentration of dissolved oxygen in co-culture medium (Prescott *et al.*, 1973, Avery *et al.*, 1995). It was proposed that increasing the concentration of dissolved oxygen may increase the metabolic activity of amoeba and hence, the amount of nutrients released into co-culture medium to foster growth of *L. monocytogenes* (Sinclair and Alexander, 1989, Gonzalez *et al.*, 1990).

The results presented in Figures 3.2 A-C indicate that average numbers of *L. monocytogenes* ( from 0 h - 96 h ) in the shaken co-cultures, with either of the three amoebae, was significantly higher than counts of their counterparts in the stationary co-cultures ( $P < 0.0005$ ). As with stationary co-cultures, bacterial survival in the shaken co-cultures varied significantly with the species of *Acanthamoeba* ( $P < 0.05$ ). The average bacterial counts for the 96 h duration of incubation was highest in co-culture with *A. polyphaga* and lowest in co-culture with *A. castellanii*. Unlike in the stationary co-cultures however, *L. monocytogenes* grew in the shaken co-cultures in which any of the three *Acanthamoebae* species was present particularly, over the first 24 h or 48 h of incubation (Figure 3.2A-C).

The pattern of bacterial growth in shaken co-cultures differed slightly with the species of *Acanthamoeba*. For instance, in the presence of *A. castellanii* (Figure 3.2A), the number of *L. monocytogenes* tripled from  $2.4 \times 10^7$  CFU/ml in the inoculum at 0 h to  $7.4 \times 10^7$  CFU/ml by the end of 24 h incubation ( $P < 0.0001$ ). Thereafter, the number started to fall, and by the end of 96 h incubation viable bacteria had significantly ( $P < 0.001$ ) dropped by about 0.5 logs CFU/ml from the starting number. Similarly, the numbers of *Listeria* in presence of *A. culbertsoni* (Figure 3.2B) doubled from  $2.6 \times 10^7$

CFU/ml in the inoculum to  $5.2 \times 10^7$  CFU/ml after 24 h incubation ( $P < 0.0001$ ), before the number slightly fell. However, unlike in co-culture with *A.*

*castellanii*, the number of viable *Listeria* in co-culture with *A. culbertsoni* did not fall below the original number of bacteria. On the other hand, CFU of *L. monocytogenes* in co-culture with *A. polyphaga* significantly ( $P < 0.001$ ) increased over 48 h (from  $2.4 \times 10^7$  CFU/ml at 0 h to  $8.1 \times 10^7$  CFU/ml at 48 h) incubation before the number also gradually fall (Figure 3.2C). Nevertheless, the final bacterial count by the end of 96 h incubation was significantly higher than the count recorded at 0 h ( $P < 0.05$ ).

Although the CFU counts of *L. monocytogenes* in co-culture with three *Acanthamoebae* sp, incubated with shaking, were higher than counts in the corresponding co-cultures that were incubated without shaking, the reverse was observed when *L. monocytogenes* was incubated in amoeba-free medium (Figure 3.2A-C). Nonetheless, bacterial numbers significantly declined ( $P < 0.05$ ) from 0 h-96 h in amoebae-free medium incubated with shaking as were in amoeba-free medium incubated without shaking (Figure 3.2A-C).

The results of amoebae trophozoite counts in shaken co-culture are also presented alongside the results of listerial survival in co-cultures (Figure 3.2A-C). The same pattern of results that were obtained in stationary cultures was also observed here, except for some few variations in the order of survival in cultures. Whereas the average *A. polyphaga* trophozoite counts (0 h-96 h) in shaking cultures (Figure 3.2C) was significantly higher ( $P < 0.05$ ) than those of *A. castellanii* (Figure 3.2A), it was comparable ( $P > 0.05$ ) with those of *A. culbertsoni* (Figure 3.2B). The average *A. polyphaga* trophozoites count in stationary cultures (Figure 3.2C). On the other hand, was significantly higher ( $P < 0.001$ ) than counts of *A. castellanii* (Figure 3.2A) or *A. culbertsoni* (Figure 3.2B) over the same period. In addition, the average *A. culbertsoni*

trophozoite counts (0 h-96 h) in cultures incubated with agitation (Figure 3.2B) was significantly higher ( $P < 0.05$ ) than those of *A. castellanii* (Figure 3.2A) while in non-shaking culture counts of *A. culbertsoni* were comparable ( $P > 0.05$ ) with those of *A. castellanii* (Figure 3.2A).

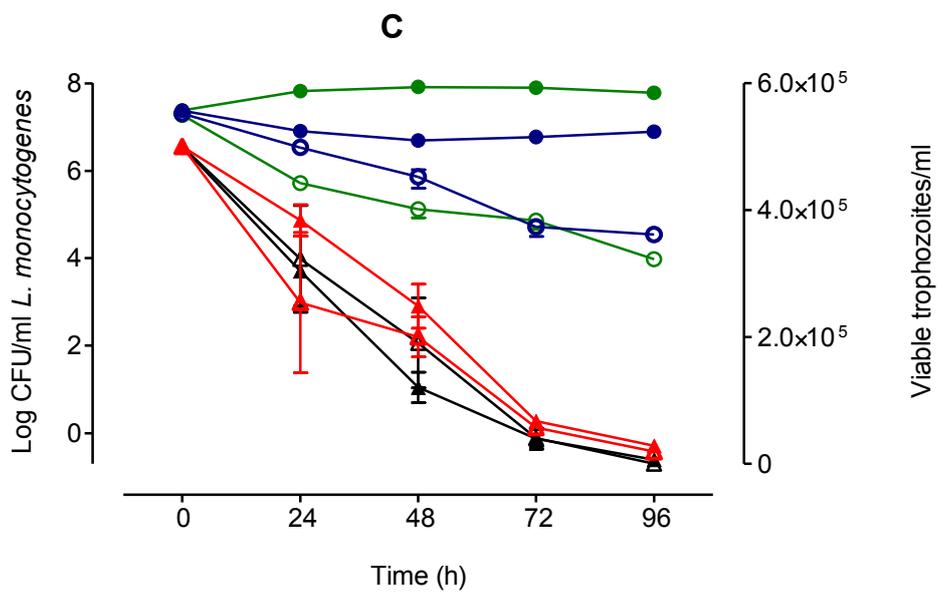
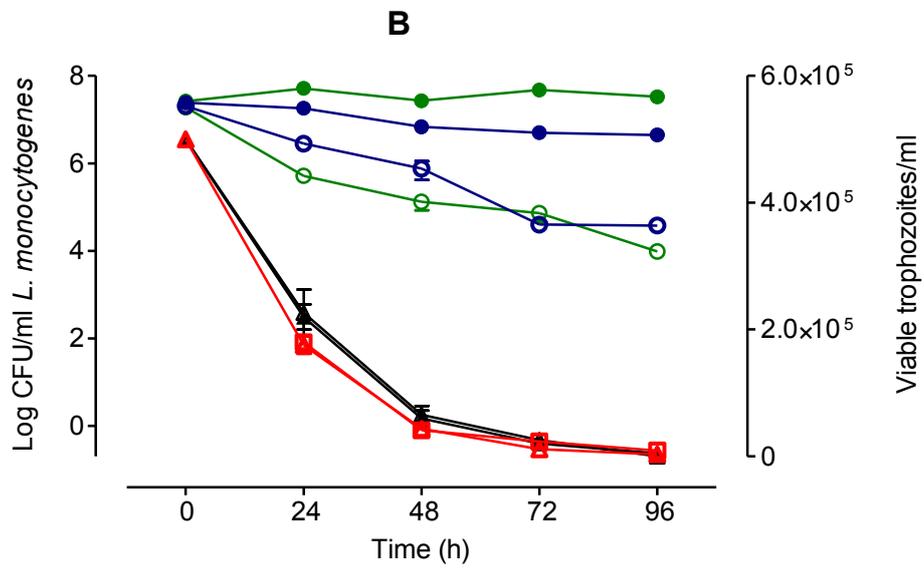
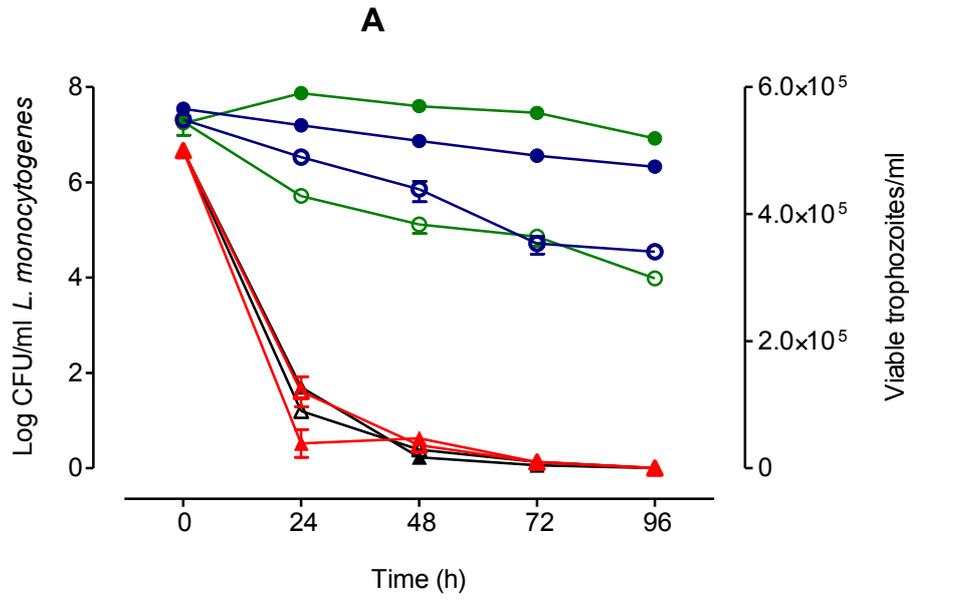


Figure 3.2 Effect of shaking incubation on survival of *L. monocytogenes* Scott A in co-culture with (A) *A. castellanii*, (B) *A. culbertsoni* and (C) *A. polyphaga*. Mean counts of *L. monocytogenes* in the presence of amoeba incubated with (●) and without shaking (◐). Mean counts of *L. monocytogenes* incubated alone with (◑) and without shaking (◒). Amoeba trophozoites counts in the presence of *L. monocytogenes* incubated with (▲) and without shaking (★). Amoeba trophozoite counts incubated alone with (☆) and without shaking (△). Error bars represent standard error of means of three replicate experiments.

For both shaken and stationary co-cultures, the numbers of *Acanthamoebae* trophozoites declined with time of incubation even when numbers of viable bacteria were still high in the medium (Figure 3.2A-C). The decrease in number of trophozoites was due in part to the massive encystment of the amoebae trophozoites that was observed in co-culture. This suggests that *L. monocytogenes* may not be a suitable food source for *Acanthamoeba*. To test this hypothesis *A. castellanii* trophozoites were fed with *E. coli* for 24 h at 32°C and afterward numbers of amoebae trophozoites were counted and compared with those fed with *L. monocytogenes*. The results are shown in Figure 3.3 and Figure 3.4. The *A. castellanii* trophozoites that were fed with *E. coli* doubled in number ( $P < 0.0005$ ) over 24 h of incubation (Figure 3.3 and Figure 3.4A-B). In contrast, the number of trophozoites fed with *L. monocytogenes* fell by a half during the same period due to encystment (Figure 3.3 and Figure 3.4C-D)( $P < 0.05$ ).

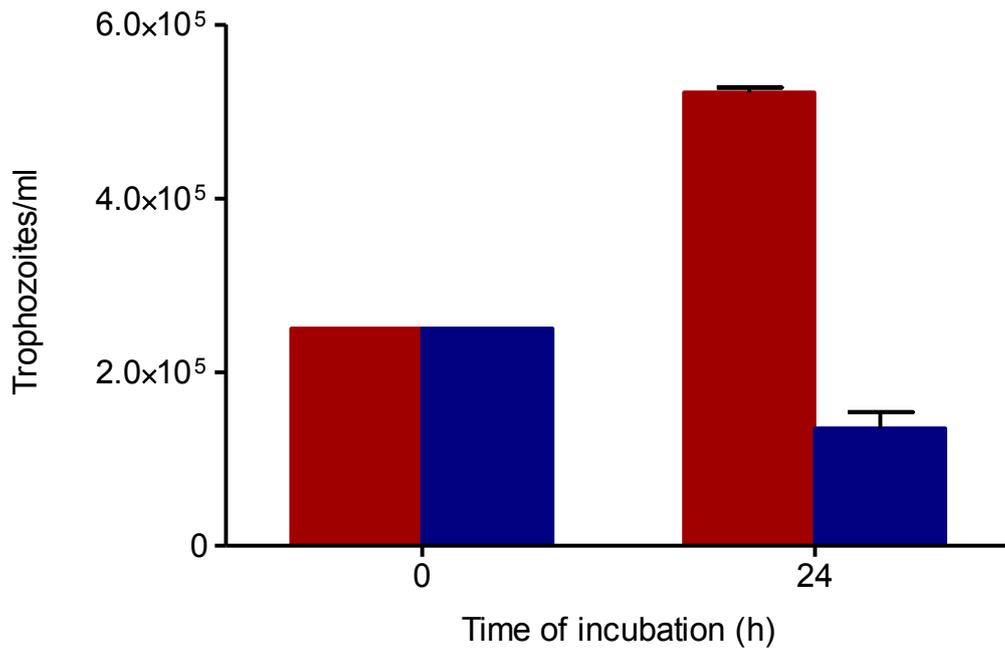


Figure 3.3 Food preference by *Acanthamoeba*.

The number of trophozoites present when *A. castellanii* was fed with *Escherichia coli* (■) or *L. monocytogenes* (■). Error bars represent standard error of mean counts for triplicate experiments.

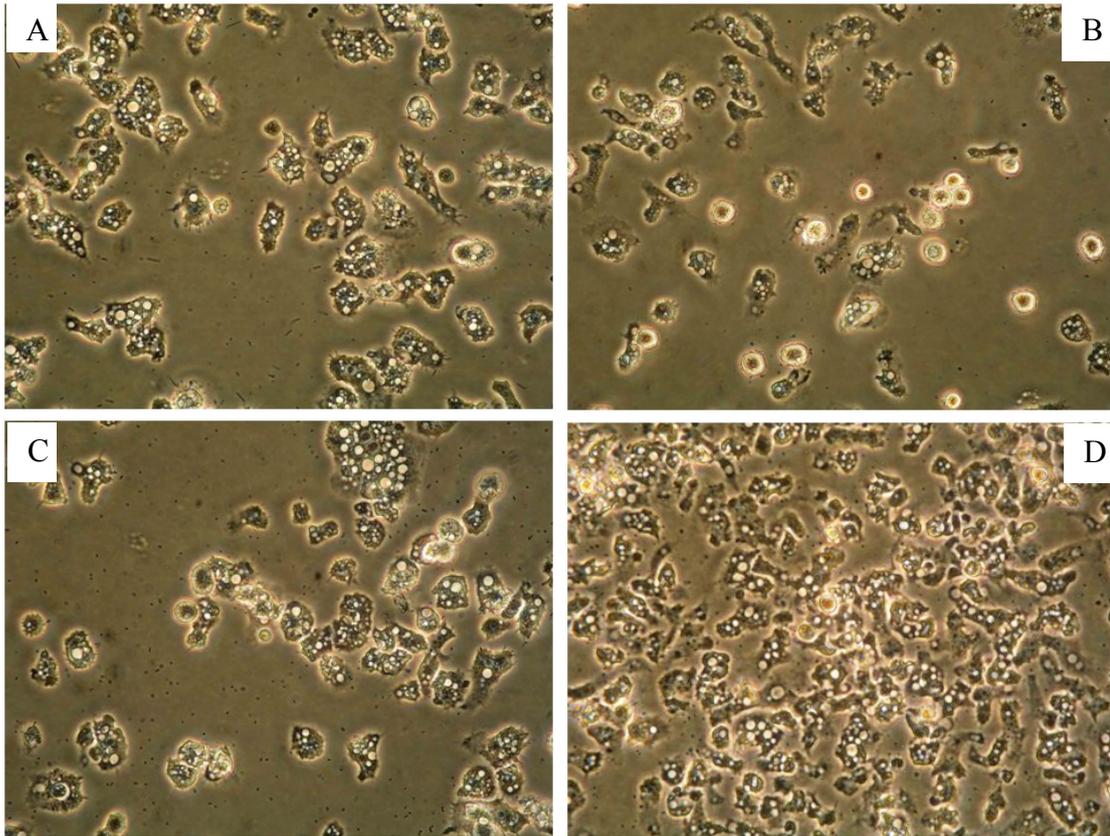


Figure 3.4 Comparing growth of *A. castellanii* fed with *L. monocytogenes* Scott A and those fed with *E. coli*. (A) Cells fed with *L. monocytogenes* after 1 h incubation reduced in numbers after 2h incubation (B). While those fed with *E. coli* after 1 h of incubation (C) doubled in their numbers after 24 h of incubation (D). Magnification  $\times 400$

### 3.3.3 *Listeria monocytogenes* thrives on amoeba by-products

In order to test if *L. monocytogenes* had enhanced survival in the presence of amoeba products, *Acanthamoeba* sp ( $5 \times 10^5$  cells/ml) were first incubated for 24 h in a fresh RS at 32°C without agitation. Then, amoeba cells and cell debris were removed by passing the medium through a membrane filter (0.2 µm pore size). The filtrate, now called conditioned-medium (CM), was then used for growth of *Listeria*.

Shown in Figure 3.5A and B are the results of determining the viability of *L. monocytogenes* in amoeba CM incubated without and with shaking respectively. Growth patterns similar to that of *Listeria* grown in co-culture with amoeba cells were seen. As with co-culture with cells, the numbers of viable *L. monocytogenes* in conditioned medium were significantly ( $P < 0.05$ ) higher than the numbers recorded in non-nutrient control medium at either incubation condition (Figure 3.5). In addition, bacteria persisted in this medium for the 96 h duration of incubation, whereas the number in the control declined throughout the incubation period, regardless the condition of incubation ( $P < 0.05$ ). However, unlike in co-culture with cells, incubation with shaking did not enhance survival of *Listeria* in CM except for *A. castellanii* CM ( $P > 0.05$ ). Moreover, differences in species of *Acanthamoeba* did not influence survival of *L. monocytogenes* in CM incubated with agitation ( $P > 0.05$ ), unlike survival in presence of agitated cells (Figure 3.1, Figure 3.2).

Bacterial numbers in *A. castellanii* and *A. culbertsoni* CM, incubated without shaking, increased significantly ( $P < 0.05$ ) over 96 h incubation (Figure 3.5A), while those in *A. polyphaga* CM also significantly increased ( $P < 0.05$ ) from 0 h to 48 h but then declined significantly ( $P < 0.05$ ) thereafter (Figure 3.5A). The pattern of growth was, however, slightly different when cultures were incubated with shaking (Figure 3.5B). Here, bacteria increased ( $P < 0.05$ ) in all the conditioned media incubation by 24 h of

incubation but thereafter numbers fell. However, following the decline in *A. culbertsoni* CM, bacterial numbers started to mount again from 48 h to 96 h of incubation ( $P < 0.05$ ).

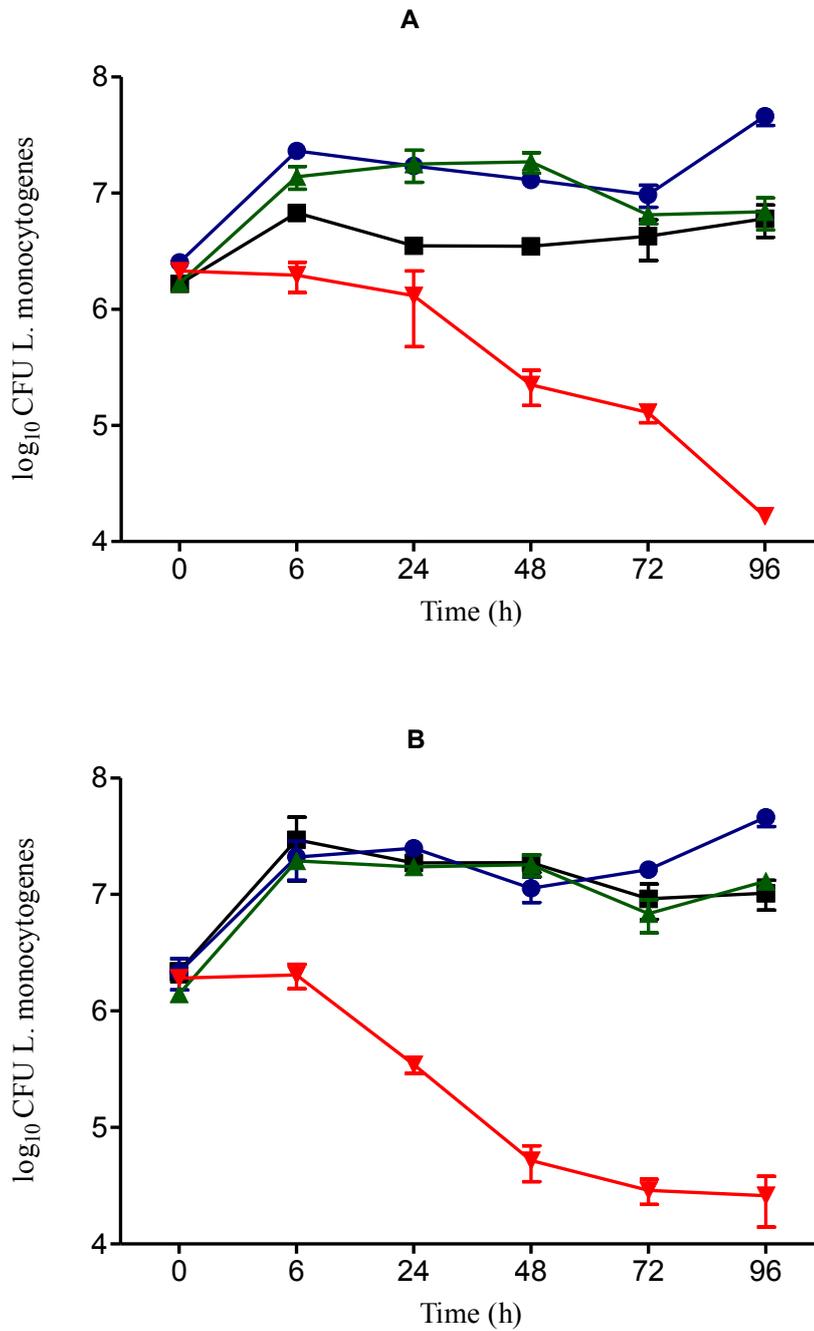


Figure 3.5 Survival of *L. monocytogenes* Scott A in condition medium.

*A. castellanii* conditioned medium (●), *A. culbertsoni* CM (■), *A. polyphaga* CM (▲) and in a (■) 1/4 strength Ringer's solution (▼). Cultures were incubated without shaking (A) or with shaking at 100 rpm (B). Error bars represent standard error of mean of listerial counts for three replicate experiments.

The majority of *A. castellanii* trophozoites used to produce CM formed cysts by the end of the 24 h incubation (Figure 3.6A, B) whereas only a few of the *A. culbertsoni* trophozoites encysted over this period (Figure 3.6C, D). On the other hand, very few cysts were formed from *A. polyphaga* trophozoites after 24 h incubation (Figure 3.6E, F). The encystment of amoebae trophozoites, particularly those of *A. castellanii* that were used in making conditioned medium suggests that the nutrients in the conditioned medium could have been derived from the waste expelled by the encysting amoebae. Therefore, to show that metabolically active amoeba trophozoites are capable of releasing nutrients, conditioned medium was made by pre-incubating *A. castellanii* in RS for 1 h and the medium was used to grow *L. monocytogenes* as described in section 2.7. As shown in Figure 3.7, the number of *L. monocytogenes* in medium pre-conditioned with *A. castellanii* for 1 h increased significantly ( $P < 0.05$ ) by 24 h of incubation, before the numbers started to decline. On the other hand, the number of bacteria in RS declined significantly ( $P < 0.05$ ) by 24 h of incubation and continued to decline thereafter.

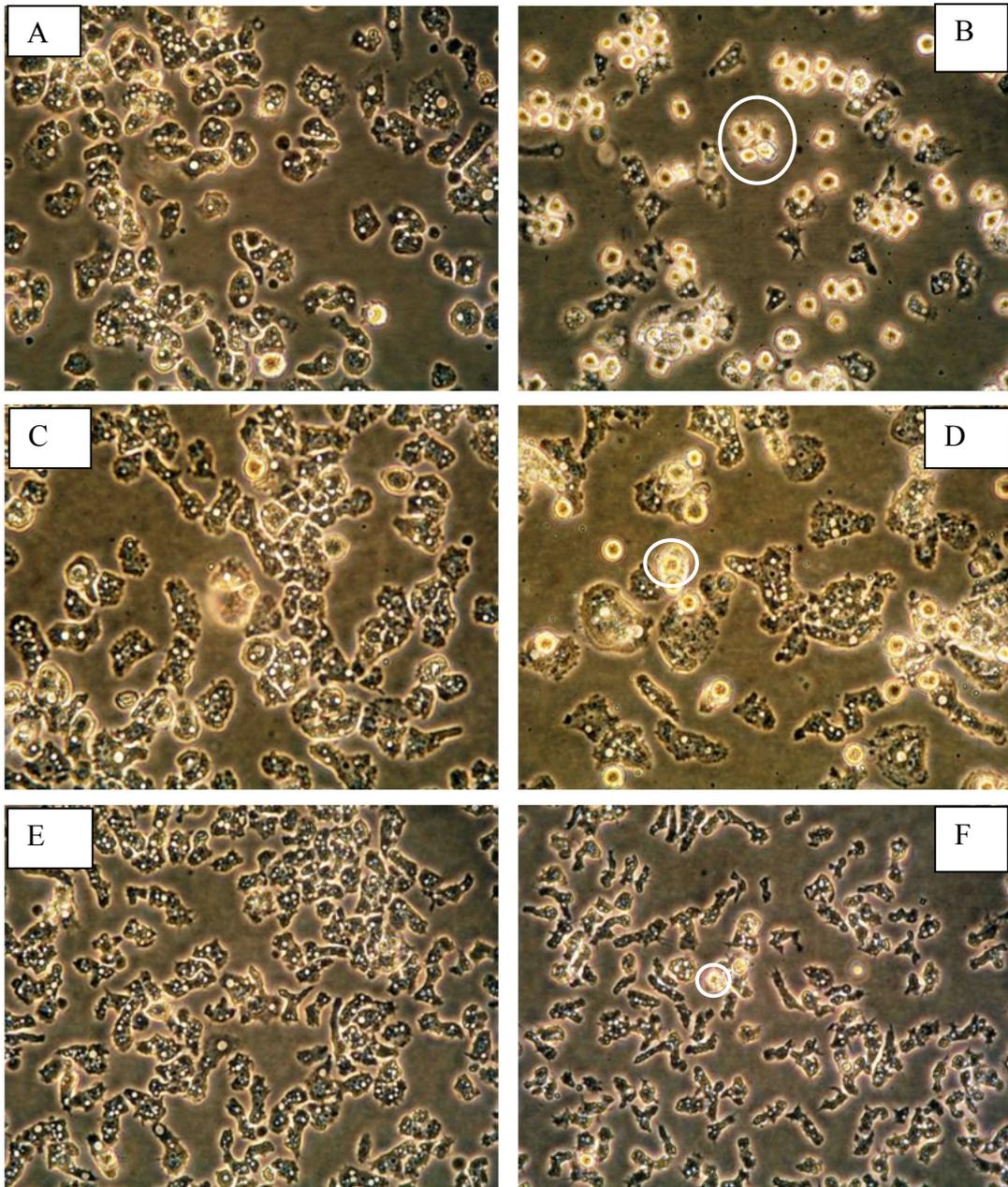


Figure 3.6 Phase contrast microscopy of *Acanthamoebae* used in preparing conditioned-media: *A. castellanii* (A and B), *A. culbertsoni* (C and D) and *A. polyphaga* (E and F) incubated in fresh RS for 1 h (Left panel) and 24 h (Right panel). At the end of 24 h incubation, majority of *A. castellanii* (B) trophozoites have formed cysts (circles), only few of *A. culbertsoni* (D) formed cysts and an insignificant number of *A. polyphaga* (F) formed cysts during this period. Magnification  $\times 400$ .

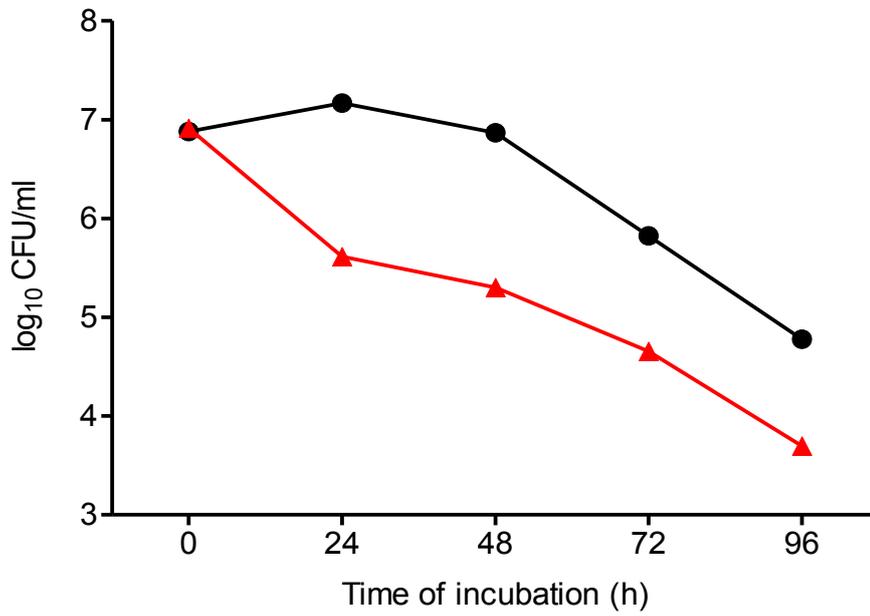


Figure 3.7 Survival of *L. monocytogenes* Scott A in *A. castellanii* conditioned medium (CM) pre-conditioned for 1 h. CFU counts of *L. monocytogenes* in conditioned medium (●); (B) CFU of *L. monocytogenes* in the RS (▲). Error bars are standard error of mean counts for triplicate experiments.

## Chapter 4. Intra-amoebal survival of *L. monocytogenes*

### 4.1 Introduction

*Listeria monocytogenes* is a soil bacterium that lives in decaying vegetation but is capable of causing gastroenteritis in healthy people, meningitis in immunocompromised individuals and abortion in pregnant women (Cossart and Archambaud, 2009). The capacity to grow in a variety of cells has made *L. monocytogenes* one of the best model to study host-pathogen interactions and intracellular parasitism (Hamon *et al.*, 2006). The life cycle of *L. monocytogenes* in these cells reflects its remarkable adaptation to intracellular survival and suggests previous adaptations for intracellular survival in other eukaryotic organisms in the environment (Gray *et al.*, 2006).

Free-living amoeba, particularly *Acanthamoebae* are the main predators of bacteria in the soil (Rodriguez-Zaragoza, 1994). Many bacterial species are able to resist predation through various adaptive mechanisms (Matz and Kjelleberg, 2005). It has been suggested that resistance to protozoan grazing is a pre-requisite in the evolution of virulence in many bacteria pathogens, including *L. pneumophila* (Cirillo *et al.*, 1999, Molmeret *et al.*, 2005), Mycobacterium (Cirillo *et al.*, 1997, Salah *et al.*, 2009), Chlamydiae (Molmeret *et al.*, 2005), *E. coli* (Adiba *et al.*, 2010), *S. enterica* (Wildschutte *et al.*, 2004) and *Serratia marcescens* (Friman *et al.*, 2009).

Many human intracellular pathogens are capable of surviving in free-living amoebae particularly *Acanthamoeba* (Greub and Raoult, 2004, Casadevall, 2008, Thomas *et al.*, 2010). Consequently, it has been proposed that bacterial species that are pathogenic to humans may likely resist digestion by free-living amoebae (Greub and Raoult, 2004,

Thomas *et al.*, 2010). However, till date no study has unambiguously shown that *L. monocytogenes* can survive or grow in free-living amoeba.

## **4.2 Hypothesis**

*Listeria monocytogenes* can survive and even multiply in *Acanthamoeba* given the right test system. To test this hypothesis the following objectives were tackled:

- a. To determine a suitable ionic detergent that can completely lyse amoeba trophozoites
- b. To determine viability of *L. monocytogenes* in concentration of detergent that completely lysed amoeba trophozoites
- c. To demonstrate uptake of *L. monocytogenes* into amoebae
- d. To demonstrate intracellular survival of *L. monocytogenes* within amoeba using gentamicin protection assay
- e. To determine the effect of manganese ions on intracellular survival of *Listeria*

## 4.3 Results

### 4.3.1 Sarcosine is effective for cell lysis of *Acanthamoeba* trophozoites

The critical step in the determination of intracellular survival of *L. monocytogenes* within amoeba was getting a right agent that can lyse the amoeba cells to liberate intracellular bacteria without killing them. In order to achieve this objective seven chemical detergents, comprising three anionic (sodium dodecyl sulphate (SDS), sarcosine and taurocholic acid) and four non-ionic (triton-x100, tween-80, saponin and nonidet-p40) detergents were tested for their ability to lyse *A. castellanii* trophozoites as described in section 2.5 of Materials and Methods. The results of that test are presented in Table 4.1. Of the seven detergents tested, only sarcosine and SDS were able to effectively lyse amoeba. The lowest concentration that resulted in complete lysis of amoeba cells was 0.06 % (w/v) for both chemicals. On the other hand, the other five detergents either caused partial or no trophozoites lysis at all the concentrations tested.

The viability of *L. monocytogenes* was also determined after exposure to the concentration of sarcosine or SDS that caused complete lysis of *Acanthamoeba* trophozoites as described in section 2.5 and the results are presented in Figure 4.1. There was no significant difference in the average counts of bacteria incubated in sarcosine and those incubated in RS for all the 3 time-points examined ( $P > 0.05$ ). This showed that the concentration of sarcosine used was not lethal to *L. monocytogenes*. By contrast, the average CFU counts of *Listeria* following exposure to SDS was significantly less than counts in RS at 10 min and 30 min of incubation ( $P < 0.05$ ). This was as a result of reduction of 1.5 logs CFU of viable bacterial numbers between 0 min and 10 min incubation and 1.8 logs CFU reduction between 10 min and 30 min incubation with SDS.

Table 4.1 Screening of detergents for lysis of *Acanthamoeba castellanii*

Detergent	<i>Acanthamoeba castellanii</i> lysis							
	Concentration of detergents ( % v/v)							
	1.00	0.50	0.25	0.13	0.06	0.03	0.02	0.00
Nonidet-p40	-	-	-	-	-	-	-	-
Saponin	-	-	-	-	-	-	-	-
Sarcosine	+	+	+	+	+	-	-	-
SDS	+	+	+	+	+	-	-	-
Taurocholic acid	-	-	-	-	-	-	-	-
Tween-80	-	-	-	-	-	-	-	-
Triton-x100	-	-	-	-	-	-	-	-

+, complete cell lysis; - partial or no cell lysis

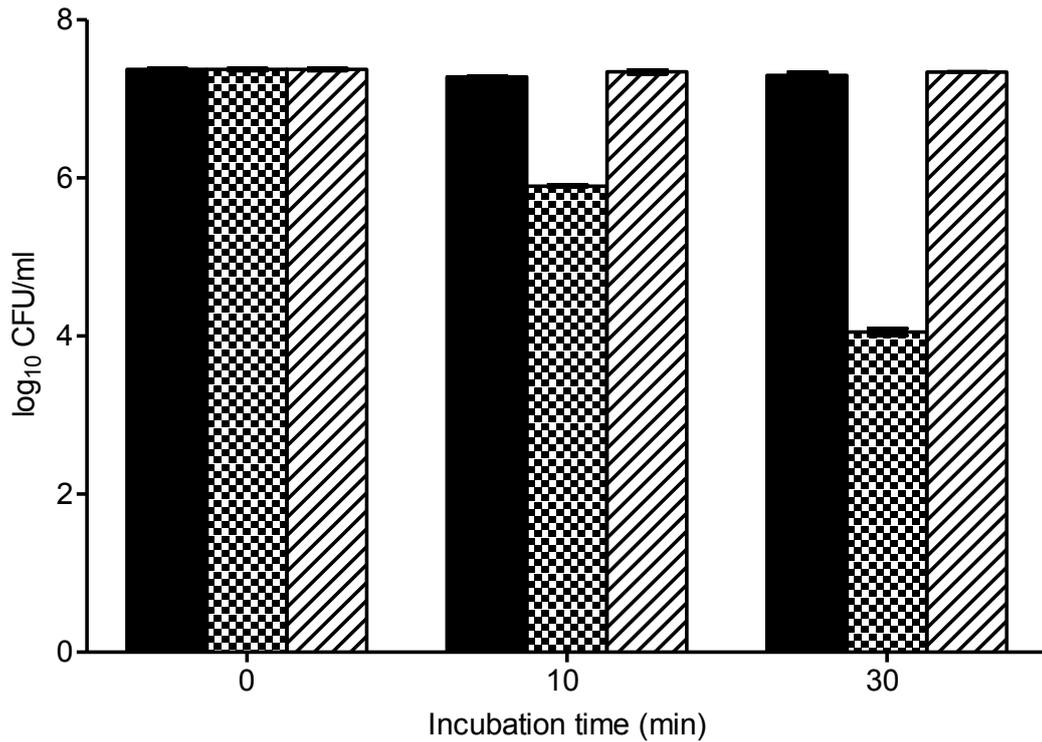


Figure 4.1 Viability of *L. monocytogenes* Scott A exposed to detergents. CFU counts in: (■) 0.06 % (w/v) sarcosine, (▣) 0.06 % (w/v) SDS and in a 1/4 quarter-strength Ringer's solution (▨). Error bars represent standard error of mean (SEM) counts of triplicate experiments.  $P > 0.05$ .

#### 4.3.2 Suitability of maintenance medium

One important aspect in the assay of intracellular survival of *L. monocytogenes* within *Acanthamoeba* was the medium to maintain cells post-infection, henceforth referred to as the maintenance medium (MM). The medium needed to prevent or reduce encystment of the infected trophozoites while at the same time maintaining the number of trophozoites at a low level that would not affect the results significantly. In addition, the medium had to be permissible for growth of *L. monocytogenes* such that, lack of growth during incubation of infected trophozoites could serve as indicator that the medium was devoid of extracellular bacteria contaminants. Incubation of uninfected *A. castellanii* trophozoites in 20 % (v/v) SK6 (section 2.8.3 and Appendix 1F) significantly

impaired ( $P < 0.05$ ) the ability of amoeba to proliferate, when compared with those propagated in the full strength SK6 medium (Figure 4.2). While the number of trophozoites in MM increased from  $2.5 \times 10^5$  to  $4.1 \times 10^5$  over 48 h of incubation, those in the full strength medium increased from  $2.5 \times 10^5$  to  $1.1 \times 10^6$  over the same period ( $P < 0.05$ ). Although the trophozoites in the 20 % SK6 medium started to encyst after 48 h of incubation, nonetheless the number at 72 h was relative unchanged from the number at start. On the other hand, no cysts were formed in the full strength growth medium, rather, the number of trophozoites continued to increase, attaining confluent growth at 72 h (Figure 4.2).

Figure 4.3 is the results of comparison of growth of *L. monocytogenes* in MM and growth in the full strength medium as determined by their optical densities. The results show that growth was comparable in the two media over 10 h of incubation. This suggests that the 20 % SK6 medium can support the growth of *L. monocytogenes*.

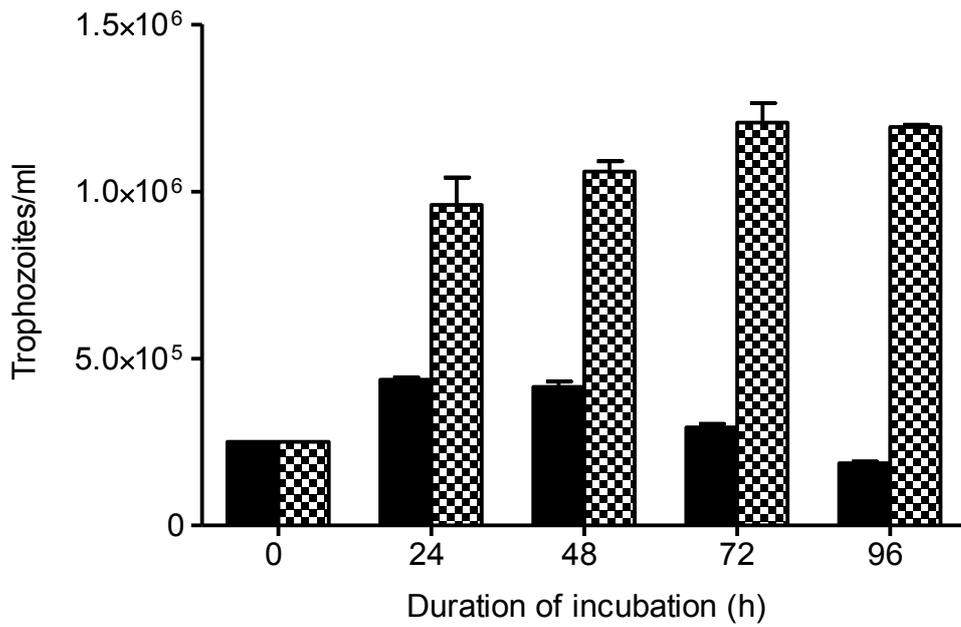


Figure 4.2 Survival of *A. castellanii* in 20 % SK6 medium used for maintaining amoebae after infection (■), compared with growth in full strength SK6 medium used to grow axenic amoebae (▣).

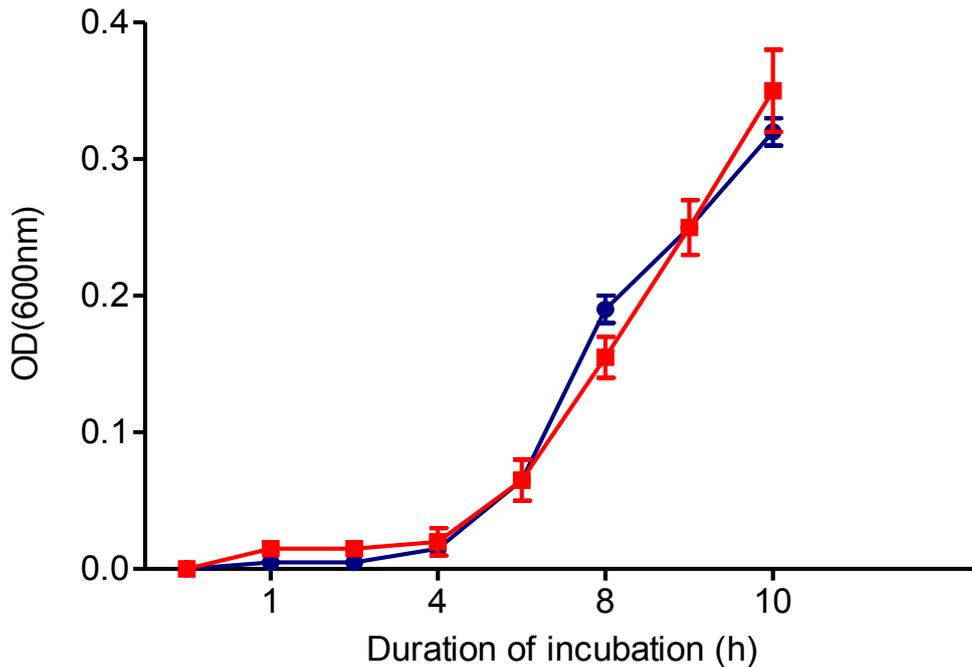


Figure 4.3 Growth curve of *L. monocytogenes* Scott A in maintenance medium (20 % SK6) (●) and in full strength SK6 medium (■) were almost the same after 10 h incubation. Error bars represent standard error of means for triplicate experiments.

### 4.3.3 Uptake of *Listeria* by *Acanthamoeba*

The ability of *Acanthamoeba* to phagocytose *L. monocytogenes* was examined by co-incubating *L. monocytogenes* with *A. castellanii* for a limited period, to allow infection to take place and thereafter washing cells five times and treating with gentamicin to remove non-internalised bacteria, as described in 2.8.1. The efficiency of these treatments was assessed by plating a sample of the supernatants of the first, fifth and post-gentamicin washings on TSA to detect viable bacteria.

The washing of infected cells significantly ( $P < 0.0005$ ) reduced the number *L. monocytogenes* in the supernatant by about 2.4 logs CFU that is, from  $5.6 \times 10^7$  CFU/ml in the first wash to  $2.1 \times 10^5$  CFU/ml in the fifth wash (Table 4.2).

The 1 h gentamicin treatment and subsequent washings totally eliminated the remaining extracellular bacteria as was evident by the absence of viable bacteria in the post-gentamicin supernatant and in the maintenance medium. The ratio of intracellular bacteria to amoeba after 3 h of uptake was approximately 1: 20 respectively (Table 4.2).

The effect of the size of bacterial inocula on uptake of *L. monocytogenes* by *A. castellanii* was also examined and the results are presented in Figure 4.4. The results show that the number of bacteria phagocytosed by amoeba significantly increased when the multiplicity of infection was increased from 1: 10 to 1: 400 or 1: 720 ( $P < 0.0005$ ) but not from 1:10 to 1: 100 ( $P > 0.05$ ). On the other hand, when the duration of incubation of co-culture was varied and the multiplicity of infection, MOI was fixed at 1:500, the number of intracellular *L. monocytogenes* reduced significantly ( $P < 0.05$ ) with time of incubation of co-culture (Figure 4.5). The highest number of bacteria taken up by amoeba ( $6.5 \times 10^4$  CFU) was obtained when *Listeria-Acanthamoeba* mixture was incubated for just 30 min (Figure 4.5). Subsequent incubation of co-culture beyond this time resulted in the recovery of less number of intracellular bacteria. The differences between the numbers of intracellular *Listeria* recovered when co-culture was incubated for 30 min and the numbers recovered when co-culture was incubated for 1 h or above was statistical significant ( $P < 0.05$ ). The results suggest that prolonged incubation results in killing of intracellular *L. monocytogenes* by *A. castellanii*.

The co-culture condition (Figure 4.6) was also found to influence the number of bacteria taken up by *A. castellanii*. The number of intracellular *L. monocytogenes* recovered from *Acanthamoeba* after an hour of incubation of co-culture was significantly higher in amoebae that were incubated with gentle agitation (100 rpm) than in the amoebae that

were allowed to attach to the bottom of a culture flask before they were infected and then incubated without shaking ( $P < 0.005$ ).

The ability of *A. castellanii* to ingest *L. monocytogenes* into vacuoles was demonstrated using bright field microscopy to examine infected *A. castellanii* that were stained with dyes (Figure 4.7). *Listeria monocytogenes* localised in vacuoles of *A. castellanii* were readily seen when amoeba cells were fixed with glutaraldehyde and stained with toluidine (Figure 4.7).

Table 4.2 Efficiency of washing, gentamicin treatment and uptake of *L. monocytogenes* Scott A by *A. castellanii*

Viable amoebae and <i>L. monocytogenes</i> counts in 10 ml of co-culture								
Experiment number	Amoeba trophozoites	Extracellular <i>L. monocytogenes</i> counts (CFU/ml)						Intracellular <i>L. monocytogenes</i> post-infection
		Inoculum	1 <sup>st</sup> wash	5 <sup>th</sup> wash	Reduction after washing	5 <sup>th</sup> wash as % of 1 <sup>st</sup> wash	Post-gentamicin wash	Bacteria uptake CFU/ml
1	$5.0 \times 10^5$	$8.9 \times 10^7$	$4.7 \times 10^7$	$3.2 \times 10^5$	$4.67 \times 10^7$	99.4	0	$2.0 \times 10^4$
2	$5.0 \times 10^5$	$9.4 \times 10^7$	$5.7 \times 10^7$	$3.1 \times 10^5$	$5.67 \times 10^7$	99.5	0	$2.3 \times 10^4$
3	$5.0 \times 10^5$	$1.9 \times 10^8$	$6.2 \times 10^7$	$1.6 \times 10^5$	$6.18 \times 10^7$	99.7	0	$4.6 \times 10^4$
Mean	$5.0 \times 10^5$	$1.2 \times 10^8$	$5.6 \times 10^7$	$2.1 \times 10^5$	$5.55 \times 10^7$	99.6	0	$2.0 \times 10^4$

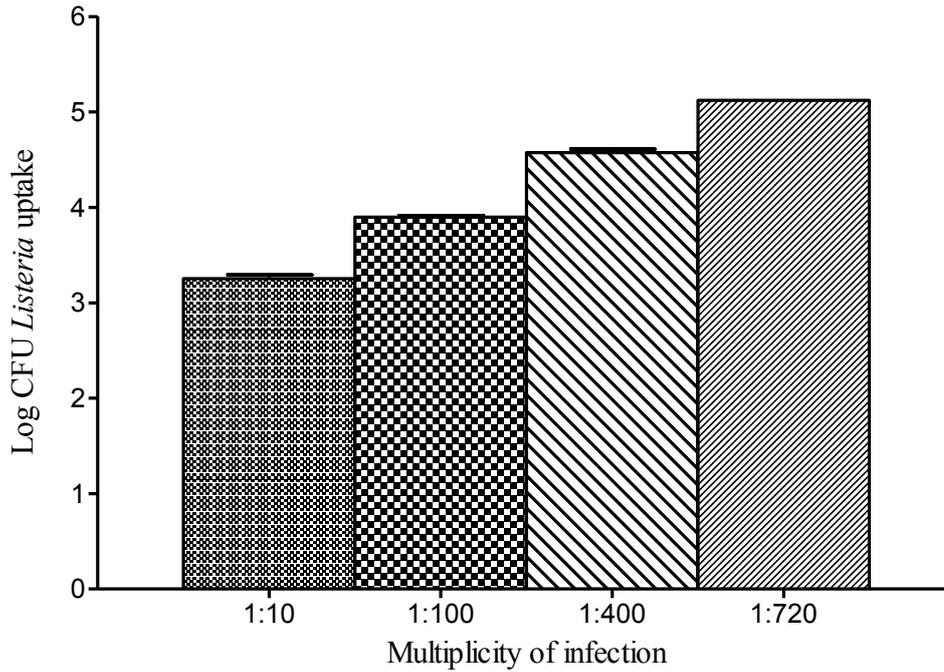


Figure 4.4 Effect of multiplicity of infection (MOI) on uptake of *L. monocytogenes* Scott A by *A. castellanii*. Number of intracellular bacteria when the multiplicity of infection was: 1 amoeba to 10 *Listeria* cells (▤), 1 amoeba to 100 *Listeria* cells (▥), 1 amoeba to 400 *Listeria* cells (▧), 1 amoeba to 720 *Listeria* cells (▨). Error bars are SEM for three replicate experiments

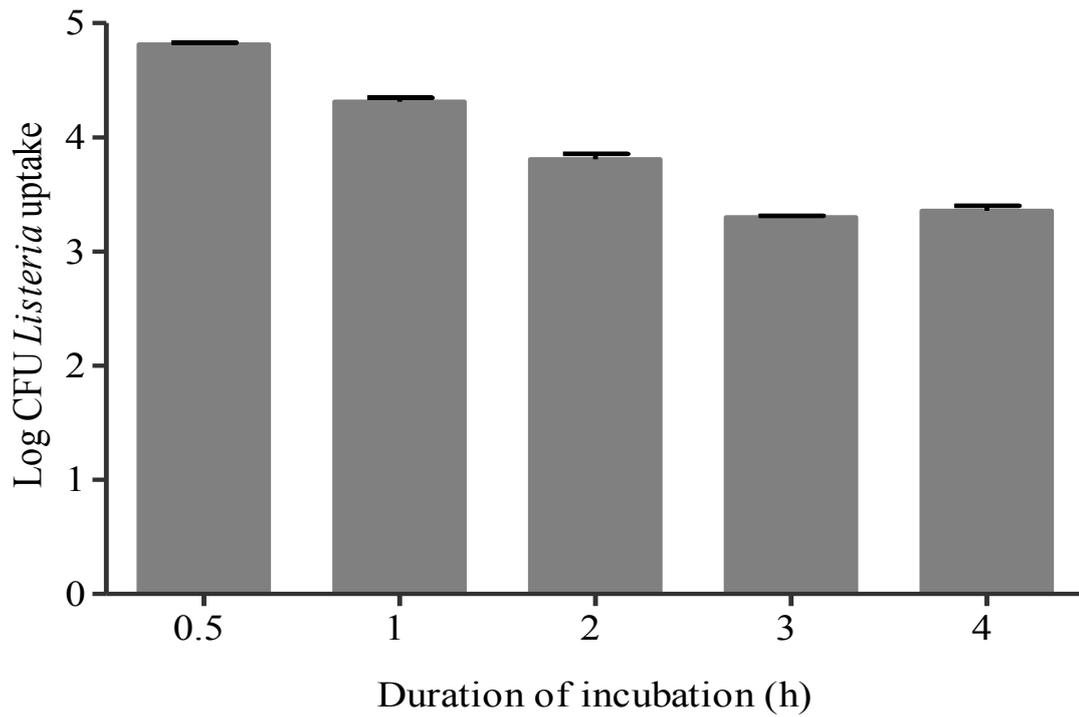


Figure 4.5 Time course for uptake of *L. monocytogenes* Scott A by *A. castellanii*.

*Acanthamoeba castellanii* and *L. monocytogenes* (MOI 1:500) were mixed and incubated for 4 h. Samples were drawn at time intervals to test for intracellular bacteria.

Error bars represent standard error of mean for replicate experiments.

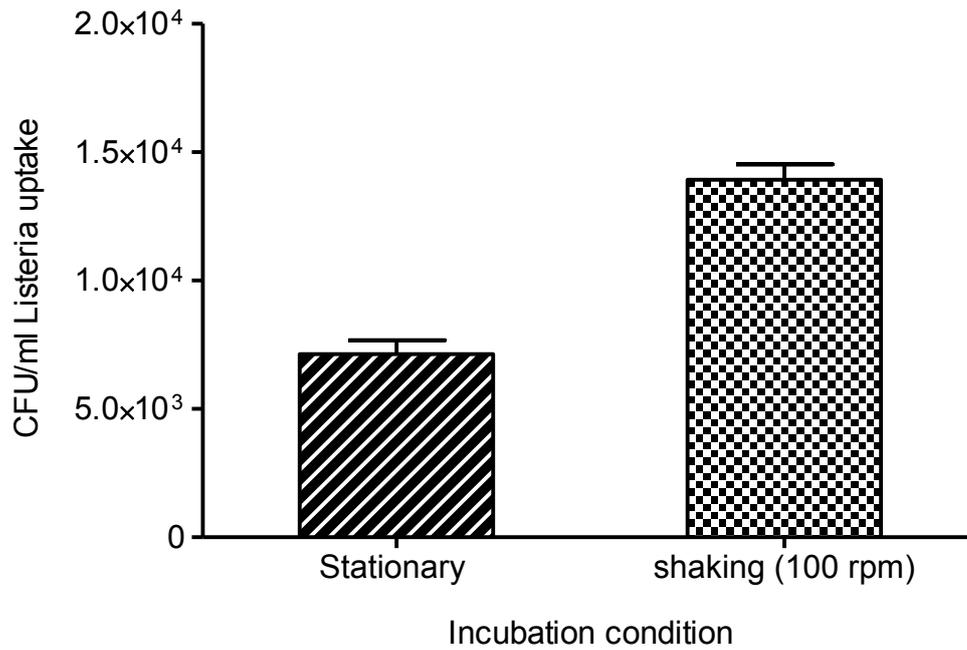


Figure 4.6 The effect of incubation condition on uptake of *L. monocytogenes* Scott A by *A. castellanii*. Intracellular *L. monocytogenes* were recovered from *A. castellanii* when co-cultures were incubated for 1 h at 32°C without shaking (stationary) or with shaking at 100 r.p.m. Error bars are standard error of mean of three replicate experiments.

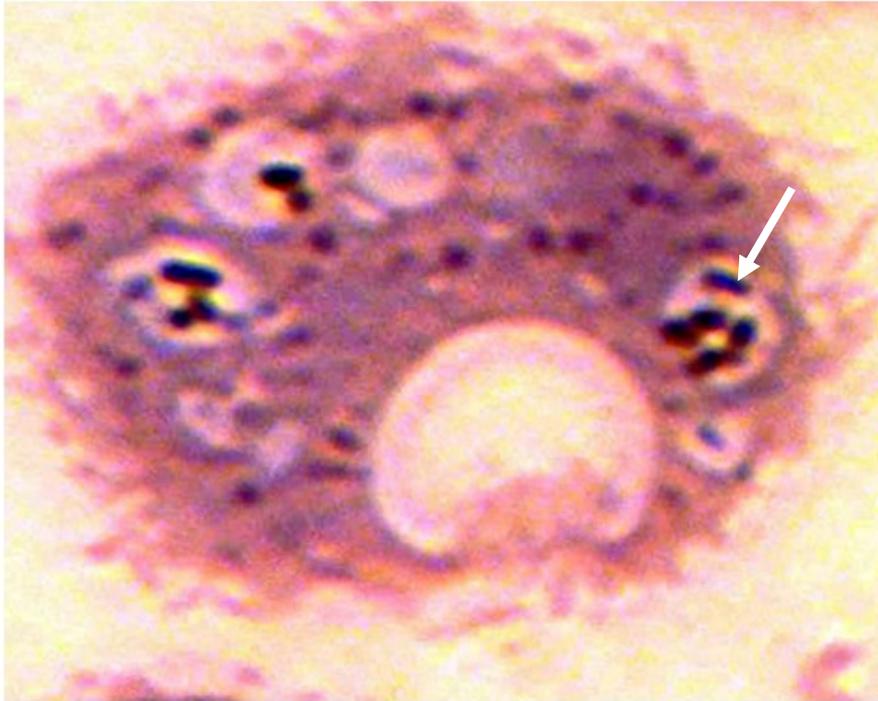


Figure 4.7 Demonstration of uptake of *L. monocytogenes* Scott A into the vacuoles of *A. castellanii* by brightfield microscopy. Samples were fixed with glutaraldehyde and stained with toluidine bue. Multiple bacteria inside a vacuole (arrow). Magnification  $\times 1000$ .

#### 4.3.4 *L. monocytogenes* survives after predation by *Acanthamoeba*

Results of intracellular survival of *L. monocytogenes* Scott A within *Acanthamoeba* are presented in Figure 4.8. The results show that intracellular bacteria persisted within *A. castellanii* and *A. polyphaga* but not *A. culbertsoni* for up to 72 h without significant change ( $P > 0.05$ ) in the overall numbers of bacteria from 0 h post-infection to the end of assay.

For the three *Acanthamoebae* examined, intracellular bacteria counts sharply dropped by more than 1 log CFU/ml by 4 h post-infection ( $P < 0.05$ ). Interestingly, thereafter the number of intracellular bacteria in *A. castellanii* gradually rose from  $1.6 \times 10^3$  CFU/ml to  $2.5 \times 10^4$  CFU/ml over 72 h ( $P < 0.05$ ), whereas numbers of intracellular *Listeria* in

*A. polyphaga* were unchanged over this time ( $P > 0.005$ ). On the other hand, no bacteria were detected in *A. culbertsoni* thereafter.

Intracellular survival was also assessed using other strains of *L. monocytogenes* in *A. castellanii* or *A. polyphaga* (Figure 4.9). Unlike Scott A that survived in *A. castellanii* and to some extent, in *A. polyphaga*, strain EGDe and 10403S did not survive after ingestion by either of the species of *Acanthamoeba* while no intracellular bacteria was recovered from *A. castellanii* or *A. polyphaga* that infected with strain C52 at any time point examined (Figure 4.9).

Although none of the other strains of *L. monocytogenes* was able to survive in *Acanthamoeba*, the numbers taken up at 0 h however varied with the strain of *Listeria* and to some extent, species of *Acanthamoeba*. For instance, the numbers of strain EGDe that were recovered from *A. castellanii* at 0 h were comparable with the numbers recovered from *A. polyphaga* ( $P > 0.05$ ) but the numbers recovered from either *A. castellanii* or *A. polyphaga* was significantly higher than the numbers of strain 10403S that were recovered from the corresponding species of *Acanthamoeba* ( $P < 0.05$ ). On the other hand, the numbers of strain 10403S that was recovered from *A. polyphaga* was significantly higher ( $P < 0.05$ ) than the numbers recovered from *A. castellanii* (Figure 4.9).

The initial decline in the number of intracellular *L. monocytogenes* Scott A in *Acanthamoeba* after uptake (Figure 4.8) could have been due to killing mediated by superoxide and other reactive oxygen intermediates (ROI) (Babior, 1984). In order to test this hypothesis *Acanthamoeba* cells were pre-treated with 10  $\mu\text{g/ml}$  of the NADPH oxidase inhibitor diphenyleneiodonium (DPI) for 15-30 min before infection. DPI was also included in the medium for the duration of the experiment. As shown in

Figure 4.10A, treatment of *A. castellanii* with DPI resulted in significantly greater recovery of intracellular *L. monocytogenes* Scott A at 4 h post-infection ( $P < 0.05$ ). This is in contrast with the significant reduction ( $P < 0.005$ ) in numbers of intracellular bacteria in untreated control during same period. A similar pattern of results were obtained when *A. polyphaga* were pre-treated with DPI and infected with Scott A (Figure 4.10B) or *A. castellanii* pre-treated with DPI and infected with strain 10403S (Figure 4.11). Although treatment with DPI enhanced intracellular survival of *L. monocytogenes* within *Acanthamoebae*, there was no evidence of increase in the numbers of intracellular bacteria after 4 h of incubation as a result of the treatment.

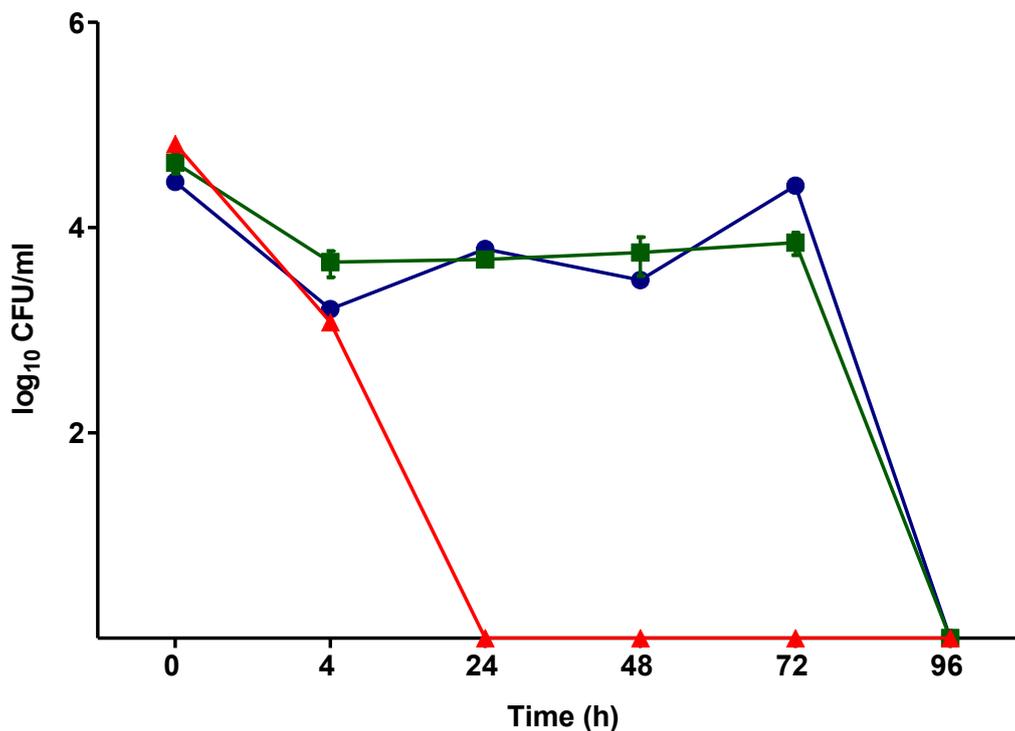


Figure 4.8 Intracellular numbers of *L. monocytogenes* Scott A in *Acanthamoeba*.

Intracellular numbers of *L. monocytogenes* in *Acanthamoeba castellanii* (●), *A. culbertsoni* (▲) and *A. polyphaga* (■). Error bars are standard error of mean counts for triple experiments

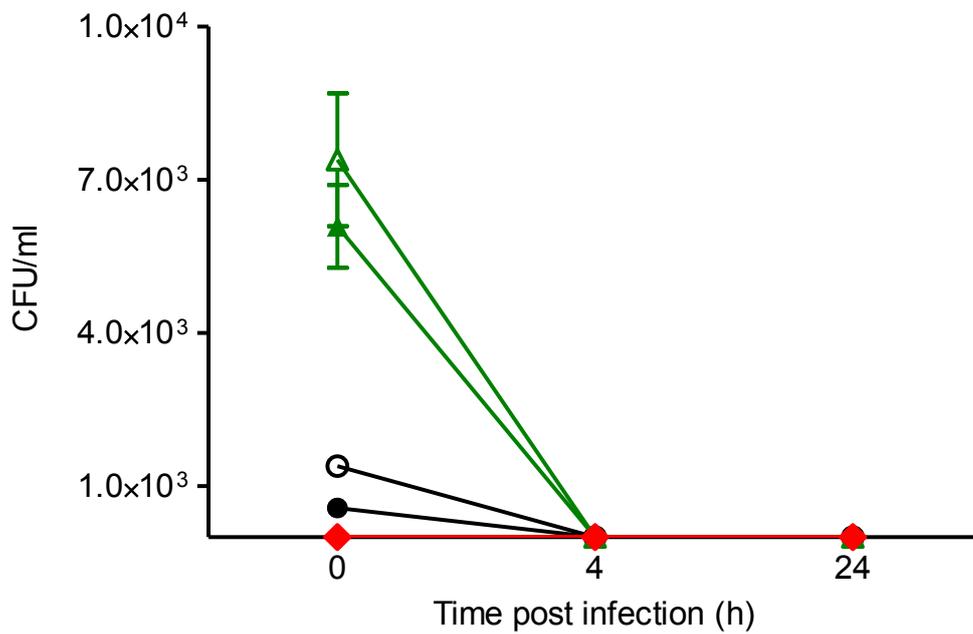


Figure 4.9 Intracellular survival of other strains of *L. monocytogenes* within *Acanthamoebae*. CFU counts of *L. monocytogenes* strain 10403S within *A. castellanii* (●) and *A. polyphaga* (⊖); CFU counts of *L. monocytogenes* strain EGDe within *A. castellanii* (▲) and *A. polyphaga* (△); CFU counts of *L. monocytogenes* strain C52 within *A. castellanii* (◆) and *A. polyphaga* (◇). Error bars represent standard error of mean counts for triplicate experiments.

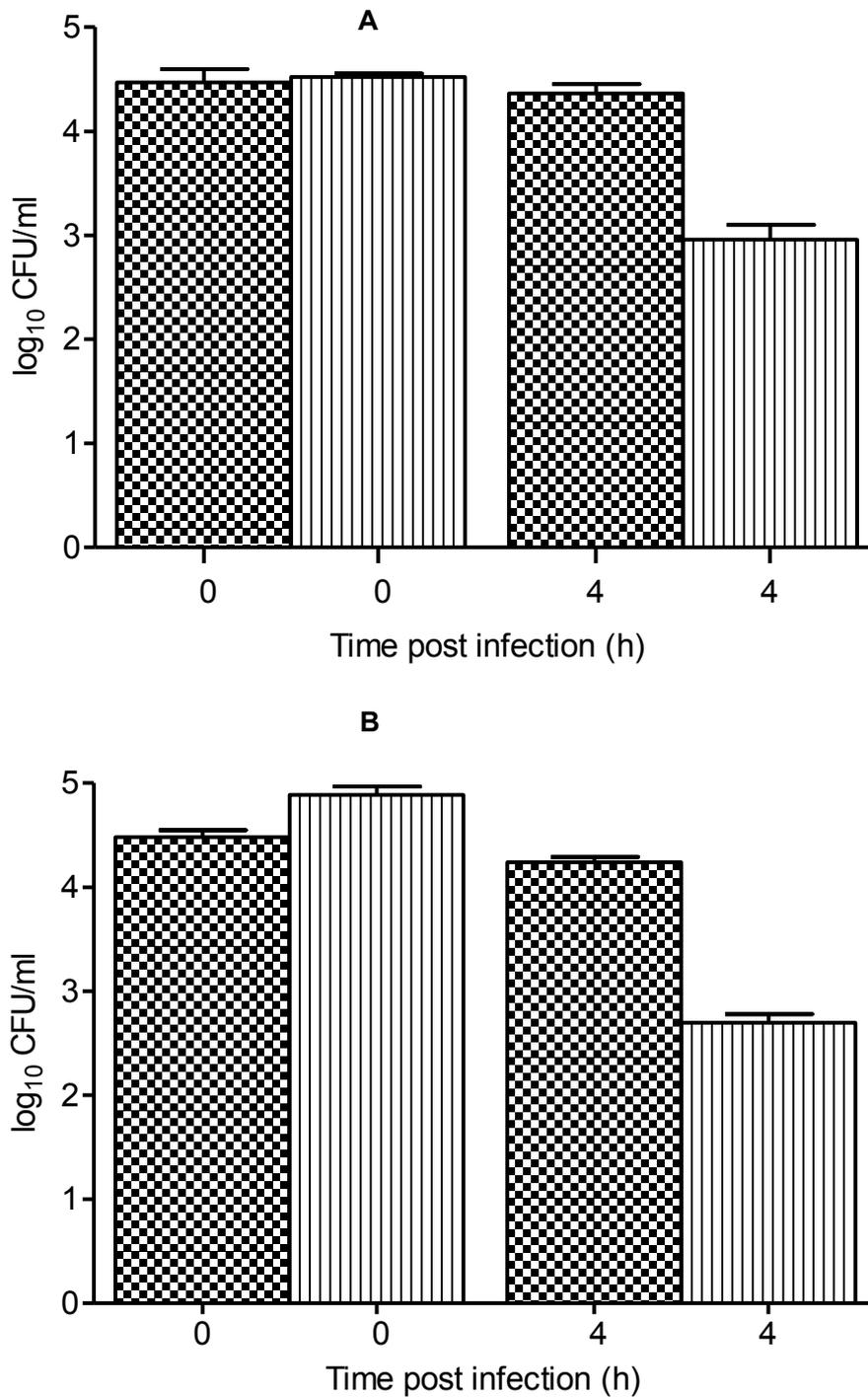


Figure 4.10 Effect of diphenyleiodonium on intracellular survival of *Listeria monocytogenes* Scott A within (A) *A. castellanii* and (B) *A. polyphaga*. Intracellular bacteria recovered from amoeba pre-treated with DPI (▣) or the untreated amoeba (▤). Error bars are standard errors of mean counts for three experiments.

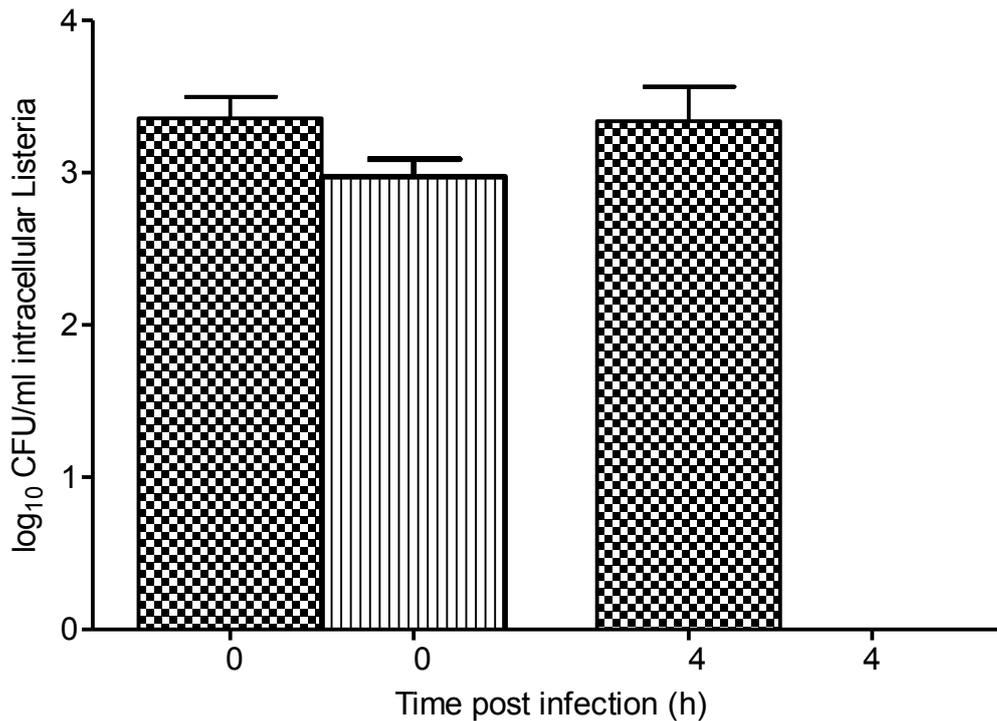


Figure 4.11 Effect of DPI on intracellular survival of *L. monocytogenes* 10403S within *A. castellanii*. Intracellular bacteria recovered from amoebae pre-treated with DPI (▣) or the untreated amoebae (▤). Error bars represent standard error of mean counts for at least three experiments.

#### 4.3.5 Manganese enhances intracellular survival of *Listeria*

*Listeria monocytogenes* produces superoxide dismutase (SOD) and levels are modulated in response to environmental factors such as oxygen and iron concentration (Welch *et al.*, 1979, Schiavone and Hassan, 1987). The SOD of *L. monocytogenes* contains manganese as a cofactor and addition of MnCl to listerial extract that has been depleted of ions was shown to restore SOD activity of the extract (Schiavone and Hassan, 1987, Vasconcelos and Deneer, 1994). MnSOD has been found to be critical in the pathogenesis of *L. monocytogenes* (Welch *et al.*, 1979, Archambaud *et al.*, 2006). It is likely therefore that incubation of *L. monocytogenes* with a source of manganese will

increase production of MnSOD and hence, increase the ability of *L. monocytogenes* to survive within *Acanthamoeba*. To test if manganese can enhance the intracellular survival of *L. monocytogenes*, bacteria were first exposed to manganese in growth medium and then tested for increased production of protein by measuring the total protein in bacterial extract as described in section 2.8.8. The bacteria pre-treated with manganese were then used to infect *Acanthamoeba*.

The *L. monocytogenes* Scott A strain that was pre-treated with manganese produced four times as much protein as the untreated control and higher amount of protein than that produced by either of the *Listeria* strains incubated in presence of manganese (Table 4.3). Similarly, the amount of protein produced by strain 10403S that was pre-treated with manganese was twice the amount produced by the untreated sample, while that produced by EGDe only marginally increased as a result of the treatment. On the other hand, the protein concentration in the extract of strain C52 that was pre-treated with manganese was less than the concentration in the untreated control (Table 4.3). With the exception of Scott A, treatment with manganese did not significantly increase the numbers of *L. monocytogenes* strains that were taken up by either of the three *Acanthamoeba* sp (Table 4.3) neither did the treatment enabled the bacterial strains to survive within amoebae as were also the case with the untreated samples (Table 4.3). On the other hand, the numbers of manganese-treated *L. monocytogenes* Scott A that were taken up by *A. castellanii* was twice ( $5.5 \times 10^4$  CFU/ml) the number taken up without pre-exposure to manganese ( $P < 0.001$ ), whereas the numbers of the manganese-treated and untreated Scott A recovered from *A. polyphaga* or *A. culbertsoni* were comparable ( $P > 0.05$ ) (Table 4.3).

Treatment with manganese significantly ( $P < 0.001$ ) enhanced intracellular survival of *L. monocytogenes* Scott A within *A. castellanii* but not in *A. polyphaga* or *A.*

*culbertsoni*, where intracellular bacteria numbers were comparable with those in untreated samples ( $P > 0.05$ )(Figure 4.12). However, as with the untreated sample, the number of intracellular bacteria fell significantly after 4 h post-infection before it gradually rose to peak at 48 h ( $P < 0.05$ ). But unlike the untreated control, the manganese-treated bacteria survived after 96 h in *A.castellanii* whereas the untreated ones only survived in *A. castellanii* for up to 72 h post-infection (Figure 4.12).

Although bacterial protein output increased when treated with manganese however, attempts to detect SOD activity in bacterial extracts was not successful.

The majority of amoeba trophozoites infected with manganese-treated *L. monocytogenes* Scott A formed cysts by 96 h post-infection (Figure 4.13A) in contrast to those infected with untreated bacteria, which lost their infection by 96 h post-infection but did not formed cysts (Figure 4.13B). On the other hand, the uninfected *A. castellanii* incubated in maintenance medium, with or without manganese, produced cysts after 96 h incubation. The number of cells in the manganese-treated sample, appeared however to have diminished after this period (Figure 4.13C and D) suggesting a cytotoxic effect of manganese.

Table 4.3 Manganese treatment, protein expression and uptake of *L. monocytogenes* by *Acanthamoeba*

<i>L.monocytogenes</i> strain	Protein concentration in extract (µg/ml)	Intracellular bacterial counts (CFU) at T= 0 h		
		<i>A. castellanii</i>	<i>A. polyphaga</i>	<i>A. culbertsoni</i>
Scott A	32	$2.8 \times 10^4 \pm$ $3.7 \times 10^3$	$6.7 \times 10^4 \pm$ $2.1 \times 10^4$	$6.5 \times 10^4 \pm$ $9.9 \times 10^3$
Scott A + Mn <sup>2+</sup>	126	$5.5 \times 10^4 \pm$ $7.4 \times 10^3$	$5.9 \times 10^4 \pm$ $1.2 \times 10^4$	$6.7 \times 10^4 \pm$ $8.9 \times 10^3$
EGDe	76	$6.1 \times 10^3 \pm$ $1.2 \times 10^3$	$5.4 \times 10^3 \pm$ $3.4 \times 10^3$	ND
EGDe + Mn <sup>2+</sup>	81	$5.0 \times 10^3$ $\pm 1.9 \times 10^2$	$3.2 \times 10^3 \pm$ $1.2 \times 10^3$	ND
10403S	51	$5.7 \times 10^2 \pm$ $1.5 \times 10^2$	$1.1 \times 10^3 \pm$ $6.1 \times 10^2$	ND
10403S + Mn <sup>2+</sup>	101	$1.3 \times 10^3 \pm$ $1.0 \times 10^2$	$1.5 \times 10^3 \pm$ $6.1 \times 10^2$	ND
C52	93	0	0	ND
C52 + Mn <sup>2+</sup>	68	0	0	ND

ND; Not done

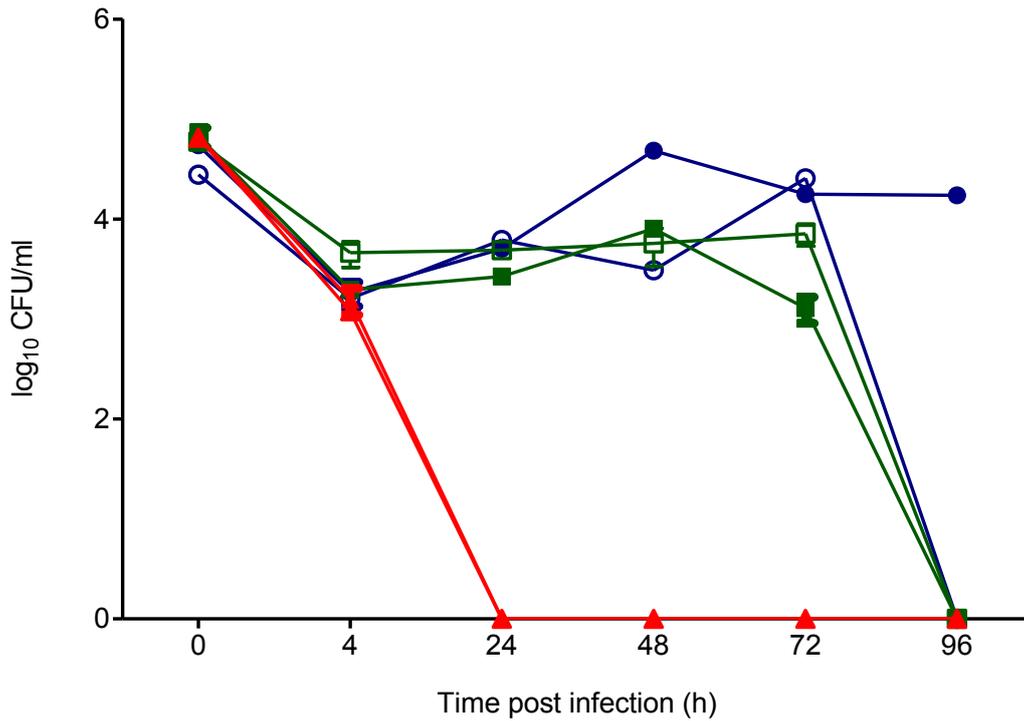


Figure 4.12 . Effect manganese on intracellular survival of *L. monocytogenes* Scott A within *Acanthamoeba* species. CFU counts of intracellular bacteria in *A. castellanii* (○), *A. culbertsoni* (△) and *A. polyphaga* (□) that were infected with manganese-treated *L. monocytogenes* compared with CFU counts of intracellular bacteria in *A. castellanii* (●), *A. culbertsoni* (▲) and *A. polyphaga* (■) that were infected with untreated *L. monocytogenes*. Error bars represent standard error of mean of replicate experiments.

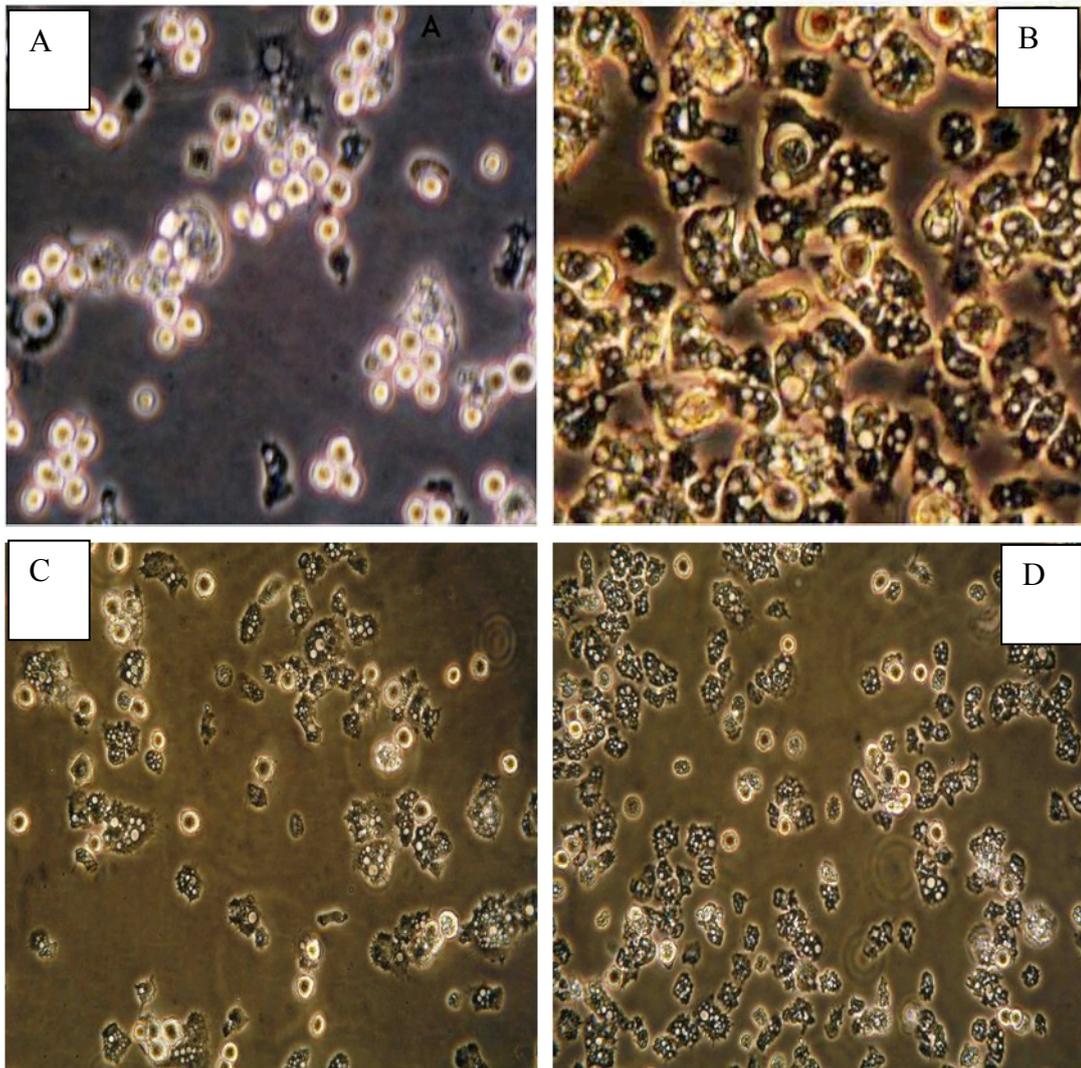


Figure 4.13 Phase contrast images of *A. castellanii* in maintenance medium (20 % SK6) after 96 h of incubation: (A) Cells infected with *L. monocytogenes* Scott A pre-treated with manganese. Intracellular bacteria were still recovered from (A) at this time even though majority the trophozoites had encysted. (B) Cells infected with untreated *L. monocytogenes*. No cysts were formed in (B) at 96 h post-infection and no intracellular bacteria were recovered. C and D are uninfected amoebae incubated in MM containing 4mM manganese (C) and without manganese (D). Some trophozoites in the uninfected controls also formed cyst during the incubation. Magnification 400×

### 4.3.6 Transmission electron microscopy results

Transmission electron microscope analysis of *A. castellanii* infected with *L. monocytogenes* Scott A at 1h post-infection confirmed uptake of *L. monocytogenes* which were mainly found in spacious, membrane-bound vacuoles of their host cell (Figure 4.14A and B). Most vacuoles contained single bacterial cells juxtaposed to other vacuoles containing one or more bacteria (Figure 4.14A). It appeared that bacteria were taken up into individual vacuoles that coalesced with time into large spacious vacuoles containing two or more bacteria (Figure 4.15A and B). A few of the bacterial cells were seen dividing in phagosomes as evident by septum formation (Figure 4.14A and Figure 4.16A). Small vesicles, presumed to be lysosomes, were seen in the vicinity of the vacuoles but there was no evidence that fusion occurred (Figure 4.16A). This suggests that *L. monocytogenes* can replicate in vacuoles of *Acanthamoeba* probably by preventing fusion of phagosome with lysosomes. Although vacuoles containing dividing bacteria were only found at 1h and 4, nonetheless, vacuoles that contained multiple bacteria were found after 24 h post-infection (Table 4.4). Although bacteria replicated in vacuoles, there was no evidence that they escaped or replicated in the cytoplasm at any time examined.

Whereas intact bacteria were found in vacuoles and some showed evidence of division, others in separate vacuoles demonstrated classical features indicative of lost cellular integrity (Figure 4.16B). For instance, their cell wall was disrupted at one or more locations and the electron dense material on their cytoplasm was considerably reduced (Figure 4.16B) compared with the healthy bacteria (Figure 4.16A). In addition, the vacuoles were often surrounded by lysosome-like vesicles some of which were partially fused with the vacuole. This suggests that some vacuoles within amoeba are permissive to bacterial replication while others are not.

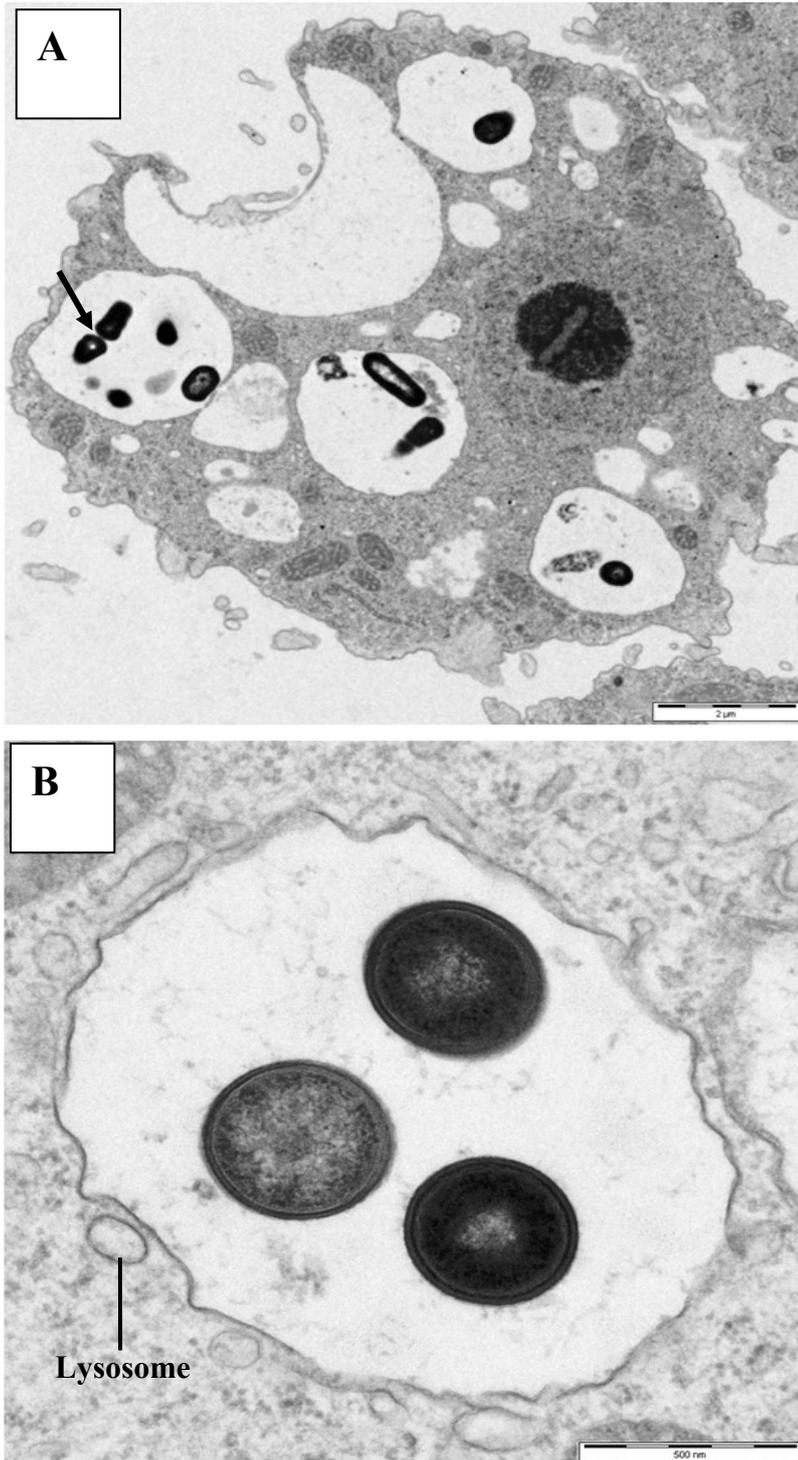


Figure 4.14 TEM of *A. castellanii* infected with untreated *L. monocytogenes* Scott A. (A) Single and multiple bacteria in vacuoles at 1 h post-infection. Arrow points to a bacterium undergoing division. Scale bar = 2  $\mu\text{m}$  (B) Bacteria in vacuoles at 24 h post-infection. Lysosome-like vesicles can be seen around the vacuoles. Scale bar = 0.5  $\mu\text{m}$ .

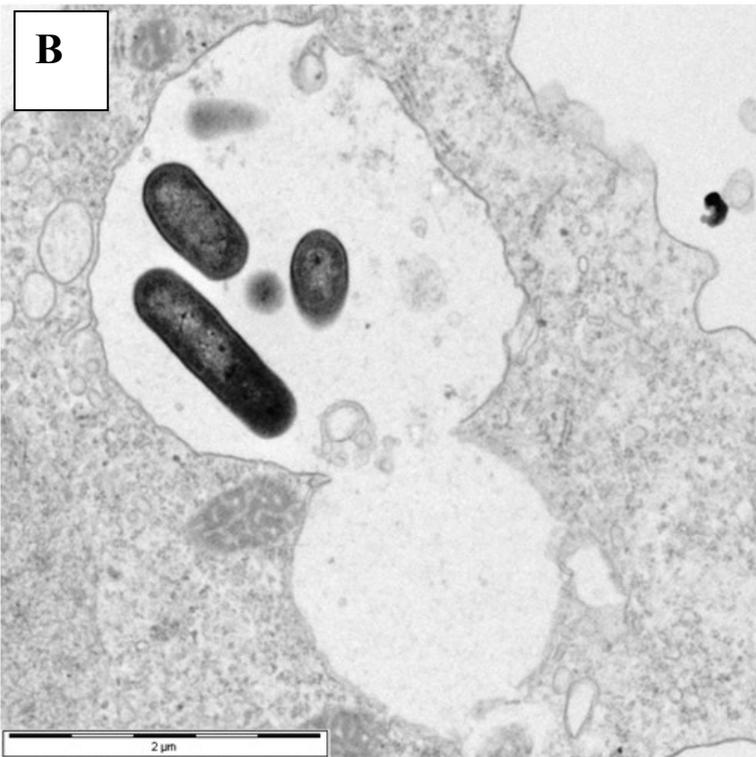
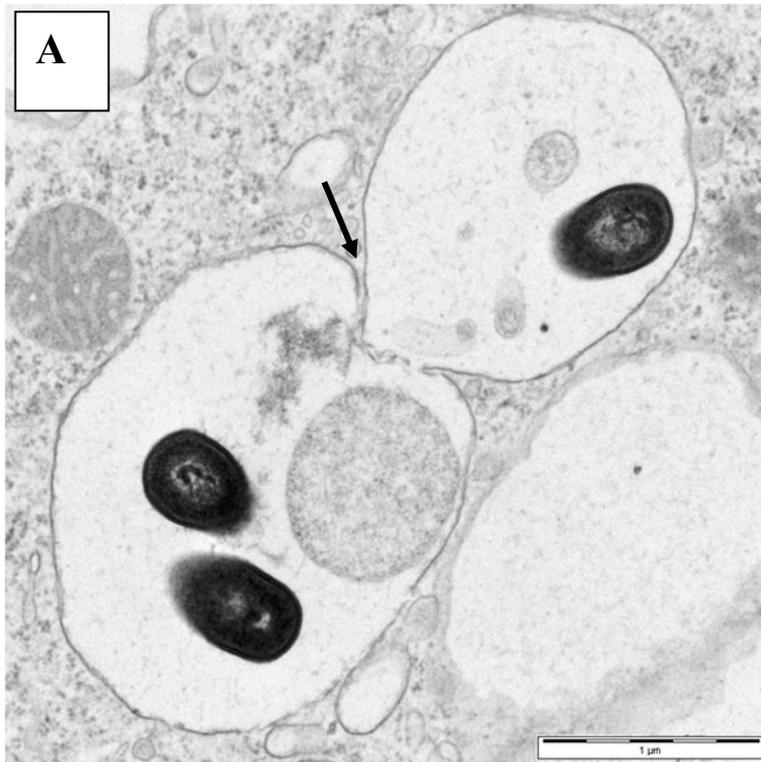


Figure 4.15 TEM micrographs of *A. castellanii* infected with *L. monocytogenes* Scott A after 4 h post-infection. (A) Two vacuoles fusing together (arrow). Scale bar = 1 μm (B) A larger vacuole formed after the fusion. Scale bar = 2 μm

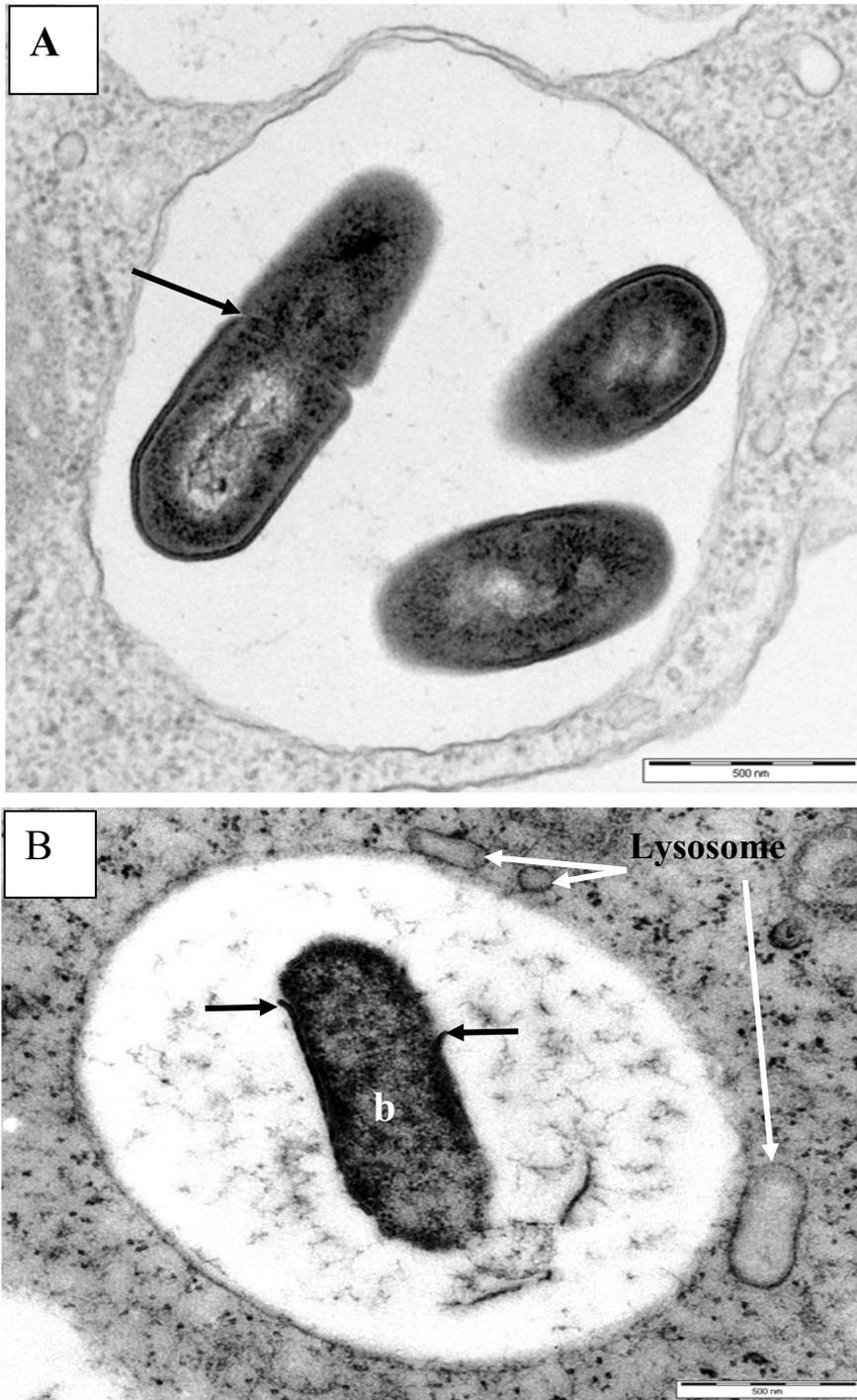


Figure 4.16 Representative TEM of *A. castellanii* infected with *L. monocytogenes* Scott A at 4 h post infection. (A) A bacterium replicating in vacuoles (arrow) and no lysosome fused with vacuole. (B) A bacterium (b) appears to be in the process of degradation within the vacuole evident by the disrupted cell wall (arrows) and depleted electron dense material in its cytoplasm. Lysosomes partly fused with the vacuole (white arrows). Scale bar = 0.5  $\mu$ m each.

The TEM results of *A. castellanii* that were infected with manganese-treated *L. monocytogenes* are shown in Figure 4.17-Figure 4.18. As with the untreated *L. monocytogenes*, the manganese- treated *L. monocytogenes* Scott A were mostly confined in vacuoles (Figure 4.17A and B). Some of the bacteria had an undulating membrane-like structures (Figure 4.17B) that were not observed in the untreated sample. Also, as with untreated samples, the manganese-treated bacteria were found replicating in vacuoles (Figure 4.17A, Figure 4.18A and Figure 4.19A) concurrently with bacterial degradation in other vacuoles (Figure 4.18B). However, the percentage of vacuoles containing replicating bacteria were higher than in cells that were infected with untreated bacteria, at all the times examined (Table 4.4).

For both treated and untreated samples, the percentage of vacuoles containing replicating bacteria declined after 4 h post infection but then increased slightly in the manganese treated sample after 24 h post-infection while none was found in the untreated sample during the same period (Table 4.4). As with the untreated sample, bacterial cells were not found free in the cytoplasm of amoeba. However, at 24 h post-infection, few bacterial cells appeared to be escaping into cytoplasm (Figure 4.19B-C). This was evident by the disruption of vacuole's membrane at one or more locations (Figure 4.19B-C) and bacterial partial contact with the cytosol (Figure 4.19C). In addition, a few bacteria occupied vacuoles presumed to be the advanced form of listerial secondary vacuoles previously shown in macrophages infected with *L. monocytogenes* (Figure 4.19D)(Tilney and Portnoy, 1989, Dussurget *et al.*, 2004).

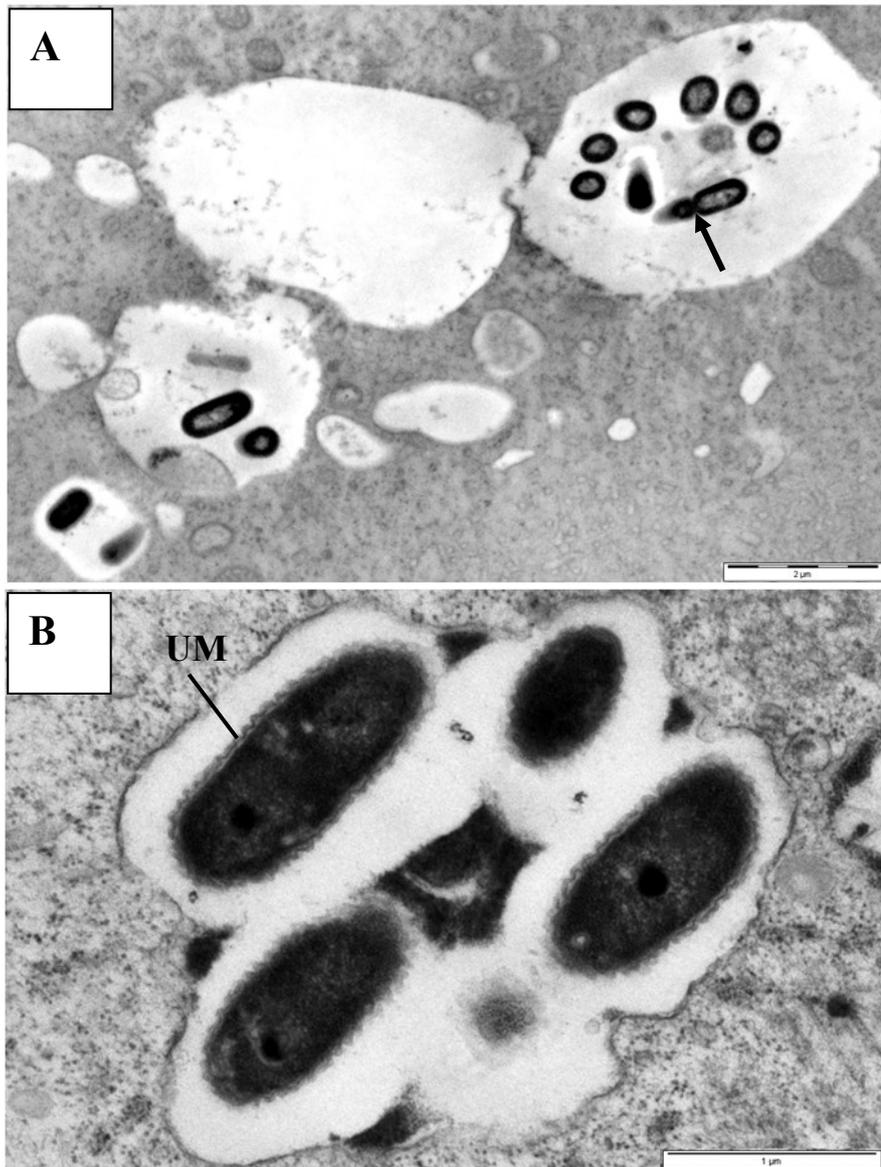


Figure 4.17 *Acanthamoeba castellanii* cells infected with manganese pre-treated *L. monocytogenes* Scott A showing (A) Bacteria in large vacuoles at 1h post-infection. Arrow points to a dividing bacterium. Scale bar = 2 $\mu$ m (B) Multiple bacteria in a vacuole at 24 h post-infection. Bacteria were coated with undulating membranes (UM). Scale bar = 1 $\mu$ m

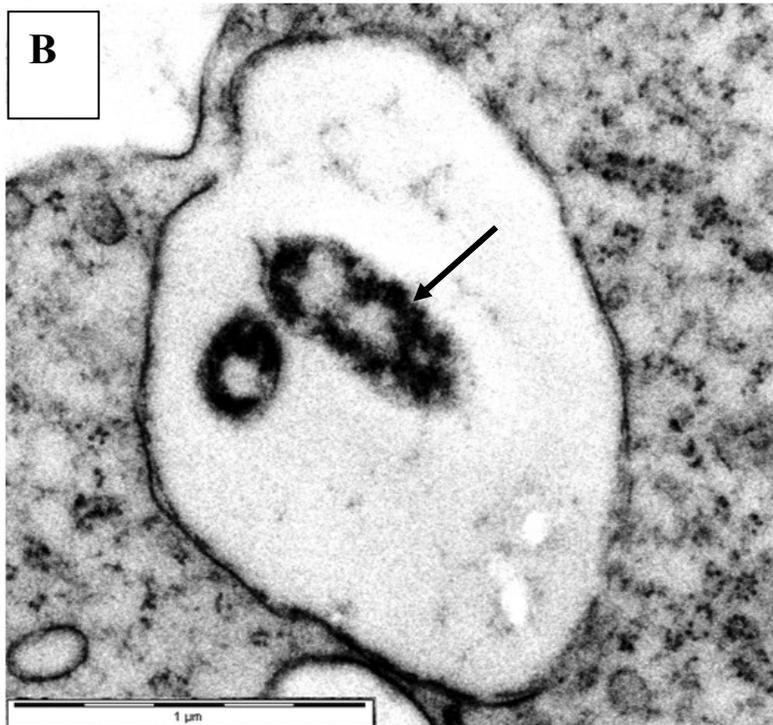
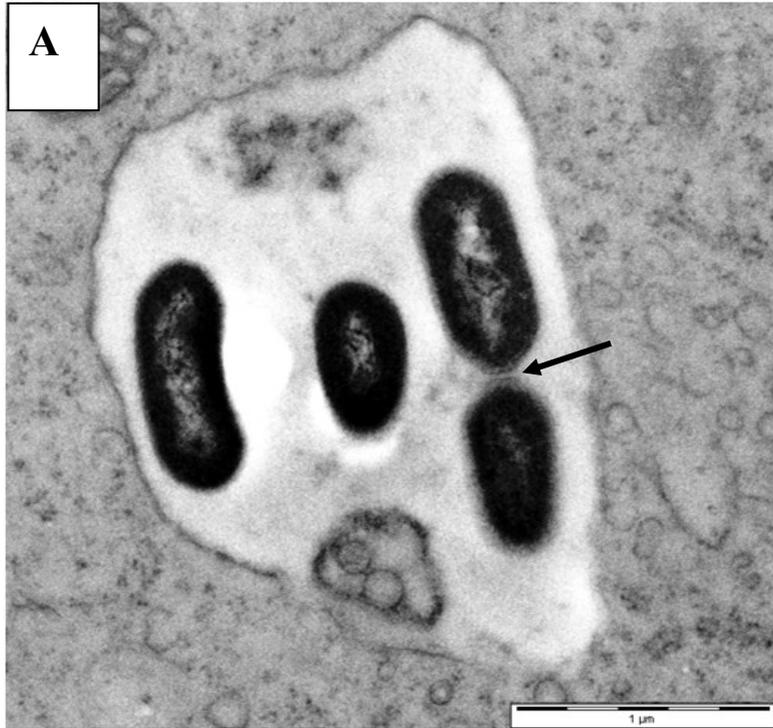
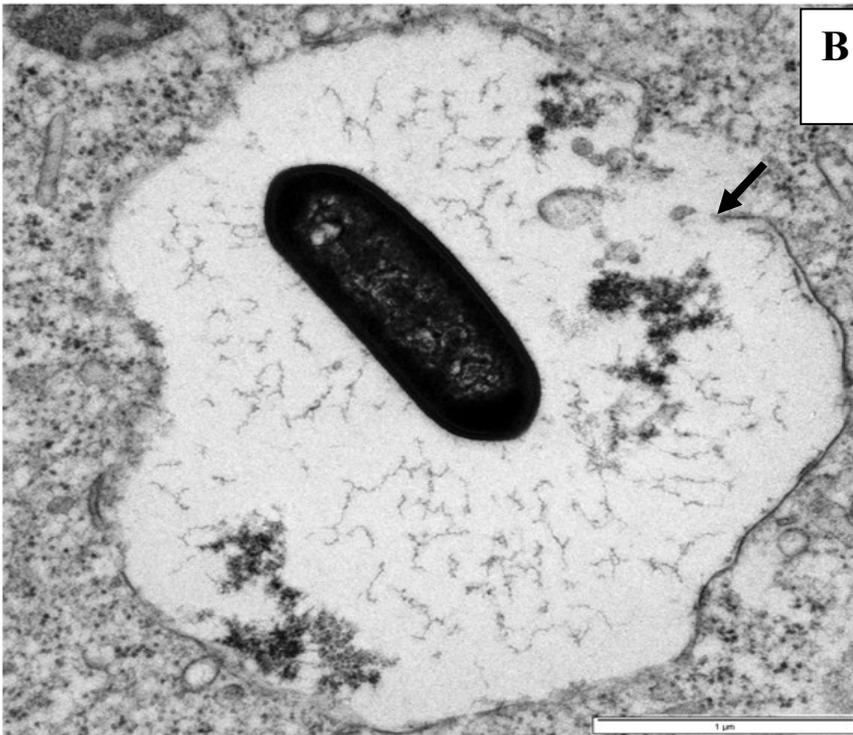
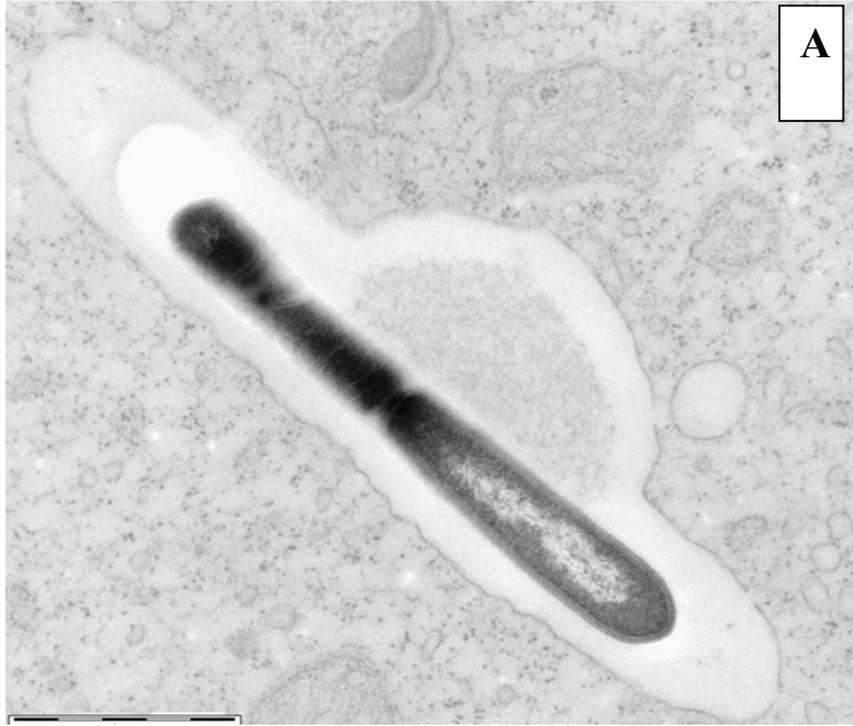


Figure 4.18 TEM micrographs of *A. castellanii* infected with *L. monocytogenes* Scott A at 4h post-infection. (A) A bacterial cell at final stage of cell division (arrow). (B) Bacteria undergoing degradation inside a vacuole. Note the cell wall (arrow) of bacterium appears to be completely destroyed.

Table 4.4 TEM analysis of *L. monocytogenes* Scott A replication within the vacuoles of *A. castellanii*

<i>L. monocytogenes</i> Scott A	Vacuoles with healthy bacteria (dividing and non- dividing)	Vacuoles with health dividing bacteria	% vacuoles with healthy dividing bacteria
Manganese- treated(time post- infection)			
1 h	56	8	14.3
4 h	42	2	4.8
24 h	70	4	5.7
Untreated (time post infection)			
1 h	57	7	12.3
4 h	142	6	4.2
24 h	88	0	0



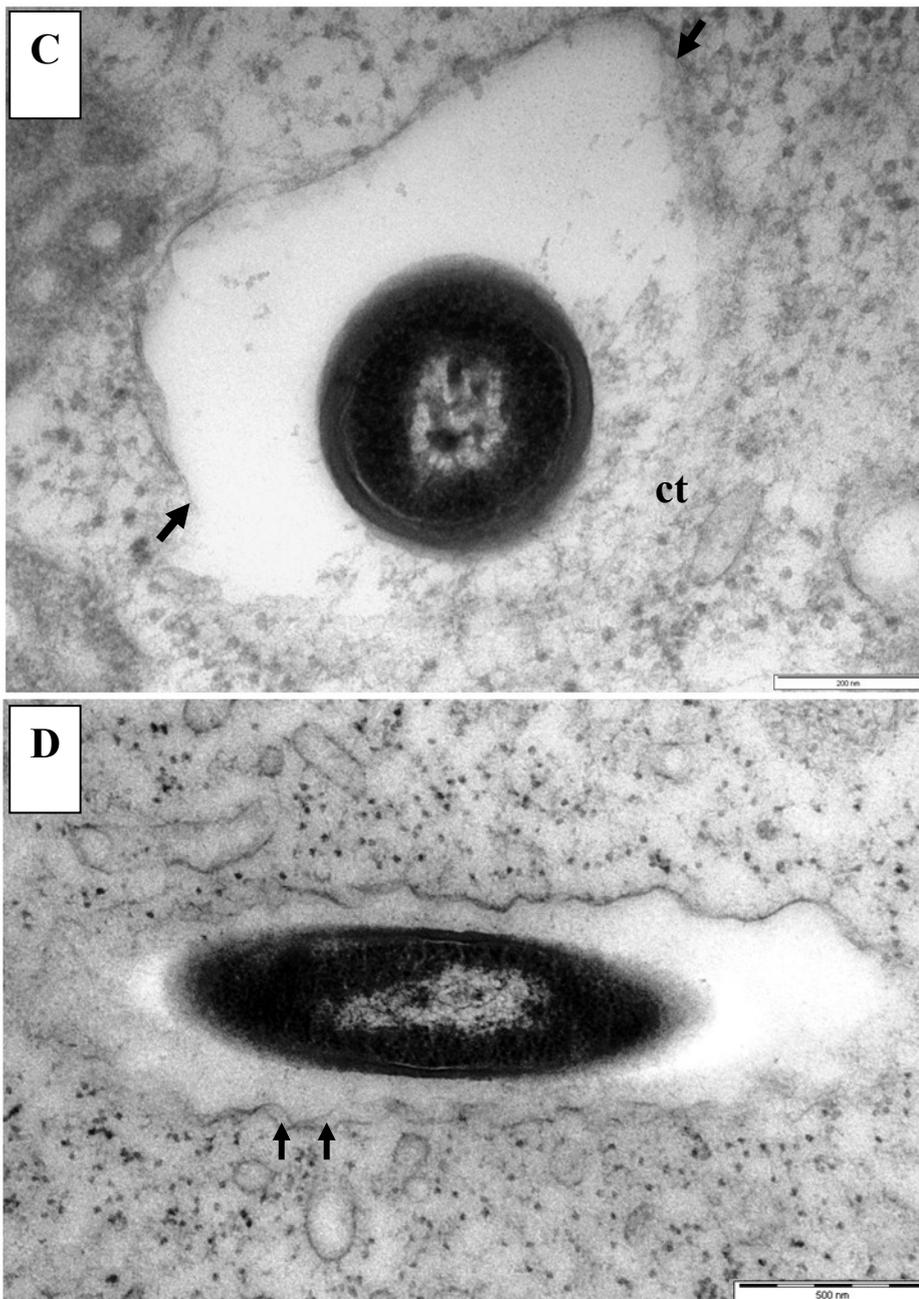


Figure 4.19 Electron micrographs of *A. castellanii* infected with manganese-treated *L. monocytogenes* Scott A at 24 h post-infection. (A) A bacterium replicating in vacuole. Scale bar = 1 $\mu$ m (B) A part of a membrane of vacuoles appeared to have been disrupted (arrow) Scale bar = 1 $\mu$ m. (C) A bacterium appeared partially in contact with the cytoplasm (ct) after disruption of most part of the vacuole's membrane (arrows). Scale bar = 0.2  $\mu$ m (D) A bacterium in a secondary-like vacuole membrane partly surrounded by two membranes (short arrows). Scale bar = 0.5 $\mu$ m

## Chapter 5. Autophagy and intra-amoebal survival of

### *Listeria monocytogenes*

#### 5.1 Introduction

Autophagy is a lysosome-mediated degradation pathway used by eukaryotic cells to recycle cytoplasm and dispose of excess or dysfunctional organelles (Dunn, 1994, Levine and Klionsky, 2004, Mizushima, 2005). The process begins with the sequestration of regions of cytosol within double-membrane-bound compartments which mature and fuse with lysosome to degrade their cytoplasmic contents (Kirkegaard *et al.*, 2004, Mizushima, 2004).

Available records suggest that autophagy is also specifically induced by some human and animal cells during infection to eliminate invading microbes, for example *Salmonella enterica* serovar Typhimurium (Birmingham *et al.*, 2006), *L. pneumophila* (Amer *et al.*, 2005, Amer and Swanson, 2005), *Mycobacterium tuberculosis* (Gutierrez *et al.*, 2004, Vergne *et al.*, 2006, Biswas *et al.*, 2008), *Helicobacter pylori* (Terebiznik *et al.*, 2009), *Brucella abortus* (Pizarro-Cerda *et al.*, 1998a, Pizarro-Cerda *et al.*, 1998b), Group A Streptococcus (Nakagawa *et al.*, 2004), *Vibrio cholera* cytotoxin (Gutierrez *et al.*, 2007), *Burkholderia pseudomallei* (Cullinane *et al.*, 2008, Gong *et al.*, 2011), *Bacillus anthracis* lethal toxin (Tan *et al.*, 2009a, Tan *et al.*, 2009b), and *Listeria monocytogenes* (Py *et al.*, 2007, Yano and Kurata, 2008, Yano *et al.*, 2008).

The involvement of autophagy in host defence against intracellular *L. monocytogenes* was first suggested by Rich and colleagues who showed that treatment of infected macrophages with chloramphenicol (an inhibitor of bacterial protein synthesis), after

bacteria escaped from phagosome resulted in the trapping of the cytoplasmic bacteria within autophagosome, and decreased in number of viable bacteria due to autophagic degradation (Rich *et al.*, 2003). Recently, Yano and co-workers also found that infection of *Drosophila* cells with *L. monocytogenes* triggered autophagy against cytoplasmic bacteria (Yano and Kurata, 2008, Yano *et al.*, 2008). They showed that autophagy induction was dependent on the recognition of listerial diaminopimelic acid-type peptidoglycan by the host cell peptidoglycan-recognition protein. In addition, they found that induction of autophagy inhibited intracellular growth of the pathogen and enhanced host cell resistance to infection (Yano and Kurata, 2008, Yano *et al.*, 2008).

Studies have shown that autophagy can also target *L. monocytogenes* in phagosomes that have been damaged by listeriolysin O (LLO) during the primary infection of mouse fibroblasts and macrophages in the early phase, prior to listerial escape into cytoplasm thereby limiting early bacterial growth (Py *et al.*, 2007, Birmingham *et al.*, 2008b, Corr and O'Neill, 2009).

Birmingham and co-workers also showed that some *L. monocytogenes* trapped in autophagic vacuoles avoided killing by inhibiting fusion of lysosome with autophagosome and modified the vacuole into a replicative niche (Birmingham *et al.*, 2008a, Birmingham *et al.*, 2008b).

Although numerous studies have suggested the involvement of autophagy in intracellular death or survival of pathogens inside cells of human and animal origin, there is no report to date of the occurrence of this phenomenon in *Acanthamoebae* infected with bacteria or other microbes.

## 5.2 Hypothesis

The hypothesis was that infection of *A. castellanii* with *L. monocytogenes* may trigger the autophagy machinery of the amoeba leading to either increase in survival of the bacterium or killing of intracellular *Listeria* within amoeba. To test the hypothesis the following objectives were undertaken

- a. To demonstrate autophagy in *Acanthamoeba* infected with *L. monocytogenes*, using transmission electron microscopy.
- b. To show that autophagy can both limit and support intracellular survival of *L. monocytogenes* within amoeba.

## 5.3 Results

### 5.3.1 Evidence of autophagy in *Acanthamoeba* infected with *Listeria*

Transmission electron microscopy studies of *A. castellanii* infected with *L. monocytogenes* Scott A revealed that a large number of vacuoles occupied by bacteria exhibit one or both key distinctive features of a classical autophagosome (Kirkegaard *et al.*, 2004, Eskelinen and Saftig, 2009) i.e. possession of a double-membrane and/or presence of undigested cytoplasmic components such as portion of cytosol and/or organelles like mitochondria and other vesicles in their lumen (Figure 5.1A). In addition, the bacterial autophagosomes were often very spacious measuring 5 µm or more in diameter (Figure 5.1A). This is in contrast with the conventional phagosomes (Figure 5.1B) which has only one membrane surrounding its tight-fitting lumen and does not contain any cytoplasmic material beside bacteria (Bowers, 1980, Touret *et al.*, 2005). Some autophagosomes contained only one bacterium (Figure 5.2A) while others contained two or more bacteria in their lumen (Figure 5.1A). Putative autophagosomes were observed in cells as early as 1 h post-infection but were also noticeable at other times examined. Autophagic-like vacuoles were also demonstrated in uninfected amoeba cells incubated in a quarter strength Ringer's solution (Figure 5.2B), suggesting a role of starvation in the induction autophagy in *A. castellanii*. However, the autophagosomes with bacteria were considerably larger than the classical autophagosomes of the uninfected cells which contained only cellular constituents (Figure 5.2B). Some bacteria in autophagosomes were partially bounded by single-membrane structures reminiscent of a phagosome membrane (Figure 5.3). This suggests that autophagy targets *L. monocytogenes* while in phagosomes. In addition, some autophagosomes appeared to be fusing with phagosome or other autophagosomes to form a larger autophagosomes containing multiple bacteria (Figure 5.4A and B).

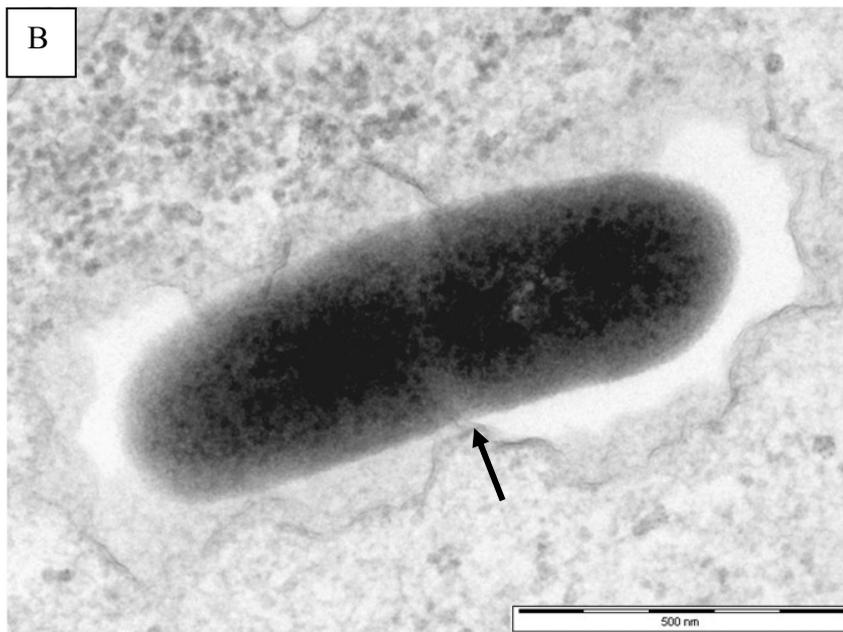
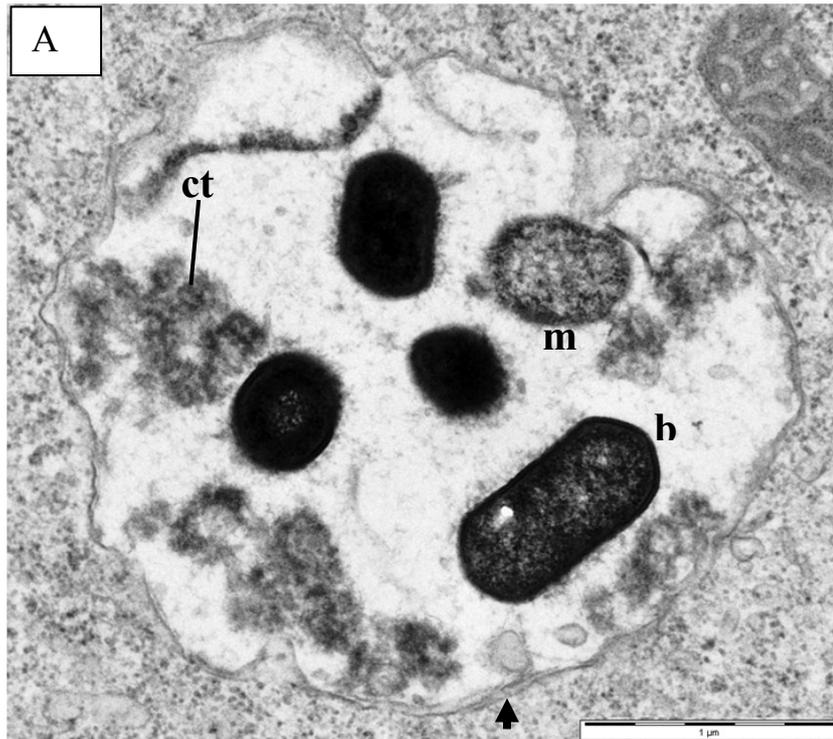


Figure 5.1 Demonstration of autophagy in *A. castellanii* infected with *L.monocytogenes* Scott A by TEM 1.(A) A spacious autophagosome at 1h post- infection sequesters multiple bacteria (b), cytoplasmic ground matter (ct) and an unhealthy mitochondrion (m) is delimited by a double membrane (short arrow). Scale bar = 1 $\mu$ m. (B) A bacterium in a relatively tight, single membrane phagosome (arrow) also at 1 h post-infection. Scale bar = 0.5 $\mu$ m

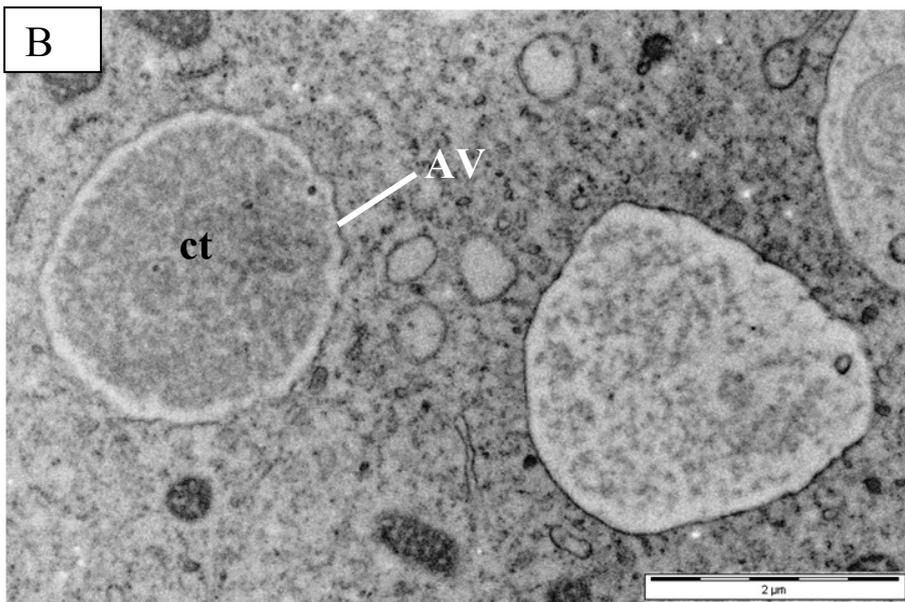
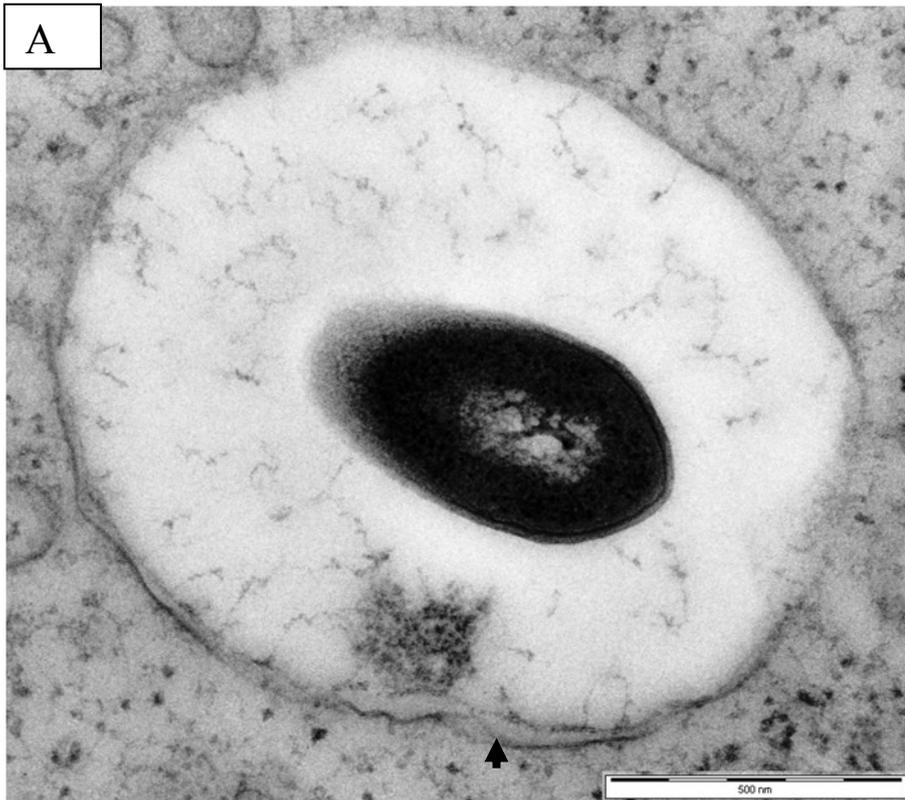


Figure 5.2 Demonstration of autophagy in *A. castellanii* (A) A single bacterium sequestered into a double-membrane autophagic vacuole (arrow) in amoeba that was infected with *L. monocytogenes* 4 h post-infection. Scale bar = 0.5μm. (B) Autophagic vacuole (AV) in an uninfected cell was identified by the presence of cytoplasmic material (ct) inside the vacuole. Scale bar 2μm

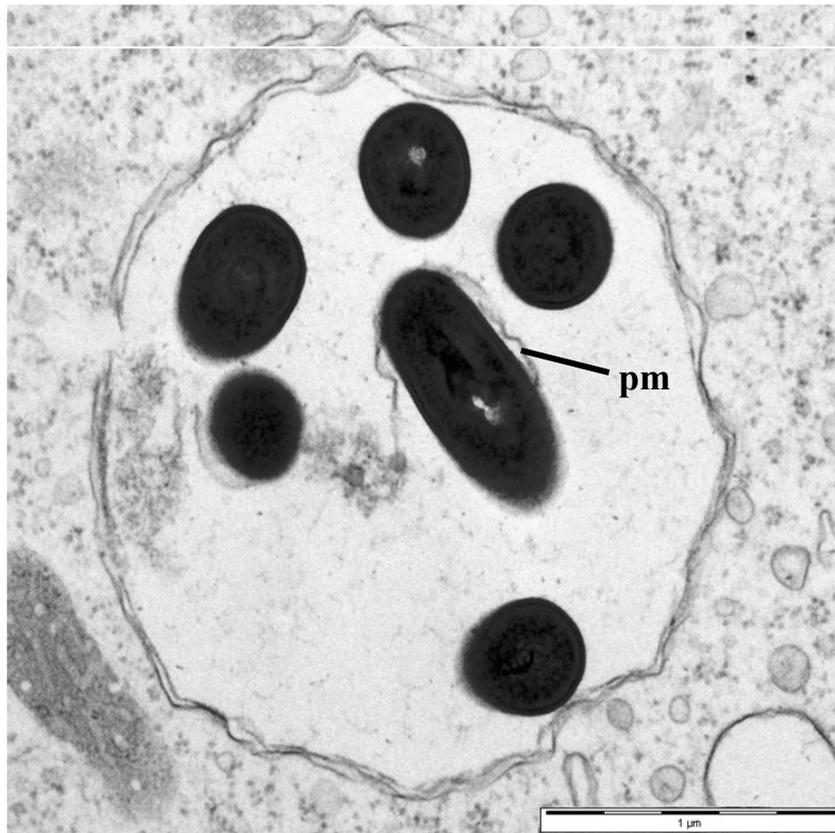


Figure 5.3 Demonstration of autophagy in *A. castellanii* infected with *L. monocytogenes*. An autophagosome containing multiple bacteria some of which were partially surrounded by a single membrane presumed to be remnant of a primary phagosome membrane (pm). Scale bar = 1 μm.

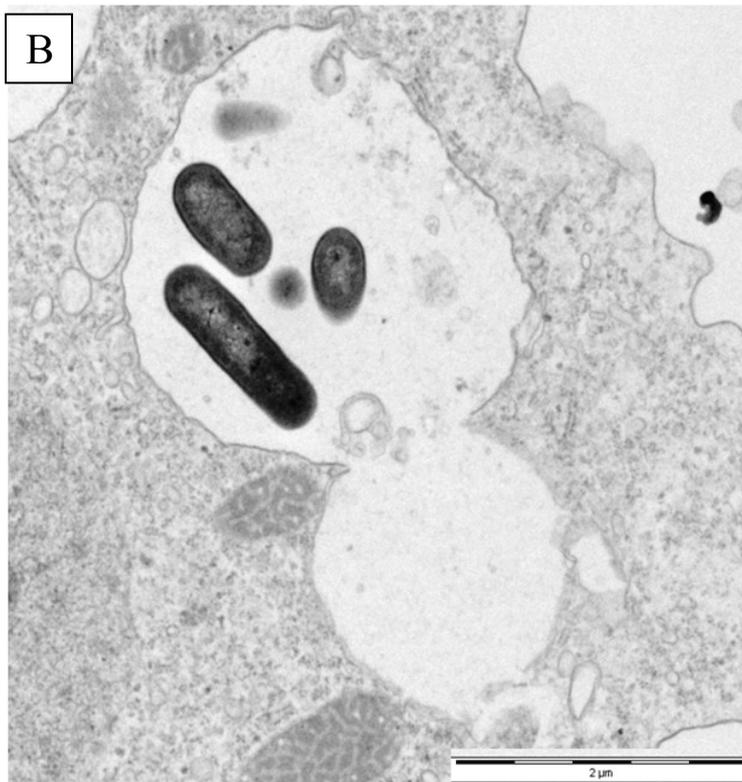
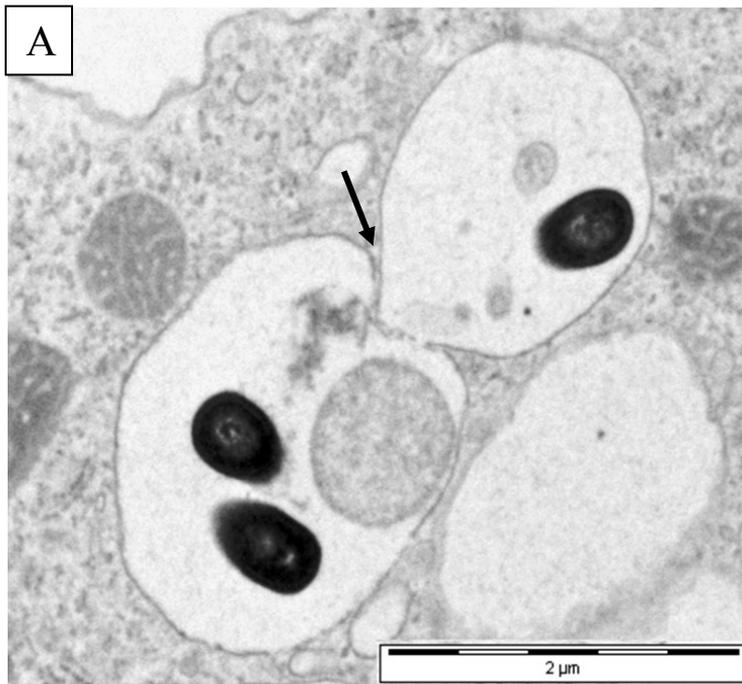


Figure 5.4 Electron micrographs of *A. castellanii* infected with *L. monocytogenes* Scott A demonstrating fusion of an autophagosome with a phagosome. (A) An autophagosome in a process of fusion with a phagosome. (B) Complete fusion had occurred resulting in a bigger hybrid autophagosome and mixing of contents of both vacuoles.

### 5.3.2 Types of autophagosomes in *Listeria*-infected *Acanthamoeba*

Four different types of autophagosomes were seen in *A. castellanii* infected with *L. monocytogenes*. Type 1 autophagosomes were characterised by a double membrane that apparently sequestering only *L. monocytogenes* in their lumen (Figure 5.5A). They were found in amoeba cells as early as 1 h after infection and declined with incubation (Table 5.1). These types of autophagic vesicles were less frequently seen than the other types (Table 5.1).

Type 2 autophagosomes contained both bacteria and undegraded portions of cytoplasmic ground substance, mitochondria and/or other vesicles and were also delimited by a double membrane (Figure 5.5B). Their percent numbers increased after 4 h of infection and then declined afterward (Table 5.1).

Type 3 autophagosomes sequestered both bacteria and cellular constituents in their lumen, but were apparently limited by a single membrane (Figure 5.5C). This type of autophagic vesicle was the most abundant of the autophagosomes observed in *A. castellanii* that were infected with *L. monocytogenes*. Their relative numbers also increased by 4 h post-infection, before the numbers decline (Table 5.1).

Type 4 autophagosomes on the other hand, sequestered only cytoplasmic components without bacteria (Figure 5.6 A and B), and these were observed in amoeba cells that were infected with *Listeria* (Figure 5.6A) as well as in cells that appear to be uninfected (Figure 5.6B). They were visible in most of the amoeba cells examined and their percent numbers generally increased after 24 h post-infection.

In addition to the four types of autophagic vesicles that were identified, there were other bacterial vacuoles that although did not have the typical features of autophagosome, but were as spacious as most of the autophagic vacuoles (Figure 5.7).

All types of autophagic vacuoles described in *A. castellanii* that were infected with untreated-*L. monocytogenes* above were also seen in amoeba cells that were infected with manganese-treated *L. monocytogenes*. The relative percentages of autophagic vacuoles in the treated samples also decreased after 24 h post-infection (Table 5.1).

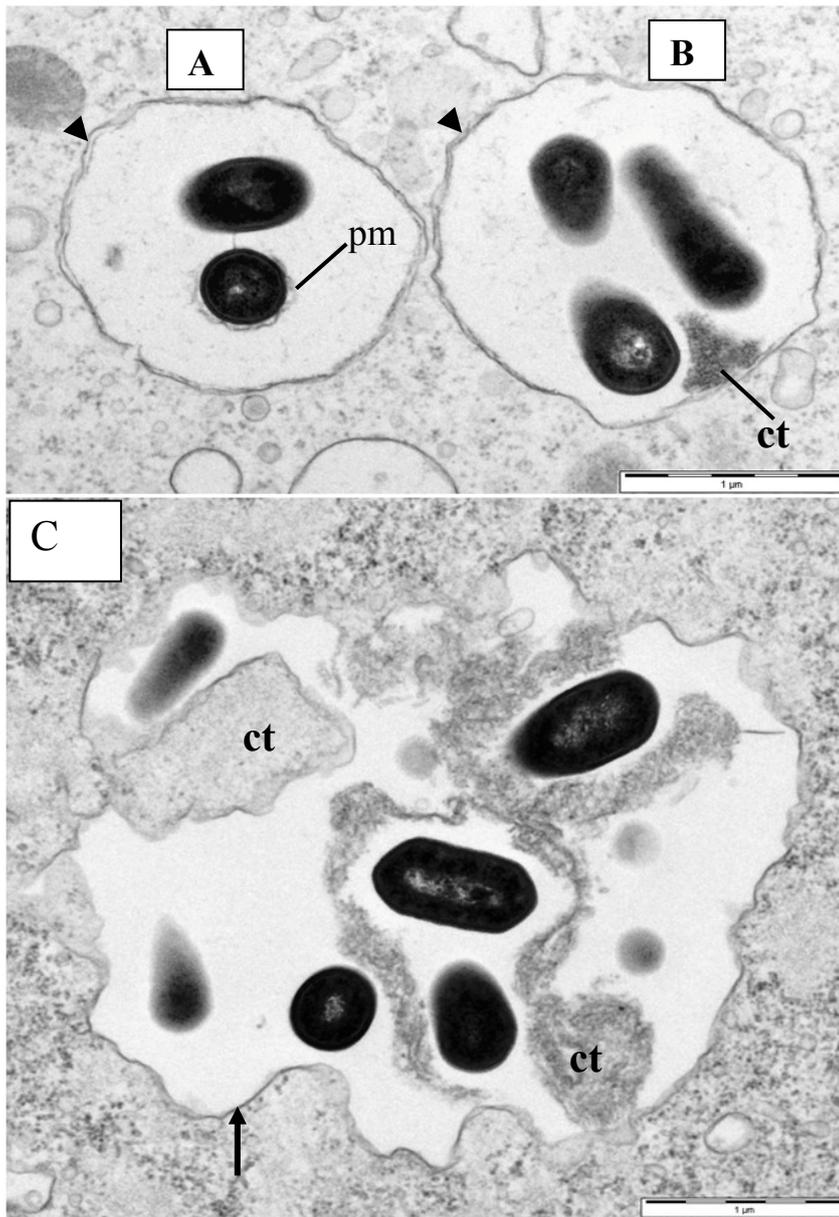


Figure 5.5 Types one to three autophagosomes found in *A. castellanii* infected with *L. monocytogenes* Scott A. (A) Type 1 is characterised by a double membrane (arrow head) containing only bacteria. Note a residue of a phagosome-like membrane (pm) partially surrounding a bacterium. (B) Type 2 is characterised by a double-membrane (arrow head) and sequestered both bacteria and cytoplasmic material (ct). (C) Type 3 is characterised by a single membrane (arrow) and contained both bacteria and cytoplasmic material (ct). Images were taken at 4 h and 1 h respectively post-infection. Scale bars = 1  $\mu$ m for both.

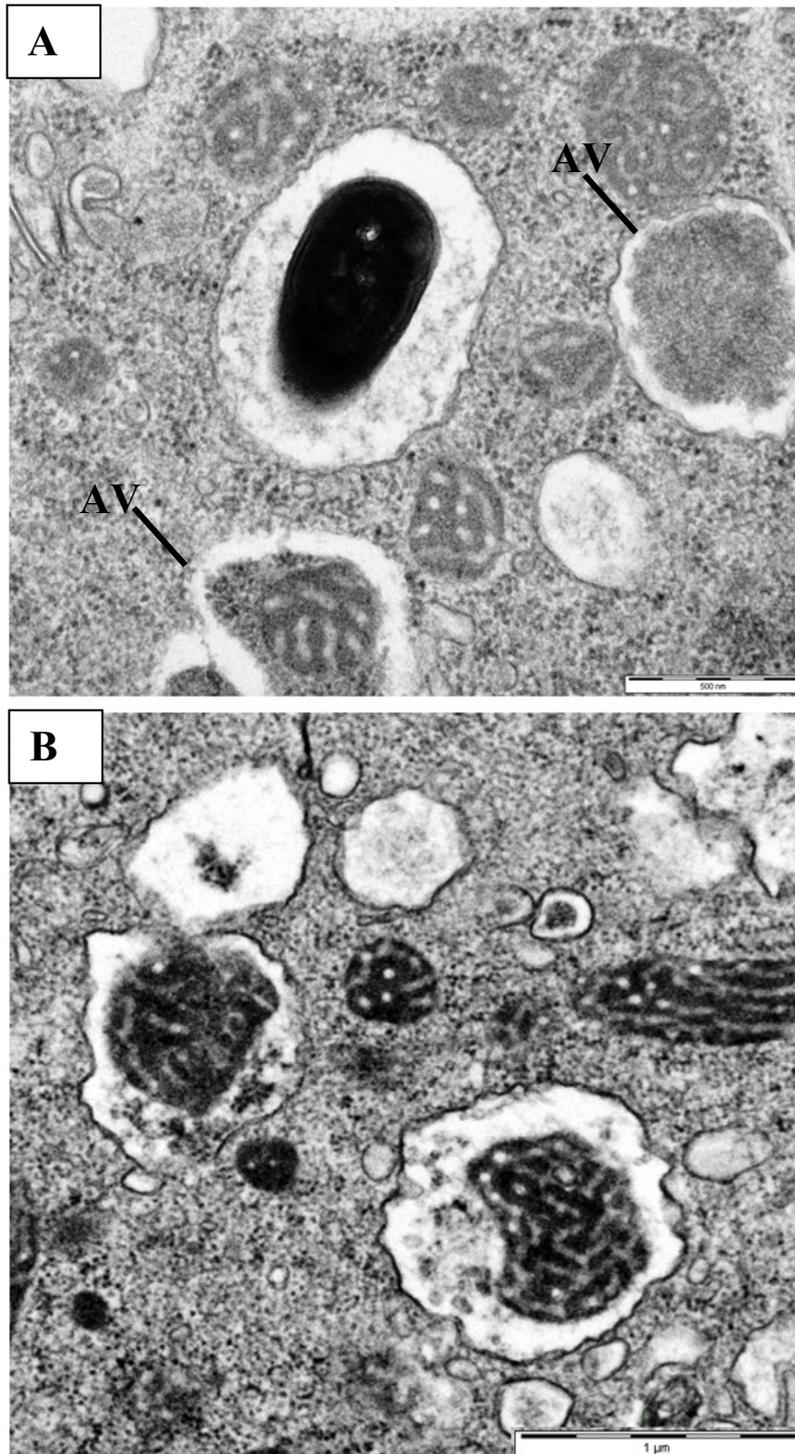


Figure 5.6 Type 4 autophagosomes (AV) sequesters only cytoplasmic material such as mitochondria (M) or cytoplasmic ground substance (ct). They occurred in infected cells (A) and in apparently uninfected cells (B). Images were taken at 24 h post-infection. Scale bars = 0.5 $\mu$ m for (A) and 1 $\mu$ m for (B).

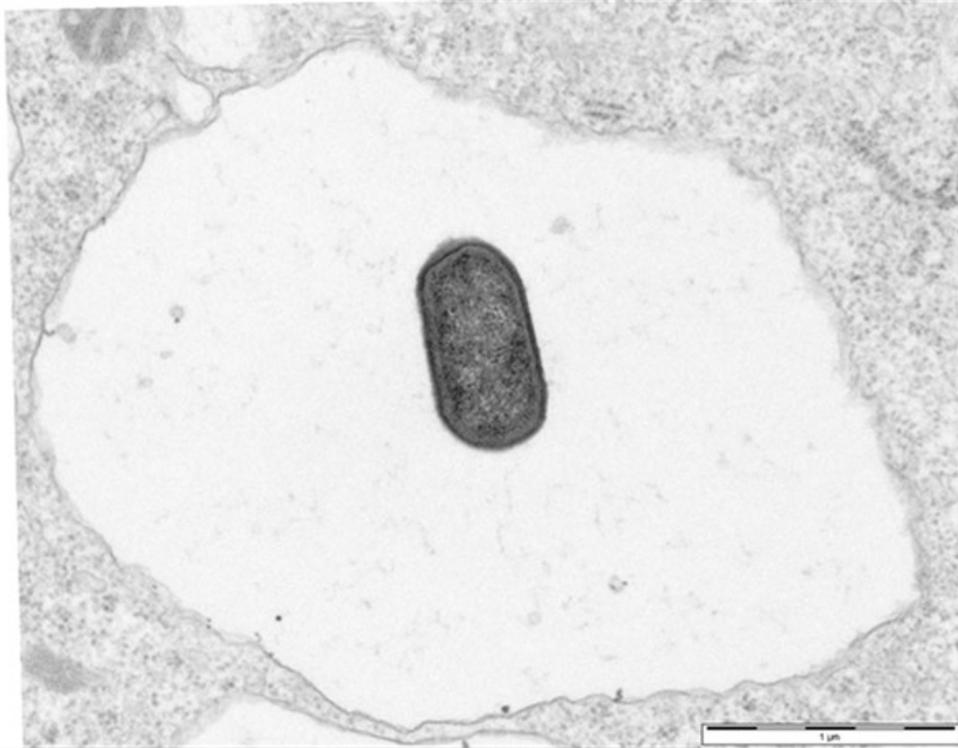


Figure 5.7 A spacious *L. monocytogenes* Scott A vacuole apparently with a single membrane and lacking cytoplasmic inclusions of a typical autophagosome. Scale bar = 1  $\mu\text{m}$ .

Table 5.1 The percent of the different types of autophagic vesicles found in *A. castellanii* infected with *L. monocytogenes* Scott A at different times post-infection

<i>Acanthamoeba</i>	Distribution of the different types of autophagosomes*				
	Type 1	Type 2	Type 3	Type 4	Total
Infected with untreated <i>L. monocytogenes</i>					
1 h	6 (6.3%)	4 (4.4%)	42 (46.2%)	39 (42.9%)	91
4 h	5 (10.2%)	6 (12.3%)	30 (61.2%)	8 (16.3%)	49
24 h	5 (3.1%)	6 (3.7%)	25 (15.3%)	127 (77.9%)	163
Infected with manganese-treated <i>L. monocytogenes</i>					
1 h	4(4.8%)	10 (11.9 %)	32(38.1%)	38 (45.2%)	84
4 h	4 (4.3%)	9 (9.6%)	27 (28.7%)	54 (57.5%)	94
24 h	2 (5.6%)	1 (2.8%)	16 (44.4%)	17 (47.2%)	36

\* Type 1 is surrounded by a double membrane and sequesters only bacteria  
 Type 2 is surrounded by a double membrane and sequester both bacteria and cytoplasmic components  
 Type 3 appeared to have a single membrane and sequester both bacteria and cytoplasmic components  
 Type 4 is bounded by either single or double membrane but only sequester cytoplasmic components.

The autophagic vacuoles in which *L. monocytogenes* were found had similar morphological characteristics with the early autophagosomes, late autophagosomes and autolysosomes previously described by some authors (Dunn, 1994, Dorn *et al.*, 2001, Eskelinen, 2005)(Figure 5.8-Figure 5.10). The early autophagosomes were apparently non-degradative and characterised by a double-membrane or in many cases, a single membrane but usually contained single or multiple intact bacteria together with cytoplasmic components and no evidence of autophagosome-lysosome fusion (Figure 5.8A and B). Some bacteria in these vacuoles exhibited septa (Figure 5.8A) suggesting bacterial replication occurs within autophagosomes.

The late autophagosome-like vacuoles also have a double membrane. The vacuoles were often surrounded by lysosomes which sometimes fused with the vacuole (Figure 5.9A). Also, some bacteria in these type of vacuoles were seen at various stages of degradation (Figure 5.9B).

The autolysosome-like vacuoles on the other hand were single-membrane degradative vacuoles which contained electron dense matter suspected to be fragments of bacteria that were degraded beyond recognition (Figure 5.10).

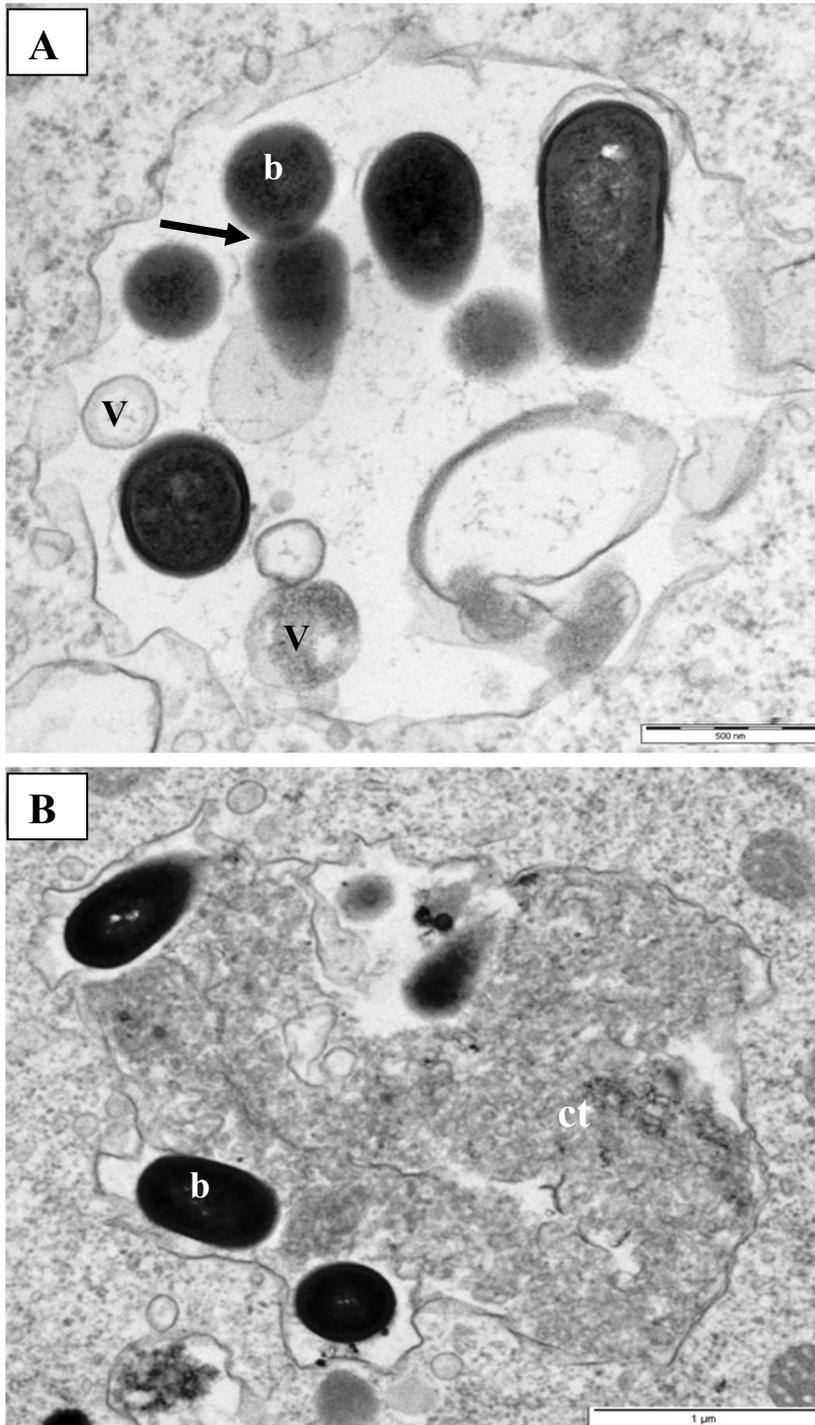


Figure 5.8 The non-degradative early autophagosome-like vacuoles sequestered multiple intact bacteria (b) and sometimes along with cytoplasm components (ct) including vesicles (V). Bacterial replication occurs in vacuoles evident by septum formation (arrow). Images were taken at: (A) 4 h post-infection; bar = 0.5 μm and (B) 24 h post-infection; bar = 1 μm.

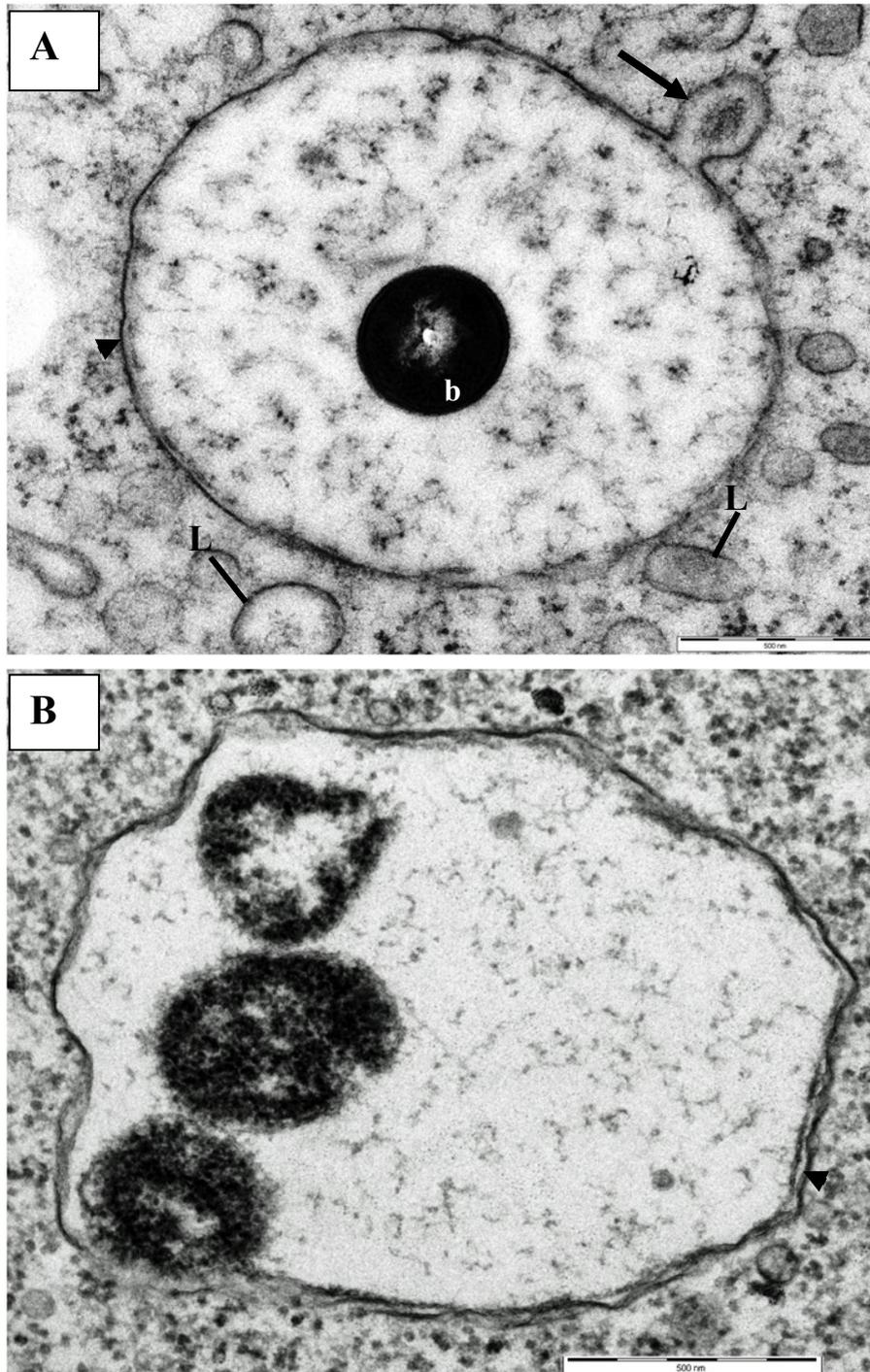


Figure 5.9 Late autophagosome-like vacuoles limited by a double-membrane (arrow head) at 4 h post-infection. (A) Onset of degradation of an apparently intact bacterium (b) in an autophagosome surrounded with numerous lysosomes (L) which also fused with the vacuole (arrow). (B) Bacteria at various stages of degradation in a late autophagosome-like vacuole. Images were captured at 4 h post-infection. Scale bars = 0.5 $\mu$ m each.

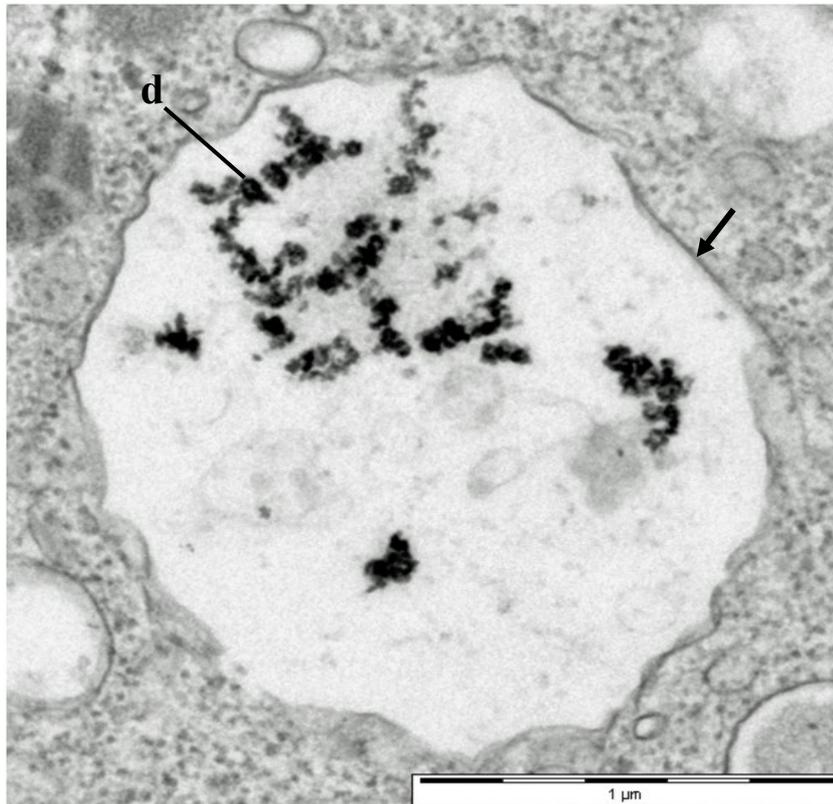


Figure 5.10 An autolysosome-like vacuole characterised by a single membrane (arrow) and containing electron-dense debris (d) presumed to be fragments of degraded bacteria at 4 h post-infection.

### 5.3.3 Role of autophagy in listerial infection

To investigate the role of autophagy in the death or survival of intracellular *L. monocytogenes*, *A. castellanii* was pre-incubated for 20-30 min with 10 mM 3-methyladenine (3-MA), an inhibitor of autophagy in mammalian cells (Stroikin *et al.*, 2004, Wu *et al.*, 2010) and then infected with *L. monocytogenes*. Intracellular *L. monocytogenes* was then assessed both by gentamicin protection assay and by electron microscopy, as described in the Materials and Methods. It was expected that if autophagosome is the intracellular niche of *L. monocytogenes* then inhibition of autophagy should result in decrease in number of bacteria able to survive relative to the

untreated control. The results of gentamicin protection assay presented in Figure 5.11 show that the number of CFU of intracellular *L. monocytogenes* in amoebae that were treated with 3-MA and in the untreated samples declined significantly after 4 h post-infection ( $P < 0.05$ ). However, the magnitude of bacterial reduction in the treated sample was significantly less than the reduction in untreated sample ( $P < 0.05$ ). The results suggest that activation of autophagy increases intracellular listerial killings by *A. castellanii*. Table 5.2 are the TEM results after the treatment of *A. castellanii* with 3-MA. The results show that proportion of bacterial autophagosomes following treatment with 3-MA reduced from 55.2 % to 30.0% after 4 h post-infection while those of the untreated samples increased from 42.3% to 55.7% during the same period (Table 5.2). The distribution of the autophagy vacuoles shows that type 1 autophagosomes were totally inhibited by 3-MA while type 2 and type 3 autophagosomes only dropped by 3 (42.9 %) and 8 (32.0%) autophagosomes respectively after 4 h post-infection (Figure 5.12).

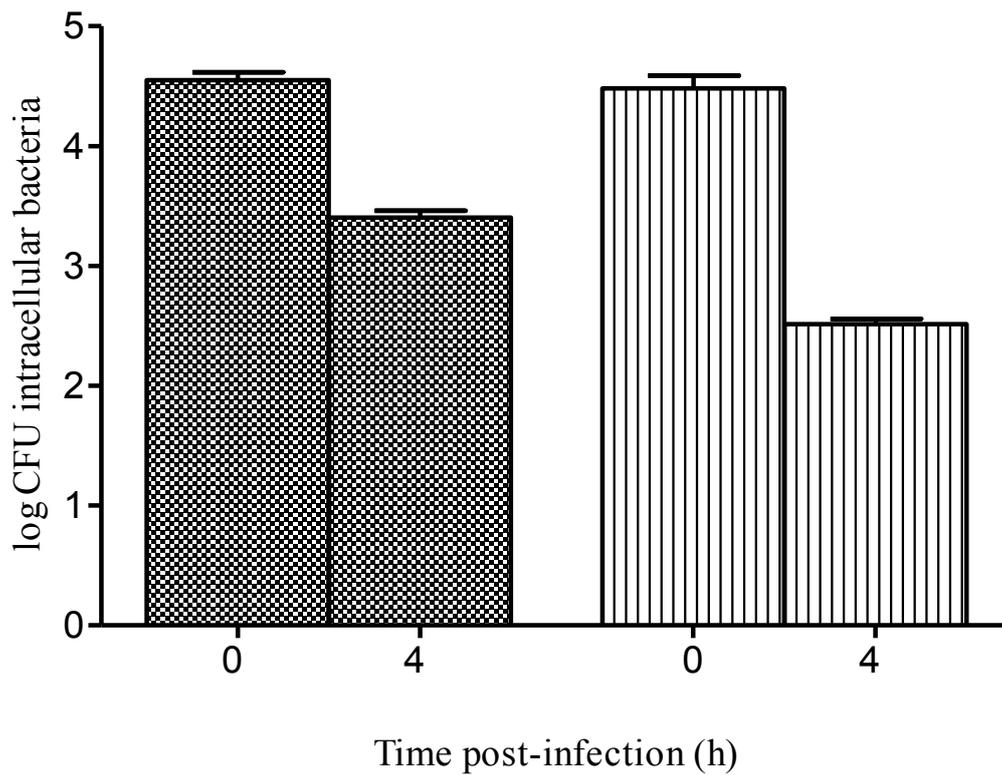


Figure 5.11 Inhibitory effect of 3-methyladenine on intracellular survival of *L. monocytogenes* Scott A in *A. castellanii*. Amoebae were treated with 10 mM of 3-methyladenine (▨) or were untreated controls (▧). Error bars represent standard error around the mean counts of three replicate experiments.

Table 5.2 Numbers of bacterial autophagosomes in *A. castellanii* pre-treated with 3-methyladenine and infected with *L. monocytogenes* Scott A

Time post-infection (h)	<i>Acanthamoeba castellanii</i>					
	3-MA-treated			Untreated		
	Total vacuoles with bacteria	Bacterial autophagosomes	% bacterial autophagosomes	Total vacuoles with bacteria	Bacterial autophagosomes	% bacterial autophagosomes
1	58	32	55.2 %	52	22	42.3 %
4	70	21	30.0 %	97	54	55.7 %

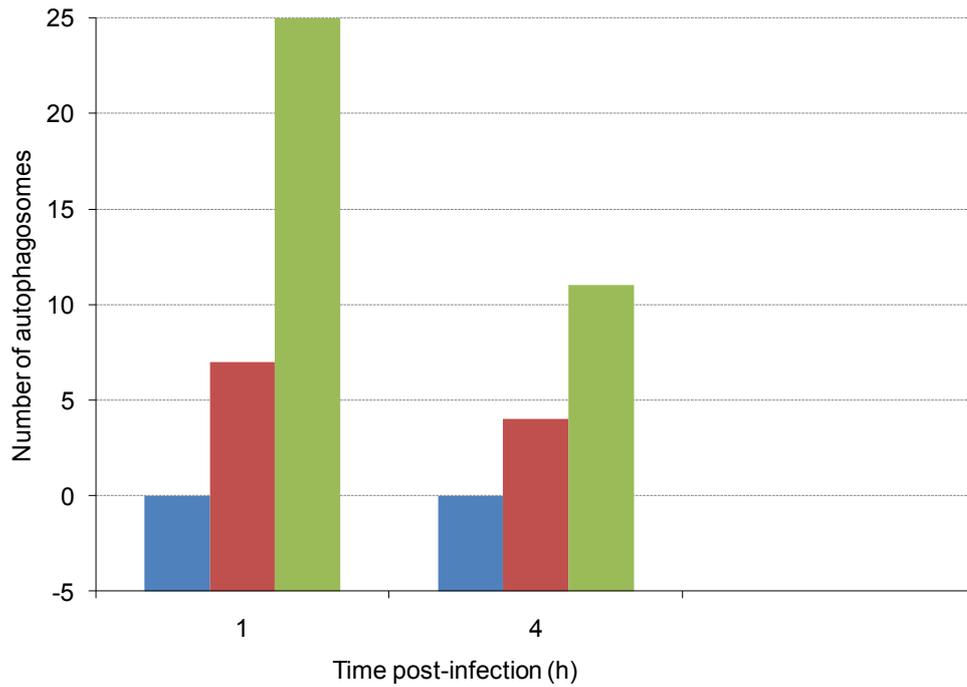


Figure 5.12 Distribution of the types of listerial autophagosomes in amoebae that were treated with 10 mM 3-methyladenine. Type 1 autophagosomes has a double membrane and sequester only bacteria (■), type 2 autophagosome has double membrane and sequester both cytoplasmic materials and bacteria (■) and type 3 autophagosomes apparently has a single membrane and sequester both bacteria and cytoplasmic components (■).

## Chapter 6 Effects of intra-amoeba survival on *Listeria*

### 6.1 Introduction

Some studies suggest that intracellular growth in amoebae may result in profound phenotypic and behavioural modifications of the intracellular bacteria (Barker and Brown, 1995, Walochnik *et al.*, 1999, Winiiecka-Krusnell and Linder, 2001). Barker and co-workers found that the intra-amoebal-grown *L. pneumophila* contained a 15-kDa outer membrane protein and a monounsaturated straight-chain fatty acid that were not found in bacteria grown in culture medium (Barker *et al.*, 1993). In addition, the amoeba-grown *L. pneumophila* were shown to be smaller in size and exhibited enhanced motility and greater resistance to chemical biocides and antibiotics than the agar-grown bacteria (Barker *et al.*, 1992, Barker and Brown, 1995, Barker *et al.*, 1995).

Cirillo and co-workers found that the amoeba-grown *L. pneumophila* were 100-fold more invasive for epithelial cells and 10-fold more invasive for macrophages and *A. castellanii* than *L. pneumophila* cells grown on agar (Cirillo *et al.*, 1994). In another study, Cirillo and colleagues found that growth in amoeba enhanced entry of *L. pneumophila* into monocytes and increased its virulence for mice (Cirillo *et al.*, 1999). Neumeister and others also showed that other species of *Legionella* replicated more efficiently in human monocytes after co-culture with *A. castellanii* (Neumeister *et al.*, 2000, Neumeister, 2004). In another study, Susa and colleagues found that intra-amoebal growth induced *de novo* synthesis of certain *L. pneumophila* antigens that were not detected in extracellularly-grown *L. pneumophila* (Susa *et al.*, 1996).

Cirillo and colleagues also reported that growth of *M. avium* in *A. castellanii* enhanced the ability of bacteria to invade and replicate in fresh *A. castellanii*, epithelial cells and macrophages and that the amoeba-grown bacteria were more virulent in the mouse than bacteria grown in broth culture (Cirillo *et al.*, 1997). In addition, *M. avium* ingested by *A. castellanii* trophozoites were found to be more resistant to treatment with chlorine and to antibiotics used as prophylaxis for *M. avium* infection in AIDS patients than the free *M. avium* (Miltner and Bermudez, 2000, Whan *et al.*, 2006).

Kings and co-workers showed that coliforms and bacterial pathogens (*Salmonella typhimurium*, *Yersinia enterocolitica*, *Shigella sonnei*, *L. gomani* and *Campylobacter jejuni*) taken up by *A. castellanii* or *T. pyriformis* trophozoites survived and grew after exposure to free-chlorine that killed free-living bacteria (King *et al.*, 1988).

Some of the intracellular pathogens that survive in amoeba trophozoites have also been shown to survive in amoeba cysts. Examples include, *L. pneumophila* (Kilvington and Price, 1990, Winiecka-Krusnell and Linder, 1999), *P. aeruginosa* (Marciano-Cabral and Cabral, 2003), *V. cholerae* (Li *et al.*, 2006), *V. mimicus* (Abd *et al.*, 2010), *Simkania negevensis* (Kahane *et al.*, 2001), *Acinetobacter baumannii* (Cateau *et al.*, 2011), *M. avium* (Adekambi *et al.*, 2006, Mura *et al.*, 2006, Ben Salah and Drancourt, 2010), *M. bovis* (Taylor *et al.*, 2003), *M. smegmatis* (Sharbati-Tehrani *et al.*, 2005), *M. xenopi* (Drancourt *et al.*, 2007), and other environmental mycobacteria (Adekambi *et al.*, 2006, Ben Salah and Drancourt, 2010).

Studies suggest that some bacteria sequestered in amoeba cysts are protected from concentrations of disinfectants that are lethal to the free bacteria (Kilvington and Price, 1990, Walochnik *et al.*, 1999, Adekambi *et al.*, 2006). For instance, Kilvington and Price found that the *L. pneumophila* trapped inside *A. polyphaga* cysts were protected from treatment with 50 mg/l free chlorine, a concentration that rapidly kills free *L.*

*pneumophila* (Kilvington and Price, 1990). Similarly, Adekambi and co-workers also found that intracellular mycobacteria in the cysts of *A. polyphaga* were able to withstand a 24 h treatment with 15 mg/l free chlorine, a concentration that was lethal to free bacteria. Furthermore, they found that bacteria were able to grow in culture medium after their release from amoeba (Adekambi *et al.*, 2006).

A naturally occurring *Listeria monocytogenes* variant (rough –colony forms or FR variants) that characteristically form atypical filament of cells have been isolated from food environments and clinical samples (Rowan *et al.*, 2000a, Rowan *et al.*, 2000b, Monk *et al.*, 2004). The rough forms of *L. monocytogenes* have been shown to have enhanced biofilm-forming abilities in a continuous flow bioreactor (Monk *et al.*, 2004).

## **6.2 Hypothesis**

The present study was designed to test the hypothesis that *L. monocytogenes* is able to survive the encystment of *A. castellanii* and thus would be protected from adverse conditions. Furthermore, that survival of *L. monocytogenes* in amoeba can influence its morphological and physiological characteristics. To test this hypothesis the following objectives were undertaken:

- a. To demonstrate survival of *L. monocytogenes* in *A. castellanii* cysts
- b. To determine the ability of *L. monocytogenes* trapped in *A. castellanii* cysts to survive chlorine treatment
- c. To demonstrate ability of amoebae-grown *L. monocytogenes* to form biofilms using a microtitre plate assay
- d. To determine susceptibility of the amoeba-grown *L. monocytogenes* to disinfectants and antibiotics using a microtitre plate assay.

## 6.3 Results

### 6.3.1 Survival of *L. monocytogenes* in *Acanthamoeba* cysts

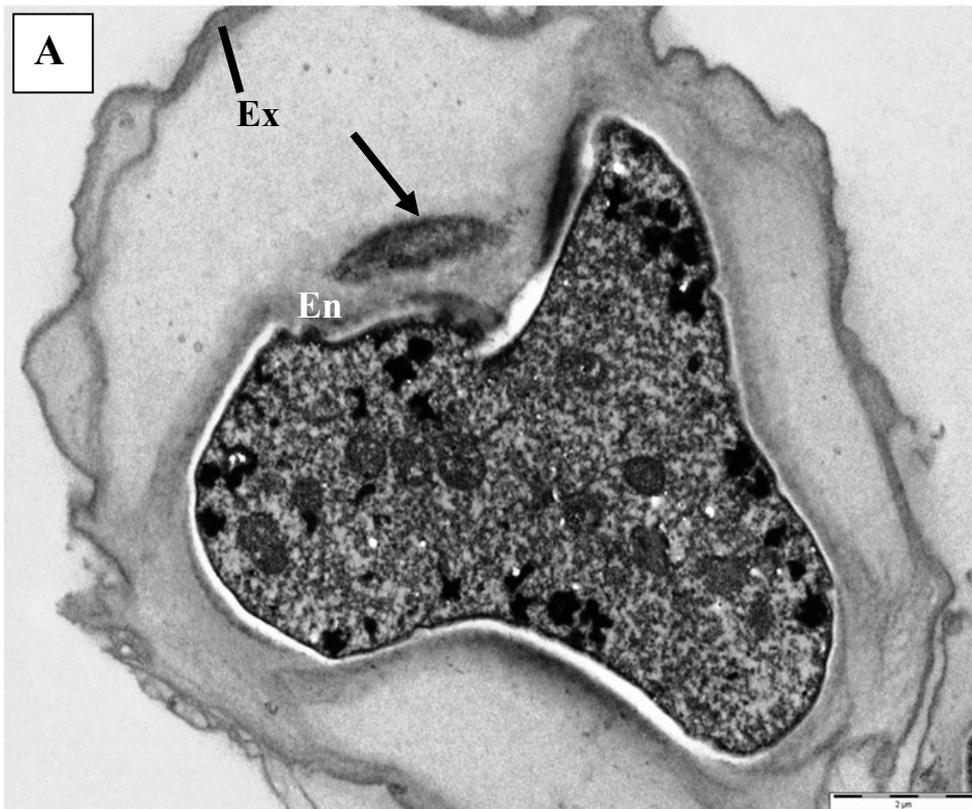
The *A. castellanii* trophozoites for this experiment were first tested for the absence of bacteria by lysing cells with sarcosine and spreading the lysate on TSA as described in section 2.8.3. The test was aimed at excluding symbiotic *L. monocytogenes* that may survive encystment to give false positive results. There was no growth on TSA after incubation of plates at 37°C for 24 h suggesting absence of *Listeria* or any bacteria culturable on TSA. To determine whether *L. monocytogenes* can survive in amoebae cysts after ingestion, both organisms were co-incubated at various times for infection to take place and thereafter amoebae were induced to form cysts as described in section 2.10. Infected trophozoites started to round up a few hours after induction and by 72 h almost all trophozoites had produced matured cysts, evident by a double wall (Figure 6.1). The 24 h acid treatment killed all extracellular *Listeria*, as was evident by the absence of viable bacteria from the final wash of the cysts when plated on TSA and incubated overnight at 37°C. In the preliminary experiments it was observed that 3 % (v/v) HCl killed *L. monocytogenes* within 5 min of exposure.

Hatching of *Acanthamoeba* cysts in most instances, occurred within 24 h-48 h incubation in SK#6 medium but was sometimes extended to 96 h. During excystment, viable bacteria trapped in the cysts were expected to be released into the growth medium. These then grew and turned the medium cloudy within 24 h of release.

Results of *L. monocytogenes* survival in *Acanthamoeba* cysts are presented in Table 6.1. Altogether, 35 time-point experiments were performed to determine whether *L. monocytogenes* can survive in amoeba cysts. Intracellular *L. monocytogenes* were recovered from 15 (43.9 %) of the time-points after hatching. Of these, 6 (54.6 %) were recovered from amoeba samples that were infected for 4 h prior to encystment and 6 (50.0 %) were from the amoeba sample infected for 1 h before encystment. On the other

hand, only 3 (25.0 %) positive results were obtained from amoeba samples that were infected for 24 h before encystment. This suggests that prolonged *L. monocytogenes*-*Acanthamoeba* co-culture reduces *Listeria* survival in amoeba cysts.

Although the cysts infected with *L. monocytogenes* hatched and released bacteria into the growth medium, nevertheless, it was difficult to locate viable bacteria in the cytoplasm or wall of the cysts using TEM. A few bacteria cells were however seen in the space between extocyst and endocyst (Figure 6.1A) and in between endocyst and the cytoplasm of matured cysts (Figure 6.1B and C). No bacteria were observed in cyst of uninfected *A. castellanii*.



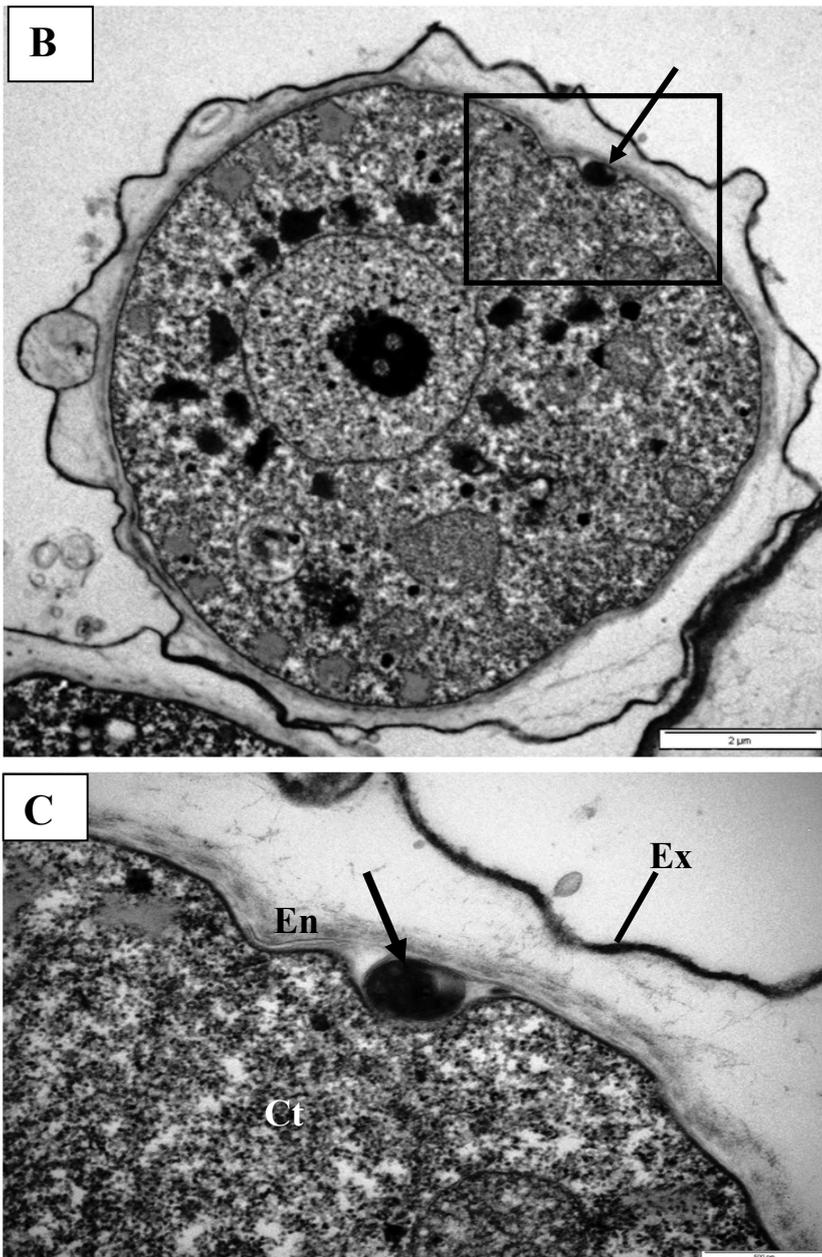


Figure 6.1 TEM of *A. ccstellanii* cysts infected with *L. monocytogenes* Scott A for 1 h prior to encystment. (A) A bacterium (arrow) in the space between exocyst (Ex) and endocyst (En) ; bar = 2μm. (B) A bacterium (box) occupying the space between endocyst (En) and cytoplasm (Ct) of a cyst; bar = 2μm. (C) An enlargement of the bacterium (arrow) from the box in 'B'; bar 0.5μm.

Table 6.1 Survival of *L. monocytogenes* Scott A in *A. castellanii* cysts treated with 3% (v/v) HCl

Analysis	Duration of exposure of <i>Listeria</i> to amoeba before encystment (h)			Total
	1	4	24	
Number of tests	12	11	12	35
Number positive for surviving bacteria	6	6	3	15
Percentage positives	50.0 %	54.6 %	25.0 %	43.9 %

### 6.3.2 *Listeria* recovered from cysts have filamentous morphology

The bacteria released from amoeba after hatching were apparently much longer and wider (Figure 6.2A) than the typical short rods of the controls grown in broth (Figure 6.2B), when cultures were viewed with a phase contrast microscope.

The morphological distinctions between the amoeba-grown *Listeria* and the control bacteria grown in laboratory medium were clearly visible when bacteria were negatively stained and viewed with TEM (Figure 6.3A-C). The bacteria recovered from amoeba cysts often formed single long nonseptated or paired filament of cells measuring up to 14  $\mu\text{m}$  long by 1  $\mu\text{m}$  wide (Figure 6.3A). Sometimes, they appeared as a long chain of bacterial cells measuring 43  $\mu\text{m}$  or more in length and consist of filamentous cells joined intermittently by the short rods (Figure 6.3B). By contrast, the bacteria grown *in vitro* were mainly coccobacillus in shape and measured between 1  $\mu\text{m}$ -2  $\mu\text{m}$  in length by 0.8  $\mu\text{m}$  wide (Figure 6.3C). Although the bacteria recovered from amoeba were morphologically distinct from *L. monocytogenes* that were grown *in vitro*, nevertheless, the amoeba-grown bacteria exhibited other characteristics suggestive of *L. monocytogenes*. For instance, the amoeba-grown bacteria were motile at 32°C although motility was not as co-ordinated as those grown in broth. In addition, they produced transparent, grey colonies on TSA and were Gram positive rods (Figure 6.4A -B). Moreover, they were confirmed as *L. monocytogenes* with API *Listeria* test kits (Figure 6.5).

*L. monocytogenes* can produce lactic and acetic acid from metabolism of glucose (Romick *et al.*, 1996) and acid stress has been shown to induce *L. monocytogenes* to form filament (Isom *et al.*, 1995, Romick *et al.*, 1996). To rule out that a pH change resulting from acid produced from metabolism of glucose (5 g/l) in the medium during the first few hours of bacterial release was responsible for the observations, *L.*

*monocytogenes* grown overnight on TSA was co-inoculated with *A. castellanii* trophozoites ( $\approx 10^3$  /ml) in a fresh SK6 (hatching medium) medium and incubated at 32°C for 3 h. The pH of medium was measured at 0 h and 3 h of incubation and the morphology of bacteria in the medium was observed using a phase contrast microscope and compared with the morphology of those grown in SK6 medium without glucose. The pH of hatching medium at the start of incubation (pH 6.7) did not change noticeably after incubation (pH 6.6). Similarly, the *L. monocytogenes* that was co-incubated with *A. castellanii* in SK6 medium containing glucose (pH 6.6) (Figure 6.6A) did not form filament of cells that were seen in the amoeba-grown bacteria, rather they exhibited the short-rod morphology of the control bacteria (Figure 6.2B) and the bacteria grown in glucose-free medium (pH 6.9) (Figure 6.6B). *Listeria* filaments also were observed in vacuoles of amoeba trophozoites infected with *L. monocytogenes* (Figure 6.7A). These were longer compared with the typical bacterial phenotype often seen in vacuoles (Figure 6.7B). This suggests that the filamentation of *L. monocytogenes* can occur during residence in *A. castellanii*.

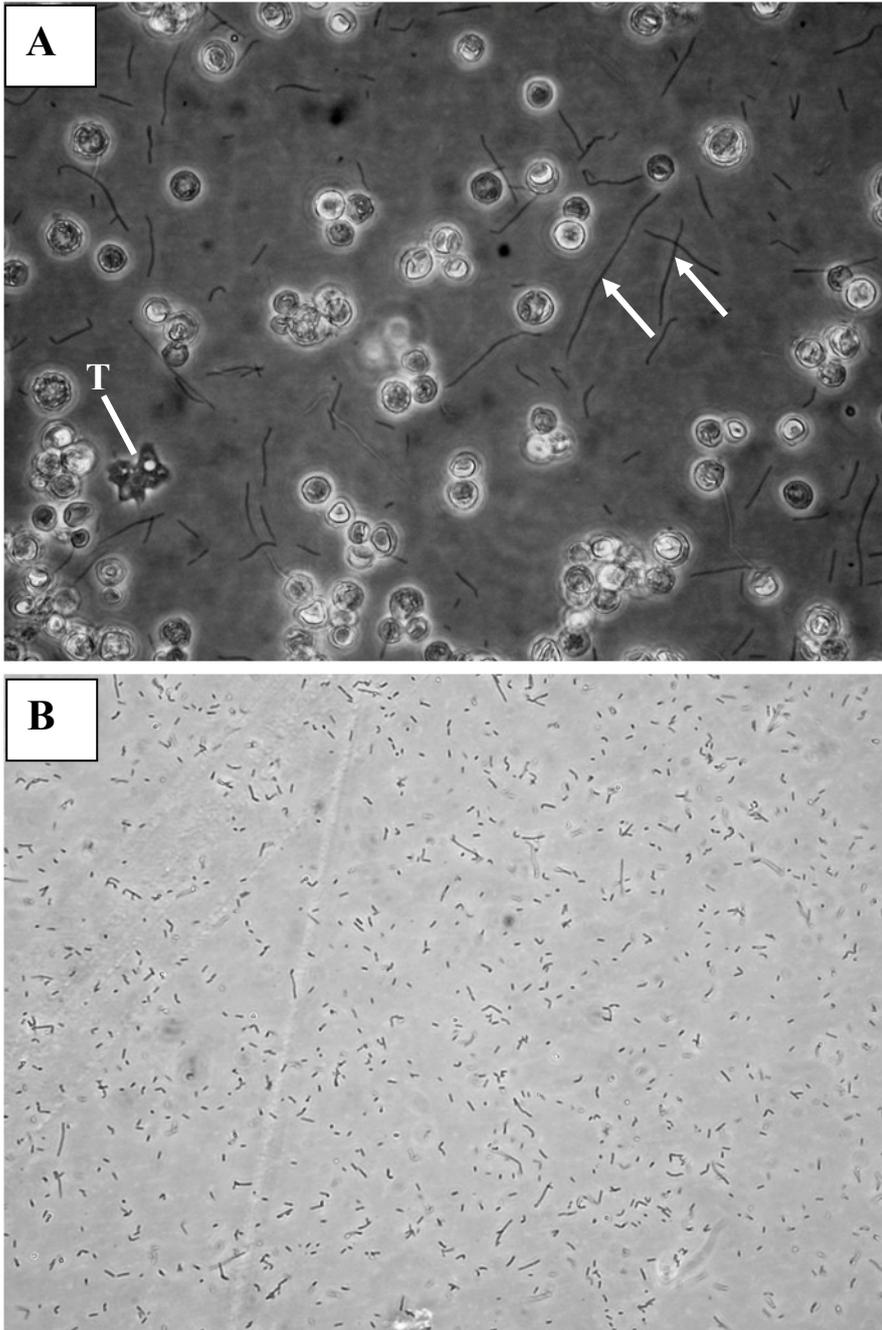


Figure 6.2 Phase contrast microscopy images of (A) Filamentous bacteria (arrows) released from *A. castellanii* cysts after about 1-2 h of hatching; 'T' is a trophozoite. (B) Short rod phenotype of *L. monocytogenes* Scott A grown *in vitro* in tryptone soya broth. Magnification  $\times 400$  for both.

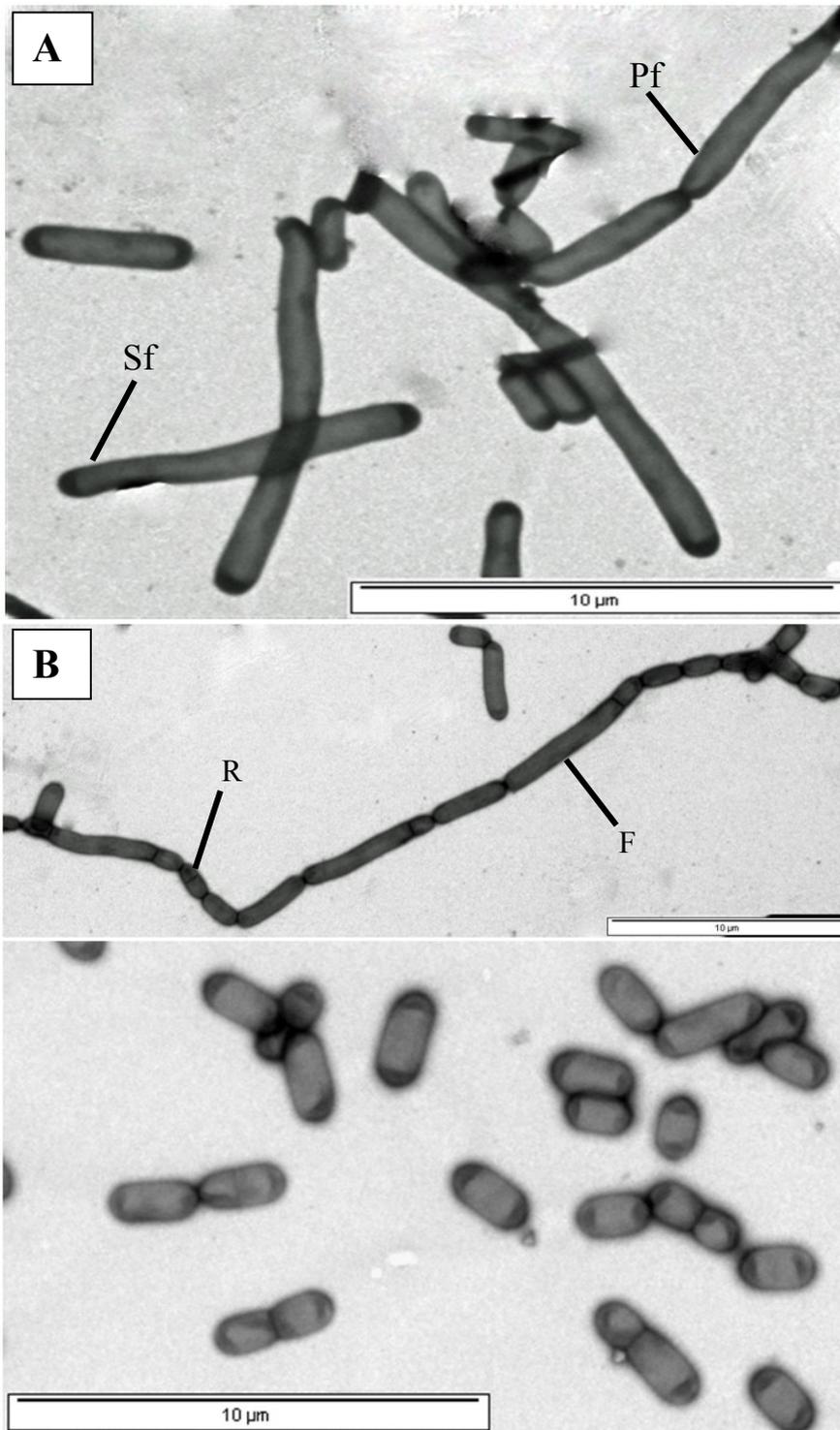


Figure 6.3 Electron micrographs of negatively-stained bacteria.  
(A and B) Filamentous bacteria recovered from amoebae cysts that were infected with *L. monocytogenes* Scott A. (A) Single filament (Sf) and paired filament (Pf) of cells. (B) A long chain consisting of filamentous (F) and short rod (R) type.  
(C) *L. monocytogenes* Scott A grown *in vitro* in TSB.

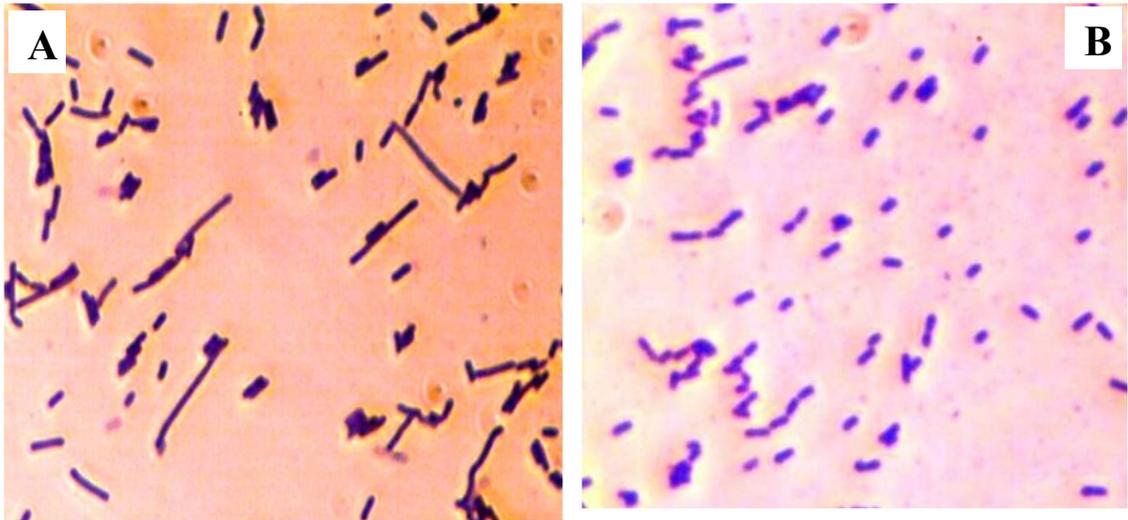


Figure 6.4 Gram positive rods of the amoeba-grown *L. monocytogenes* Scott A(A) and the *in vitro* grown *L. monocytogenes* Scott A (B). Magnification  $\times 1000$ .

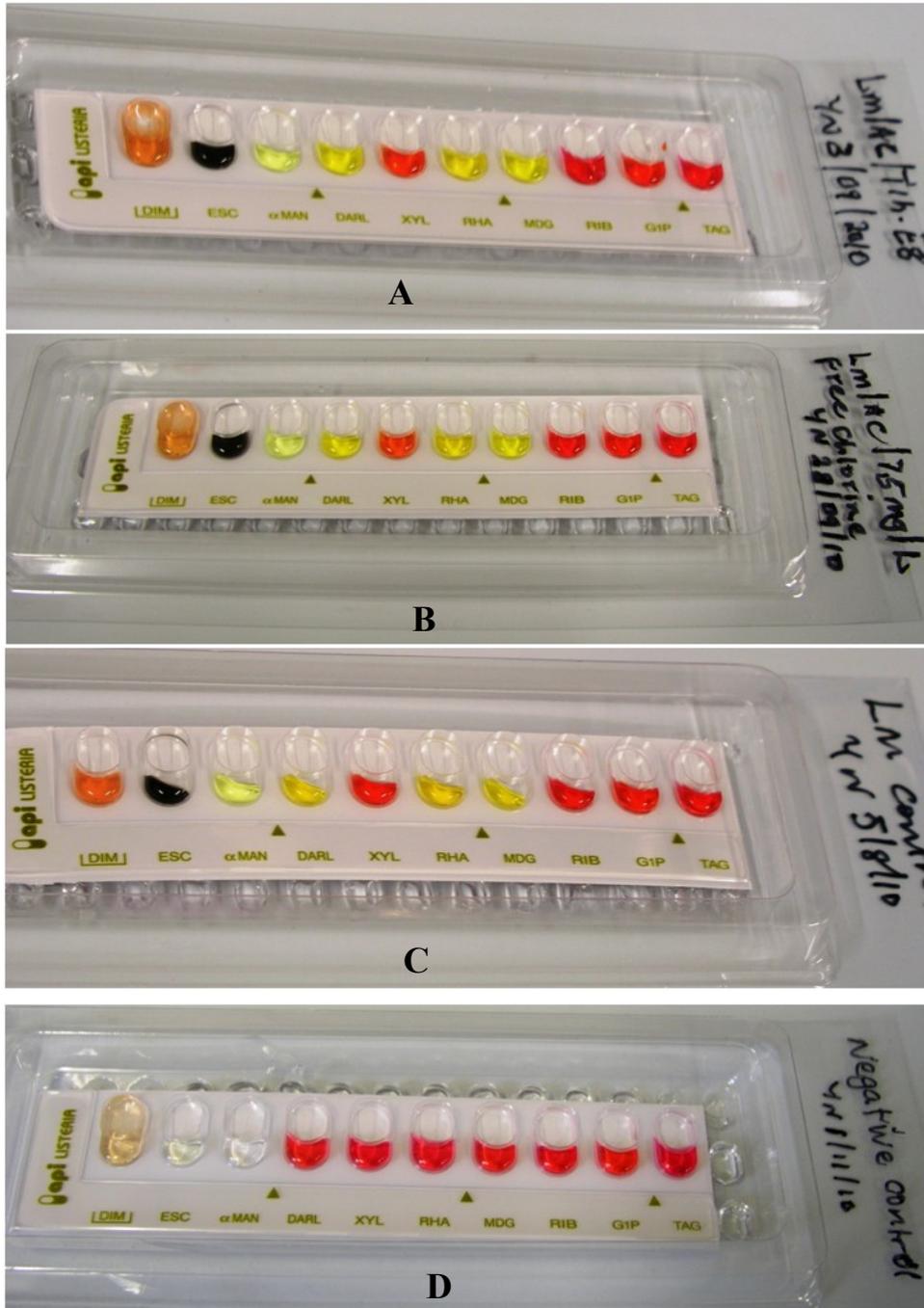


Figure 6.5 API *Listeria* test results for bacteria recovered from infected cysts (A and C). (A) Inoculated with *L. monocytogenes* Scott A recovered from cysts treated with 3 % (v/v) acid alone and (B) inoculated with *L. monocytogenes* Scott A recovered from cysts treated with acid followed by treatment with 75 mg/l free chlorine. (C) Positive control inoculated with *L. monocytogenes* Scott A bacteria grown in vitro. (D) Negative control inoculated with deionised water only.

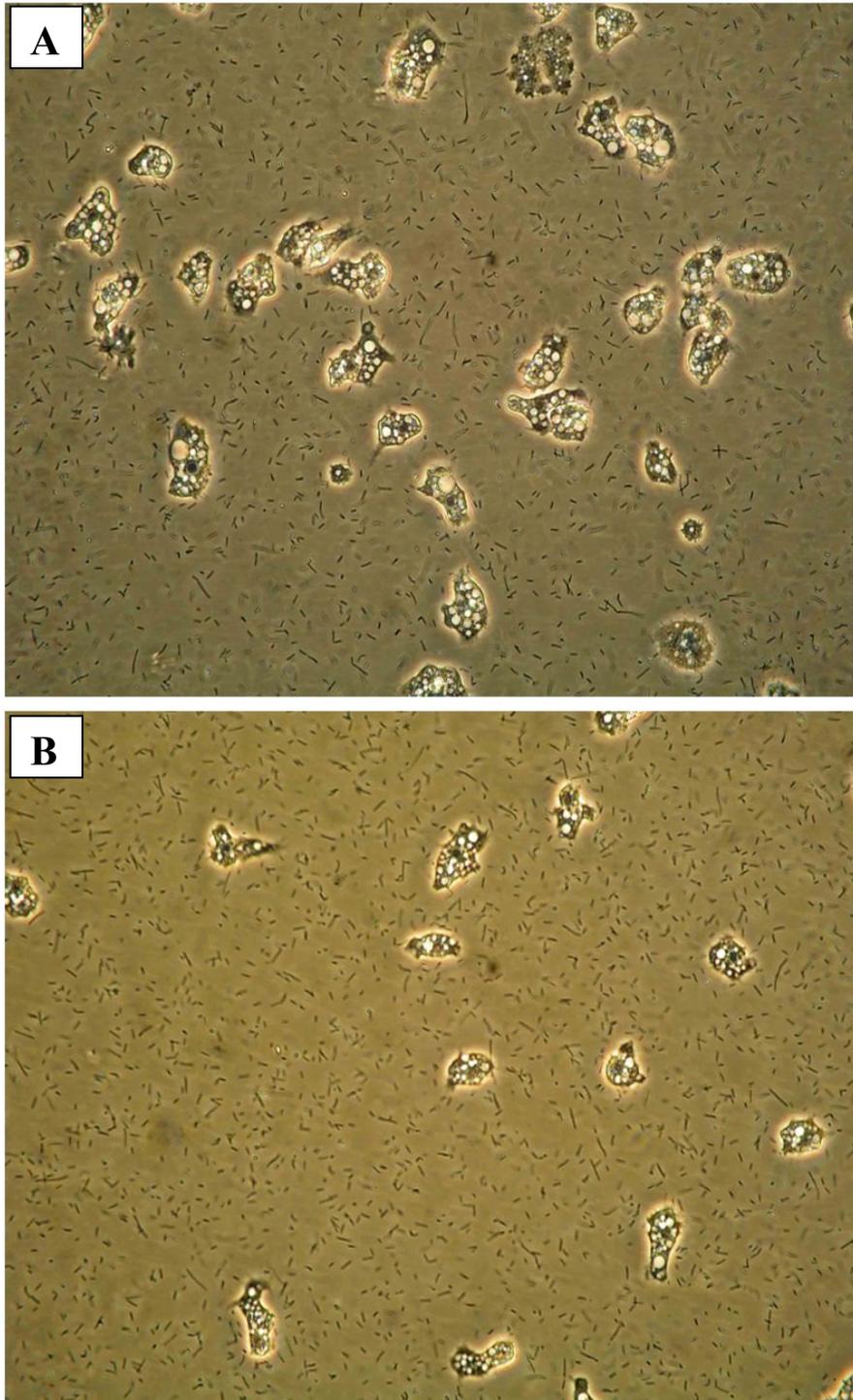


Figure 6.6 Effect of hatching medium on morphology of *L. monocytogenes* Scott A. *L. monocytogenes* Scott A co-incubated with *A. castellanii* for 3 h in (A) SK6 (hatching medium) containing 5 mg/l glucose (pH 6.6) and (B) SK6 medium without glucose (pH 6.9). *L. monocytogenes* Scott A exhibited the short rod morphology in both media. Magnification  $\times 400$  for both.

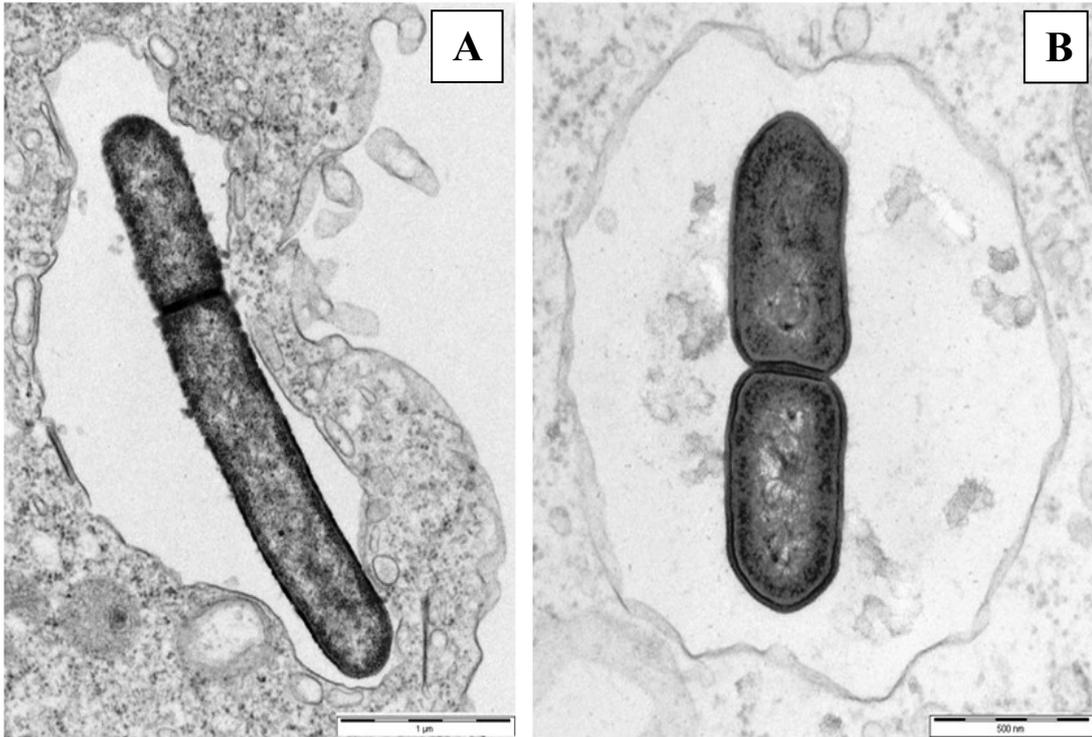


Figure 6.7 TEM images of *A. castellanii* trophozoites infected with *L. monocytogenes*

Scott A showing (A) a rare filamentous bacterium in vacuole. This contrast with the (B) short rod phenotype often seen in vacuoles. Bars; A =1μm, B = 0.5 μm.

When the amoeba-grown *Listeria* was sub-cultured in TSB, the filamentous cells reverted to the typical phenotype of *in vitro* grown bacteria (Figure 6.8). This shows that the morphological changes acquired by *L. monocytogenes* during residence in *A. castellanii* was transient.

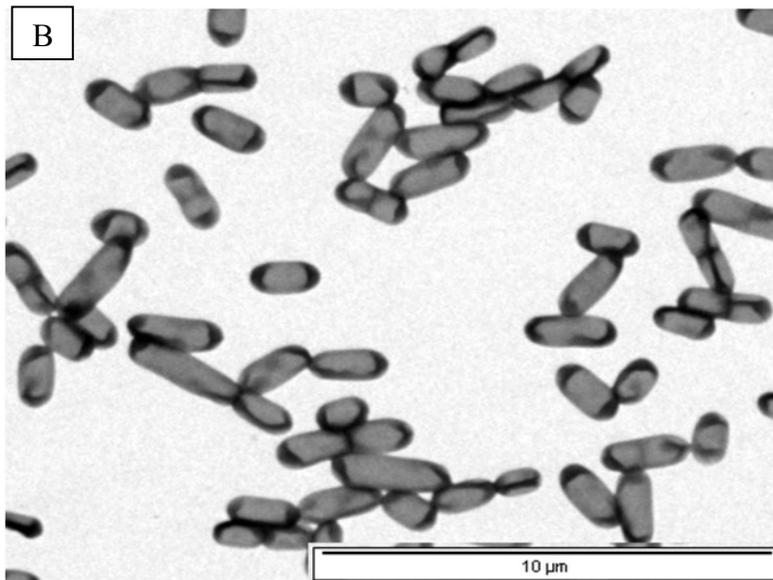


Figure 6.8 Electron micrograph of a negatively-stained *L. monocytogenes*.

A sub-culture of the bacteria isolated from cysts of *A. castellanii* infected with *L. monocytogenes* Scott A. The long filaments of cells reverted to the short rod morphology following sub-culture in TSB.

### **6.3.3 *Listeria* trapped in cysts are resistant to chlorine treatment**

To test if *L. monocytogenes* trapped in *A. castellanii* can survive treatment with chlorine, infected cysts were first treated for 24 h with acid (section 2.10) followed by treatment with various concentrations of free chlorine for another 24 h (section 2.10.1). Results of the treatment with chlorine are presented on Table 6.2. The results show that all cysts samples treated with 100 mg/l free chlorine or less hatched and at least one out of the four samples treated with each concentration of chlorine released intracellular *L. monocytogenes* into the medium after hatching. For example, all infected cyst samples treated with 50 mg/l free chlorine hatched but only 2 (50%) samples released *L. monocytogenes* into medium after hatching. On the other hand, treatment of samples with 75 mg/l or 100 mg/l free chlorine resulted in about 98-99 % kill of infected cysts yet the few cysts that survived this treatment were able to hatch though sometimes after prolonged incubation (7-14 days). Only one sample from each of these two concentrations produced *L. monocytogenes* after hatching. Bacteria released from cysts following treatment with acid and chlorine had similar morphological and biochemical characteristics as those released from cysts treated with acid alone (see section 6.3.2).

Table 6.2 Survival of *Listeria monocytogenes* Scott A in *Acanthamoeba castellanii* cysts treated with acid and then chlorine

<i>Acanthamoeba</i>		Concentration of free chlorine (mg/l).						
		0	10	20	40	50	75	100
Infected with <i>L. monocytogenes</i>	*Number of samples	4	4	4	4	4	4	4
	† <i>Listeria</i> recovered	2	2	2	1	2	1	1
Uninfected	*Number of samples	4	4	4	4	4	4	4
	† <i>Listeria</i> recovered	0	0	0	0	0	0	0

\* Samples that hatched after treatment with chlorine; † samples that were positive for *L. monocytogenes* after hatching

#### 6.3.4 Survival of *Listeria* in amoeba-cysts enhances biofilm formation

To test if intracellular survival of *L. monocytogenes* within *A. castellanii* cysts can enhance its ability to form biofilm, bacteria released into growth medium after hatching were separated from amoeba trophozoites, empty shells of the hatched cysts and unhatched cysts by centrifugation as described in section 2.10.3. The amoeba-grown (AG) *L. monocytogenes* are designated AG-1 and AG-4 to signify that they were recovered from amoebae infected with *L. monocytogenes* for 1 h and 4 h, respectively, prior to encystment.

Results of biofilm formed by *L. monocytogenes* are presented in Table 6.3. The data indicate that *L. monocytogenes* that survived encystment by *A. castellanii* (AG-1 or AG-4) produced more biofilms than those produced by the *in vitro* grown (IVG) *L. monocytogenes* bacteria ( $P < 0.05$ ). However, the difference between the amount of biofilm formed by bacteria AG-1 ( $OD_{595\text{ nm}}: 0.205 \pm 0.031$ ) and the amount produced by AG-4 bacteria ( $OD_{595\text{ nm}}: 159 \pm 0.015$ ) was not statistically significant ( $P > 0.05$ ) suggesting that duration of infection does not influence the ability of amoeba-grown *Listeria* to form biofilm. Similarly, the quantity of biofilm formed when bacteria AG-1 or bacteria AG-4 was sub-cultured in TSB (AGS-1 and AGS-4 respectively) was not significantly different ( $P > 0.05$ ) from the amount of biofilm formed by the *in vitro* grown bacteria (Table 6.3). This suggests that the amoeba-grown *L. monocytogenes* reverts to the characteristics of the bacteria grown *in vitro* upon sub-culture.

The visual inspection of the stained biofilms in a microtitre plate (Figure 6.9) and the pattern of adherence of bacteria to the bottom of a microtitre plate (Figure 6.10) support the optical density results showing that AG *L. monocytogenes* (Figure 6.9A and Figure 6.10A) produced more biofilms than the *in vitro* grown bacteria (Figure 6.9B and Figure 6.10B) or the sub-cultured bacteria (Figure 6.9C and Figure 6.10C).

Table 6.3 Biofilm formation by *L. monocytogenes* Scott A in a microtitre plate measured by crystal violet staining (n= 4).

Assay results		
<i>L. monocytogenes</i>	Mean OD <sub>595nm</sub>	Standard deviation
<sup>1</sup> AG-1h	0.21	0.03
<sup>2</sup> AGS-1h	0.11	0.01
AG-4h	0.16	0.02
AGS-4h	0.12	0.02
<sup>3</sup> IVG (control)	0.11	0.02
TSB medium only	0.09	0.01
SK6 medium only	0.08	0.01

1: Amoeba-grown *L. monocytogenes*; 2: Amoeba-grown *L. monocytogenes* sub-cultured in TSB; 3: *In vitro* grown *L. monocytogenes*

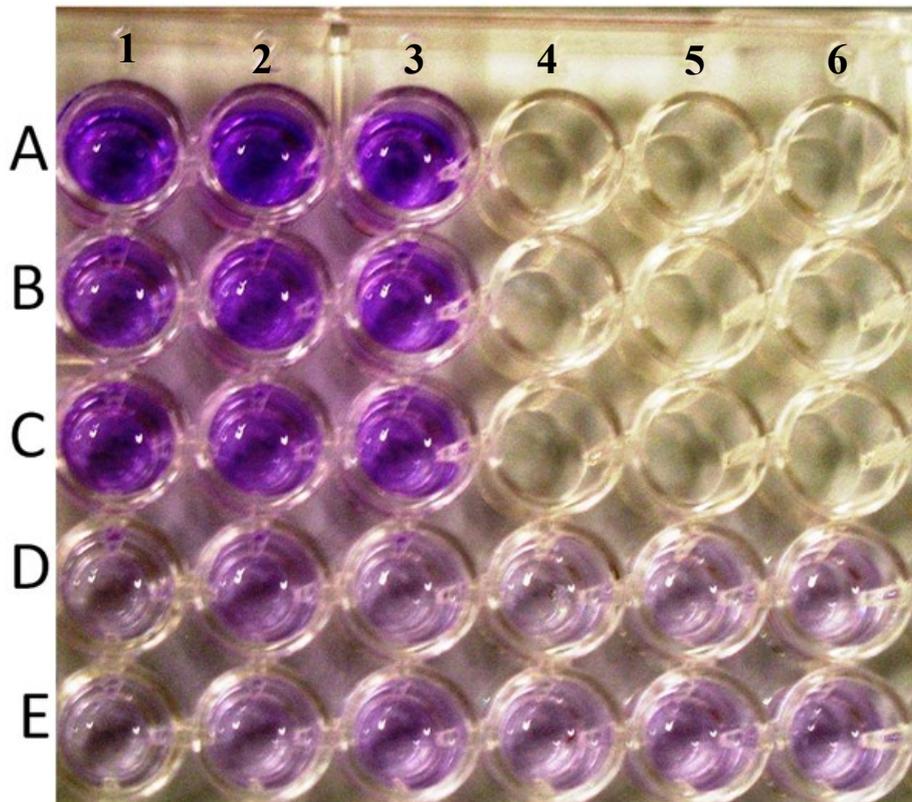


Figure 6.9 Crystal violet-stained biofilms formed by *L. monocytogenes* ScottA in wells of a microtitre. (A1-A3) Amoeba-grown (AG) *L. monocytogenes*, (B1-B3) Sub-cultured (AGS) *L. monocytogenes*, (C1-C3) The in vitro grown (IVG) *L. monocytogenes* bacteria. (D1-D6) Fresh SK6 (hatching medium) alone and, (E1-E6) fresh TSB medium alone. A4-A6, B4-B6, C4-C6 are blank;  $\times 400$ .

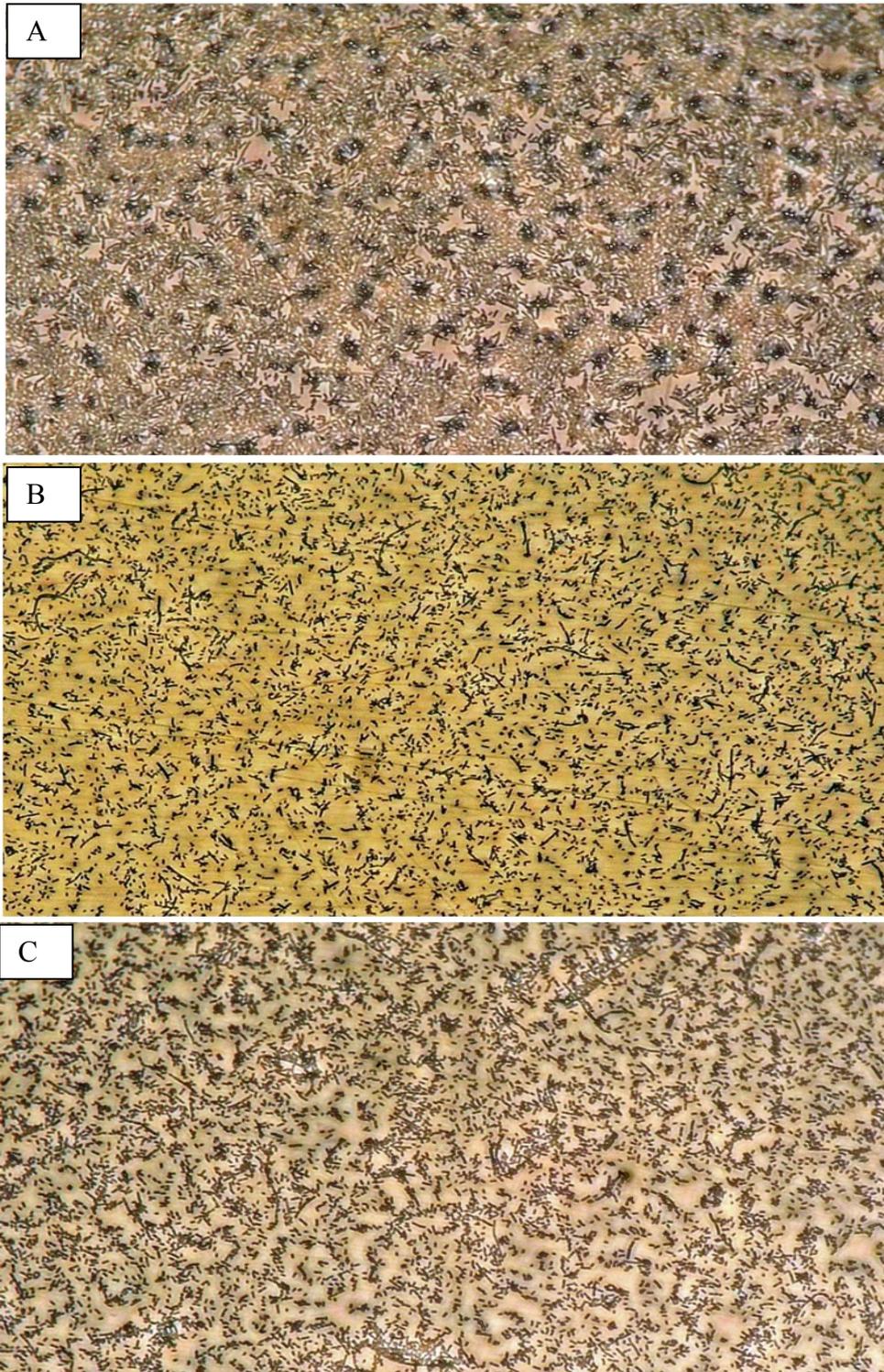


Figure 6.10 Pattern of attachment of bacteria to the bottom of wells of a 96-well microtitre plate after 2 h incubation at 37°C and 3 washings to remove unbound bacteria. (A) Amoeba-grown *L. monocytogenes* Scott A formed thicker biofilm than (B) *In vitro* grown *L. monocytogenes* Scott A or (C) *L. monocytogenes* Scott A sub-cultured in TSB after growth in amoeba. Magnification  $\times 400$  in each.

To rule out the influence of acid produced during metabolism in medium on the enhanced biofilms observed in AG bacteria, *L. monocytogenes* grown on TSA was inoculated in SK6 medium without glucose and grown at 37°C overnight. The pH of the growth medium was determined at the beginning and at the end of incubation and thereafter the amount of biofilms formed in this medium was determined, as described in section 2.10.4. This was compared with the amount of biofilm formed in SK6 medium with glucose and the amount formed in TSB. The results are shown in Figure 6.11. *Listeria monocytogenes* grown in SK6 medium containing glucose formed an equal amount of biofilm as that formed by the bacteria grown in SK6 medium without glucose, despite that the medium with glucose being more acidic (pH 4.4) than the non-glucose medium (pH 6.4) at the end of incubation. In a marked contrast, the *L. monocytogenes* that were cultivated in TSB formed twice as much biofilms as those formed by the bacteria grown in SK6 medium, with or without glucose. However, TSB medium was less acidic (pH 5.3) than SK6+glucose medium (pH 4.4) but more acidic than the SK6 medium without glucose (pH 6.4). The observations suggest that the acidity of SK6 medium does not contribute to the formation *L. monocytogenes* biofilms.

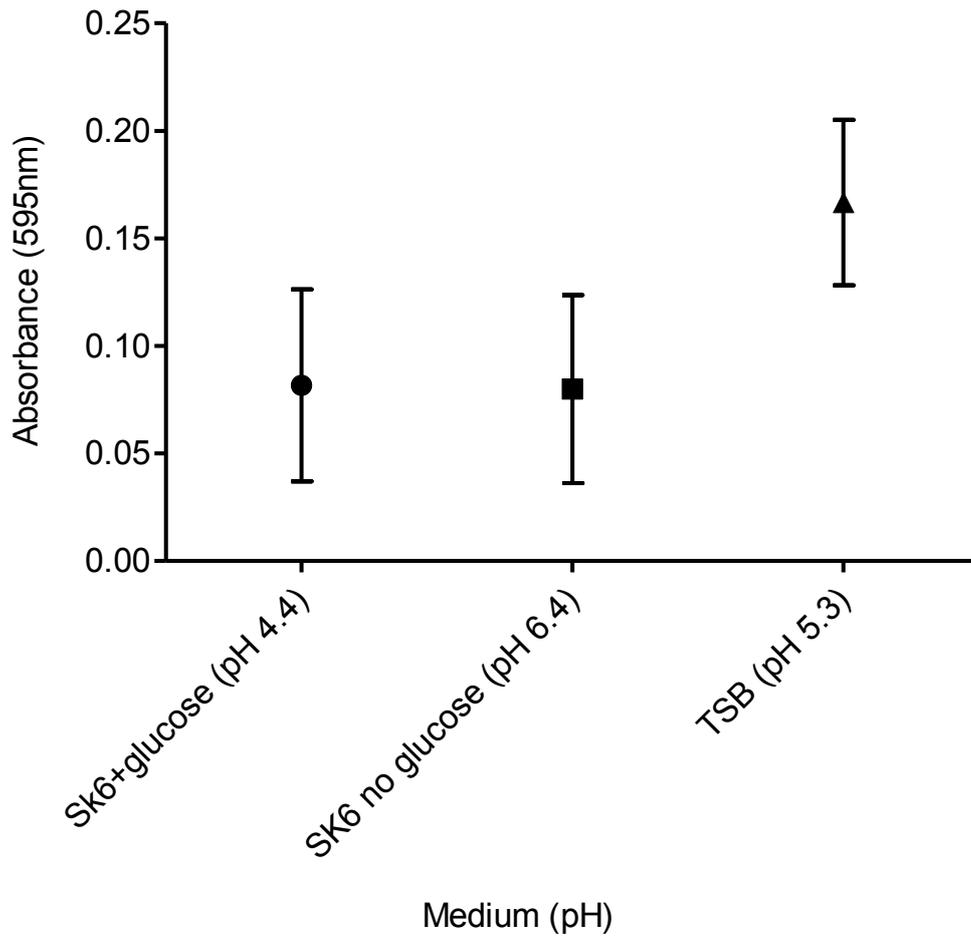


Figure 6.11 Mean plot of absorbance of crystal violet from stained *L. monocytogenes* Scott A biofilms formed in medium at different pH. Error bars represent standard deviation of triplicate experiments.

### **6.3.5 Susceptibility of *L. monocytogenes* to disinfectant**

In order to test if *L. monocytogenes* that survived encystment and released after hatching were less susceptible to disinfectants, bacteria were incubated in quaternary ammonium compounds (QACs) and a Milton complete protection sterilising fluid (MPSF), which has sodium hypochlorite (2 % w/v) as its principal ingredient. Thus, the MIC for MPSF was effectively the MIC for the sodium hypochlorite.

Results of bacterial susceptibility to QACs and MPSF are presented on Table 6.4. MIC values of QACs for amoeba-grown *Listeria* or MICs for the sub-cultured bacteria were almost the same with MIC values for *L. monocytogenes* grown in culture medium suggesting no increased resistance to QACs. On the other hand, the amoeba-grown bacteria were more resistance to MPSF than either the *in vitro* grown bacteria or the bacteria recovered from amoeba and sub-cultured in TSB.

### **6.3.6 Susceptibility of *L. monocytogenes* to antibiotics**

Antimicrobial susceptibilities of *L. monocytogenes* determined by microtitre plate dilution technique are demonstrated in Figure 6.12. The three bacteria tested showed equal susceptibilities to erythromycin and streptomycin. On the other hand, the MIC of ampicillin (MIC: 8 mg/l) for *L. monocytogenes* recovered from amoeba was considerably higher than the MICs for the culture medium-grown bacteria. (MIC: 0.25 mg/l). The most remarkable resistance by the amoeba-grown bacteria occurred with gentamicin and penicillin where the MIC was 32 mg/l for each antibiotic. This contrast the MICs of 1 mg/l and 0.5 mg/l produced by *in vitro*-grown bacteria in gentamicin and penicillin respectively. The resistance of the amoeba-grown bacteria to ampicillin and penicillin was completely lost after sub-culture in TSB.

Table 6.4 Minimum inhibitory concentration of quaternary ammonium compounds and Milton sterilising fluid for *L. monocytogenes* Scott A

		Mean MIC of disinfectants (mg/l) $\pm$ SD					
<i>L.monocytogenes</i>		Benzalkonium	Benzethonium	Chlorhexidine	Cetrimide	Cetylpyridinium	Milton
Recovered from amoeba		0.98 $\pm$ 0	0.98 $\pm$ 0	0.98 $\pm$ 0	0.98 $\pm$ 0	0.98 $\pm$ 0	350 $\pm$ 122
Sub-cultured after recovery from amoeba		0.98 $\pm$ 0	0.98 $\pm$ 0	1.18 $\pm$ 0.4	1.18 $\pm$ 0.4	0.98 $\pm$ 0	250 $\pm$ 0
<i>In vitro</i> -grown		0.98 $\pm$ 0	0.98 $\pm$ 0	1.18 $\pm$ 0.4	1.18 $\pm$ 0.4	0.98 $\pm$ 0	250 $\pm$ 0

N = 5

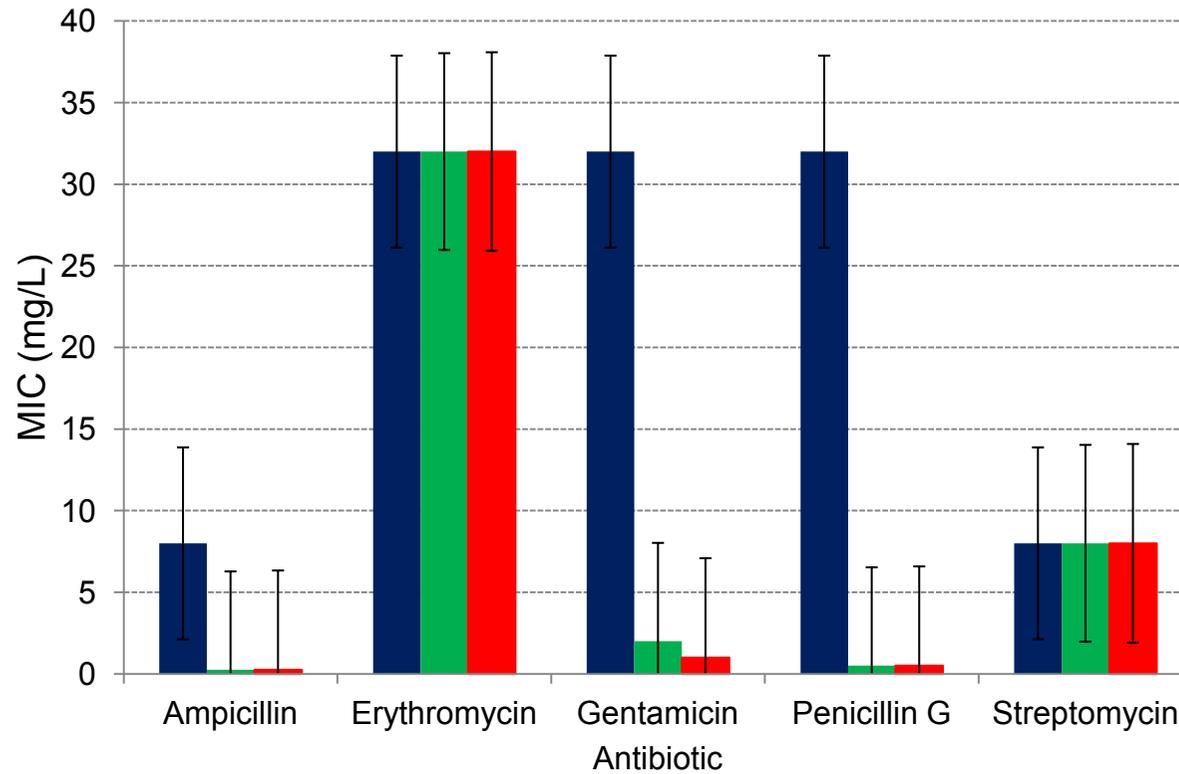


Figure 6.12 Minimum inhibitory concentration of antibiotics for *L. monocytogenes* Scott A. Amoeba-grown *L. monocytogenes*

(■); *L. monocytogenes* grown in amoeba and then sub-cultured into TSB (■); *in vitro*-grown *L. monocytogenes* (■). Error bars represent standard error of mean of three separate experiments.

## **Chapter 7. Discussion and conclusion**

### **7.1 *Listeria-Acanthamoeba* interactions in a co-culture**

Free-living amoebae live in close association with bacteria in the same ecological niche, where they are involved in complex interactions (Harf, 1993). The outcome of such interactions will depend, among other things, on species of amoeba and bacteria, growth state, relative abundance of species, and environmental conditions (for example, temperature)(Harf, 1993, Wang and Ahearn, 1997, Walochnik *et al.*, 1999, Pickup *et al.*, 2007b). The interaction of amoeba with bacteria can ultimately result in the destruction of the bacteria, destruction of amoebae, or alternatively, a symbiotic relationship may develop (Marciano-Cabral, 2004).

The present work was designed to investigate interactions between *L. monocytogenes* and free-living amoebae of the genus *Acanthamoeba* and how that may impact on the environment.

#### **7.1.1 Extracellular listerial growth and survival in the presence of**

##### ***Acanthamoeba***

The results of the findings showed that *L. monocytogenes* can be maintained at a high number in the presence of amoebae, while in the absence of amoebae the number of *L. monocytogenes* decreases dramatically. These results suggest that amoebae can enhance the growth of *L. monocytogenes* probably by excreting molecules into the medium to support growth of extracellular bacteria. The findings confirm the previous results on survival of *L. monocytogenes* in co-culture with *Acanthamoeba* (Zhou *et al.*, 2007, Huws *et al.*, 2008, Akya *et al.*, 2009b, 2010)and *T. pyriformis* (Pushkareva and

Ermolaeva, 2010). Similar findings have also been reported for *Mycobacterium avium*, *Helicobacter pylori*, *Burkholderia cepacia*, *Staphylococcus aureus*, *Vibrio cholerae*, *V. parahaemolyticus* and *Acinetobacter baumannii* (Steinert *et al.*, 1998, Marolda *et al.*, 1999, Winiecka-Krusnell *et al.*, 2002, Huws *et al.*, 2008, Laskowski-Arce and Orth, 2008, Cateau *et al.*, 2011). It is well documented that protozoa are able to excrete mineral nutrients particularly, nitrogen and phosphorus as by-products when they graze on bacteria (Sinclair *et al.*, 1981, Petropoulos and Gilbride, 2005, Tso and Taghon, 2006, Pogue and Gilbride, 2007). In addition, protozoa also excrete compounds such as vitamins, amino acids and nucleotides that can enhance growth and persistence of bacteria in the presence of their predators (Levrat *et al.*, 1992, Ratsak *et al.*, 1996).

Although *L. monocytogenes* survived longer in co-culture with *Acanthamoeba* than when they were suspended alone in RS, there was no evidence that bacteria grew when cultures were incubated without shaking, rather, they slowly declined with incubation. These observations may be partly due to increased rate of bacterial death above the rate of growth as a result of heavy predation by amoeba, and partly due to exhaustion of nutrients in the co-culture medium. The results are in agreement with the results obtained by Akya and colleagues (Akya *et al.*, 2009b). However, the overall numbers of bacteria that declined in the presence of either of the *Acanthamoeba* sp after 5 d incubation ( maximum =1.2 logs CFU) were much smaller than the decline (4 logs CFU) reported by Akya and colleagues during the same time period(Akya *et al.*, 2009b). On the other hand, the results contradicted those obtained by other workers who suggested that *L. monocytogenes* numbers increased in co-culture with *Acanthamoeba* when cultures were incubated in same condition (Zhou *et al.*, 2007, Huws *et al.*, 2008). These contradictions may be related to differences in the strain of *L. monocytogenes* used. Alternatively, the temperature of incubation may account for these variations.

Whereas the analyses in this study were conducted at 32°C, those of Akya and colleague were conducted at 22°C, while Zhou and colleagues and Huws and colleagues conducted their study at 37°C. Although *L. monocytogenes* can grow between the temperatures of 1-45°C, the optimum temperature of growth is between 30 and 37°C (Smith and Marmer, 1990, Rowan and Anderson, 1998). The temperature of incubation not only affects growth of bacteria as a pure culture, it is also a major factor that can determine the fate of some bacteria or protozoa in co-culture (Sherr *et al.*, 1988, Marolda *et al.*, 1999, Ohno *et al.*, 2008). Sherr and colleagues found that digestion of fluorescence-labelled bacteria by phagotrophic flagellates and ciliates increased exponentially at 12-22°C but decreased at higher temperatures (Sherr *et al.*, 1988). Similarly, Ohno and colleagues recently reported that *L. pneumophila* multiplied in greater numbers within *A. castellanii* when cultures were incubated at 25°C, but were rapidly eliminated by the amoeba when cultures were incubated at temperature below 20°C (Ohno *et al.*, 2008). On the other hand, Marolda and co-workers found that *Acanthamoeba* trophozoites rapidly developed into cysts within 24-48 h upon incubation at 37°C while those infected with *B. cepacia* at 37°C rapidly declined because of lysis. They however showed that amoeba infection proceeded normally between 20-30°C (Marolda *et al.*, 1999).

The data presented here also suggest that survival of *L. monocytogenes* co-culture depends on the species of *Acanthamoeba*, with the rate of bacterial decline in the presence *A. castellanii* being significantly more than the rate of decline in presence of *A. culbertsoni* or *A. polyphaga*. This observation may be attributed to the rate of reduction in the number of viable amoebae trophozoites due to encystment. The rate varied with the species of amoeba and was more in *A. castellanii* co-culture than *A. culbertsoni* or *A. polyphaga* co-culture. The results were in complete contrast with those of Akya and

colleagues who suggested that the rate of bacterial reduction in the presence of *A. polyphaga*, *A. castellanii* or *A. lenticulata* were the same regardless the species of *Acanthamoeba*.

The observed enhancement of bacterial survival when co-cultures were incubated with shaking may be attributed to increased input of waste products in the medium due to increased rate of oxidative metabolism by *A. castellanii* when the concentration of dissolved oxygen had increased following shaking. Avery and co-workers reported that phagocytosis of beads by *A. castellanii* increased markedly upon rotary agitation of the cell suspension (Avery *et al.*, 1995). In addition, other reports have shown that protozoan grazing of bacteria increases rate of release of nutrients to facilitate growth of the remaining bacterial population (Ratsak *et al.*, 1996, Tso and Taghon, 2006). It is conceivable therefore, that shaking of co-cultures could have resulted in increased uptake of *L. monocytogenes* and hence, more nutrients secretion by amoebae. It is likely that the observations made here are the regular occurrence in aquatic habitats where free-living amoebae and *L. monocytogenes* can be found (Rodriguez-Zaragoza, 1994, Thomas *et al.*, 2004, Thomas *et al.*, 2008). This is so because shaking incubation mimics the conditions in most of the aquatic environments, in particular the constant motion of water.

### **7.1.2 Survival of *Acanthamoeba* in co-culture with *Listeria***

The experimental results described here, which showed that amoeba trophozoites declined in numbers in co-culture with *L. monocytogenes*, confirm the previous observations made by others for *Acanthamoeba* sp (Zhou *et al.*, 2007, Huws *et al.*, 2008) and *T. pyriformis* (Pushkareva and Ermolaeva, 2010) and were attributed to the encystment of protozoa. The results however, differed from those of Akya and colleagues and Ly and Muller who suggested that the number of amoebae trophozoites

increased during the first few days of co-culture with *L. monocytogenes* before it declined (Ly and Muller, 1990a, Ly and Muller, 1990b, Akya *et al.*, 2009b). This discrepancy may be attributed to the different sources of *Acanthamoeba* cultures used which probably had affected the ability of amoebae to form cyst. Recent reports suggest that the ability of *Acanthamoeba* sp to form cyst is reduced or even lost upon prolong sub-culture of axenic cultures (Köhler *et al.*, 2008, Moon *et al.*, 2011). While in this study a maximum of five sub-cultures of amoeba cultures were done before a fresh culture was made, the number of sub-cultures carried out by Akya and colleagues and Ly and Muller were not specified (Ly and Muller, 1990a, Ly and Muller, 1990b, Akya *et al.*, 2009b).

Several factors have been reported in the literature to induce cyst formation in amoebae (Crump, 1950, Weisman, 1976, Byers, 1979, Khunkitti *et al.*, 1998, Hughes *et al.*, 2003, Cordingley and Trzyna, 2008). However, the most likely factor that was responsible for the encystment of *Acanthamoeba* in co-culture with *L. monocytogenes* is starvation. Two lines of evidence from this study support this hypothesis. First, the rate of reduction of trophozoites in the presence of *L. monocytogenes* was not significantly different from the rate observed in the absence of *L. monocytogenes*. Second, the culture of *A. castellanii* trophozoites fed with live *E. coli*, yielded more than 50 % trophozoites overnight while those fed with *L. monocytogenes* fell by 50 % during same period.

It is interesting to note that although there were still a large number of *L. monocytogenes* to feed on, the amoeba continued to encyst in co-culture. This is probably because *L. monocytogenes* is not a suitable food source for the amoebae. Previous studies showed that Gram positive bacteria are not suitable to most *Acanthamoeba* sp. because of the thickness of Gram positive bacteria cell wall which slows down digestion (Gonzalez *et al.*, 1990, Weekers *et al.*, 1993, Wang and Ahearn,

1997, Ronn *et al.*, 2002, Pickup *et al.*, 2007b). Consequently, amoeba responds to unsuitable food source by forming cysts (Pickup *et al.*, 2007a, de Moraes and Alfieri, 2008).

It is not clear if other factors such as listeriolysin O (LLO) toxin secreted by *L. monocytogenes* during cell infection (Portnoy *et al.*, 1988, Gedde *et al.*, 2000) played any role in the encystment of amoeba because recent findings by Pushkareva and Ermolaeva suggest that LLO can stimulate *T. pyriformis* to encyst during co-culture with *L. monocytogenes* (Pushkareva and Ermolaeva, 2010). Since *T. pyriformis* like *Acanthamoeba* do not have cholesterol in their phagosome membranes which is required for the in vitro activity of LLO, it is possible that LLO produced by extracellular *L. monocytogenes* into co-culture medium contributed to encystment of amoebae. Previous workers found that *L. monocytogenes* was able to produce LLO in broth cultures (Moors *et al.*, 1999).

### **7.1.3 Survival of *L. monocytogenes* in amoeba conditioned-medium**

The present data show that *L. monocytogenes* is able to grow in *Acanthamoeba*-conditioned media. This ability strongly suggests that *L. monocytogenes* benefitted from by-products secreted by amoeba. The data confirm the results previously obtained by others (Huws *et al.*, 2008, Akya *et al.*, 2009b). The results provide further evidence that *L. monocytogenes* is a saprophytic bacterium (Vazquez-Salinas *et al.*, 2001). A similar phenomenon has been reported for *M. avium*, *B. cepacia* and *A. baumannii* during co-culture with *Acanthamoeba* (Steinert *et al.*, 1998, Marolda *et al.*, 1999, Cateau *et al.*, 2011).

The fact that in this study *L. monocytogenes* grows much better in amoeba-conditioned medium than in co-culture with amoeba cells clearly suggests that the presence of *L. monocytogenes* or any bacteria in co-culture is not necessary for the release of growth-stimulating substances by *Acanthamoeba*. This proposal is strongly supported by the

observation that the filtrates of both mixed culture of *Pseudomonas putida* and *A. castellanii* and of axenic culture of *A. castellanii* were able to stimulate *P. putida* to produce pyoverdine, a siderophore that facilitates iron uptake in *Pseudomonas* under condition of iron deficiency (Meyer *et al.*, 1990, Levrat *et al.*, 1992). Levrat and colleagues concluded that the substances in the filtrates that caused the production of pyoverdine were stimulatory factors (Levrat *et al.*, 1992). The finding is however in disagreement with that of the previous workers who suggested that the inability of *H. pylori* or *V. parahaemolyticus* or *L. pneumophila* to survive in *Acanthamoeba*-conditioned medium was because the presence of bacteria was required for the production of amoeba-growth factors (King *et al.*, 1988, Barker *et al.*, 1992, Winiecka-Krusnell *et al.*, 2002, Laskowski-Arce and Orth, 2008). It is either that the amoeba factors in conditioned medium that can support growth of these bacteria were quickly metabolised or they were volatile and required continuous production.

The observation that *L. monocytogenes* counts were lower in *A. castellanii*-conditioned medium than in *A. culbertsoni* or *A. polyphaga* conditioned media despite there being more cysts in *A. castellanii* culture suggests that by-products from encysting amoebae did not contribute much to the growth of *Listeria* in co-culture as proposed by some workers (Akya *et al.*, 2009b). Indeed, the observed ability of *L. monocytogenes* to grow in medium pre-conditioned with *A. castellanii* for only 1 h during which time, no cysts were formed, clearly suggests that growth stimulating factors released by actively metabolising amoeba trophozoites could be the sole substance that supported the growth of *L. monocytogenes* in co-culture with *Acanthamoeba*.

## **7.2 Intracellular survival of *Listeria* within *Acanthamoeba***

*Listeria monocytogenes* is saprophytic bacterium that can cause disease in man and animals. However, in contrast to most other saprophytic bacteria that are occasionally pathogenic, *L. monocytogenes* rather behaves like other obligate pathogens in its ability to multiply in a variety of mammalian host cells (Cossart and Toledo-Arana, 2008, Pizarro-Cerda and Cossart, 2009). It is suggested that *L. monocytogenes* acquired its ability to survive in mammalian cells from previous adaptations in unknown environmental hosts such as free-living amoebae (Gray *et al.*, 2006). Unfortunately, the potential hosts for *L. monocytogenes* in the environment have not been identified.

### **7.2.1 Factors that may affect intracellular survival of *L. monocytogenes* within *Acanthamoeba***

The data presented in this study suggest that *A. castellanii* is a potential host for *L. monocytogenes* Scott A. This conclusion is based on the results of gentamicin protection assay which showed intracellular *L. monocytogenes* Scott A numbers in *A. castellanii* increased from 4 h to 72 h post-infection. Moreover, the intracellular survival of *L. monocytogenes* in *A. castellanii* was confirmed by the TEM results which clearly showed bacteria replicating in vacuoles and remaining intact 24 h post-infection. The results are at odds with findings of previous workers (Huws *et al.*, 2008, Akya *et al.*, 2009a, 2009b, 2010).

Several factors in this study, which were lacking in previous studies, could have contributed to the observed ability of *L. monocytogenes* Scott A to survive in *A. castellanii*. Firstly, intracellular survival assays in this study were all done at 32°C against the 37°C, 15°C or 22°C reported in previous studies (Huws *et al.*, 2008, Akya *et al.*, 2009a, 2009b, 2010). It has been suggested that 37°C incubation can cause rapid

encystment of *Acanthamoeba* leading to the reduction or death of intracellular bacteria (Marolda *et al.*, 1999, Greub and Raoult, 2004). Indeed, Cirillo and co-workers found that *Mycobacterium marinum* were able to replicate in *A. castellanii* at 32°C but were killed when cultures were incubated at 37°C (Cirillo *et al.*, 1997). On the other hand, incubation at 22°C or below has been associated with complete elimination of some intracellular bacterial pathogens by *Acanthamoeba*. For example, *L. pneumophila* were able to grow in *A. castellanii* at 35°C but were killed by the amoeba at 22°C incubation (Nagington and Smith, 1980, Anand *et al.*, 1983, Ohno *et al.*, 2008).

Secondly, the strain of *L. monocytogenes* (*L. monocytogenes* Scott) used in this study differed from the strains used by previous workers (Huws *et al.*, 2008, Akya *et al.*, 2009a, 2009b, 2010). The differences may therefore be related to variability of virulence and epidemic potential existing in *L. monocytogenes* strains (Roche *et al.*, 2003, Velge and Roche, 2010). Indeed, *L. monocytogenes* Scott A is an epidemic strain that has been associated with high prevalence in outbreaks of human listeriosis (Kathariou, 2002). This suggests that *L. monocytogenes* Scott A could potentially survive in many cells including *Acanthamoeba*.

Strain variability may also account for the inability to show that other strains of *L. monocytogenes* survive in *A. castellanii* or *A. polyphaga* of this study.

Similarly, Dey and colleagues have recently shown that strain variability was responsible for the failure of *L. pneumophila*, Paris to grow in the amoeba, *Willaertia magna* whilst others (*L. pneumophila*, Philadelphia and *L. pneumophila*, Lens) belonging to the same serotype were able to grow in the amoeba unhindered (Dey *et al.*, 2009).

Thirdly, *Acanthamoebae* in this study were disrupted with 0.06 % sarcosine to release intracellular *L. monocytogenes*. This concentration of detergent was found to completely lyse *Acanthamoeba* cells without significantly affecting the viability of *L. monocytogenes*. In contrast, Huws and colleagues and Akya and colleagues disrupted their cells with 0.1% (v/v) and 0.3 % (v/v) triton-X100 respectively (Huws *et al.*, 2008, Akya *et al.*, 2009a, 2009b, 2010). Both concentrations of triton-X100 were tested in this study and found to be insufficient for *Acanthamoeba* lysis. The inability of triton-X100 to cause complete lysis implies that bacteria would remain trapped in *Acanthamoeba* so producing false negative results.

Fourthly, the *L. monocytogenes* inocula used to infect *Acanthamoebae* in the present study were cultivated in solid culture medium as against liquid culture medium used by Huws and colleagues and Akya and colleagues (Huws *et al.*, 2008, Akya *et al.*, 2009a, 2009b, 2010). Tezcan-Merdol and colleagues showed that *Salmonella* strains grown on solid medium and those grown in liquid broth were taken up by *A. rhysodes* with different efficiencies (Tezcan-Merdol *et al.*, 2004).

Lastly, in this study *Acanthamoeba* were maintained in 20 % (v/v) growth medium after infection, to reduce encystment of amoeba, which can lead to death of intracellular bacteria (Greub and Raoult, 2004). In contrast, the previous workers incubated their infected cells in buffers which do not have nutrients to support amoebae (Huws *et al.*, 2008, Akya *et al.*, 2009a, 2009b, 2010). Consequently, *Acanthamoeba* would rapidly encyst due to starvation (Cordingley and Trzyna, 2008)so affecting the ability of *L. monocytogenes* to survive within amoeba (Greub and Raoult, 2004).

Zhou and others have previously shown that *L. monocytogenes* strains were able to survive in *A. castellanii* over 72 h after predation but found no evidence of bacterial replication within amoeba (Zhou *et al.*, 2007). However, there is a strong indication, from the data presented by Zhou and colleagues that intracellular *L. monocytogenes* were killed over 72 h incubation (Zhou *et al.*, 2007). Indeed, when the procedures described by Zhou and co-workers were followed in this study to test if *L. monocytogenes* 10403S could produce similar results in *A. castellanii*, bacteria failed to survive after predation just as was earlier obtained using our test procedures. It is likely that the findings by Zhou co-workers were due to extracellular bacteria that survived treatment with gentamicin and remained in the growth medium.

Ly and Muller also suggested that *L. monocytogenes* could survive and multiply within *Acanthamoebae* and exit after 8 days by lysing amoeba (Ly and Muller, 1990a). However, they did not show evidence to unequivocally support growth of *L. monocytogenes* within amoeba cells. In addition, they incubated their co-cultures in tap water at 36°C for 6-8 days during which time this, and other studies, found that most, if not all *Acanthamoeba*, had formed cysts (Huws *et al.*, 2008, Akya *et al.*, 2009b, 2010). Moreover, Ly and Muller treated their infected cells with only 0.5µg/ml gentamicin for 1 h. Based on the current observations 0.5µg/ml gentamicin is too low to kill extracellular *L. monocytogenes* within 1 h. Therefore, it is highly likely that their findings were due to extracellular bacteria rather than intracellular bacteria. Indeed, Ly and Muller failed to perform a test to show distinction between extracellular and intracellular listerial growth. The results of this study and those of other workers have shown that extracellular *L. monocytogenes* can thrive on amoeba by-products to

maintain a high number in presence of *Acanthamoeba* (Zhou *et al.*, 2007, Huws *et al.*, 2008, Akyu *et al.*, 2009b, 2010).

Furthermore, Ly and Muller used non-axenic *Acanthamoebae* for their test (Ly and Muller, 1990a). There is high probability that the *Acanthamoeba* sp used in Ly and Muller's study were already harbouring obligate endosymbiotic bacteria that could interfere with their results. Indeed, Fritsche and co-workers found that 24 % of *Acanthamoeba* isolates from clinical and environmental sources harboured obligate intracellular bacteria (Fritsche *et al.*, 1993).

### **7.2.2 Similarities between intracellular Survival of *L. monocytogenes* in *Acanthamoeba* and survival in macrophages**

The pattern of intracellular survival observed here, in which intracellular *L. monocytogenes* Scott A numbers in *A. castellanii* first plummeted after uptake before the numbers began to rise again, is consistent with previously observed growth of *L. monocytogenes* in resident (non-activated) macrophages (Portnoy *et al.*, 1989, de Chastellier and Berche, 1994, Inoue *et al.*, 1995). The authors attributed their findings to concomitant intracellular killing and survival of *L. monocytogenes* occurring in the same macrophages (Portnoy *et al.*, 1989, de Chastellier and Berche, 1994, Inoue *et al.*, 1995).

It is generally believed that *L. monocytogenes* grows in resident (non-activated) macrophages but is killed in activated macrophages through generation of reactive oxygen or reactive nitrogen species (ROS or RNS). The oxygen radicals prevent bacteria from escaping phagosome into cytoplasm. Consequently, lysosomes fuse with the phagosome to kill bacteria (Harrington-Fowler *et al.*, 1981, Higginbotham *et al.*, 1992, Myers *et al.*, 2003, Shaughnessy and Swanson, 2007). However, studies have

shown that a large proportion ( $\approx 80\%$ ) of *L. monocytogenes* taken up into resident macrophages do not succeed in escaping the primary phagosome and are degraded in phagolysosomes within 2 h of infection as a result of ROS activity. Only about 14-28% eventually escapes into cytoplasm to multiply (Portnoy *et al.*, 1989, de Chastellier and Berche, 1994, Inoue *et al.*, 1995).

Since *Acanthamoeba castellanii* have previously been shown to produce ROS similar to those of macrophages (Davies *et al.*, 1991, Davies and Edwards, 1991), it can be speculated that ROS produced by *A. castellanii* were also responsible for intracellular killing of *L. monocytogenes* in this study. The speculation was supported by the present observations that pre-treatment of *A. castellanii* with DPI before infection enhanced intracellular survival of *L. monocytogenes*, particularly during the early phase of intracellular infection. DPI is an inhibitor of NADPH oxidase that catalyses the production of superoxide in macrophages and polymorphonuclear leukocytes (Hancock and Jones, 1987, O'Donnell *et al.*, 1993, Babior, 1999). Previous studies have shown that the inhibitory effect of DPI abrogated generation of ROS in macrophages, leading to increased survival of intracellular *L. monocytogenes* (Myers *et al.*, 2003).

The precise mechanism by which ROS could inhibit escape of *L. monocytogenes* from phagosomes of *Acanthamoeba* was not tested. However, the most obvious mechanism would be to block the action of LLO, which mediates escape of *L. monocytogenes* from phagosomes of macrophages (Gedde *et al.*, 2000, Henry *et al.*, 2006, Schnupf *et al.*, 2006). LLO contains one cysteine residue in the highly conserved undecapeptide sequence which binds to cholesterol in phagosome membrane of macrophages (Mengaud *et al.*, 1988, Pinkney *et al.*, 1989, Stachowiak *et al.*, 2009). This cysteine residue is oxygen-labile and can readily be oxidized and thus affect activity of the toxin

to render it inactive (Billington *et al.*, 2000). Unfortunately, it was not possible to test if LLO is also required for intracellular survival of *L. monocytogenes* within *Acanthamoeba*. The inability was due to lack of *hly* mutants of *L. monocytogenes* Scott A. The other *L. monocytogenes* strain that was included in the intracellular survival assay, and for which a *hly* mutant has been made was 10403S. Unfortunately, the wild-type 10403S and the mutant were not able to survive inside *Acanthamoeba*.

Mansfield and colleagues found that *L. monocytogenes* was able to establish lethal infections in the adults and larvae of *Drosophila melanogaster* (Mansfield *et al.*, 2003). Furthermore, they showed that the bacterial gene products (including LLO) necessary for intracellular replication and cell-to-cell spread within mammalian cells were similarly required for survival within the insect cells (Mansfield *et al.*, 2003).

Most recently, Pushkareva and Ermolaeva also found that expression of LLO supported growth and cytotoxic effect of *L. monocytogenes* in co-culture with another protozoa, *T. pyriformis* (Pushkareva and Ermolaeva, 2010). In addition, they showed that *L. monocytogenes* mutants deficient in LLO expression were impaired in growth and failed to produce cytotoxic effect, while replenishment of *hly* gene in the mutant strain restored toxicity to the protozoan (Pushkareva and Ermolaeva, 2010). These findings suggest that LLO may not only be required for survival in mammalian cells but in other cell types as well.

The inability to find *L. monocytogenes* that escaped to the cytoplasm of *A.castellanii* may be attributed to lack of cholesterol in the phagosomes membrane of the amoeba (Ulsamer *et al.*, 1971). Listeriolysin O requires cholesterol in phagosomes membrane of mammalian cells for binding and pore-forming activity (Bavdek *et al.*, 2007).

However, *A. castellanii* do not have cholesterol in its membrane instead, the membrane contains two major sterols, ergosterol and 7-dehydrostigmasterol (Smith and Korn, 1968, Raederstorff and Rohmer, 1985). Bavedek and co-workers have recently demonstrated the ability of LLO to bind to ergosterol *in vitro* although, the level of binding was not comparable with that with cholesterol (Bavdek *et al.*, 2007). Whether LLO could also bind to ergosterol *in vivo* to produce pores that will enable *L. monocytogenes* escape to cytoplasm at some point is not yet understood.

Although in this study, no bacterial replication occurred in the cytoplasm of *A. castellanii* infected with *L. monocytogenes*, there was evidence that bacteria replicated in vacuoles. This is in contrast with the usual occurrence in mammalian cells in which *L. monocytogenes* escapes from phagosomes into the cytoplasm within 30 min of uptake (Myers *et al.*, 2003). Interestingly, Birmingham and colleagues have recently suggested that a small percentage ( $\approx 13\%$ ) of *L. monocytogenes* that infect macrophages are unable to escape phagosome due to insufficient LLO activity to drive escape into the cytoplasm (Birmingham *et al.*, 2008a, Birmingham *et al.*, 2008b). Nevertheless, Birmingham and others found that such bacteria were able to replicate in vacuoles of macrophage because their LLO activity though not sufficient to produce enough pores to escape to cytoplasm, was sufficient to block fusion of lysosome with phagosome (Birmingham *et al.*, 2008a, Birmingham *et al.*, 2008b). Consequently, *L. monocytogenes* modified the vacuoles into spacious vacuoles within which they replicated slowly over 72 h (Birmingham *et al.*, 2008a, Birmingham *et al.*, 2008b). Since most of the vacuoles containing *L. monocytogenes* in the present study appeared very spacious, it is conceivable that they could also have been formed in a similar way moreso that bacteria appeared unable to escape to cytoplasm of *A. castellanii*.

So far, the pattern of intra-amoebal listerial growth in the gentamicin protection assay, the evidence of ROS activity in *A. castellanii* infected with *L. monocytogenes* and the ability of *L. monocytogenes* to replicate in vacuoles of *A. castellanii* suggest that there are similarities with intracellular survival of *L. monocytogenes* within macrophages (Portnoy *et al.*, 1989, Myers *et al.*, 2003, Birmingham *et al.*, 2008a). Previous workers also found that intracellular survival of *L. pneumophila* and *M. avium* in *Acanthamoeba* shares many similarities at the cellular and molecular level with growth of bacteria within macrophages (Cirillo *et al.*, 1997, Gao *et al.*, 1997, Swanson and Hammer, 2000). These similarities add support to the idea that the ability of *L. monocytogenes* and other intracellular bacteria to parasitize macrophages and cause human disease may be a consequence of their adaptation for intracellular survival within free-living amoebae (Brown and Barker, 1999, Molmeret *et al.*, 2005, Gray *et al.*, 2006, Hilbi *et al.*, 2007, Salah *et al.*, 2009).

### **7.2.3 Exposure to Mn<sup>2+</sup> augmented intracellular growth of *L. monocytogenes* Scott A in *A. castellanii***

Previous studies in mammalian cells suggest that superoxide dismutase (SOD) is a key determinant in the intracellular survival of *L. monocytogenes* (Welch *et al.*, 1979, Archambaud *et al.*, 2006, Cossart and Toledo-Arana, 2008). *L. monocytogenes* produces SOD which detoxifies ROS produced by their host cells to enable bacteria escape to cytoplasm (Myers *et al.*, 2003). Furthermore, manganese was found to restore SOD activity in *L. monocytogenes* extract that was depleted of metal ions (Vasconcelos and Deneer, 1994). Since manganese could restore SOD activity of bacterial extract *in vitro*, one can speculate that exposure of *L. monocytogenes* to manganese may enhance SOD activity in live listerial cells to enable bacterial survival in *Acanthamoeba*. Consistent with this, is the present observations that treatment with manganese enhanced

intracellular growth and prolonged survival of survival of *L. monocytogenes* Scott A within *A. castellanii*.

The primary step to determine SOD activity in bacterial extract is to measure the total protein of the extract. The results show that *L. monocytogenes* Scott A exposed to manganese produced four times as much protein as the unexposed bacteria. This suggests that manganese affect gene expression of *L. monocytogenes*. However, attempts to detect activity of SOD in bacterial protein extract failed. The lack of success may be attributed to insensitivity of the method to detect SOD activity or perhaps, due to experimental errors rather than absence of SOD in the extracts.

### **7.3 Involvement of autophagy in intracellular survival of *Listeria***

Autophagy, the process whereby eukaryotic cells digest their own organelles and proteins, may have evolved as a mechanism to survive periods of starvation, but is increasingly exploited by some cells as host defence mechanisms against invading microbes in a process that has been termed ‘xenophagy’ (Reggiori and Klionsky, 2002, Levine, 2005, Mizushima *et al.*, 2008, Sanjuan and Green, 2008, Orvedahl and Levine, 2009). The hallmark of a classical autophagy is the formation of a double membrane vacuole that contains cytoplasmic material and/or organelles to be degraded (Reggiori and Klionsky, 2002, Klionsky *et al.*, 2008, Mizushima *et al.*, 2010).

#### **7.3.1 The relevance of TEM in the study of autophagy**

There are many methods for identifying and quantifying autophagosomes formation including electron microscopy, light microscopy, biochemical assays and autophagic flux (Eskelinen, 2008, Klionsky *et al.*, 2008, Yla-Anttila *et al.*, 2009, Mizushima *et al.*, 2010, Kaminsky *et al.*, 2011). However, TEM remains one of the most widely used and sensitive techniques for quantitative and qualitative analysis of autophagy in eukaryotic cells (Eskelinen, 2008, Klionsky *et al.*, 2008, Barth *et al.*, 2010, Mizushima

*et al.*, 2010). The advantages of this method are that it is sensitive and relatively easy to identify autophagosomes based on their morphological characteristics. In addition, the method does not depend on the availability of specific antibodies or probes (Eskelinen, 2008, Yla-Anttila *et al.*, 2009, Mizushima *et al.*, 2010). However, the method requires a high level of skill and takes more time than most other methods. In addition, the correct interpretation of electron microscopy pictures in some instances requires special expertise that can only be gained by experience (Klionsky *et al.*, 2008, Kaminsky *et al.*, 2011).

### **7.3.2 Evidence of autophagy in *Acanthamoeba***

The TEM results presented here show that autophagy is induced or upregulated in *A. castellanii* during infection with *L. monocytogenes*. This is the first experimental evidence that demonstrated the involvement of autophagy in intracellular survival of bacteria within amoeba. The results are however consistent with *L. monocytogenes* being targeted to autophagosome after invasion of mouse embryonic fibroblasts (MEFs) and macrophages during the early stages of infection (Birmingham *et al.*, 2007, Py *et al.*, 2007, Birmingham *et al.*, 2008a, Birmingham *et al.*, 2008b). Similarly, autophagy has been shown to be induced following infection of mammalian cells by many intracellular pathogens, including *L. pneumophila*, *M. tuberculosis*, *S. typhimurium*, *S. flexneri*, *P. gingivalis*, *Coxiella burnetii*, *B. abortus*, Hepatitis B virus, Coxsackievirus and *T. gondii* (Dorn *et al.*, 2001, Gutierrez *et al.*, 2004, Ogawa *et al.*, 2005, Birmingham *et al.*, 2006, Deretic and Levine, 2009).

### **7.3.3 Possible triggers of autophagy in *Acanthamoeba***

Autophagy is induced by a variety of intracellular and extracellular stimulus, including starvation, protein aggregation, damaged organelles and infection (Liu and Lenardo,

2007, Sasnauskiene *et al.*, 2009). Several lines of evidence from this study suggest that starvation could possibly be a major trigger of autophagy in *Acanthamoeba*.

Firstly, there was evidence of autophagy in non-infected cells. Secondly, non-bacterial autophagosomes were also observed in infected cells. Thirdly, cytoplasmic components were found in the lumen of listerial and non-listerial autophagosomes. Indeed, the sequestration of cytoplasmic components into autophagosomes is a defining characteristic of autophagy induced by starvation (Mizushima, 2005, Klionsky *et al.*, 2008). It appears autophagy was induced primarily to provide amoeba with nutrients for survival but was then diverted to fight infection by *L. monocytogenes*. *M. tuberculosis*, which is capable of surviving in phagosome of macrophages by preventing fusion with lysosomes, was shown to be destroyed in vacuoles when autophagy was induced by starvation or rapamycin (Gutierrez *et al.*, 2004, Vergne *et al.*, 2006). Dorn and colleagues had earlier reported that after 90 min of internalisation into vacuoles of human coronary artery endothelial (HCAEA) cells, *P. gingivalis* were found in early and late autophagosomes containing undegraded cytoplasm and cytoplasmic vesicles (Dorn *et al.*, 2001, 2002). They were, however, not certain what induced the autophagy and whether induction of autophagy preceded the internalisation of bacteria (Dorn *et al.*, 2001, 2002). It now appears that cell starvation could have been responsible for inducing autophagy in Dorn and colleagues study.

The question is, how were bacteria taken into autophagosomes after activation? It is possible that autophagy targeted *L. monocytogenes* while in the cytoplasm of amoeba as was demonstrated for *L. monocytogenes* in macrophages after treatment with chloramphenicol (Rich *et al.*, 2003) and in the infection of *Drosophila* S2 cells (Yano and Kurata, 2008, Yano *et al.*, 2008) or in the case of *F. tularensis* which were captured into autophagosomes after an initial replication in the cytoplasm of macrophages

(Checroun *et al.*, 2006). However, the lack of bacteria that escaped into cytoplasm of *Acanthamoeba* makes it unlikely for autophagy to trap *L. monocytogenes* in cytoplasm of amoeba. Alternatively, bacteria may have been targeted in intact vacuoles. Consistent with this is the present observation of autophagosomes fusing with other bacterial vacuoles. In support of this observation, Gutierrez and others found that *M. tuberculosis* were targeted in intact vacuoles of macrophages when autophagy was induced by starvation or rapamycin (Gutierrez *et al.*, 2004, Vergne *et al.*, 2006, Liu and Modlin, 2008). The apparent fusion of autophagosomes with phagosomes suggests that there was a convergence between endocytic and autophagic pathways in amoeba consistent with previous observations that endosomes fuse primarily with the early autophagosomes within 10 min of endocytosis (Liou *et al.*, 1997, Jing and Tang, 1999). The fusion of phagosome with autophagosome and mixing of their contents probably provided avenue for the transport of bacteria to autophagosomes. Alternatively, the phagosomes with bacteria were sequestered into autophagosomes. This is also consistent with the present observation in autophagosomes of bacteria that appeared partly bounded with phagosome membrane.

The signals that mediated the activation of autophagy in *Acanthamoeba* are not known. However, it is possible that reactive oxygen species (ROS) generated during phagocytosis of *L. monocytogenes* contributed to the induction of autophagy. The hypothesis is supported by the current indirect demonstration that ROS played a major role in intracellular *L. monocytogenes* killings within *Acanthamoeba* (Section 7.2.2).

Previous workers have shown that the Nox2 NADPH oxidase-derived ROS, which are necessary for elimination of micro-organism in phagocytes (Rada *et al.*, 2008) are required for activation of antibacterial autophagy (Scherz-Shouval *et al.*, 2007, Rada *et al.*, 2008, Huang and Brumell, 2009, Huang *et al.*, 2009). In addition, accumulating data

suggest that starvation can induce mitochondria to form ROS and these were shown to serve as signalling molecules in the starvation-induced autophagy (Chen *et al.*, 2007, Scherz-Shouval and Elazar, 2007, Chen and Gibson, 2008, Chen *et al.*, 2009, Scherz-Shouval and Elazar, 2009, 2011). Moreover, ROS has also been found to regulate autophagy induced through damage of mitochondria (Chen *et al.*, 2007, Chen and Gibson, 2008).

There is likelihood that the different types of autophagosome observed in *A. castellanii* were probably formed due to different regulation by ROS produced by mitochondria on one hand and the ones produced during phagocytosis of *L. monocytogenes* on the other hand. While the starvation-induced autophagosome often encloses cytosol or organelles particularly mitochondria in their lumen (Klionsky *et al.*, 2008), those formed specifically for defence purposes seldom have these features (Levine, 2005, Klionsky *et al.*, 2008). The differences in ROS regulation probably accounted for the disappearance of Type 1 autophagosomes following treatment with 3-MA.

Another possible stimulus that triggered amoebae autophagic machinery to sequester *L. monocytogenes* into autophagosomes is the disruption of the integrity of phagosome by LLO during the process of *L. monocytogenes* escape into cytoplasm.

Previous studies using mammalian cells showed that autophagy can target *L. monocytogenes* in phagosomes that have been damaged by LLO during the primary phase of infection prior to escape into cytoplasm and that phagosome perforation is necessary for induction of autophagy (Birmingham *et al.*, 2007, Py *et al.*, 2007, Birmingham *et al.*, 2008a, Birmingham *et al.*, 2008b). Similarly, damage to *Salmonella typhimurium* -containing vacuoles and *Toxoplasma gondii* parasitophorus vacuoles in macrophages was shown to be the signal that triggered host cell autophagy against the pathogens (Birmingham *et al.*, 2006, Ling *et al.*, 2006).

Although it was earlier speculated (section 7.2.2 ) that the inability of *L. monocytogenes* to escape into cytoplasm of amoeba was due to lack of cholesterol in the phagosomes membrane for binding and pore forming activity of LLO, nevertheless, it is possible that LLO binds to ergosterol, the major sterol found in membranes of amoebae (Smith and Korn, 1968, Raederstorff and Rohmer, 1985) to produce pores in the phagosomes. This is because previous reports have shown that LLO recognition and binding to cholesterol is determined by the most exposed 3 $\beta$ -hydroxyl group of cholesterol which incidentally is structurally similar to the 3 $\beta$ -hydroxyl group found in ergosterol (Bavdek *et al.*, 2007). Moreover, Bavdek and colleagues also found that LLO binds to ergosterol although at a relatively low level compared with binding to cholesterol.

The binding and pore formation of LLO in phagosome could result in leakage of vacuolar contents into the cytosol, thus triggering autophagy. Study has shown that perforations made on phagosomes of macrophage by LLO shortly before *L. monocytogenes* escaped into cytoplasm creates small pores through which small molecules such as calcium ions and protons leak into cytosol (Shaughnessy *et al.*, 2006). It is possible that increased concentration of cytosolic calcium was the signal that triggered autophagy to the damaged phagosomes. This is because calcium signalling is responsible for the regulation or modification of virtually all processes in healthy cells (Ferrari *et al.*, Berridge *et al.*, 2000). Indeed, Høyer-Hansen and co-workers found that elevation in the concentration of free cytosolic calcium is a potent inducer of autophagy in mammalian cells (Høyer-Hansen *et al.*, 2007).

#### **7.3.4 Purpose for the activation autophagy in *A. castellanii***

The observation that autophagy increased intracellular *L. monocytogenes* death in *Acanthamoeba*, particularly after 4 h of infection, suggests that autophagy was purposely stimulated to eliminate *L. monocytogenes* infection. The finding is consistent

with the role of autophagy as innate immune barrier to infection (Shintani and Klionsky, 2004, Mizushima, 2005, Amano *et al.*, 2006, Whitmarsh and Hunter, 2008, Yano and Kurata, 2008, Corr and O'Neill, 2009, Levine *et al.*, 2011). The finding also confirmed previous observations made in mammalian cells which suggest that activation of autophagy attenuates *L. monocytogenes* infection during the early phase of primary infection prior to escape but not after bacteria have escaped into the cytoplasm where *L. monocytogenes* evades autophagy largely, due to expression of ActA (Birmingham *et al.*, 2007, Py *et al.*, 2007, Birmingham *et al.*, 2008b, Yoshikawa *et al.*, 2009). Autophagy induction has similarly been shown to play a protective role in infections caused by other microbes, including Group A Streptococcus, *S. aureus*, *H. pylori*, *B. pseudomallei*, *S. flexneri*, *V. cholerae* among others (Deretic, 2005, Deretic and Levine, 2009).

### **7.3.5 *L. monocytogenes* replicated in autophagosomes**

The fact that in this study intact bacteria were found in early autophagosomes after 24 h of infection and some even showed evidence of replication is an indication that the vacuoles were permissive for growth of *L. monocytogenes*. The observations suggest that some autophagosomes that harboured *L. monocytogenes* did not mature to autolysosomes. *L. monocytogenes* may inhibit autophagosome maturation in a fashion similar to that of phagosomes of macrophages (Shaughnessy *et al.*, 2006). Birmingham and co-workers recently found that a small population of *L. monocytogenes* that were taken up by autophagosomes of macrophages were unable to escape into cytoplasm due to insufficient LLO activity but were able to prevent maturation of the vacuoles through pore creation that prevented acidification of the vacuoles. Furthermore, they found that the compartments of the vacuoles were neutral (pH  $7.3 \pm 0.29$ ) and supported *L.*

*monocytogenes* growth at a generation time of 8 h (Birmingham *et al.*, 2008a, Birmingham *et al.*, 2008b).

Since *L. monocytogenes* can efficiently replicate in the cytoplasm of mammalian host cells (Goebel and Kuhn, 2000, Goetz *et al.*, 2001), it is expected that the bacterium can utilise the nutrients derived from the cytosolic substances that were sequestered within the autophagosomes of *Acanthamoeba*.

#### **7.4 Influences of intracellular survival of *L. monocytogenes* in *Acanthamoeba***

*Listeria monocytogenes* is a food borne pathogen that is of major concern to the food industry (Gandhi and Chikindas, 2007). The bacterium can persist on food processing surfaces through formation of biofilms that are resistant to disinfections (Lee Wong, 1998, Norwood and Gilmour, 2000, Pan *et al.*, 2006, Carpentier and Cerf, 2011).

Previous researchers have demonstrated that the surface properties of *L. monocytogenes* such as, hydrophobicity, morphology, surface charge, extracellular protein content, flagella and extracellular polysaccharides, are significant factors in the cell attachment and biofilm formation by the organism (Briandet *et al.*, 1999a, Bereksi *et al.*, 2002, Monk *et al.*, 2004, Chae *et al.*, 2006). These physiological properties of *L. monocytogenes* have been shown to be altered by other factors, including growth temperature, pH, salinity, bacterial cell density and composition of growth medium to influence biofilm formation (Bereksi *et al.*, 2002, Gravesen *et al.*, 2005, Chae *et al.*, 2006, Giotis *et al.*, 2007, Van Houdt and Michiels, 2010).

One potential way by which the surface properties of *L. monocytogenes* could be altered is through intracellular growth in free-living amoeba such as *Acanthamoeba*. Previous workers showed that the surface properties of *L. pneumophila* were altered as

a result of their growth within *Acanthamoeba* (Barker *et al.*, 1992, Barker *et al.*, 1993, Barker and Brown, 1995, Barker *et al.*, 1995, Susa *et al.*, 1996). However, the role of *Acanthamoeba* in promoting the survival of *L. monocytogenes* in food processing environments has not been explored.

The objective of this study was to determine if *L. monocytogenes* can survive in cysts of *Acanthamoeba* and whether survival in cysts can influence the morphological and physiological characteristic of bacteria.

#### **7.4.1 Ability of *Listeria* inside amoeba cysts to withstand chlorination**

The results indicate that *L. monocytogenes* can survive the encystment of the host *A. castellanii* and grow in culture medium when released from cysts. The results are in disagreement with those of Ly and Muller who suggested that encystment of amoeba leads to death of intracellular *L. monocytogenes* (Ly and Muller, 1990a). This difference may be attributed to the species of *Acanthamoeba* used. While *A. castellanii* was used in the current study, Ly and Muller used an unspecified species of *Acanthamoeba* which they isolated from the environment (Ly and Muller, 1990a).

Another reason that may account for the difference is the duration of incubation in co-culture. Whereas in this study co-cultures were incubated for a maximum of one day, Ly and Muller incubated theirs for 34 days (Ly and Muller, 1990a). The present study found that the ability of *L. monocytogenes* Scott A to survive encystment reduced when co-cultures were incubated beyond 4 h.

The fact that *L. monocytogenes* Scott A could survive in *A. castellanii* cysts have potential consequences. Firstly, it could provide a means by which *L. monocytogenes* can spread and colonise new habitats when cysts are blown through the air (Barker and Brown, 1994). Secondly, *L. monocytogenes* could be protected from adverse environmental conditions such as extreme temperatures, desiccation and biocides (De

Jonckheere and van de Voorde, 1976, Sriram *et al.*, 2008, Coulon *et al.*, 2010) and this could lead to persistence of the organisms in the environment (Carpentier and Cerf, 2011). Consistent with the possibility of amoeba cysts protecting *L. monocytogenes* from biocides is the present results which show that *L. monocytogenes* sequestered in *A. castellanii* cyst were able to withstand treatment with up to 100 mg/l free chlorine. This is in contrast to free *L. monocytogenes* which were previously shown to be killed after 30 s exposure to 5 mg/l free chlorine (Norwood and Gilmour, 2000).

A similar high level of resistance to chlorine was reported for *L. pneumophila* in *A. polyphaga* cysts (Kilvington and Price, 1990) and mycobacterium sp in *A. polyphaga* (Adekambi *et al.*, 2006). However, chlorine resistance of *L. monocytogenes* in *A. castellanii* was twice (100 mg/l) the resistance reported for *L. pneumophila* in *A. polyphaga* (Kilvington and Price, 1990). The observed differences with the present results may be ascribed to the inherent differences in characteristics of the two *Acanthamoeba* sp that influenced their level of tolerance to treatment with chlorine differently. The difference may also be due to the longer (maximum 14 days) period that amoeba cysts were incubated for hatching in this study compared with the 7 days hatching period by Kilvington and Price in their study (Kilvington and Price, 1990). In the present study, it was observed that cyst samples treated with 75 mg/l of chlorine and above sometimes took more than 7 days to hatch.

It is possible that the sequestration of *L. monocytogenes* inside cysts of *Acanthamoeba* and their resistance to disinfection may contribute to the persistence of bacteria in food processing environments (Pan *et al.*, 2006, Carpentier and Cerf, 2011).

#### **7.4.2 Altered *L. monocytogenes* morphology**

Results of this study suggest that *L. monocytogenes* that survived encystment of *A. castellanii* and freed after hatching can form filaments of cells that are many times

longer than cells grown in bacteriological medium. Previous reports demonstrated that *L. monocytogenes* form filamentous cells when exposed to adverse conditions, such as high concentrations of NaCl in the presence of acid (Isom *et al.*, 1995, Bereksi *et al.*, 2002) or absence of acid (Brzin, 1973, Isom *et al.*, 1995, Jørgensen *et al.*, 1995), acid conditions, i.e. pH 5.0 (Isom *et al.*, 1995), high hydrostatic pressure (Ritz *et al.*, 2001), sub-lethal alkaline i.e pH above 9.0 (Isom *et al.*, 1995, Giotis *et al.*, 2007), increased CO<sub>2</sub> environments (Nilsson *et al.*, 2000, Jydegaard-Axelsen *et al.*, 2005), in the presence of antimicrobial agents such as trimethoprim and co-trimoxazole (Minkowski *et al.*, 2001), above-optimum growth temperature, i.e. 42.8°C (Rowan and Anderson, 1998), high-intensity pulsed-plasma gas discharge condition (Rowan *et al.*, 2009) and sucrose plus bacteriocin (Ratti *et al.*, 2010).

Intracellular growth of *L. pneumophila* in *Acanthamoeba* species has similarly been shown to induce altered morphology (Barker and Brown, 1995). For example, they showed that the amoeba-grown *L. pneumophila* were, flagellated and motile whereas those grown *in vitro* were predominantly filamentous and non-motile (Barker *et al.*, 1992, Cirillo *et al.*, 1994, Barker and Brown, 1995).

The mechanism involved in the formation of filaments by the *L. monocytogenes* that survived encystment is not yet known. It is possible that the filaments were formed as an adaptive strategy by *L. monocytogenes* to evade subsequent ingestion by *Acanthamoeba*. Hahn and co-workers found *Flectobacillus* switched to filamentous state as a survival response in defence against flagellates grazing (Hahn *et al.*, 1999). On the contrary, Rowan and others found that *L. monocytogenes* filaments isolated from food and clinical samples demonstrated wild-type levels of adherence, invasion and cytotoxicity to human cells (Rowan *et al.*, 2000a, Rowan *et al.*, 2000b, Rowan *et al.*, 2009). Alternatively, the *L. monocytogenes* filaments were formed in response to the

adverse conditions inside the *Acanthamoeba* trophozoites and cysts. Minkowski and co-workers previously demonstrated the ability of intracellular *L. monocytogenes* to form filaments (Minkowski *et al.*, 2001). They showed that *L. monocytogenes* exposed to sub-inhibitory concentration of trimethoprim and co-trimoxazole while inside macrophages formed filament of cells (Minkowski *et al.*, 2001)

The observed reversion of *L. monocytogenes* filaments to the normal forms when amoeba-grown bacteria were sub-cultured in TSB is probably due to the removal of stress conditions. Previous workers suggested that removal of deleterious stresses can result in slow return filamentous *L. monocytogenes* to normal cells forms within 24 h (Brzin, 1973, Isom *et al.*, 1995, Minkowski *et al.*, 2001).

#### **7.4.3 Enhanced biofilm-forming abilities**

The experimental results described here show that *L. monocytogenes* Scott A, which survived encystment of *A.castellanii*, have enhanced biofilm-forming abilities on release. The enhanced ability may be associated with the observed changed in the morphological characteristic of amoeba-grown *L. monocytogenes* i.e. filamentation of bacterial cells. A naturally occurring filamentous *L. monocytogenes* isolated from a continuous-flow bioreactor was previously shown to have a 100-fold greater ability to colonise stainless steel surface than the short rods phenotype and this ability was attributed to their filamentous morphology (Monk *et al.*, 2004).

The enhanced ability of amoeba-grown *L. monocytogenes* to form biofilm may also be attributed to cell surface changes that occurred during residence in *Acanthamoeba*.

Numerous studies suggest that bacterial surface properties, such as hydrophobicity and cell surface charge are major factors in the attachment of bacteria to surfaces (van Loosdrecht *et al.*, 1987, Dickson and Koohmaraie, 1989, Mafu *et al.*, 1991, Briandet *et*

*al.*, 1999b, Chavant *et al.*, 2002, Di Bonaventura *et al.*, 2008). The fatty acid contents of bacteria determines its hydrophobicity (Moorman *et al.*, 2008) while bacterial surface charge is attributed to cell wall constituents such as phosphate, carboxylate groups and proteins (Pelletier *et al.*, 1997). It has been suggested that changes in the composition of *L. monocytogenes* fatty acid and proteins could alter the hydrophobicity and surface charge of the bacterium (Moorman *et al.*, 2008)

Previously, it was shown that the surface properties of *L. pneumophila* were altered as a result of their intracellular survival in *Acanthamoebae* (Barker *et al.*, 1993). Bacterial surfaces were coated with amoebae-derived monounsaturated straight-chain fatty acid and a 15kDa outer membrane protein (Barker *et al.*, 1993). It is conceivable that *L. monocytogenes* also acquired amoeba-derived fatty acid and protein while inside *A. castellanii* to alter their hydrophobicity and surface charge.

Alternatively, the composition of *L. monocytogenes* fatty acids and proteins possibly changed as a result of bacterial response to stress caused by the microbicidal substances produced by the host amoeba, such as ROS, acid and harsh degradative enzymes (Davies and Edwards, 1991, Akya *et al.*, 2009a). Studies have suggested that *L. monocytogenes* membrane fatty acids and proteins composition changed in adaptation to various stress conditions, including high salinities (Esvan *et al.*, 2000), acid condition (pH 5.5) (Mastronicolis *et al.*, 2010), disinfectants (Bisbiroulas *et al.*, 2011) and intraphagosomal signals in macrophages (Rouquette *et al.*, 1998, Olsen *et al.*, 2005, Sun and O'Riordan, 2010).

Previous reports suggested that exposure of *L. monocytogenes* Scott A in growth medium supplemented with glucose and/lactic acid enhanced their ability to form biofilms (Briandet *et al.*, 1999b, Pan *et al.*, 2010). In contrast, the present results show

that growth in medium containing glucose at pH 4.4 reduced the ability of *L. monocytogenes* Scott A to form biofilms. The data agree with those of Tresse and colleagues and Bereksi and colleagues who found that the surface of *L. monocytogenes* Scott A were less hydrophobic and their biofilm-forming abilities were reduced when bacteria were pre-incubated in lactic acid or HCl plus NaCl at pH 5 than those grown at pH 7 (Bereksi *et al.*, 2002, Tresse *et al.*, 2006).

The observation that the enhanced biofilm-forming abilities of *L. monocytogenes* grown in amoeba were lost when bacteria were sub-cultured in broth was probably due to the reversion from filamentous to normal cell types following the sub-culture. A similar observation was made by Monk and colleagues for *L. monocytogenes* filament derived from a bioreactor and suggested that the reversion was due to absence of stress conditions in growth medium (Monk *et al.*, 2004).

The fact that amoeba-grown *L. monocytogenes* have enhanced biofilm-forming abilities has important implications for food industry because of the reported resistance of biofilms to various disinfectants (Norwood and Gilmour, 2000, Pan *et al.*, 2006). In addition, there is risk of bacteria detaching to contaminate food products.

#### **7.4.4 Increased resistance to biocides**

The data presented here show that growth in *A. castellanii* increased resistance of *L. monocytogenes* to Milton complete protection sterilising fluid (MPSF). Barker and co-workers also found that *L. pneumophila* grown in *A. polyphaga* were more resistant to three disinfectants namely, polyhexamethylene biguanide (PHMB), benzisothiazolone (BIT) and 5-chloro-N-methylisothiazolone (CMIT) than bacteria grown in broth (Barker *et al.*, 1992).

Milton solution is used to sterilise breast feeding equipment and baby's feeding utensils. The main active ingredient of this solution is 2 % (w/v) sodium hypochlorite (NaOCl) and the recommended treatment specified by the manufacturers is 30 ml in 5 L of water (12 ppm NaOCl) for 15 min (<http://www.milton-tm.com/english.html> ). Unfortunately, the present results found that 12 ppm NaOCl was neither sufficient to kill *L. monocytogenes* grown *in vitro* nor the ones that were grown in *Acanthamoeba*.

The reason for the observed lack of resistance when amoeba-grown *L. monocytogenes* were exposed to quaternary ammonium compounds (QACs) is not known. It is likely that the active sites of QACs on *L. monocytogenes* were not adversely affected by any change that had occurred on listerial surface consequent to their residence in *A. castellanii*. Alternatively, *L. monocytogenes* strain or serotype used here is probably very susceptible to QACs. Mallupudi and co-workers while screening *L. monocytogenes* isolates for benzalkonium resistance found that serotype 4b produced the least resistance compared to other serotypes (Mullapudi *et al.*, 2008)

Although the ability of *L. monocytogenes* in biofilms to resist QACs or NaOCl was not tested, previous studies found *L. monocytogenes* biofilms demonstrated high resistance to these biocides (Norwood and Gilmour, 2000, Pan *et al.*, 2006). It is expected that the biofilms formed by the amoeba-grown *L. monocytogenes* to be resistant to disinfectants.

The data of this study show that growth of *L. monocytogenes* within *A. castellanii* induces a phenotype with increased resistance to some antimicrobial compounds, such as ampicillin, gentamicin and penicillin compared with the level of resistance of cells grown *in vitro*. Previously, Barker and colleagues reported *L. pneumophila* grown in *A. polyphaga* were 1000-fold more resistant to ciprofloxacin, rifampin and erythromycin compared with the control bacteria grown *in vitro* (Barker *et al.*, 1995).

Similarly, Miltner and Bermudez also found that *M. avium* residing in trophozoites of *A. castellanii* were protected from the antimicrobials, including rifabutin, clarithromycin and azithromycin usually (Miltner and Bermudez, 2000). In addition, they also found that *M. avium* retrieved from *A. castellanii* after infection demonstrated increased resistance to these drugs compared to the bacteria grown in culture medium (Miltner and Bermudez, 2000).

The treatment of choice for *Listeria* infections remain the administration of ampicillin or penicillin G in combination with an aminoglycoside such as gentamicin (Charpentier and Courvalin, 1999, Granier *et al.*, 2011). However, there are increasing cases of resistance of *L. monocytogenes* isolates from food and environment to these drugs (Arslan and Özdemir, 2008, Ayaz and Erol, 2010).

Published data show that several mechanisms are involved in resistance of bacteria to antimicrobial agents (Mah and O'Toole, 2001, Granier *et al.*, 2011). However, the mechanism of resistance in the present study will most probably be associated with residence of *L. monocytogenes* within *Acanthamoeba*. It was mentioned earlier that intracellular survival of *L. monocytogenes* within *Acanthamoeba* may alter its surface property through acquisition of fatty acids and protein from the host amoeba. Besides, there are also possibilities of listerial surface to be modified as a result of stress inside amoeba (O'Driscoll *et al.*, 1996, Galdiero *et al.*, 1997, Juneja *et al.*, 1998, Rouquette *et al.*, 1998). Alterations to bacterial surface, particularly those affecting phospholipid content, LPS or surface charge (protein composition) has been shown to reduce permeability to antimicrobial molecules (Mah and O'Toole 2001, Brown et al 1990).

## **7.5 Future work- Improvements on intracellular survival studies**

1. The present work was to determine the potential role of *Acanthamoeba* in the survival of *L. monocytogenes* in the environment. Although the results showed

that *A. castellanii* could potentially serve as host for *L. monocytogenes* Scott A in the environment, it will be interesting to sample different environments, particularly the food processing areas for *Acanthamoeba* trophozoites or cysts that harbours *L. monocytogenes*.

2. The inability to detect SOD activity in extract of *L. monocytogenes* exposed to manganese may be partly due to impurities present in the extract. In future, the SOD in bacterial extract will first be purified in an affinity column before quantifying the SOD activity. In addition, SOD purified from bacterial extract would be immunoprecipitated and analysed by western blotting (Archambaud *et al.*, 2006).
3. Listeriolysin O is the key enzyme that is required for intracellular survival of *L. monocytogenes* in mammalian cells (Portnoy *et al.*, 1988, Gedde *et al.*, 2000). It was not possible to establish the role of LLO in *Acanthamoeba* in this study because of lack of hly mutant produced from *L. monocytogenes* Scott strain. In future studies hly of Scott A strain would be constructed. In addition, the intracellular gene expression profile of *L. monocytogenes* within *Acanthamoeba* will be examined using whole genome microarray analysis (Chatterjee *et al.*, 2006) to determine the genes that are required for intracellular survival within *Acanthamoeba*.
4. In this study, *L. monocytogenes* were not found in cytoplasm of *Acanthamoeba* but were seen in vacuoles resembling autophagosomes. It is possible that bacteria escaped from vacuoles and were immediately targeted by autophagy. One way to test if bacteria escaped from phagosomes before they were sequestered into autophagosomes is to label bacteria in the vacuole with specific antibodies to ubiquitin, a marker for bacteria with previous contact with

cytoplasm (Perrin *et al.*, 2004, Birmingham *et al.*, 2008a) in addition to labelling the vacuole with autophagic markers.

5. Intracellular *L. monocytogenes* replication within vacuoles of *Acanthamoeba* would also be determined using immunofluorescence assay. Infected cells would be incubated with bromodeoxyuridine (BrdU), an analogue of thymidine and intracellular bacteria will be labelled with anti-BrdU (Birmingham *et al.*, 2008a).
6. The surface changes of *L. monocytogenes* probably influenced its ability to resist biocides and also to form biofilm. In future, the fatty acid and protein profile of amoeba-grown *L. monocytogenes* would be analysed to determine possible changes in bacteria (Barker *et al.*, 1993, Kim *et al.*, 2009). In addition, the test for the resistance of amoeba-grown *L. monocytogenes* to biocides would preferably be carried out on bacteria in biofilms rather than planktonic bacteria.

## 7.6 Conclusion

In conclusion, the present results have shown that *L. monocytogenes* can grow and remained in high numbers in the presence *Acanthamoeba* as well as in *Acanthamoeba* conditioned medium. *Acanthamoeba* on the other hand continued to formed cyst in the presence of *L. monocytogenes* suggesting that the bacteria are not suitable for *Acanthamoeba*. Incubation of cultures with agitation greatly enhanced growth and survival of *Listeria* in co-culture. In addition, *L. monocytogenes* Scott A were also found to survive and replicate inside vacuoles of *A. castellanii* over 72 h. Intracellular *L. monocytogenes* within *Acanthamoeba* was host-specific. Although bacteria replicated in vacuoles, they were unable to escape into cytoplasm probably due to lack of cholesterol in phagosome membrane which is required for pore-forming activity of LLO. Exposure of *L. monocytogenes* to manganese before infection enhanced growth and survival within *A. castellanii*. The fact that manganese enhanced intracellular survival of *L.*

*monocytogenes* in *A. castellanii* is of great significance because manganese is widely distributed in the environment including soil and water where *L. monocytogenes* and amoeba can be found (Dijkstra, 1982, World Health, 2004, Ivanek *et al.*, 2006, Thomas *et al.*, 2008).

The present study also demonstrated for the first time that autophagy is involved in infection of *Acanthamoeba* caused by *L. monocytogenes*. Starvation and damage to phagosomes were probably the main stimuli responsible for induction of autophagy during infection with *L. monocytogenes* while ROS were potentially the signalling molecules that mediated the process. Although it appeared autophagy was induced to control infection by *L. monocytogenes*, some bacteria subverted autophagic killing and replicated within the vacuoles. Cumulative evidence from this study suggests that *L. monocytogenes* can survive encystment of its host *A. castellanii*. Survival in amoeba not only altered the morphology of *L. monocytogenes* but also influenced its ability to form biofilms and increased resistance to some biocides. Intracellular survival of *L. monocytogenes* within *Acanthamoeba* may be the primary mechanism for dissemination and persistence of the organism in the food environments where *L. monocytogenes* contamination of food products is a major problem (Aarnisalo *et al.*, 2000, Norwood and Gilmour, 2000).

## APPENDIX 1. Media and Reagents recipes

### A. Bovine serum albumin

Bovine serum albumin powder (Europa; EQBAH62) -----1 g

Deionised water -----10 ml

Dissolve by layering powder on surface of liquid-capped tube and rock gently to dissolve. Store at -20°C

### B. Dey- Engley(DE) neutralizing broth

DE/neutralizing broth (Difco 281910) -----7.25 g

Deionised water ----- 250 ml

Autoclave at 121°C for 15 min and store at 4°C

### C. 3-Methyladenine (0.01M)

3- Methyladenine salt -----0.15 g

DMSO-----1 ml

Vortex to mix then:

Add deionised water -----9 ml

Dissolve in water bath at 70°C for 1 min, Store at 4°C

### D. Neff's encystment medium

i) Prepare 100 ml stock solutions of the following

CaCl<sub>2</sub>.2H<sub>2</sub>O (Sigma C3306)-----0.5 M

MgSO<sub>4</sub>.6H<sub>2</sub>O (Sigma M2393)-----1 M

NaHCO<sub>3</sub> (BDH 10247 4V)-----1M

TRIS (Sigma T1503)-----1 M

Phenol red (Sodium salt, Sigma P5530)-----1.5 % (w/v)

Autoclave at 121°C for 15 min

ii) Add the following into clean 1000 ml Duran bottle

KCl<sub>2</sub> (Sigma P-9541) -----7.46 g (0.1 M)

MgSO<sub>4</sub> (1M) -----8 ml (0.008M)

NaHCO<sub>3</sub> (1M) -----1 ml (0.001 M)

TRIS (1M) -----20 ml (0.02 M)

CaCl<sub>2</sub> (0.5 M) -----0.4 ml (0.004M)

Phenol red (1.5 % w/v) -----25 µl

Deionised water -----to make up 1000 ml

Allow to dissolve and adjust pH 8.9-9.0 at 20-25°C then filter sterilize with a disposable unit (0.2µm pore size). Store at room temperature for use within 2 months.

iii) Peptone water sugar

Peptone water

Bacto peptone (BD: 211677) -----12.5 mg

NaCl-----1.25 mg

Deionised water-----225 ml

Adjust to pH 7.1-7.3 then add

Bromocresol purple (0.08 % w/v) ----- 2.5 ml

Autoclave at 121°C for 15 min then add

Dissolve 2.5 g mannose in 22.5 ml H<sub>2</sub>O

Aliquot 10 ml in tubes and store at 4°C

**E. *Acanthamoeba axenic* growth medium (SK-#6)**

i) Basal medium

Biosate (BBL: BD 211862) -----	20.0 g
D-glucose (Sigma, G7021) -----	5.0 g
KH <sub>2</sub> PO <sub>4</sub> (anhydrous: Fluka, 60219) -----	0.3 g
Vitamin B <sub>12</sub> (10µg//ml: Sigma, B4051) -----	100 µl
L-Methionine (5 mg/ml: Fluka, 64319)-----	3 ml
Deionised water -----	make up 1000 ml

Aliquot in 250 ml volumes in Duran bottles and autoclave at 121°C for 15 min

Store at room temperature for and use within 4 weeks.

ii) For use add 225 ml of basal medium:

Penicillin + streptomycin (10,000 U/ml: Sigma, P4333) -----	1 ml
---	------

Store complete medium at 4°C and use within 4 weeks

**F. Maintenance medium**

SK#6 basal medium-----	50 ml
Deionsied water-----	250 ml

Autoclave at 121°C for 15 min and store at room temperature

**G. Superoxide dismutase analysis kit**

**1)216 mM potassium phosphate buffer**

K <sub>2</sub> HPO <sub>4</sub> .3H <sub>2</sub> O (Sigma; P5504) -----	24.65 g
Deionised water-----	500 ml

Adjust the pH to 7.8 at 25°C with 1M NaOH or 1M HCl

**2) 10.7 mM Ethylenediaminetetraacetic acid (EDTA)**

EDTA disodium salt dihydrate (Sigma; E5134)-----40 mg

Deionised water-----10 ml

**3) 1.1 mM Cytochrome C solution**

Cytochrome C powder (Sigma; C2037) -----29.2 mg

Deionised water -----2 ml

**4) 0.108 mM Xanthine solution**

Xanthine (Sigma; X4002) -----1.64 mg

Deionised water-----90 ml

Dissolve using magnetic stirrer adding drops of 1 NaOH until all the xanthine has dissolved. Transfer solution a 100 ml volumetric flask and top up to 100 ml mark with deionised water.

**5) Xanthine oxidase enzyme solution (XOD).**

a) Xanthine oxidase (Sigma X1875;) -----28  $\mu$ l

Deionised water-----172  $\mu$ l

Place on ice.

Immediately before use prepare a 0.05 units/ml as follows:

b) Xanthine oxidase solution (5 units/ml) -----10  $\mu$ l

Deionised water----- 990  $\mu$ l

**6) Superoxide dismutase solution**

Immediately before use, prepare 10 units/ml solution of SOD as follows:

Superoxide dismutase (Sigma; S5639)1452 u/mg -----6.89  $\mu$ l

Cold deionised water-----993 $\mu$ l

**7) Reaction cocktail**

Deionised water -----23.0 ml

$\text{KH}_2\text{PO}_4 \cdot 3\text{H}_2\text{O}$ (216 mM)	-----	25.0 ml
EDTA (10.7 mM)	-----	1.0 ml
Cytochrome C (1.1 mM)	-----	1.0 ml
Xanthine (0.108 mM)	-----	50.0 ml

Mix and adjust the pH to 7.8 at 25°C with 1 M NaOH or 1M HCl if necessary.

## APPENDIX 2. Statistical Analyses

### A. Survival of *Listeria* in presence of *Acanthamoeba*

i) Table Analyzed: Lm in presence of *A. castellanii* vs Lm alone 32°C

Two-way RM ANOVA      Matching by cols

Source of Variation	% of total variation	P value
Interaction	4.78	< 0.0001
Time	81.73	< 0.0001
Culture type	12.67	< 0.0001
Subjects (matching)	0.1166	0.6236

Source of Variation	P value summary	Significant?
Interaction	***	Yes
Time	***	Yes
Culture type	***	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	1697000000000000	424300000000000	27.39
Time	4	2900000000000000	725000000000000	467.9
Culture type	1	4496000000000000	4496000000000000	434.6
Subjects (matching)	4	4139000000000000	1035000000000000	0.6678
Residual	16	2479000000000000	154900000000000	

Number of missing values 0

ii) Table Analyzed: Lm in presence of *A. culbertsoni* vs Lm alone 32°C

Two-way RM ANOVA Matching by cols

Source of Variation	% of total variation	P value
Interaction	5.83	< 0.0001
Time	77.86	< 0.0001
Culture type	15.88	< 0.0001
Subjects (matching)	0.0781	0.4933

Source of Variation	P value summary	Significant?
Interaction	***	Yes
Time	***	Yes
Culture type	***	Yes
Subjects (matching)	ns	No

Source of Var.	Df	Sum-of-squares	Mean square	F
Interaction	4	13260000000000	3314000000000	66.26
Time	4	177100000000000	44270000000000	885.2
Culture type	1	36130000000000	36130000000000	813.2
Subjects	4	1777000000000	4430000000	0.8882
Residual	16	800300000000	50020000000	

Number of missing values 0

iii) Table Analyzed: Lm in presence of *A. polyphaga* vs Lm alone 32°C

Two-way RM ANOVA Matching by cols

Source of Variation	% of total variation	P value
Interaction	1.04	0.0813
Time	86.66	< 0.0001
Culture type	10.41	0.0002
Subjects (matching)	0.2651	0.6367

Source of Variation	P value summary	Significant?
Interaction	ns	No
Time	***	Yes
Culture type	***	Yes
Subjects (matching)	ns	No

Source of var.	Df	Sum-of-squares	Mean square	F
Interaction	4	19200000000000	4801000000000	2.529
Time	4	1607000000000000	401800000000000	211.7
Culture type	1	1930000000000000	1930000000000000	157.0
Subjects	4	49170000000000	12290000000000	0.6475
Residual	16	303700000000000	18980000000000	

iv) Table Analyzed: Lm in presence of *A. castellanii* vs Lm alone 32°C +shaking

Two-way RM ANOVA      Matching by cols

Source of Variation	% of total variation	P value
Interaction	32.97	< 0.0001
Time	21.91	< 0.0001
Culture type	42.04	< 0.0001
Subjects (matching)	0.6167	0.4364

Source of Variation	P value summary	Significant?
Interaction	***	Yes
Time	***	Yes
Culture type	***	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	5144000000000000	1286000000000000	53.44
Time	4	3418000000000000	8544000000000000	35.51
Culture type	1	6559000000000000	6559000000000000	272.7
Subjects (matching)	4	9622000000000000	2405000000000000	0.9997
Residual	16	3850000000000000	2406000000000000	
Number of missing values	0			

v) Table Analyzed: Lm in presence of *A. culbertsoni* vs Lm alone 32°C +shaking

Source of Variation	% of total variation	P value
Interaction	16.80	< 0.0001
Time	6.08	< 0.0001
Culture type	75.01	< 0.0001
Subjects (matching)	1.1519	0.0094

Source of Variation	P value summary	Significant?
Interaction	***	Yes
Time	***	Yes
Culture type	***	Yes

Subjects (matching)		**	Yes	
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	1849000000000000	4623000000000000	70.62
Time	4	6698000000000000	1674000000000000	25.58
Culture type	1	8257000000000000	8257000000000000	260.5
Subjects (matching)	4	1268000000000000	3170000000000000	4.842
Residual	16	1047000000000000	6547000000000000	
Number of missing values	0			

vi) Table Analyzed: Lm in presence of *A. polyphaga* vs Lm alone 32°C+shaking

Two-way RM ANOVA      Matching by cols

Source of Variation	% of total variation	P value
Interaction	16.94	< 0.0001
Time	4.37	0.0047
Culture type	73.96	0.0002
Subjects (matching)	1.6754	0.1162
Source of Variation	P value summary	Significant?
Interaction	***	Yes
Time	**	Yes
Culture type	***	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	5831000000000000	1458000000000000	22.16
Time	4	1505000000000000	3761000000000000	5.719

Culture type	1	2546000000000000	2546000000000000	176.6
Subjects (matching)	4	5767000000000000	1442000000000000	2.192
Residual	16	1052000000000000	6577000000000000	
Number of missing values	0			

iv) Table Analyzed: Effect of culture condition 1-*A. castellanii*

Two-way RM ANOVA      Matching by cols

Source of Variation	% of total variation	P value
Interaction	31.03	< 0.0001
Time	39.58	< 0.0001
Incubation condition	28.08	< 0.0001
Subjects (matching)	0.2210	0.5356

Source of Variation	P value summary	Significant?
Interaction	***	Yes
Time	***	Yes
Incubation condition	***	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	4088000000000000	1022000000000000	114.0
Time	4	5214000000000000	1303000000000000	145.4
Incubation condition	1	3700000000000000	3700000000000000	508.3
Subjects (matching)	4	2911000000000000	7278000000000000	0.8121
Residual	16	1434000000000000	8962000000000000	
Number of missing values	0			

vii) Table Analyzed: Effect of culture condition 2-*A. culbertsoni*

Two-way RM ANOVA		Matching by cols		
Source of Variation	% of total variation	P value		
Interaction	25.30	< 0.0001		
Time	40.82	< 0.0001		
Incubation condition	32.61	< 0.0001		
Subjects (matching)	0.2180	0.5236		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Time	***	Yes		
Incubation condition	***	Yes		
Subjects (matching)	ns	No		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	3111000000000000	7779000000000000	96.69
Time	4	5019000000000000	1255000000000000	156.0
Incubation condition	1	4010000000000000	4010000000000000	598.3
Subjects (matching)	4	2681000000000000	6703000000000000	0.8332
Residual	16	1287000000000000	8045000000000000	
Number of missing values	0			

viii) Table Analyzed: Effect of culture condition 3-*A. polyphaga*

Two-way RM ANOVA		Matching by cols	
Source of Variation	% of total variation	P value	
Interaction	16.31	< 0.0001	
Time	6.44	0.0025	
Incubation condition	71.22	0.0003	

Subjects (matching)	2.1023	0.1230		
Source of Variation	P value summary		Significant?	
Interaction	***		Yes	
Time	**		Yes	
Incubation condition	***		Yes	
Subjects (matching)	ns		No	
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	4912000000000000	1228000000000000	16.60
Time	4	1939000000000000	4847000000000000	6.553
Incubation condition	1	2144000000000000	2144000000000000	135.5
Subjects (matching)	4	6330000000000000	1583000000000000	2.139
Residual	16	1183000000000000	7397000000000000	
Number of missing values	0			

ix) Table Analyzed: Lm in the presence of *A. castellanii*-32°C

One-way analysis of variance

P value < 0.0001

P value summary \*\*\*

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 250.6

R squared 0.9901

ANOVA Table	SS	df	MS
Treatment (between columns)	2144000000000000	4	5360000000000000
Residual (within columns)	2139000000000000	10	2139000000000000

Total 2165000000000000 14

Tukey's multiple

Comparison	Mean Diff.	q	Significant?		
Test		P < 0.05?	Summary	95%	CI of diff
0 h vs 24 h	19030000	22.54	Yes	***	15100000 to 22960000
0 h vs 48 h	27340000	32.38	Yes	***	23410000 to 31270000
0 h vs 72 h	31070000	36.79	Yes	***	27140000 to 35000000
0 h vs 96 h	32540000	38.54	Yes	***	28610000 to 36470000
24 h vs 48 h	8310000	9.841	Yes	***	4380000 to 12240000
24 h vs 72 h	12030000	14.25	Yes	***	8103000 to 15960000
24 h vs 96 h	13510000	16.00	Yes	***	9577000 to 17440000
48 h vs 72 h	3723000	4.409	No	ns	-206600 to 7653000
48 h vs 96 h	5197000	6.154	Yes	**	1267000 to 9127000
72 h vs 96 h	1473000	1.745	No	ns	-2457000 to 5403000

x) Table Analyzed: Lm in the presence of *A. culbertsoni*-32°C

One-way analysis of variance

P value < 0.0001

P value summary \*\*\*

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 550.4

R squared 0.9955

ANOVA Table SS df MS

Treatment (between columns)	972300000000000	4	243100000000000
Residual (within columns)	441600000000000	10	441600000000000
Total	976800000000000	14	

Tukey's Multiple

Comparison	Mean Diff.	q	Significant?	
Test		P < 0.05	Summary	95% CI of diff
0 h vs 24 h	6433000	16.77	Yes ***	4648000 to 8219000
0 h vs 48 h	17670000	46.05	Yes ***	15880000 to 19450000
0 h vs 72 h	19420000	50.62	Yes ***	17640000 to 21210000
0 h vs 96 h	20030000	52.20	Yes ***	18240000 to 21810000
24 h vs 48 h	11230000	29.28	Yes ***	9448000 to 13020000
24 h vs 72 h	12990000	33.86	Yes ***	11200000 to 14780000
24 h vs 96 h	13590000	35.43	Yes ***	11810000 to 15380000
48 h vs 72 h	1757000	4.579	No ns	-28960 to 3542000
48 h vs 96 h	2360000	6.151	Yes **	574400 to 4146000
72 h vs 96 h	603300	1.573	No ns	-1182000 to 2389000

xi) Table Analyzed: Lm in the presence of *A. polyphaga*-32°C

One-way analysis of variance

P value < 0.0001

P value summary \*\*\*

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 63.12

R squared 0.9619

ANOVA Table	SS	df	MS
Treatment (between columns)	7007000000000000	4	1752000000000000
Residual (within columns)	2776000000000000	10	2776000000000000
Total	7285000000000000	14	

### Tukey's Multiple

Comparison	Mean Diff.	q	Significant?		
Test			P < 0.05?	Summary	95% CI of diff
0 h vs 24 h	15520000	16.14	Yes	***	11050000 to 20000000
0 h vs 48 h	18560000	19.30	Yes	***	14090000 to 23040000
0 h vs 72 h	17580000	18.28	Yes	***	13110000 to 22060000
0 h vs 96 h	15720000	16.34	Yes	***	11240000 to 20200000
24 h vs 48 h	3041000	3.162	No	ns	-1435000 to 7518000
24 h vs 72 h	2062000	2.144	No	ns	-2414000 to 6539000
24 h vs 96 h	196700	0.2045	No	ns	-4280000 to 4673000
48 h vs 72 h	-978900	1.018	No	ns	-5455000 to 3498000
48 h vs 96 h	-2844000	2.957	No	ns	-7321000 to 1632000
72 h vs 96 h	-1866000	1.940	No	ns	-6342000 to 2611000

xii) Table Analyzed: Lm in presence of *A. castellanii*- 32°C+shaking

One-way analysis of variance

P value < 0.0001

P value summary \*\*\*

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 118.4

R squared 0.9793

ANOVA Table	SS	df	MS
Treatment (between columns)	7158000000000000	4	1790000000000000
Residual (within columns)	1511000000000000	10	1511000000000000
Total	7309000000000000	14	

Tukey's Multiple

Comparison	Mean Diff.	q	Significant?		
Test		P < 0.05?	Summary	95% CI of diff	
0 h vs 24 h	-49570000	22.09	Yes	***	-60010000 to -39120000
0 h vs 48 h	-15670000	6.980	Yes	**	-26110000 to -5221000
0 h vs 72 h	-4567000	2.035	No	ns	-15010000 to 5879000
0 h vs 96 h	15730000	7.010	Yes	**	5288000 to 26180000
24 h vs 48 h	33900000	15.10	Yes	***	23450000 to 44350000
24 h vs 72 h	45000000	20.05	Yes	***	34550000 to 55450000
24 h vs 96 h	65300000	29.10	Yes	***	54850000 to 75750000
48 h vs 72 h	11100000	4.946	Yes	*	654800 to 21550000
48 h vs 96 h	31400000	13.99	Yes	***	20950000 to 41850000
72 h vs 96 h	20300000	9.045	Yes	***	9855000 to 30750000

xiii) Table Analyzed: Lm in the presence of *A. culbertsoni*-32°C+shaking

One-way analysis of variance

P value 0.0001

P value summary \*\*\*

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 18.14

R squared 0.8789

ANOVA Table	SS	df	MS
Treatment (between columns)	1678000000000000	4	41940000000000
Residual (within columns)	2313000000000000	10	23130000000000
Total	1909000000000000	14	

Tukey's Multiple

Comparison	Mean Diff.	q	Significant?		
Test			P < 0.05	Summary	95% CI of diff
0 h vs 24 h	-25600000	9.220	Yes	***	-38520000 to -12680000
0 h vs 48 h	-500000	0.1801	No	ns	-13420000 to 12420000
0 h vs 72 h	-20900000	7.527	Yes	**	-33820000 to -7978000
0 h vs 96 h	-6867000	2.473	No	ns	-19790000 to 6055000
24 h vs 48 h	25100000	9.040	Yes	***	12180000 to 38020000
24 h vs 72 h	4700000	1.693	No	ns	-8222000 to 17620000
24 h vs 96 h	18730000	6.747	Yes	**	5812000 to 31660000
48 h vs 72 h	-20400000	7.347	Yes	**	-33320000 to -7478000
48 h vs 96 h	-6367000	2.293	No	ns	-19290000 to 6555000
72 h vs 96 h	14030000	5.054	Yes	*	1112000 to 26960000

xiv) Table Analyzed: Lm in the presence of *A. polyphaga*-32°C+shaking

One-way analysis of variance

P value 0.0019

P value summary \*\*

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 9.545

R squared 0.7924

ANOVA Table	SS	df	MS
Treatment (between columns)	6389000000000000	4	1597000000000000
Residual (within columns)	1673000000000000	10	1673000000000000
Total	8062000000000000	14	

#### Tukey's Multiple

Comparison	Mean Diff.	q	Significant?		
Test			P < 0.05	Summary	95% CI of diff
0 h vs 24 h	-42030000	5.628	Yes	*	-76790000 to -72730000
0 h vs 48 h	-58060000	7.775	Yes	**	-92820000 to -23310000
0 h vs 72 h	-54300000	7.270	Yes	**	-89060000 to -19540000
0 h vs 96 h	-36800000	4.927	Yes	*	-71560000 to -20390000
24 h vs 48 h	-16030000	2.147	No	ns	-50790000 to 18730000
24 h vs 72 h	-12270000	1.642	No	ns	-47030000 to 22490000
24 h vs 96 h	5233000	0.7007	No	ns	-29530000 to 39990000
48 h vs 72 h	3767000	0.5043	No	ns	-30990000 to 38530000
48 h vs 96 h	21270000	2.847	No	ns	-13490000 to 56030000
72 h vs 96 h	17500000	2.343	No	ns	-17260000 to 52260000

**B. Survival of *Listeria* in amoeba-conditioned medium**

i) Table Analyzed: Lm in *A. castellanii* CM vs Lm alone 32°C

Two-way RM ANOVA      Matching by cols

Source of Variation    % of total variation    P value

Interaction    18.39    0.0117

Time    13.06    0.0443

Medium    47.52    0.0009

Subjects (matching)    2.4427      0.6289

Source of Variation    P value summary      Significant?

Interaction                    \*                    Yes

Time                            \*                    Yes

Medium                        \*\*\*                    Yes

Subjects (matching)        ns                    No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	3865000000000	7730000000000	3.957
Time	5	2744000000000	5489000000000	2.810
Medium	1	9988000000000	9988000000000	77.82
Subjects (matching)	4	5134000000000	1283000000000	0.6570
Residual	20	3907000000000	1953000000000	

Number of missing values    0

ii) Table Analyzed: Lm in *A. culbertsoni* CM vs Lm alone-32°C

Two-way RM ANOVA      Matching by cols

Source of Variation    % of total variation    P value

Interaction	27.03	< 0.0001
Time	24.41	< 0.0001
Medium	41.81	< 0.0001
Subjects (matching)	0.6121	0.7369
Source of Variation	P value summary	Significant?

Interaction	***	Yes
Time	***	Yes
Medium	***	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	1805000000000000	361000000000000	17.62
Time	5	1630000000000000	326100000000000	15.91
Medium	1	2793000000000000	2793000000000000	273.3
Subjects (matching)	4	4088000000000000	102200000000000	0.4987
Residual	20	4098000000000000	204900000000000	
Number of missing values	0			

Parameter

iii) Table Analyzed: Lm in *A. polyphaga* CM vs Lm alone-32°C

Two-way RM ANOVA      Matching by cols

Source of Variation	% of total variation	P value
Interaction	18.89	0.0046
Time	17.76	0.0061
Medium	45.08	0.0012
Subjects (matching)	2.6900	0.5026

Source of Variation	P value summary	Significant?
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Interaction	**	Yes
Time	**	Yes
Medium	**	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	3744000000000000	748700000000000	4.853
Time	5	3520000000000000	704000000000000	4.563
Medium	1	8933000000000000	8933000000000000	67.03
Subjects (matching)	4	5330000000000000	1333000000000000	0.8637
Residual	20	3086000000000000	1543000000000000	

Number of missing values 0

iv) Table Analyzed: Lm in *A. castellanii* CM vs Lm alone -32°C+shaking

Two-way RM ANOVA Matching by cols

Source of Variation	% of total variation	P value
Interaction	13.54	0.1036
Time	14.41	0.0869
Incubation condition	35.70	0.0225
Subjects (matching)	10.9269	0.1121

Source of Variation	P value summary	Significant?
Interaction	ns	No
Time	ns	No
Incubation condition	*	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
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Interaction	5	6743000000000000	1349000000000000	2.130
Time	5	7180000000000000	1436000000000000	2.268
Incubation condition	1	1778000000000000	1778000000000000	13.07
Subjects (matching)	4	5443000000000000	1361000000000000	2.150
Residual	20	1266000000000000	6330000000000000	

v) Table Analyzed: Lm *A. culbertsoni* CM vs Lm alone-32°C+shaking

Two-way RM ANOVA      Matching by cols

Source of Variation    % of total variation    P value

Interaction	23.27	< 0.0001
Time	20.76	< 0.0001
Culture type	45.52	0.0011
Subjects (matching)	2.5431	0.2115

Source of Variation    P value summary      Significant?

Interaction	***	Yes
Time	***	Yes
Culture type	**	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	1761000000000000	3522000000000000	11.76
Time	5	1571000000000000	3142000000000000	10.49
culture type	1	3445000000000000	3445000000000000	71.59
Subjects (matching)	4	1925000000000000	4811000000000000	1.607
Residual	20	5988000000000000	2994000000000000	

Number of missing values    0

vi) Table Analyzed: Lm in *A. polyphaga* CM vs Lm alone-32°C +Shaking

Two-way RM ANOVA      Matching by cols

Source of Variation	% of total variation	P value
Interaction	18.48	< 0.0001
Time	16.84	< 0.0001
Culture type	57.71	< 0.0001
Subjects (matching)	0.9219	0.5623

Source of Variation	P value summary	Significant?
Interaction	***	Yes
Time	***	Yes
Culture type	***	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	4121000000000000	824300000000000	12.21
Time	5	3757000000000000	751400000000000	11.13
Culture type	1	12870000000000000	12870000000000000	250.4
Subjects (matching)	4	2057000000000000	514100000000000	0.7619
Residual	20	1350000000000000	674800000000000	
Number of missing values	0			

**C. Intracellular survival of *Listeria* in *Acanthamoeba***

i) Table Analyzed: Lm Scott A +Mn<sup>2+</sup>/*A.castellanii* vs Lm/*A.castellanii*

Two-way RM ANOVA      Matching by cols

Source of Variation	% of total variation	P value
Interaction	26.21	< 0.0001
Time	57.43	< 0.0001
Treatment	13.87	0.0007
Subjects (matching)	0.6367 0.1864	

Source of Variation	P value summary	Significant?
Interaction	***	Yes
Time	***	Yes
Treatment	***	Yes
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	3042000000	608400000	56.45
Time	5	6666000000	1333000000	123.7
Treatment	1	1610000000	1610000000	87.14
Subjects (matching)	4	73900000	18470000	1.714
Residual	20	215600000	10780000	

Number of missing values    0

ii) Table Analyzed: Lm Scott A+Mn<sup>2+</sup>/*A. polyphaga* vs Lm/*A. polyphaga*

Two-way RM ANOVA      Matching by cols

Source of Variation    % of total variation    P value

Interaction	0.73	0.9694
Time	78.72	< 0.0001
Treatment	0.00	0.9831
Subjects (matching)	3.6307	0.3960
Source of Variation	P value summary	Significant?

Interaction	ns	No
Time	***	Yes
Treatment	ns	No
Subjects (matching)	ns	No

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	167400000	33490000	0.1735
Time	5	17960000000	3592000000	18.61
Treatment	1	105200	105200	0.0005079
Subjects (matching)	4	828400000	207100000	1.073
Residual	20	3860000000	193000000	
Number of missing values	0			

iii) Table Analyzed: Lm Scott A/A. *castellanii*

One-way analysis of variance

P value < 0.0001

P value summary \*\*\*

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 78.27

R squared 0.9690

ANOVA Table	SS	df	MS
Treatment (between columns)	1950000000	4	487600000
Residual (within columns)	62290000	10	6229000
Total	2013000000	14	

#### Tukey's Multiple

Comparison	Mean	Diff.	q	Significant?	Test	P < 0.05?	Summary	95% CI of diff
0 h vs 4 h	26230	18.21	Yes	***			19530 to 32940	
0 h vs 24 h	21650	15.02	Yes	***			14940 to 28350	
0 h vs 48 h	24750	17.18	Yes	***			18050 to 31460	
0 h vs 72 h	2367	1.642	No	ns			-4339 to 9073	
4 h vs 24 h	-4587	3.183	No	ns			-11290 to 2119	
4 h vs 48 h	-1480	1.027	No	ns			-8186 to 5226	
4 h vs 72 h	-23870	16.56	Yes	***			-30570 to -17160	
24 h vs 48 h	3107	2.156	No	ns			-3599 to 9813	
24 h vs 72 h	-19280	13.38	Yes	***			-25990 to -12570	
48 h vs 72 h	-22390	15.54	Yes	***			-29090 to -15680	

iv) Table Analyzed: Lm Scott A+Mn<sup>2+</sup>/*A.castellanii*

One-way analysis of variance

P value < 0.0001

P value summary \*\*\*

Are means signif. different? (P < 0.05) Yes

Number of groups 6

F 77.60

R squared 0.9700

ANOVA Table	SS	df	MS
Treatment (between columns)	7346000000	5	1469000000
Residual (within columns)	227200000	12	18930000
Total	7573000000	17	

### Tukey's Multiple

Comparison	Mean	Diff.	q	Significant?	P	
Test				< 0.05?	Summary	95% CI of diff
0h vs 4h	52630	20.95		Yes	***	40690 to 64560
0h vs 24 h	49430	19.68		Yes	***	37490 to 61360
0h vs 48 h	6300	2.508		No	ns	-5635 to 18230
0h vs 72 h	36800	14.65		Yes	***	24870 to 48730
0h vs 96h	37230	14.82		Yes	***	25300 to 49170
4h vs 24 h	-3200	1.274		No	ns	-15130 to 8735
4h vs 48 h	-46330	18.44		Yes	***	-58260 to -34390
4h vs 72 h	-15830	6.300		Yes	**	-27760 to -3892
4h vs 96h	-15390	6.128		Yes	**	-27330 to -3458
24 h vs 48 h	-43130	17.17		Yes	***	-55060 to -31190
24 h vs 72 h	-12630	5.026		Yes	*	-24560 to -691.7
24 h vs 96h	-12190	4.854		Yes	*	-24130 to -258.3
48 h vs 72 h	30500	12.14		Yes	***	18570 to 42430
48 h vs 96h	30930	12.31		Yes	***	19000 to 42870
72 h vs 96h	433.3	0.1725		No	ns	-11500 to 12370

v) Table Analyzed: Lm Scott A/ *A. polyphaga*

One-way analysis of variance

P value 0.0161

P value summary \*

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 5.164

R squared 0.6738

ANOVA Table	SS	df	MS
Treatment (between columns)	6973000000	4	1743000000
Residual (within columns)	3376000000	10	337600000
Total	10350000000	14	

Tukey's Multiple

Comparison Mean Diff. q Significant?

Test	P < 0.05? Summary				95% CI of diff
0 h vs 4 h	54840	5.170	Yes	*	5476 to 104200
0 h vs 24 h	54550	5.142	Yes	*	5179 to 103900
0 h vs 48 h	53730	5.066	Yes	*	4366 to 103100
0 h vs 72 h	52300	4.930	Yes	*	2929 to 101700
4 h vs 24 h	-296.7	0.02797	No	ns	-49660 to 49070
4 h vs 48 h	-1110	0.1046	No	ns	-50480 to 48260
4 h vs 72 h	-2547	0.2401	No	ns	-51910 to 46820
24 h vs 48 h	-813.3	0.07667	No	ns	-50180 to 48550

24 h vs 72 h	-2250	0.2121	No	ns	-51620 to 47120
48 h vs 72 h	-1437	0.1354	No	ns	-50800 to 47930

vi) Table Analyzed: Lm Scott A+Mn<sup>2+</sup>/A. polyphaga

One-way analysis of variance

P value 0.0001

P value summary \*\*\*

Are means signif. different? (P < 0.05) Yes

Number of groups 5

F 18.71

R squared 0.8822

ANOVA Table	SS	df	MS
Treatment (between columns)	9831000000	4	2458000000
Residual (within columns)	1313000000	10	131300000
Total	11140000000	14	

Tukey's Multiple

Comparison Mean Diff. q Significant?

Test		P < 0.05?	Summary	95% CI of diff
0 h vs 4 h	65240 9.861	Yes	***	34450 to 96040
0 h vs 24 h	64530 9.753	Yes	***	33740 to 95320
0 h vs 48 h	59210 8.949	Yes	***	28420 to 90000
0 h vs 72 h	65920 9.963	Yes	***	35130 to 96710
4 h vs 24 h	-713.3 0.1078	No	ns	-31510 to 30080

4 h vs 48 h	-6033	0.9119	No	ns	-36830 to 24760
4 h vs 72 h	675.3	0.1021	No	ns	-30120 to 31470
24 h vs 48 h	-5320	0.8041	No	ns	-36110 to 25470
24 h vs 72 h	1389	0.2099	No	ns	-29400 to 32180
48 h vs 72 h	6709	1.014	No	ns	-24080 to 37500

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