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Evaluation of bacterial ligands involved in receptor-mediated phagocytosis in *Tetrahymena pyriformis*

Mara Adela Boboc

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Declaration

This dissertation is entirely my own work and has not been submitted in full or in part for the award of a higher degree at any other educational institution.

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ABSTRACT

This study examined the feeding of *Tetrahymena pyriformis* on eleven strains of bacteria evaluating the importance of receptor-ligand interactions in prey uptake and food vacuole formation. Three prey 'types' were employed: live cells (full complement of surface ligands), heat-killed (dead) cells (disrupted receptor ligands) and, latex beads (no ligands). The ciliates were fed for five minutes with each prey type in monocultures (2×10^7 cells/ml) and in 50:50 mixtures and the number of vacuoles/cell, prey/cell and prey/vacuole were determined after fixation of the ciliates.

Prey uptake and vacuole formation in *T. pyriformis* was highest with live cells, followed by dead cells and then beads; except *Synechococcus* sp., *Serratia marcescens* and *Staphylococcus aureus* which behaved like beads after heat-treatment. There was no evidence of *T. pyriformis* actively selecting one prey type over another in any of the 50:50 mixtures, and the ciliate showed to deposit different prey types in the same vacuole, suggesting only one route of internalization of different types of prey.

Significant trends regarding vacuole formation were observed in prey mixtures: (i) live and dead cells always controlled vacuole formation in mixture with beads, and (ii) live and dead cells together showed a synergistic effect on vacuoles/cell (but did not show synergy with regards to prey uptake). The latter observation led to the proposal that different receptor-ligand interactions might be in place for vacuole formation (involving a vacuole formation factor, VFF) and prey uptake (involving a prey uptake factor, PUF). Moreover, when heat-stabilities of PUF and VFF were analysed, data for *Pseudomonas aeruginosa* (VFF heat-stable, PUF heat-labile) and *Salmonella enterica* 12694, *Pseudomonas fluorescens*, *Klebsiella pneumoniae* (VFF heat-labile, PUF heat-stable) also suggested distinct PUF and VFF factors, even if similar heat-stabilities were recorded for most bacteria tested (either both intact, partially or completely destroyed).

Removal of *A. hydrophila*, *S. marcescens* and *Synechococcus* sp. S-layers by lithium chloride showed no direct implication of this ligand in prey uptake by *T. pyriformis*. However, experiments

involving trypsin and formaldehyde fixation, which damage cell-associated proteins but not carbohydrates, suggested that the uptake could be mediated by a carbohydrate ligand (for Gram-negative bacteria) or by a protein (for Gram-positive bacteria). Analysis of capsular heat stability of all strains tested suggested that capsules could be positively involved in the uptake of Gram-negative bacteria by the ciliate, but not Gram-positive.

Finally, the presence of sugar receptors and scavenger receptors in *T. pyriformis* was examined. Pre-incubation of *T. pyriformis* with up to 200mM sugar concentration of mannose, N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) suggested that receptors with binding affinities for all three sugars are involved in uptake of live and dead *S. enterica* 74. The uptake of live *S. enterica* 74 was higher than dead cells and beads until a particular sugar concentration which led them to become equivalent to dead cells (Mannose at 20mM, GalNAc at 50mM and GlcNAc at 125mM), and then at an even higher sugar concentration, when the uptake became equivalent to beads (Mannose at 175mM; GalNAc/GlcNAc at 150mM). A similar trend was observed for vacuole formation, suggesting a sequential blocking of receptors for the attachment to live cells. No evidence was found for the involvement of scavenger receptors in the uptake of beads or live and dead Gram-negative *S. enterica* 74 by *T. pyriformis*.

CHAPTER 1: INTRODUCTION

1.1 General introduction to Protists

Protists are unicellular eukaryotic cells ranging from 1 to 5000 μm , with a wide variety of cell shapes, locomotion types, feeding mechanisms and cellular membrane composition. They are either free-living, parasitic or endosymbionts and consist of groups commonly referred to as protozoa, algae and slime molds (reviewed in Corliss, 2002; Roger and Simpson, 2009).

Free-living protists are ubiquitous (Lee and Patterson, 1998; Fenchel and Finlay, 2004; Foissner, 2006; Foissner et al., 2008; Simon et al., 2015), existing in both the plankton and in biofilms (Parry, 2004), and in environments which have extreme ranges of temperature (Amaral-Zettler et al., 2002; Lovejoy et al., 2006; Wolf et al., 2015), oxygen levels (Dawson and Pace, 2002), salinity (Esteban and Finlay, 2003; Kolisko et al., 2010; Laybourn-Parry and Bell, 2014), and pH (Moser and Weisse, 2011). Their biodiversity has been studied since the 17th century and while molecular tools and high-throughput sequencing have helped in identifying new species, the taxonomic classification of protists still remains problematic, mainly because of the high number of organisms that have yet to be discovered (Adl et al., 2007; Cuvelier et al., 2008).

Most protists are phagocytic heterotrophs, while photoautotrophic and mixotrophic species are found in a lower abundance (Raven, 1997; Raven et al., 2009; Lee et al., 2014; Mitra et al., 2014; Mitra et al., 2016). There are three 'types' of free-living protists, recognized on the basis of their mode of feeding and locomotion. Amoebae use pseudopodia to crawl over solid surfaces and engulf bacteria. Flagellates use their undulating flagellum/flagella to generate water currents for swimming and to draw food towards the cell where it is ingested with pseudopodia. Ciliates use multiple cilia for swimming and drawing food towards their cytostome (mouth) (Berger and Corliss, 1979; Cavalier-Smith, 1998, 2002; Pawlowski and Burki, 2009), where poly-kinetid membranelles push bacterial cells toward the oral cavity (Fenchel, 1987; Verni and Gualtieri, 1997). Of all three types, the ciliates are the most diverse and complex.

Ciliates consist of more than 8,000 morphospecies and are often considered some of the most evolved free-living protists (Foissner and Berger, 1996; Foissner, 2008). They are characterized by the large number of cilia on their surface membrane which confers motility and helps in prey grazing. Ciliates are found primarily in marine and freshwater environments (Foissner, 2006; Weisse, 2007; Lischke et al., 2015; Liu et al., 2017) and most of them are heterotrophic, with a high bacterial ingestion rates in both pelagic and benthic zones (Fenchel, 1987). As the cell size is normally larger than flagellates and amoebae, ciliates are able to consume a wider range of prey (Jezbera et al., 2005), including algae, bacterial cells, bacterial aggregates and other microzooplankton (Parry, 2004), making them important predators of bacteria in aquatic and terrestrial ecosystems (Sherr and Sherr, 1987, 2002).

Protists play an essential role in nature due to: (i) their contribution in nutrient recycling as part of the 'microbial loop'; (ii) their effect on bacterial communities by consumption of bacterioplankton and, (iii) their role as food for metazoan zooplankton (Stoecker and Capuzzo, 1990; Verity and Vernet, 1992; Sautour et al., 2000; Calbet and Saiz, 2005).

The microbial loop describes the trophic process at the basis of the aquatic food web in which dissolved organic matter is returned to the higher trophic levels by incorporation of bacterial biomass through feeding processes (Pomeroy, 1974; Azam et al., 1983). Heterotrophic protists, particularly flagellates and ciliates, are the major consumers of bacteria in both aquatic and terrestrial ecosystems (Sherr and Sherr, 2002, 2007; Calbet and Landry, 2004). Their high grazing rates contribute to higher rates of bacterial mortality (Sanders et al., 1992; Sherr and Sherr, 2002), but they also increase the release of undigested products and organic and inorganic matter into the environment (Nagata and Kirchman, 1992; Strom et al., 1997; Nagata, 2000), including ammonia and phosphorus (Caron and Goldman, 1990; Dolan, 1997).

Grazing on bacterial communities can significantly affect bacterial biomass and alter the morphology and taxonomic composition of their communities (Jürgens and Sala, 2000; Hahn and Hoefle, 2001; Jürgens and Matz, 2002; Matz and Kjelleberg, 2005; Pernthaler, 2005), including changes in biofilms (Parry et al., 2007; Boehme et al., 2009). As bacteria and protists have co-

evolved for almost 1.5 billion years (Javaux et al., 2001), these interactions have been important driving forces for bacterial evolution (Jürgens and Matz, 2002; Matz and Kjelleberg, 2005; Erken et al., 2013). Even if grazing on bacteria increases the nutrient release by protists as a result of 'slopping feeding' (e.g., peduncle feeding of dinoflagellates), which in return stimulates the growth of some microbial communities (Matz and Jürgens, 2003), most of the time intense grazing by heterotrophic protists puts selection pressure on the evolution of virulence factors and defense mechanisms in bacteria, increasing the number of pathogenic microbes in the environment (Fenchel, 1987; Hahn and Hoefle, 2001; Sherr and Sherr, 2002). The traits that provide grazing resistance can also enhance protection and pathogenicity against other phagocytic cells like mammalian immune cells (macrophages, neutrophils, dendritic cells) (Brown and Barker, 1999), or against other eukaryotic hosts like plants and animals (Adiba et al., 2010).

1.2 Phagocytosis

Cells have evolved multiple mechanisms for particle engulfment which include pinocytosis, clathrin-dependent endocytosis and phagocytosis (Silverstein, 1995; Allen and Aderem, 1996b). Phagocytosis is the actin-dependent engulfment of a particle, larger than 0.5 μ m, which is recognized by receptors on the phagocytic cell (Aderem and Underhill, 1999). Protists use phagocytosis for the acquisition of food (Cardelli, 2001) whereas in higher organisms, specialised phagocytes such as macrophages and dendritic cells play a central role in tissue turnover and disease by the recognition and destruction of pathogens, apoptotic and necrotic cells (Brown et al., 2015).

Phagocytosis in both specialised phagocytes and protists follow the same well-organized multi-step process: the particle binds to the phagocytic receptor(s) which initiates rearrangement and polymerization of actin filaments and membrane trafficking inside the phagocyte (Aderem and Underhill, 1999; Greenberg and Grinstein, 2002). The particle is then internalized, and an early phagosome is formed. This matures and fuses with endosomes and lysosomes to become a phagolysosome, which is a highly acidic hydrolytic vacuole (Figure 1.1).

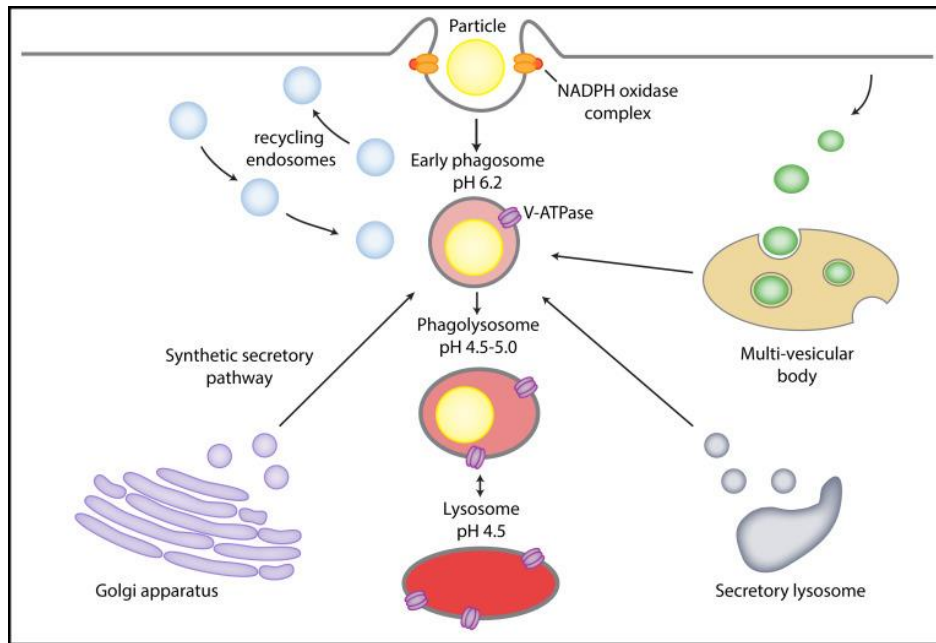


Figure 1.1. *Prey processing in macrophages.* The particle is first recognized by receptors found on the cell membrane and it is engulfed into an early phagosome. Once lysosomes fuse with the phagosome it becomes a phagolysosome and the particle is degraded by the increased acidic environment, and the presence of digestive enzymes in this vacuole (Figure taken directly from Russell, 2011).

The lower pH in the phagolysosome is due to the presence of reactive oxygen and nitrogen species, formed by the action of membrane-bound NADPH oxidase complexes and V-ATPase pumps (Forgac, 1999; Cross and Segal, 2004), and enzymes such as acid phosphatases (Eskelinen et al., 2003) and cysteine proteases (Temesvari et al., 1999). All these steps help in breaking down the ingested particle after which, the undigested material is in ciliates, excreted at the cytoproct (Fenchel, 1987; Verni and Gualtieri, 1997) (Figure 1.2). The phagosome membrane then fuses with the external membrane and it is re-cycled to form new phagosomes/food vacuoles. Vacuole maturation and recycling are all facilitated by actin filaments (Sugita et al., 2009; Botelho and Grinstein, 2011).

The first and crucial step in phagocytosis is the recognition and binding to the prey particle by the phagocyte. Given its importance in the medical area, more research has been directed at how phagocytes in the mammalian's immune system recognize particles and trigger the appropriate response (Chaplin, 2003; Vance et al., 2009) while less attention has been given to the molecular mechanisms of prey recognition in protists (Hamels et al., 2004; Montagnes et al., 2008).

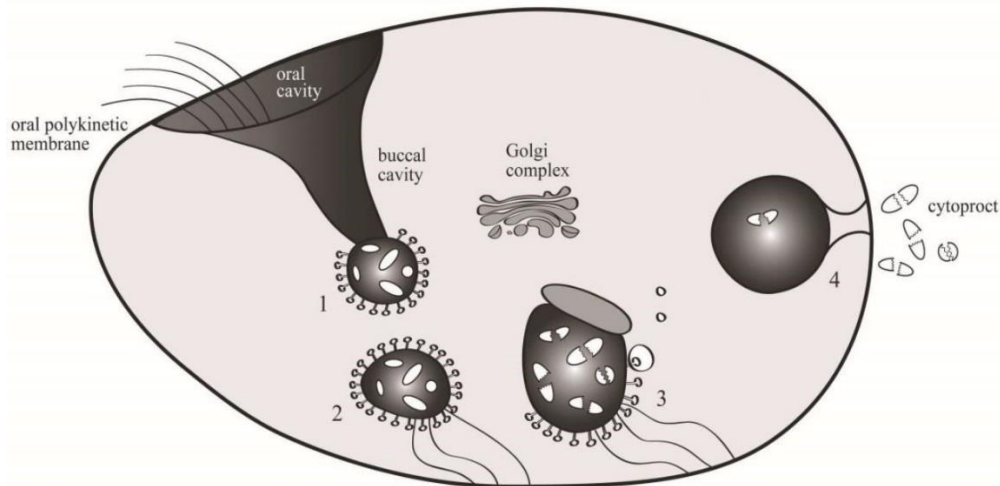


Figure 1.2. *Prey processing in Tetrahymena pyriformis.* The oral membrane is packed with cilia which generate water currents to draw in prey from suspension. 1) The particle enters the oral cavity and the food vacuole forms around the prey (phagosome); 2) The food vacuole is pulled away from the cytostome by the actin filaments (Allen and Aderem, 1996a); 3) Lysosomes from the Golgi apparatus attach and form the phagolysosome, which releases hydrolytic enzymes that degrade the prey's cellular membrane (Nilsson, 1977); 4) Once all the nutrients are extracted from the prey, the food vacuole travels towards the cytoproct area; the undigested material is excreted and the empty vacuole fuses with the cellular membrane (Fenchel, 1987) (adapted from Lutz, 2014).

Particle recognition involves a complex network of interacting receptors called Pattern Recognition Receptors (PRRs) that are either soluble or bound to the membrane of the phagocyte (Janeway, 1989, 1992; Cambi et al., 2005). The main PRRs present in mammalian cells are shown in Table 1.1. Even though these PRRs are commonly found in phagocytes, not all are activating factors for phagocytic processes, e.g., Toll-like receptors, NOD-like receptors and Peptidoglycan recognition proteins (Takeda and Akira, 2005; Kashyap et al., 2011). Instead, these 'PRRs' *modulate* the phagocytic response but cannot initiate particle engulfment once the phagocytic cell binds to the particle. True PRRs have the ability to recognize evolutionary conserved bacterial motifs termed Pathogen-Associated Molecular Patterns (PAMPs) (reviewed in Janeway and Medzhitov, 2002; Underhill and Ozinsky, 2002).

Table 1.1. *Main pattern recognition receptors found in mammalian phagocytes.* *C-type lectin receptors. NOD-like receptors= Nucleotide oligomerization domain-like receptors; PGRP= Peptidoglycan recognition proteins; Mac-1=Macrophage-1 antigen; DC-SIGN= Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin. Dectin receptors bind to β -glucans from fungal cell walls (East and Isacke, 2002), while the other C-type lectins bind bacterial sugar entities.

Receptor type	Role in mammalian phagocyte	Reference
Toll-like receptors	Pro-inflammatory receptors, mostly transmembrane	Takeda and Akira, 2005; Ribes et al., 2010
NOD-like receptors	Pro-inflammatory cytosolic receptors	Kim et al., 2004; Le Bourhis et al., 2007
PGRPs	Activates a bactericidal response upon binding with bacteria	Dziarski, 2004; Royet et al., 2011
Fc-mediated receptors	Opsonic phagocytosis	Huber et al., 1976
Complement receptors		Janeway et al., 2001
Mac-1 integrin	Both opsonic and non-opsonic phagocytosis	Chen et al., 2010; Xie et al., 2010
Mannose receptor*	Non-opsonic phagocytosis	Stahl and Ezekowitz, 1998
Dectin receptor*		East and Isacke, 2002
DC-SIGN receptor*		Feinberg et al., 2001; Geurtsen et al., 2010
Scavenger receptors		Areschoug and Gordon, 2009; Peruń et al., 2016

1.3 Pathogen-Associated Molecular Patterns (PAMPs)

PAMPs can be a large variety of ligands, from bacterial capsules, lipopolysaccharides, peptidoglycan, lipoteichoic acids and lipoproteins (Schwandner et al., 1999; Fiset et al., 2003; Albiger et al., 2007), to flagellin (Hayashi et al., 2001), microbial DNA (Hemmi et al., 2000) and mannans in the yeast walls (Lebrun et al., 2003). Of these, the bacterial lipopolysaccharide layer, peptidoglycan and lipoteichoic acids are the most common activators of phagocytic cells in mammals, and due to their location on the cell surface, have the potential to be important ligands for attachment to protozoa.

1.3.1 Lipopolysaccharides

Lipopolysaccharides (LPS) are vital components for the structural and functional integrity of the outer membrane of Gram-negative bacteria (Rietschel et al., 1996). Due to its expression on the cell surface, and its absence in Gram-positive bacteria, LPS serves as a primary target for the innate immune system cells, such as macrophages, to recognize and ingest Gram-negative bacteria (Holst et al., 1992).

LPS comprises three covalently linked structures (Figure 1.3): (i) lipid A, a highly hydrophobic component anchored in the outer membrane, (ii) the *O*-antigen (or *O*-polysaccharide chain), which is the outermost part of the LPS and extends out from the cell surface, and (iii) the core oligosaccharide, which links lipid A to the *O*-antigen (Rietschel et al., 1994). Two types of LPS have been described: smooth (S)-LPS, which has all three regions, and rough (R)-LPS, which lacks the *O*-antigen (Alexander and Rietschel, 2001; Caroff and Karibian, 2003; Steimle et al., 2016). Some bacteria such as *Yersinia pestis* (Knirel et al., 2005), *Campylobacter jejuni* (Kilcoyne et al., 2014) and *Stenotrophomonas maltophilia* (Rahmati-Bahram et al., 1996), express both forms of LPS, depending on temperature; a modification that is believed to help the bacteria survive the elevated temperatures inside the human body. The amoeba *Naegleria gruberi* has been shown to have reduced feeding on rough-LPS *Salmonella* strains while the ciliate *Tetrahymena pyriformis* exhibits no preference for rough or smooth *Salmonella* strains (Wildschutte et al., 2004).

LPS is highly diverse, mainly due to the O-polysaccharide region which consists of one to eight glycosyl residues and differs between strains by means of its repeating carbohydrate composition, positions of bonds and location of non-sugar substituents (Wang et al., 2010). The outer core is also variable between bacterial strains, being formed by common sugars such as mannose, glucose, galactose, N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc) (Holst, 2011). In contrast, the polysaccharide portion of lipid A is highly conserved and composed of a $\beta(1-6)$ -linked glucosamine disaccharide backbone which is mostly phosphorylated at positions 1' and 4' of the saccharides and acylated at positions 2' and 3' of each monosaccharide (Homma et al., 1985; Galanos and Freudenberg, 1993).

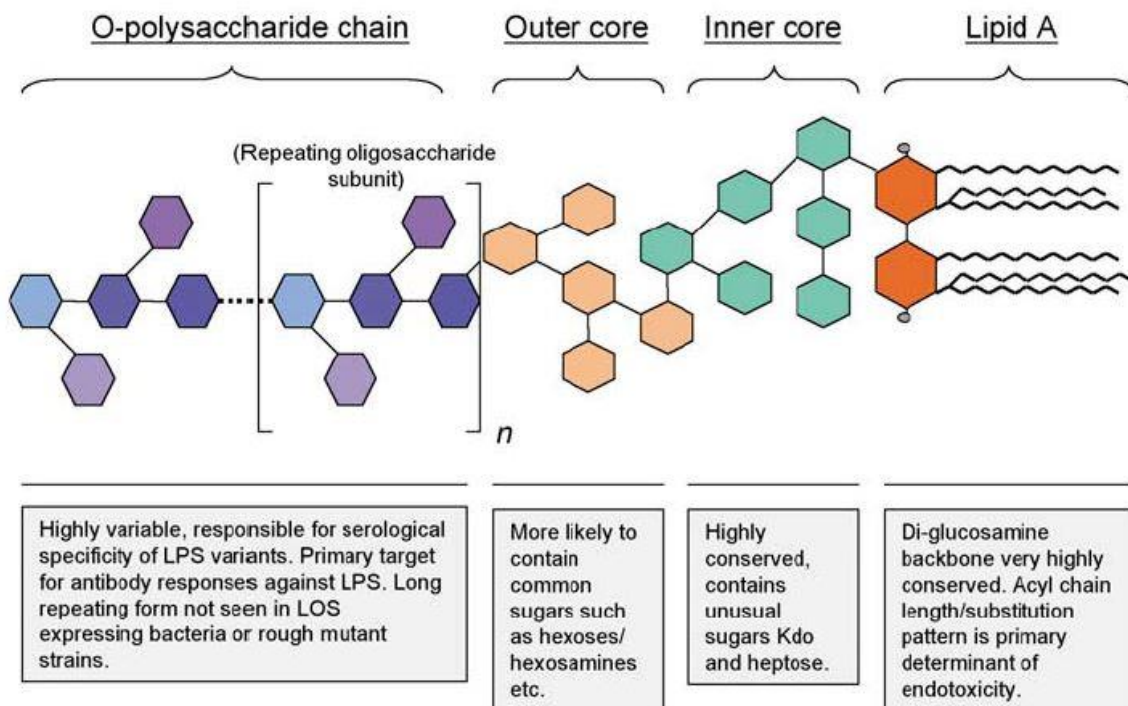


Figure 1.3. Lipopolysaccharide structure in Gram-negative bacteria (Erridge et al., 2002).

All three LPS components are important in recognition and uptake by macrophages. Firstly, the brain angiogenesis inhibitor 1 (BAI1) receptor expressed on the macrophage cell surface (Park et al., 2007) was shown to bind specifically to the core oligosaccharide of *Escherichia coli* and *Salmonella* spp. which was demonstrated by experiments with mutants lacking either the O-

polysaccharide (*E. coli*) or the LPS core (*Salmonella*) (Das et al., 2011). No Gram-positive strain tested (*Staphylococcus aureus*, *Streptococcus* spp. and *Streptococcus pneumoniae*) bound to the receptor, suggesting that BAI1 selectively recognizes LPS of Gram-negative bacteria (Das et al., 2011). Similarly, DC-SIGN, a transmembrane C-type lectin which promotes adherences and phagocytosis of bacteria in dendritic cells (Zhang et al., 2006a) and alveolar macrophages (Soilleux et al., 2002), interacts directly to the core region of *E. coli* LPS (Klena et al., 2005). A later study demonstrated that this interaction occurs between the GlcNAc residues in the core LPS of *E. coli* K12, *Haemophilus ducreyi*, *Neisseria gonorrhoeae* or *Salmonella typhimurium*, and not mannose residues, which are known ligands of this receptor, because none of these bacteria possessed mannose residues in their core polysaccharide (Zhang et al., 2006b).

On the other hand, two well-described phagocytic receptors, the Mannose receptor and the Scavenger receptor A (SR-A), were shown to bind to the O-antigen of *Klebsiella pneumoniae* (Zamze et al., 2002) and the lipid A of *E. coli* (Hampton et al., 1991) respectively. Further information on these two receptors is presented in Sections 1.4.1 and 1.4.2.

1.3.2 Peptidoglycan

Peptidoglycan (PG) is an essential cell wall component of both Gram-positive and Gram-negative bacteria, that preserves the cell's integrity and serves as anchoring layer for other cell membrane components such as teichoic acids (Rogers et al., 1980). PG is a glycoprotein oligomer that comprises 30-70% of cell walls of Gram-positive bacteria but only 10% of the cell walls of Gram-negative bacteria, constituting a thin layer between the cytoplasmic membrane and LPS (Schleifer and Kandler, 1972; Rosenthal and Dziarski, 1994; Beveridge, 1999). Both Gram-positive and Gram-negative PG consists of a polymer of $\beta(1-4)$ -linked N-acetylglucosamine (NAG/GlcNAc) and N-acetylmuramic acid (NAM/MurNAc), crossed-linked by short peptides bridges (Schleifer and Kandler, 1972). While the overall composition and biogenesis of the PG is conserved in all bacteria, interspecies variations occur in the glycan strands length, in amino acids positions and composition of the peptide stems (Doyle and Dziarski, 2001).

Binding of professional phagocytes to PG has been documented to have a pro-inflammatory effect, rather than activating phagocytosis of bacteria, mainly due to intense interactions with (i) Toll-like receptors and their co-receptor CD14 (Kusunoki et al., 1995; Takeuchi et al., 1999; Dziarski et al., 1998), (ii) cytosolic NOD-like receptors (Girardin et al., 2003; Viala et al., 2004), or (iii) Peptidoglycan recognition proteins (PGRPs) (Kang et al., 1998; Liu et al., 2001; Dziarski, 2004).

No information could be found regarding transmembrane C-type lectins such as the Mannose receptor and DC-SIGN, or indeed Scavenger receptors, binding to PG moieties without opsonization, in mammalian phagocytes. However, in the fruit fly *Drosophila melanogaster*, two phagocytic receptors were suggested to bind to PG and activate phagocytosis, i.e., the peptidoglycan recognition protein LC (Rämet et al., 2002) and *Eater*, a scavenger receptor (Chung and Kocks, 2011). The exact PG component that binds to these receptors is not yet identified, as PG samples were often contaminated with other cell wall components such as lipoteichoic acids (Travassos et al., 2004), which can activate receptors on their own.

1.3.3 Lipoteichoic acids (LTAs)

LTAs are amphiphilic polymers hydrophobically attached via lipid anchors to the cytoplasmic membrane of Gram-positive bacteria and comprise alternating units of polyhydroxy alkane joined by phosphodiester bonds (Fischer, 1990). LTAs extend into the peptidoglycan until they reach the cell surface (Fischer, 1990, 1994) where, together with wall teichoic acids (WTA) which are not anchored into the cytoplasmic membrane, form a negatively charged network that extends on the surface membrane of all Gram-positive bacteria (Greenberg et al., 1996). Functionally, LTA is analogous to the LPS in Gram-negative bacteria, whereby both maintain membrane integrity (Fischer, 1988), control autolytic enzymes (Neuhaus and Baddiley, 2003) and serve as ligands for innate immune cells (Wicken and Knox, 1975; Ginsburg, 2002; Weidenmaier and Peschel, 2008; Percy and Gründling, 2014).

Four types of LTA (I-IV) have been described (Table 1.2) (Percy and Gründling, 2014). The most common is type I LTA, which is found in most Gram-positive bacteria such as *S. aureus* (Duckworth

et al., 1975; Kiriukhin et al., 2001), *Bacillus subtilis* (Schirner et al., 2009), *Streptococcus* sp. (Koch et al., 1984) and *Listeria monocytogenes* (Uchikawa et al., 1986).

Only two phagocytic receptors have demonstrated binding to un-opsonized LTA: Complement receptors (CRs) and Scavenger receptors (SRs). Zeng et al. (2016) showed that CRs from Kupffer cells, a subtype of tissue-resident macrophages located in the liver, bound directly to the LTA of *S. aureus*, *L. monocytogenes* and *Bacillus cereus*. Addition of purified LTA showed a dose-dependent reduction in CR-*S. aureus* binding, with 23-58µM completely blocking binding to both mouse and human CRs. *S. aureus* mutants lacking LTA were incapable of binding to CRs independent of opsonisation (Zeng et al., 2016) and peptidoglycan itself showed no binding to CRs (Zeng et al., 2016).

Table 1.2. Main types of bacterial Lipoteichoic acids (LTAs) and their structural characteristics. Type II LTA was found in *Streptococcus lactis* strain Kiel 42172, while type III LTA was found in *Clostridium innocuum*. *only found so far in *Clostridium difficile* and *Peptostreptococcus anaerobius*; ~only found in *Streptococcus pneumoniae*.

LTA type	Structural characteristics	References
Type I	Unbranched 1-3 linked glycerolphosphate (Gro-P) backbone with glucolipid anchor	Koch et al., (1984) and others mentioned in text
Type II	α-Gal(1-6)-α-Gal(1-3)- Gro-P repeating unit in the backbone	Koch and Fischer, 1978
Type III	α-Gal(1-3)-Gro-P repeating unit and an αβ-glucosamine(1-3)- α-Glc(1-3)-diacylglycerol lipid anchor	Fischer, 1990
Type IV	* α-D-N-acetylglucosamine (GlcNAc)- α-DGlcNAc repeating units OR ~ pentasaccharide 2-acetamino-4-amino-2,4,6-trideoxygalactose, glucose, and ribitolphosphate followed by two N-acetylgalactosamine moieties	Reid et al., 2012 Stortz et al., 1990, Gisch et al., 2013

Three Scavenger receptors have also been shown to recognize LTA: SR-A (Dunne et al., 1994; Thomas et al., 2000; Peiser et al., 2002), the macrophage receptor with collagenous structure (MARCO) (van der Laan et al., 1997; Arredouani et al., 2004) and CD36, a class B Scavenger receptor (Hoebe et al., 2005; Stuart et al., 2005). While transfected cells with CD36 or SR-A gave non-phagocytic cells the ability to bind both Gram-positive and Gram-negative bacteria (Peiser et al., 2000; Stuart et al., 2005), macrophages deficient in either CD36 or SR-A showed an impaired ability to phagocytose *S. aureus* (Peiser et al., 2000; Stuart et al., 2005).

1.3.4 Bacterial alteration of PAMPS

Some strains of bacteria have developed resistance to phagocytosis by modifying their PAMPS.

1.3.4.1 Modifications in LPS

Modifications in LPS occur in any of the three structural components: lipid A, core oligosaccharide or the *O*-antigen. Most modifications of LPS are present in the (normally) highly conserved lipid A (Raetz et al., 2007). These changes can be subdivided in four categories: (i) modification of the phosphate residue (addition or substitution of phosphate groups; dephosphorylation) as observed in *Neisseria meningitidis* (Cox et al., 2003), *S. typhimurium* (Helander et al., 1994; Trent et al., 2001a; Reynolds et al., 2006), *Salmonella* spp. (Bentala et al., 2002) and *E. coli* (Touzé et al., 2007), (ii) alterations in the fatty acyl chain length or residues found in *E. coli* (Jia et al., 2004), *S. typhimurium* (Guo et al., 1998), *Legionella pneumophila* (Robey et al., 2001) and *Bordetella bronchiseptica* (Pilione et al., 2004), (iii) addition of aminoarabinose as observed in *S. typhimurium* (Guo et al., 1997; Ernst et al., 2001) and *E. coli* (Trent et al., 2001b) and, (iv) incorporation of palmitate, mostly found in *E. coli* K12 and *S. typhimurium* (Brozek et al., 1987; Bishop et al., 2000).

Impairment of *O*-antigen synthesis in *Salmonella* spp. (Wildschutte et al., 2004) and *Synechococcus* spp. (Zwirgmaier et al., 2009; Simkovsky et al., 2012) has been shown to provide resistance from grazing by amoebae (Simkovsky et al., 2012) and nanoflagellates (Zwirgmaier et al., 2009). Conversely, March et al. (2013) demonstrated that mutants of *K. pneumoniae*, which

lacked a heptose chain in the *O*-antigen and the first glucose of the LPS core, were shown to be more prone to phagocytosis by alveolar macrophages and the amoeba *Dictyostelium discoideum*.

1.3.4.2 Modifications in peptidoglycan

The PG layer is a highly dynamic component of all bacterial cell walls (Horcajo et al., 2012) and most pathogenic bacteria use mechanisms to modify their PG to avoid recognition by phagocytes in the immune system (Bera et al., 2005; Bera et al., 2006). There are four categories of PG modification, with the majority of these resulting in the bacterium being able to survive lysozyme degradation inside the food vacuole: (i) *O*-acetylation of NAM/MurNAc in position Carbon 6 (C-6) (Bera et al., 2005; Dillard and Hackett, 2005), (ii) N-deacetylation of NAG/GlcNAc in position C-2 (Vollmer and Tomasz, 2000; Severin et al., 2004; Hébert et al., 2007; Meyrand et al., 2007), (iii) N-glycosylation of NAM/MurNAc in position C-2 (Lederer et al., 1975; Raymond et al., 2005) and, (iv) modification of the stem peptides (L-Alanine or D-Glutamic, L-ornithine acid substitution) (Mahapatra et al., 2008; Bernard et al., 2011b; Slamti et al., 2011).

Modifications of the glycan backbone (via *O*-acetylation, N-deacetylation or N-glycosylation) results from the catalytic activity of PG-hydrolytic enzymes and are more often found in Gram-positive bacteria (Vollmer, 2008). For example, pathogenic strains of *S. aureus* (Bera et al., 2005; Bera et al., 2007), *Enterococcus faecalis* (Hébert et al., 2007), *Bacillus* spp. (Laaberki et al., 2011; Guariglia-Oropeza and Helmann, 2011) and *Lactobacillus* spp. (Courtin et al., 2006; Bernard et al., 2011a) have shown resistance to lysozyme degradation due to *O*-acetylation of PG - carried out by the enzyme *O*-acetyl transferase A which acetylates the NAM/MurNAc residues (Bera et al., 2005; Velga et al., 2005; Hébert et al., 2007; Guariglia-Oropeza and Helmann, 2011; Bernard et al., 2012).

In Gram-negative bacteria such as *N. meningitidis*, *Helicobacter pylori* and *Campylobacter* spp., *O*-acetylation of NAM is performed by *O*-acetyl transferase-B enzyme (Laaberki et al., 2011). *O*-acetylation of NAG/GlcNAc residues of PG is very rare, with only one bacterium, *Lactobacillus plantarum*, so far identified as having both NAG/GlcNAc and NAM/MurNAc acetylated (Bernard et al., 2011a).

N-deacetylation, performed by PG-deacetylase A (PgdA), is found in Gram-positive bacteria such as *B. cereus* (Araki et al., 1971), *E. faecalis* (Hébert et al., 2007), *S. pneumoniae* (Vollmer and Tomasz, 2000) and *Lactococcus lactis* (Meyrand et al., 2007) while N-glycosylation of NAM/MurNAc is in the Gram-positive genera *Mycobacterium*, *Micromonospora*, *Tsukamurella*, *Nocardia* and *Rhodococcus* (Raymond et al., 2005; Vollmer, 2008). Both mechanisms have been shown to protect bacteria against lysozyme activity (Vollmer and Tomasz, 2000; Veiga et al., 2007) and intracellular activation of pro-inflammatory NOD-like receptors (Boneca et al., 2007).

Finally, amination of PG was documented in the Gram-positive bacteria *L. lactis* (Courtin et al., 2006), *Lactobacillus plantarum* (Bernard et al., 2011b) and *Enterococcus faecium* (Bellais et al., 2006).

1.3.4.3 Modifications in Lipoteichoic acids

Three types of modification of LTA have been shown to affect bacterial resistance towards immune cells: (i) D-alanylation, (ii) glycosylation and, (iii) addition of phosphocholine residues. The latter has only been documented in *S. pneumoniae* (Tomasz, 1967; Denapaite et al., 2012), where an increase in choline residues resulted in avoidance of complement deposition and antimicrobial peptides (Peschel et al., 1999; Mukerji et al., 2012).

D-alanylation is a more common LTA modification, resulting from the action of enzymes carrying the D-alanine residue from the cytoplasm onto the LTA (Perego et al., 1995; Debabov et al., 2000; Du et al., 2008). The number of added D-alanine residues differs according to environmental factors such as an increase in pH (MacArthur and Archibald, 1984; Koch et al., 1985), temperature (Hurst et al., 1975; Koch et al., 1985) or NaCl concentration (Fischer and Rösel, 1980; Madiraju et al., 1987), which lead to a decrease in D-alanylation. Addition of D-alanine residues changes the highly-negatively charged surface membrane to a more positive surface, making the bacteria a less desirable target for phagocytic receptors that use a positive-charged groove to bind ligands (for example Scavenger Receptors [SRs], Section 1.4.2). Indeed, substitution of 59% of *S. aureus* LTA with alanine led to low affinity binding with SRs from Chinese hamster ovary (CHO) cells,

transfected with bovine macrophage SR (Greenberg et al., 1996), suggesting that alanylation could be used by bacteria to avoid receptor binding to immune cells.

Incorporation of glycosyl residues has been proposed as the main modification for type I LTAs (Schirner et al., 2009; Brown et al., 2013). The added sugar differs between genera. For example, *Bacillus* strains are glycosylated by both GlcNAc and α -Gal additions (Weidenmaier and Peschel, 2008), whilst *Listeria* spp. strains are glycosylated with only α -Gal (Hether and Jackson, 1983) and *S. aureus* strains are glycosylated with only GlcNAc (Arakawa et al., 1981). The roles of these modifications have not been fully described, but it was suggested that the sugar additions aid host invasion (Greenberg et al., 1996).

1.4 Pattern Recognition Receptors (PRRs) in immune cells

PRRs recognize both opsonized and non-opsonized particles. Opsonized phagocytosis is triggered when the phagocytic receptors recognize particles coated with opsonins, i.e., soluble molecules such as antibodies, surfactant proteins, mannose binding proteins. This facilitates an accurate, diverse and rapid activation of cellular activities that leads to particle internalization and particle destruction (reviewed in Aderem and Underhill, 1999; Underhill and Ozinsky, 2002; Stuart and Ezekowitz, 2005).

Compared to the opsonized phagocytosis, non-opsonized phagocytosis has received far less attention, but it is most akin to phagocytosis by protists. Internalization of particles is triggered by the direct recognition of PAMPs on the microbial cell surface without the need of enhanced stimuli given by opsonins. Two main families of receptors are involved in non-opsonic phagocytosis: C-type lectins (Harb et al., 1998; Alsam et al., 2005; Stahl and Ezekowitz, 1998; Ariizumi et al., 2000a) and Scavenger Receptors (Platt et al., 1996; Hoebe et al., 2005; Peruń et al., 2016).

1.4.1 C-type lectins

The C-type lectin superfamily comprises a large group of receptors that are characterized by the presence of one or more C-type lectin-like domains (CTLDs) (Drickamer and Fadden, 2002). They primarily recognize carbohydrate ligands from bacterial LPS and from fungal cell walls (Stahl and Ezekowitz, 1998; East and Isacke, 2002). The mechanism is calcium-dependent, and lock-and-key, due to their conserved extracellular carbohydrate recognition domains (CRDs) (Sharon, 1984; Holmskov et al., 1994). The carbohydrates they recognize include mannose, N-acetylglucosamine (GlcNAc), L-fucose and glucose - which are recognized by the EPN (Glutamic acid-Proline-Asparagine) motif present in the CRD, and galactose and N-acetylgalactosamine (GalNAc) - recognized by the QPD (Glutamine-Proline-Aspartic acid) motif from the CRD (Drickamer and Fadden, 2002).

The C-type lectins are functionally diverse and are involved in cell adhesion, tissue integration, platelet activation, complement activation, intracellular pathogen recognition, endocytosis and phagocytosis (reviewed in Dambuza and Brown, 2015; Brown, et al., 2018). This superfamily includes transmembrane receptors: Dectin-1 and Dectin-2 (Ariizumi et al., 2000a; Ariizumi et al., 2000b), the Mannose Receptor (Ezekowitz et al., 1990; Stahl and Ezekowitz, 1998) and DC-SIGN (Feinberg et al., 2001; Geurtsen et al., 2010), but also soluble molecules which act as opsonins, such as the mannose-binding lectin (Kuhlman, 1989; Brouwer et al., 2008) and surfactant proteins (Pikaar et al., 1995; Benne et al., 1997).

1.4.1.1 Receptors that bind mannose and GlcNAc

Bacterial mannose and GlcNAc residues are recognized by macrophages using two main receptors: Mannose receptor (MR) and DC-SIGN.

The MR is a type I 180-kDa transmembrane C-type lectin that is expressed on immune cells of myeloid lineage, particularly dendritic cells and macrophages (Ezekowitz et al., 1990; Sallusto et al., 1995; Stahl and Ezekowitz, 1998). MR consists of five domains: an extracellular cysteine-rich region, a fibronectin type II repeat domain, eight CRDs, a transmembrane region and a short

carboxy-terminal cytoplasmic tail (Lennartz et al., 1989; Ezekowitz et al., 1990; Taylor et al., 1990). Recognition of ligands is performed by the CTLDs (Taylor et al., 1992; Mullin et al., 1997) where the cytoplasmic tail is crucial for the endocytic and phagocytic functions (Stahl and Ezekowitz, 1998; Ezekowitz et al., 1990).

The presence of conserved Glu-Pro-Asp amino acid sequences in recognition domain 4 alone enables the MR to bind mannose and fucose with high affinity in a Ca^{2+} dependent manner (Largent et al., 1984; Taylor et al., 1992). MR also binds N-acetylglucosamine (GlcNAc) and GalNAc₄-SO₄ with low affinity but does not bind galactose or GalNAc alone (Largent et al., 1984; Iobst and Drickamer, 1994; Fiete et al., 1998; Roseman and Baenziger, 2000).

Once the ligand has bound, the cytoplasmic tail is activated and proteins such as F-actin, protein kinase C, MARKs (microtubule-affinity regulating kinases), and Myosin I are recruited to the site of binding (Allen and Aderem, 1996a). Further activation of local F-actin polymerization, Cdc42 and Rho (common phagocytic GTPases) promote PAK1 activation which leads to vacuole formation (Zhang et al., 2005). In fact, removal of cytoplasmic tail in MR transfected cells COS-1 showed significant reduction in the uptake of *Candida albicans* (Ezekowitz et al., 1990), further exemplifying that the cytoplasmic tail is necessary for phagocytic engulfment. The O-antigen of *Klebsiella pneumoniae* (Taylor et al., 1992; Zamze et al., 2002), the LPS (core oligosaccharide or O-antigen; the exact structural component has not been analyzed) and the capsule of *S. pneumoniae* (Zamze et al., 2002) and mannose-capped lipoarabinomannan (ManLAM) from the *Mycobacterium tuberculosis* cell surface (Kang et al., 2005), all have mannose and GlcNAc residues.

DC-SIGN is a type-II transmembrane C-type lectin, expressed by the majority of dendritic cells and decidual and specialized tissue macrophages (Geijtenbeek et al., 2000; Soilleux et al., 2002). This receptor has more of an endocytic function (Serrano-Gómez et al., 2004; Cambi et al., 2008; Iyori et al., 2008; Zhang et al., 2008), with only a few studies demonstrating that DC-SIGN transfected HeLa cells can lead to phagocytosis of *E. coli* (Zhang et al., 2006a; Iyori et al., 2008) and *Y. pestis* (Zhang et al., 2008). Binding with mannose residues of *M. tuberculosis*, *C. albicans*,

Aspergillus fumigatus, *H. pylori* (Geijtenbeek et al., 2003; Serrano-Gómez et al., 2004; Cambi et al., 2008) and GlcNAc residues of *E. coli*, *H. ducreyi*, *N. gonorrhoeae* and *S. typhimurium* (Zhang et al., 2006b, Cambi et al., 2008) has been demonstrated.

DC-SIGN binds preferentially to complex mannose and GlcNAc residues (Feinberg et al., 2001; Mitchell et al., 2001; Geijtenbeek et al., 2002), while the MR recognizes mostly terminal mannose, fucose and GlcNAc (Largent et al., 1984; Taylor et al., 1992; Fiete et al., 1998). This is due to the distinct branching and spacing in carbohydrate-recognition domains between the two receptors (Cambi and Figdor, 2003). Different binding affinities is not the only difference between these two receptors, as they also regulate endosomal trafficking differently. The DC-SIGN cytoplasmic tail possesses an additional tri-acidic cluster and di-leucine signaling motif that targets endocytosed molecules to lysosomes and delivers the particles to the major histocompatibility complex (MHC) class II-positive late endosomes (Figdor et al., 2002), whereas the MR tail has only one tyrosine residue which recruits proteins to the site of attachment and delivers the bound particle to early endosomes (Kruskal et al., 1992).

1.4.1.2 Receptors that bind GalNAc

GalNAc residues are building blocks in the cell walls of both Gram-positive and Gram-negative bacteria, where they are part of the glycosaminoglycans, teichoic acids and lipopolysaccharides (Abeygunawardana et al., 1989; Michael et al., 2002; Fox et al., 2003; Freymond et al., 2006; Rush et al., 2010; Liu et al., 2014; Whitworth and Imperiali, 2015; Leker et al., 2017). GalNAc residues are recognized by three main C-type lectin receptors in macrophages: (i) the Asialoglycoprotein receptor (ASGR) (Spiess and Lodish, 1985; Porat et al., 1995; Rensen et al., 2001; Tanowitz et al., 2017), (ii) the macrophage galactose receptor (MGL) (Kawasaki et al., 1986; Oda et al., 1989; Sato et al., 1992; van Sorge et al., 2009) and, (iii) the Mannose receptor (Fiete et al., 1998; Roseman and Baenziger, 2000; Mi et al., 2001).

ASGR binds both galactose and GalNAc residues (Schwartz, 1984; Weiss and Ashwell, 1989; Figdor et al., 2002), with a 50-fold higher affinity for the latter (Baenziger and Maynard, 1980). ASGR is a type-II transmembrane C-type lectin present in Kupffer cells and mouse peritoneal

macrophages (Kolb-Bachofen et al., 1984; Ozaki et al., 1995; Tanaka et al., 2017). Desialylated *Streptococcus agalactiae*, with exposed Gal and GalNAc residues, were cleared at a faster rate than cells with non-exposed Gal/GalNAc residues, and the clearance was inhibited significantly by addition of galactosylated but not mannosylated glycoproteins (Cundell and Tuomanen, 1994).

In comparison, the MGL receptor, which is expressed in macrophages and monocyte-derived dendritic cells (Higashi et al., 2002a, Higashi et al., 2002b; Raes et al., 2005; van Vliet et al., 2005; van Vliet et al., 2008a; van Vliet et al., 2008b) binds only GalNAc residues in the glycoproteins or glycosphingolipids of microorganisms (Yamamoto et al., 1994a; van Vliet et al., 2005; van Vliet et al., 2009; van Sorge et al., 2009). Addition of anti-MGL antibodies (600µM) or GalNAc monosaccharides (100mM) to dendritic cells showed blocking of the uptake of *N. gonorrhoeae* (van Vliet et al., 2009). Also, COS-1 cells transfected with MGL indicated that human MGL is sufficient for binding GalNAc residues from N or O-linked glycans (Suzuki et al., 1996).

Finally, the Mannose Receptor (Section 1.4.1.1.) can bind to terminal GalNAc₄-SO₄ but not GalNAc or Gal residues (Fiete et al., 1998; Roseman and Baenziger, 2000). However, this binding has been shown to only be important in the clearance of lutropin hormone from the human body (Fiete et al., 1991; Mi et al., 2001), with no role in phagocytosis described to date.

1.4.2 Scavenger receptors (SRs)

Scavenger receptors (SRs) are another class of surface membrane glycoprotein which act as PRRs. They were originally discovered as receptors that bind and internalize oxidized low-density lipoproteins; being involved in the recognition of modified self-molecules from self (Brown and Goldstein, 1979). Further research showed that SRs could recognize microbial cell structures such as lipopolysaccharides (LPS) and lipoteichoic acids from bacterial cell walls leading to phagocytosis (Thomas et al., 2000; Mukhopadhyay and Gordon, 2004; Plüddemann et al., 2006; Plüddemann et al., 2007; Areschoug and Gordon, 2009; Perún et al., 2016).

SRs have been classified into 10 classes (A-J), with Class A SRs (SR-A) having cysteine-rich domains similar to the C-type lectins and being the most commonly involved SRs in macrophage phagocytosis, particularly SR-A/CD204 (Platt et al., 1996; Platt et al., 1999; Peiser et al., 2000; Thelen et al., 2010). Direct binding experiments with purified bacterial cell wall components indicate that SR-A recognizes two main ligands: lipoteichoic acids from Gram-positive bacteria (Dunne et al., 1994) and lipid A from the LPS of Gram-negative bacteria (Hampton et al., 1991). Bone-marrow derived macrophages deficient in SR-A showed reduced phagocytosis rates on heat-killed fluorescence-labeled *S. aureus* (Peiser et al., 2000). Expression of SR-A in transfected CHO cells and murine macrophage RAW 264.7 led to internalization of heat-killed *S. aureus* and heat-killed *E. coli* (and its purified LPS) respectively (Peiser et al., 2000). However, Peruñ et al. (2016) showed that SR-A was only involved in the phagocytosis of live *E. coli* cells but not live *S. aureus* cells. Also, SR-A was not involved in the uptake of heat-killed cells of both strains *unless* they were labelled with a fluorochrome; suggesting that commercially available heat-killed fluorochrome stained cells (which are commonly used) do not represent the true dynamics of SR-ligand binding. DeLoid et al. (2009) examined the SR-binding and phagocytosis of *S. aureus* when live or heat-killed (unstained) and compared it to the uptake of latex beads. SRs were only found to be involved in the uptake of latex beads.

Class B scavenger receptors such as CD36 also bind both LTA from Gram-positive bacteria (Stuart et al., 2005; Hoebe et al., 2005) and lipid part of LPS (independent of the O-antigen presence) of Gram-negative bacteria (Baranova et al., 2008; Olonisakin et al., 2016). Macrophages deficient of CD36 showed impaired ability to phagocytose *S. aureus* or purified *S. aureus* LTA (Stuart et al., 2005), while transfected cells with CD36 bound and internalized both *S. aureus* and *E. coli* (Stuart et al., 2005). Direct binding experiments also showed that CD36 binds preferentially to rough LPS (lacking the O-antigen) (Biedroń et al., 2016), suggesting a deeper ligand that enhances recognition and phagocytosis by macrophages upon binding of CD36 (Baranova et al., 2008; Olonisakin et al., 2016). Conversely, Peruñ et al. (2016) showed that CD36 was not involved in the phagocytosis of live or heat-killed *S. aureus* and *E. coli*.

Thus, there is a lack of agreement as to the exact role of SRs in the uptake of bacterial cells and although this might be partly due to the type of prey cell used (live, heat-killed, heat-killed and stained etc.), Peruń et al. (2016) also suggested that differences might be due to the type of phagocyte used in experiments.

1.5 Pattern Recognition Receptors (PRRs) in protists

Filter-feeding ciliates were once thought to feed indiscriminately on bacterial prey, or be less selective than flagellates and amoebae (Fenchel, 1980), until Verity (1991) showed that the ciliate *Euplotes* selected a preferred prey based on chemical cues given off by that prey. Since then, in addition to chemical cues, prey selectivity in has been shown to be influenced by numerous criteria such as, prey strains (Thurman et al., 2010a, b; Jezbera, et al., 2005; Pickup et al., 2007b; Dopheide et al., 2011), prey size (Andersson et al., 1986; Chrzanowsky and Šimek, 1990; González et al., 1990; Holen and Boraas, 1991; Epstein and Shiaris, 1992) and motility (Monger and Landry, 1992; Matz and Jürgens, 2005), hydrophobicity and charge (Krieger, 1994; Hammer et al., 1999; Matz et al., 2002), prey nutritional quality (Kremp and Anderson, 2004; Shannon et al., 2006) and predator feeding history (Ayo et al., 2009). But probably one of the most fundamental of these characteristics is the biochemical composition of the prey cell surface.

Evidence for receptor-recognition of prey in protists has been increasing, albeit slowly, through the years to demonstrate that it plays an important role in prey recognition and selectivity in amoeba (Allen and Dawidowicz, 1990; Venkataraman et al., 1997b; Sakaguchi et al., 2001), dinoflagellates (Hansen and Calado, 1999; Tillmann, 2004; Boenigk, 2005; Wootton et al., 2007) and ciliates (Wilks and Sleigh, 2004; Dürichen et al., 2016).

1.5.1 C-type lectins

The presence of specific C-type lectins in protists has been studied using, in the main, two methods. Sugar blocking experiments pre-incubate the protist with a specific sugar, to block their respective receptor, and compares the uptake of prey (with receptor blocking) to that in the absence of the sugar (Allen and Dawidowicz, 1990; Wootton et al., 2007). Alternatively, the

presence of carbohydrate-binding sites on/in the protist cell can be visualized using fluorescent derivatives of plant lectins, which are carbohydrate-binding proteins that bind specifically to their homologous sugar residues (Lis and Sharon, 1986; Costas and Rodas, 1994; Takahashi et al., 2002; Wilks and Sleight, 2004; Roberts et al., 2006). The most commonly used lectins are Concanavalin A (ConA) that binds D-mannose (Goldstein et al., 1965), Wheat germ agglutinin (WGA) that binds N-acetylglucosamine (GlcNAc) (Wright, 1992), and Peanut agglutinin (PNA) that binds N-acetylgalactosamine (GalNAc) (Macartney, 1986).

1.5.1.1 The mannose receptor

Bracha et al. (1982) were the first to demonstrate that the uptake of live and glutaraldehyde-fixed *E. coli* and *Serratia marcescens* by the amoeba *Entamoeba histolytica* involved a mannose receptor, with bacterial attachment being reduced by 66% with 100mM mannose. Glutaraldehyde fixation of the bacteria would not be expected to greatly modify the outer surface of these, since aldehydes react poorly, if at all, with carbohydrates, as demonstrated on yeast (Hayat, 1981). Same *E. histolytica* strain was also found to shun bacteria that did not have a mannose binding capacity, such as strains of *Shigella* (*sonnei*, *flexneri*, and *dysenteriae*), *Bacteroides fragilis*, *S. aureus*, and *Micrococcus luteus*; once coated in ConA lectin, these bacteria were taken in comparably more than wild-type (Bracha et al. 1982).

Allen and Davidowicz (1990) identified a mannose-binding receptor in the amoeba *Acanthamoeba castellanii* that bound and internalized heat-killed yeast (*Saccharomyces cerevisiae*). Phagocytosis was significantly reduced with exogenous mannose, in a dose dependent manner, yielding an IC₅₀ of 10mM. However, blocking the mannose receptor had no significant effect on the uptake of latex beads. Alsam et al. (2005) also showed that this amoeba uses the mannose receptor to bind heat-killed *E. coli* with ingestion being reduced by 80% in the presence of 100mM mannose. The *A. castellanii* mannose receptor was first cloned in 2004 by Garate et al. and it is a 400-kDa protein that comprises multiple 130-kDa subunits. Despite extensive BLAST searches, no significant matches were retrieved; the receptor lacks sequence

identity to well characterized lectin carbohydrate recognition domains of C-type lectins, mammalian galectins, and plant lectins.

Wootton et al. (2007) demonstrated that the marine dinoflagellate *Oxyrrhis marina* uses a mannose binding lectin to ingest live algal prey, *Isochrysis galbana*. Blocking the receptor with 20 μ M of Mannose-BSA significantly reduced ingestion by 60%; blocking with GlcNAc-BSA or GalNAc-BSA had no effect. When presented with an equal mix of mannose-coated and GalNAc-coated beads, *O. marina* ingested twice as many mannose-coated beads, but when pre-incubated with mannose-BSA, the dinoflagellate lost its ability to discriminate between the different types of sugar-coated beads. Wootton et al. (2007) also reported that after a 1-hour incubation with FITC-labelled mannose-BSA, the label was found to be concentrated at a specific region of the dinoflagellate cell, in approximately the same position as the non-permanent cytostome, described by Höhfeld and Melkonian (1998).

Lectin binding experiments involving ciliates have given conflicting results with regards to the position of mannose-rich sites on the cells. Wilks and Sleight (2004) used ConA to visualize the presence of mannose residues on the surface of the ciliate *Euplotes mutabilis* and found a sequence of binding over time which they interpreted as a sequence of ingestion events: (i) staining of the cytostome only, (ii) simultaneous staining of the cytostome and vacuoles, (iii) staining of the vacuoles only, (iv) no staining. Conversely, Dürichen et al. (2016) reported that ConA only bound to food vacuole membranes of *T. pyriformis* throughout their full life cycle.

1.5.1.2 The N-acetylglucosamine (GlcNAc) receptor

Wootton et al. (2007) demonstrated that *O. marina* does not use a GlcNAc receptor to ingest live *I. galbana* as incubation with 20 μ M of GlcNAc-BSA did not significantly reduce ingestion rate.

Lectin binding experiments, like those for mannose, have given conflicting results with regards to the position of GlcNAc-rich sites on the cells. Wilks and Sleight (2004) observed a reproducible pattern of WGA binding in *E. mutabilis*. All cells showed labelling of the 8–9 oral membranelles after only 10 seconds. Labelling of the cytostome area in 45% cells was observed after 30 seconds

then some cells showed simultaneous binding of the cytostome and food vacuole (maximum of 43% cells at 10 minutes). At 30 minutes, 42% of the cells showed staining of the food vacuole only and at 60 minutes no binding was observed. In contrast, Roberts et al. (2006) observed no binding of WGA to *E. vannus* but did record binding to the surface of *O. marina* and *Goniomonas amphinema*. The most recent lectin binding study (Dürichen et al., 2016) showed that WGA bound specifically to patches of the vacuole membrane of *T. pyriformis*, unlike ConA which labeled the whole food vacuole. And, whereas ConA bound to all food vacuoles, WGA only bound to young vacuoles near the cytostome. The authors did not observe any WGA binding at the cytostome.

1.5.1.3 The N-acetylgalactosamine (GalNAc) receptor

The first Gal/GalNAc receptor to be described in protists was for *Entamoeba histolytica*. It is a 170 kDa transmembrane lectin, with carbohydrate binding domains located extracellularly (Petri et al., 1987). *E. histolytica* trophozoites primarily use this lectin as an adherence molecule to bind to host cells (Ravdin and Guerrant, 1981; Petri et al., 1987). Such adhesion can be blocked in the presence of anti-lectin antibodies (Petri et al., 1990; Cheng et al., 2000), 4.5mM GalNAc (Ravdin and Guerrant, 1981; Ravdin et al., 1985), and 56mM galactose (Ravdin et al., 1985). *E. histolytica* has also been shown to use the Gal/GalNAc lectin to bind to sugar moieties on bacteria such as live *E. coli* serotype O55, a bacterial strain rich in Gal and GalNAc residues on its cell surface (Bär et al., 2015).

Vermamoeba (Hartmannella) vermiformis has been shown to use a similar 170kDa transmembrane lectin to bind and internalize gentamicin-killed *Legionella pneumophila* (Venkataraman et al., 1997a; Venkataraman et al., 1997b; Harb et al., 1998). All three studies showed that 100mM of galactose or GalNAc (but not mannose, glucose or lactose) partially blocked (70-89%) the binding and uptake of *L. pneumophila*. Interestingly, addition of antibodies against the Gal/GalNAc lectin found in *E. histolytica* also inhibited the uptake of *L. pneumophila* by *V. vermiformis*, indicating that the lectins possess similar epitopes for the carbohydrate residues.

In contrast to these results, pre-incubation for 30 minutes with 20 μ M of GalNAc-BSA did not affect the uptake of live *I. galbana* by *O. marina* (Wootton et al., 2007); which was considered to be due to only a small number of GalNAc residues on the algal cell surface (demonstrated by a lack of PNA binding to its cell surface) (Wootton et al., 2007).

Roberts et al. (2006) found that the binding of PNA and MAA (*Maackia amurensis* agglutinin) to GalNAc residues occurred only in the food vacuoles of *E. vannus*, while Wilks and Sleight (2004) showed that the GalNAc-binding lectin SBA (*Glycine max* [Soy Bean]) bound to the cytostome and food vacuoles of *E. mutabilis*. Ramoino (1997), using PNA, and Cho (2002), using SBA, have shown intense GalNAc presence in the cilia of *Paramecium primaurelia* and on the cell surface of the dinoflagellates *Gymnodinium*, *Prorocentrum* and *Alexandrium*.

1.5.2 Scavenger Receptors

The only study which demonstrates a role for SRs in the uptake of prey by protists is that of Sattler et al. (2018) who showed that the amoeba *Dictyostelium discoideum* possesses homologous receptors to the mammalian class B SRs, LIMP-2 and CD36, i.e. LmpA and LmpB. LmpA was found in late endosomes and phagolysosomes and played a role in the binding and phagocytosis of Gram-negative bacteria, Gram-positive bacteria and beads; although a much higher affinity was recorded for Gram-positive bacteria. LmpB was localized in lipid rafts of the plasma membrane and early phagosomes and it was exclusively involved in the uptake of Gram-positive bacteria and mycobacteria (Sattler et al. 2018).

1.6 Rationale for the current study and specific objectives

There is growing evidence to suggest that C-type lectins are involved in the phagocytosis of prey by protists but there is just one study that has examined whether protists possess scavenger receptors (Sattler et al., 2018). With regards to the prey used in receptor-mediation experiments, these have differed greatly from the use of inert beads (mainly in macrophage studies) to the use of live and/or heat-killed-stained cells (mainly in protist studies). There are no published reports which *directly* compares the role of receptors in the uptake of different 'prey types', here defined

as Live cells, Dead cells and Beads. This study aimed to address this paucity of information by comparing prey uptake and vacuole formation in the ciliate *Tetrahymena pyriformis* when feeding on these three prey types. This study also extended a typical single predator-single prey study to include eleven strains of bacteria, to help elucidate any general trends.

Ciliates such as *Tetrahymena* spp. are important model organisms in bacteria-protist research and they are easy to culture and have rapid growth rates (Montagnes et al., 2012). An added advantage is their simplicity because they do not have numerous growth forms like *Dictyostelium* spp. (Cotter et al., 1976) or produce cysts like *Acanthamoeba* spp. (Byers et al., 1991), and certain cellular pathways are very similar to higher phyla cells such as macrophages (Aderem and Underhill, 1999; Greenberg and Grinstein, 2002). The latter is particularly pertinent to the current study, as most information on receptor-mediated prey recognition originates from macrophage studies.

T. pyriformis is an avid bacteriovore (Curds and Cockburn, 1968) and as such, the prey used in the current study were bacteria. Live bacterial cells are difficult to visualize within protists so many studies have fluorescently stained cells to allow visualization. The most common method has been to stain cells with DTAF (5-[4,6-dichlorotriazinyl] aminofluorescein), following heat-killing (Sherr et al., 1987) but since 2010, live bacterial prey transformed to express a fluorescent protein have been used in protist-prey feeding experiments (Thurman et al., 2010b; Dopheide et al., 2011). Both these methods allow for a single bacterial strain to be prepared in two ways (dead and live) in order to directly compare the effect of heat-killing on prey uptake.

Heat-killing is known to be an invasive process and results in altered surface characteristics which are potential ligands for recognition by protozoa (Matz et al., 2002; Wilks and Sleigh, 2004). Thus, for the purpose of this study, Live bacterial cells are considered to possess a full complement of surface ligands and heat-killed cells are considered to possess damaged ligands. Polystyrene beads were included in this study as particles that possess no surface ligands at all.

Using these three prey ‘types’, the specific aims of the current study were to,

- (i) Compare the ingestion rates and vacuole formation rates when *T. pyriformis* feeds on the three prey types in monoculture, and evaluate whether there was any significant trend with regards to prey surface ligand availability.
- (ii) Determine whether *T. pyriformis* could actively select a specific prey type within a prey mixture, and evaluate whether there was any significant trend with regards to prey surface ligand availability.
- (iii) Compare the effects of heat and chemical treatments of bacteria on prey uptake by *T. pyriformis*; specifically, via removing the bacterial S-layer, removing bacterial surface proteins and fixing bacterial cells with formaldehyde.
- (iv) Using *Salmonella enterica* 74 only (live and dead), together with beads, evaluate whether *T. pyriformis* possesses scavenger receptors.
- (v) Using *Salmonella enterica* 74 only (live and dead), together with beads, determine whether blocking three C-type lectins, with their specific sugar targets (mannose, GlcNAc and GalNAc), affects the processing of any of the three prey types and evaluate if there was any significant trend with regards to prey surface ligand availability.

CHAPTER 2: MATERIALS AND METHODS

2.1 Maintenance of organisms and fluorescently-labelled microspheres

Experiments studying feeding in *Tetrahymena pyriformis* involved three types of prey: (i) 'Live' bacteria which included heterotrophic strains previously transformed by J. Parry to express the Red Fluorescent Protein (RFP) and the autotrophic *Synechococcus* sp. S-KH3 ('Pico 3'), (ii) their heat-killed counterparts which were stained with 5-([4,6-Dichlorotriazin-2-yl]amino) fluorescein (DTAF) ('Dead') and, (iii) fluorescently-labelled green/yellow 0.49µm diameter microspheres ('Beads') (Polysciences).

2.1.1 Live heterotrophic bacteria

Heterotrophic bacteria were maintained as streak plates on their preferred agar and incubated for 3 days at their preferred temperature (Table 2.1). Bacterial suspensions were freshly prepared on the day of the experiment using sterile water (Milli-Q).

2.1.2 Live autotrophic bacterium ('Pico')

Synechococcus strain S-KH3 (Pico 3) (Dillon and Parry, 2009) was grown in BG11 broth (Appendix), on a rotary shaker (0.00118 g) at room temperature (*ca.* 23°C). The bacterium was maintained in a 16:8 natural light:dark cycle and sub-cultured into fresh BG11 medium 3 days prior to each experiment.

2.1.3 Heat-killed-DTAF stained bacteria ('Dead')

Nine non-RFP expressing strains (^ in Table 2.1) and Pico 3 were heat-killed and stained following Sherr et al. (1987). *Klebsiella aerogenes* was omitted from this procedure as it was the food source for the ciliate during routine maintenance (Section 2.1.5) and was not used in experiments. Bacterial suspensions were prepared in 10ml sterile water and 5mg of DTAF (Sigma) was added. The suspensions were vortexed and placed in a water bath at 60°C for 2 hours. The cells were then centrifuged at 3500rpm for 10 minutes, the supernatant removed, and the pellet

re-suspended in 10 ml of solution 1 (Appendix) followed by vortexing and sonication for 10 minutes (this was one 'wash'). The cells were washed three times with solution 1 and then suspended in solution 2 (Appendix), left overnight at 4°C and then washed three times with Chalkley's medium (Appendix). Cell concentration was determined (Section 2.2.2) before aliquoting the suspensions (300µL) into Eppendorf tubes and storing them at -20°C. For experiments, the aliquots were thawed at room temperature, vortexed and sonicated for 10 minutes.

The only RFP-expressing strain used for DTAF-staining was *Escherichia coli* AKN132 due to lack of a non-RFP expressing counterpart. In this case, the suspension was heat-killed for 8 hours at 70°C (to denature the RFP); DTAF staining then followed the procedure described above.

2.1.4 Fluorescently labeled microspheres ('Beads')

A suspension of inert fluorescently-labelled yellow/green microspheres (Fluoresbrite™ Polyscience Inc.) of 0.49µm diameter (0.065µm³ biovolume) were prepared in sterile water (Milli-Q), their concentration determined (Section 2.2.2) and stored at 4°C. Prior to experiments, they were sonicated for 5 minutes.

2.1.5 *Tetrahymena pyriformis*

The ciliate *Tetrahymena pyriformis* (Culture Collection of Algae and Protozoa [CCAP] 1630/1W) was maintained in 400ml Chalkley's medium and fed with approximately 3 ml of a live, non-RFP expressing *K. aerogenes* suspension (OD600 ca. 0.5). The culture was left at room temperature (ca. 23°C) and fed 3 days prior to any experiment. On the day of an experiment, sixteen tubes (containing 15ml suspension) were centrifuged at 2000rpm for 15 minutes. The top 14ml was carefully removed and the remaining 1ml from each tube was combined. This yielded a *T. pyriformis* cell concentration of between 1.2-2.3x10⁵ cells/ml.

Table 2.1. Bacteria used in the experiments alongside their source, strain, preferred medium, temperature of incubation, biovolume and motility status (M-Motile, NM-Non-motile). LB-Luria-Bertani agar (Appendix 1), NCIMB-National Collection of Industrial Marine and Food Bacteria, NCTC-National Collection of Type Culture, ATCC-American Type Culture Collection, MAST-MAST Laboratories, Bootle. Gentamicin (Gn), Ampicillin (Amp), Chloramphenicol (C) at 6µg/ml (6), 60µg/ml (60), 100µg/ml (100). *Non-RFP strain used to routinely sub-culture *Tetrahymena pyriformis*. ^Non-RFP strains used for DTAF-staining (Section 2.1.3). Biovolume data courtesy of Wong (2017).

Bacterium	Source	Strain	Medium	Temp (°C)	Biovolume (µm ³)	Motility
<i>Aeromonas hydrophila</i> (RFP)	NCIMB	9240	LB+Gn60	35°C	1.016±0.061	M
^ <i>Aeromonas hydrophila</i>	NCIMB	9240	LB	37°C		
<i>Escherichia coli</i> (RFP)	P. Hill	AKN132	LB+Amp100	30°C	0.511±0.025	M
* <i>Klebsiella aerogenes</i>	NCTC	9528	LB	25°C	0.918±0.043	
<i>Klebsiella pneumoniae</i> (RFP)	NCTC	5055	LB+Gn60	35°C	1.701±0.101	NM
^ <i>Klebsiella pneumoniae</i>	NCTC	5055	LB	37°C		
<i>Pseudomonas aeruginosa</i> (RFP)	NCIMB	10412	LB+Gn60	25°C	0.655±0.031	M
^ <i>Pseudomonas aeruginosa</i>	NCIMB	10412	LB	37°C		
<i>Pseudomonas fluorescens</i> (RFP)	R. Pickup	FH1	LB+Gn60	30°C	0.785±0.047	M
^ <i>Pseudomonas fluorescens</i>	R. Pickup	FH1	LB	30°C		
<i>Salmonella enterica</i> (RFP)	NCTC	74	LB+Gn60	35°C	0.759±0.033	M
^ <i>Salmonella enterica</i>	NCTC	74	LB	37°C		
<i>Salmonella enterica</i> (RFP)	NCTC	12694	LB+Gn60	25°C	0.512±0.028	M
^ <i>Salmonella enterica</i>	NCTC	12694	LB	37°C		
<i>Serratia liquefaciens</i> (RFP)	MAST	132(R)	LB+Gn60	25°C	0.641±0.031	M
^ <i>Serratia liquefaciens</i>	MAST	132(R)	LB	37°C		
<i>Serratia marcescens</i> (RFP)	NCIMB	1377	LB+Gn60	25°C	0.444±0.022	M
^ <i>Serratia marcescens</i>	NCIMB	1377	LB	37°C		
<i>Staphylococcus aureus</i> (RFP)	NCTC	6571	Lb+C6	35°C	0.272±0.022	NM
^ <i>Staphylococcus aureus</i>	NCTC	6571	LB	30°C		
<i>Synechococcus</i> sp. (Pico 3)	K. Harper	S-KH3	BG11 broth	23°C	0.588±0.031	M

2.2 Counting cells and beads

2.2.1 Counting non-fluorescent bacteria

Non-fluorescent bacterial suspensions were ten-fold diluted down to 10^{-4} . The suspensions (1ml) were stained with 2 drops of 1% 4,6-Diaminide-2-phenylindole dihydrochloride (DAPI) for 30 minutes at room temperature (*ca.* 23°C). A known volume of stained sample (usually 300µl at 10^{-3} dilution) was filtered onto a 0.2µm-pore black filter (Whatman, Millipore) above a vacuum pump. The filter was placed onto a slide with immersion oil underneath (cell side facing up). Another drop of immersion oil was placed on the center of the filter, before the cover slip was attached. A final drop of oil was placed on the center of the coverslip. The filter was observed with UV excitation using an epifluorescence microscope (final magnification x1600). At least 400 cells were counted in multiple Whipple Grids held within the eye piece. The total number of cells in the undiluted suspension was calculated using Equation 1 where the area of 1 Whipple Grid is $1/23068^{\text{th}}$ the area of cells collected on the filter.

Equation 1:

$$\frac{\text{Average number of cells/grid}}{\text{Known volume added on filter}} \times 23068 \times \text{dilution factor}$$
$$= \frac{\text{cells}}{\text{ml}} \text{ in undiluted suspension}$$

2.2.2 Counting fluorescent bacteria and beads

Red-fluorescing bacteria were counted as described in Section 2.2.1 with the DAPI-staining/UV excitation giving the total concentration of cells and green excitation giving the concentration of red-fluorescing cells. Only bacterial suspensions with $\geq 95\%$ red-fluorescing cells were used in experiments. For DTAF-stained bacteria, beads and Pico 3, DAPI staining was not necessary and the former two were counted under blue excitation while the latter was counted under green excitation.

2.2.3 Counting *Tetrahymena pyriformis*

T. pyriformis cells were fixed with glutaraldehyde (0.5%v/v final concentration) and loaded into four haemocytometers. The number of cells in all nine squares of a haemocytometer grid was recorded. The cell concentration was determined using Equation 2.

Equation 2:

$$\frac{\text{Total no. of cells in 4 haemocytometer grids}}{36} \times 10^4 = \text{cells/ml}$$

2.2.4 Counting prey within *T. pyriformis* cells

After fixing the ciliate cells, 8µl was placed on a slide, a cover slip applied and then a drop of immersion oil. The sample was observed under the appropriate excitation(s) for the given prey inside the ciliate using an epifluorescence microscope (final magnification x1600). The number of prey and the number of vacuoles inside 10 ciliates was determined in each sample and the average of three replicas deduced. The number of prey/vacuoles was calculated by dividing the number of prey/cell by the number of vacuoles/cell in each sample prior to averaging.

When the ciliate concentration was too low for this method (around 1.2×10^5 cells/ml), 100µl of sample was filtered onto a 1.2µm white filter (Whatman, Millipore) which was applied to a slide and cells counted as above. Counting RFP-prey within the ciliate was performed on the same day or the following morning of the experiment due to fading of the red fluorescence after that time.

2.3 Description of a standard feeding experiment

On the day of an experiment, the ciliate was prepared and counted as described in Sections 2.1.5 and 2.2.3. The 3 days-old RFP-strains and Pico 3 cell concentrations were determined as described in Section 2.2.2. The DTAF-bacteria (of known concentration) and kept at -20°C, were thawed at room temperature and sonicated for 5 minutes and vortexed for another 5 minutes to break up any aggregates. The bead suspension (of known concentration), stored at 4°C, was also sonicated for 5 minutes and vortexed for another 5 minutes.

T. pyriformis was fed with a prey type in triplicate; final experimental volumes were normally 100 μ l (in Eppendorf tubes). The prey was added to yield a starting concentration of 2×10^7 cells/ml. The ciliate was fed for 5 minutes at room temperature (*ca.* 23°C) before fixing with glutaraldehyde (0.5% v/v final concentration). The number of prey/cell, vacuoles/cell and prey/vacuole was then determined for each replicate as described in Section 2.2.4. All experiments were repeated twice unless otherwise stated.

2.4 Specifics of the feeding experiments not involving blockers

2.4.1 Ingestion of monocultures

T. pyriformis was fed with a single bacterial strain at 2×10^7 cells/ml in two forms (Live and Dead) alongside beads, in triplicate.

2.4.2 Ingestion of mixed prey types

These experiments were performed alongside the monoculture feeding experiment (Section 2.4.1), to compare the uptake of prey in mixtures with the uptake of prey alone. For each bacterial strain, *T. pyriformis* was fed a 50:50 mixture as follows: Live and Dead cells, Live cells and Beads, Dead cells and Beads, each at a concentration of 2×10^7 cells/ml (total prey concentration 4×10^7 cells/ml).

2.4.3 Effect of S-layer 'stripping' on ingestion

Three of the RFP-expressing bacteria, *Aeromonas hydrophila*, *Serratia marcescens* and *Synechococcus* sp. (Pico 3), possess an S-layer (Live S+). Each was treated with lithium chloride (Sigma) to remove their S-layer (Live S-) following Tarao et al. (2009). Ten ml of bacterial suspension was centrifuged for 10 minutes at 3500rpm. The cells were re-suspended in 10ml of phosphate buffered saline (PBS) containing 0.5M of lithium chloride. The suspension was incubated at 16°C for 30 minutes. Every 5 minutes, the tubes were shaken gently, without taking them out from the incubator. After centrifugation, incubation with lithium chloride was repeated. The suspensions were then centrifuged again, the supernatant was removed and replaced with PBS alone (one 'wash'). All bacterial suspensions were washed 6 times with PBS to

remove any remaining lithium chloride. *T. pyriformis* was fed for 5 minutes with monocultures of Live S+ and Live S- as described in Section 2.4.1.

2.4.4 Effect of proteolytic ‘shaving’ of surface-exposed proteins on ingestion

Although all live bacteria will have surface-associated proteins (P+), two strains were tested here, *Salmonella enterica 74* and *Serratia marcescens*, in order to observe the effect of removing surface exposed proteins (P-) on uptake by *T. pyriformis*. The method followed Wentzel et al. (2001).

Bacterial suspensions were washed once by centrifugation at 3500rpm for 10 minutes and the supernatant was replaced with PBS. Sequencing grade trypsin (Roche Diagnostics GmbH) was added at 50µg/ml (final concentration) and samples were gently shaken before incubating at 37°C for 10 minutes. Samples were then washed 3 times with PBS. *T. pyriformis* was fed for 5 minutes with monocultures of Live P+, Live P- and Dead cells as described in Section 2.4.1.

2.4.5 Effect of Formaldehyde fixing on ingestion

Three RFP-expressing bacteria were used for this experiment, *S. enterica 74*, *S. marcescens* and *Staphylococcus aureus*. Suspensions were fixed with formaldehyde (2% final concentration) for 30 minutes at room temperature (ca. 23°C), then washed six times with PBS, by centrifuging for 10 minutes at 3500rpm and replacing the supernatant with PBS. *T. pyriformis* was fed for 5 minutes with monocultures of Live, Fixed and Dead cells as described in Section 2.4.1.

2.5 Specifics of experiments involving blockers

These experiments were performed with *S. enterica 74* (Live and Dead) and Beads. The same monoculture experiments (Section 2.4.1) were performed but prior to adding the prey, the ciliate was incubated with a compound which blocked certain receptors (and included a control which was not blocked). These experiments evaluated the blocking of sugar receptors (Section 2.5.1) and scavenger receptors (Section 2.5.2).

2.5.1 Blocking sugar receptors

Three sugars were used to block their respective receptors in *T. pyriformis*: D(+)-Mannose (Sigma), N-Acetyl-D-galactosamine (GalNAc, Sigma) and N-Acetyl- α -D-glucosamine (GlcNAc, Sigma). The sugars were dissolved in sterile water (Milli-Q) to give a stock solution of 1M. They were prepared fresh on the day of the experiment.

The ciliate was pre-incubated at room temperature (*ca.* 23°C) with each of the sugars at concentrations: 0mM, 2mM, 20mM, 50mM, 100mM, 125mM, 150mM, 175mM, and 200mM for 30 minutes before performing a standard monoculture feeding experiment (Section 2.4.1). In addition, ciliate cell concentration was determined at 5 minutes to ensure the sugars, at high concentrations, were not toxic (and additional 400mM was included). Experiments were performed three times, each time in triplicates (n=90 ciliate cells per prey type).

2.5.2 Blocking scavenger receptors

The effects of dextran sulfate (DS) (Sigma), a general blocker of all scavenger receptors (SRs), and chondroitin sulfate-Bovine (CS) (Sigma), which is structurally similar to DS but does not bind avidly to SRs, on ciliate ingestion were evaluated following Perú et al. (2016). They were prepared fresh on the day of the experiment. The ciliate was pre-incubated with 400 μ g/ml (final concentration) of DS or CS for 30 minutes (*ca.* 23°C) before performing a standard monoculture feeding experiment (Section 2.4.1).

2.6 Statistics

One-way ANOVA followed by a post-hoc Bonferroni test was used to analyze data with more than two variables (astatsa.com/OneWay_Anova_with_TukeyHSD), while T-tests were used when only two variables were compared (Microsoft Excel). The data containing prey biovolume versus P/C, V/C, P/V or vacuole biovolume was analyzed using regression testing tools and the correlation coefficient test from Microsoft Excel. The confidence limits for all tests mentioned above were 95% (P value ≤ 0.05) and 99% (P value ≤ 0.01).

CHAPTER 3: RESULTS

3.1 Feeding experiments

3.1.1 General Overview

Tetrahymena pyriformis was fed with live RFP-expressing bacteria (full complement of surface properties, e.g., receptors, capsule etc.) and their heat-killed-DTAF-stained counterparts (assumed to have disrupted surface properties) in monoculture and 50:50 mixture experiments. An inert prey, fluorescent microspheres (Beads), was also included to represent a particle with no biological surface properties. Three parameters were determined after 5 minutes feeding: prey/cell (P/C), vacuoles/cell (V/C) and prey/vacuole (P/V) and, unless otherwise stated, experiments were repeated twice.

The average data for all 11 bacterial strains and Beads, when fed to *T. pyriformis* as monocultures and in 50:50 mixtures, are presented in Figure 3.1.

Beads: All three parameters (V/C, P/C and P/V), in monoculture, were significantly lower with Beads ($P < 0.01$), compared to Live and Dead prey (Figure 3.1). Beads were also ingested to a significantly lesser extent in the 50:50 Bacterium:Bead mixtures (Figure 3.1A). In these mixtures, vacuole formation appeared to be governed by the prey bacterium, not the Beads. With Live prey, 3.73 vacuoles/cell were formed in mixture (Figure 3.1B) which is not significantly different ($P = 0.82$) to the number formed with Live prey alone (3.75 V/C, Figure 3.1B), but significantly higher ($P < 0.01$) than with Beads alone (2.46 V/C, Figure 3.1B). With Dead prey, 3.3 vacuoles/cells were formed in mixture (Figure 3.1B) which is not significantly different ($P = 0.39$) to the number formed with Dead prey alone (3.21 V/C, Figure 3.1B) but once again significantly higher ($P < 0.01$) than with Beads alone (2.46 V/C, Figure 3.1B).

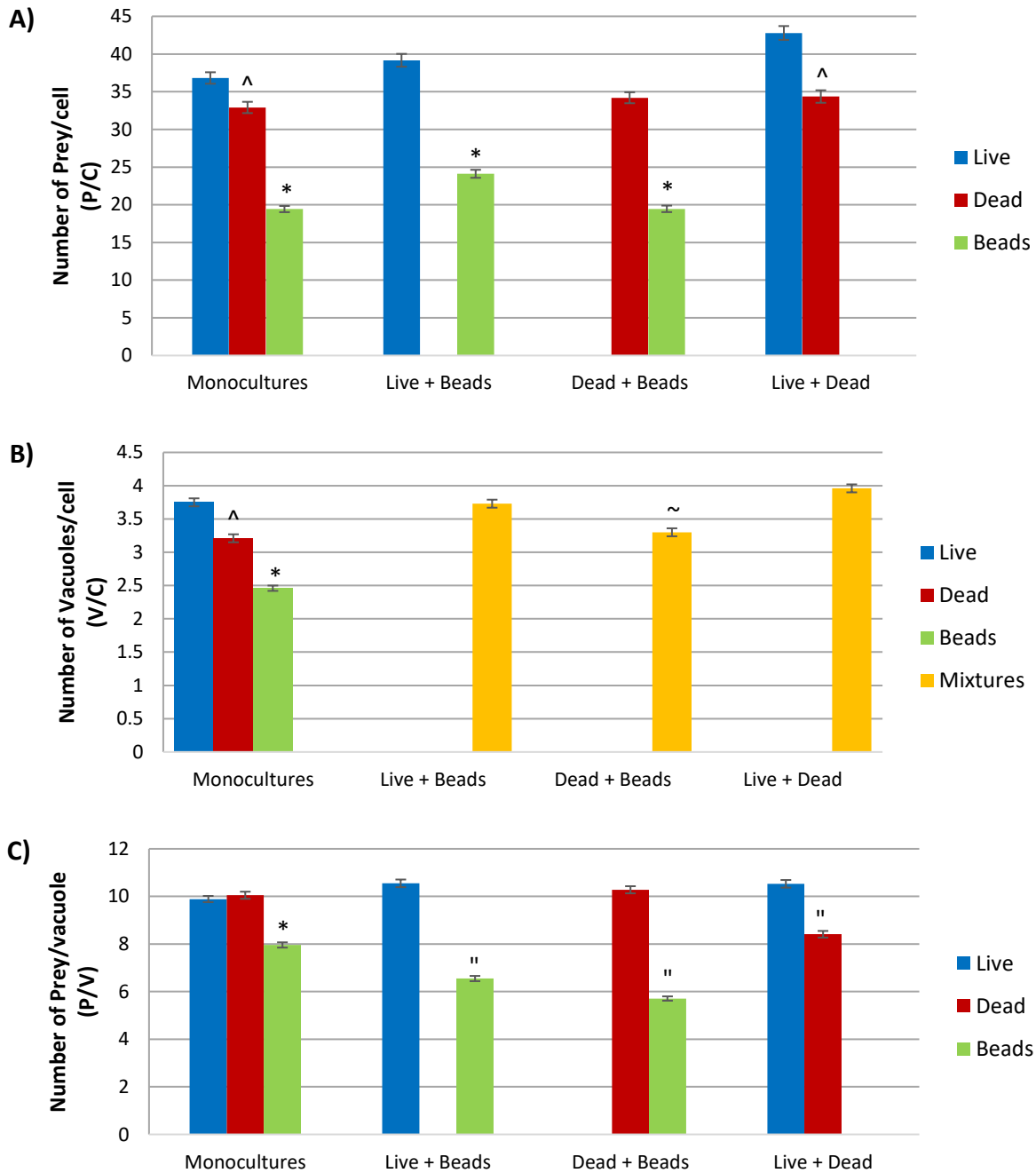


Figure 3.1. *Tetrahymena pyriformis* was fed with each prey type at 2×10^7 cells/ml in monocultures and in 50:50 mixtures before determining: prey/cell (A), vacuoles/cell (B) and prey/vacuole (C). Error bars=SEM. The data is presented as averages of 60 ciliate cells per prey type from each strain. *significantly lower than Live ($P < 0.01$) and Dead ($P < 0.01$) in monocultures or in mixtures. [^]significantly lower than Live ($P < 0.01$) and higher than Beads ($P < 0.01$) in monocultures; significantly lower than Live ($P < 0.01$) in mixture. [~]significantly lower to V/C in Live+Beads and Live+Dead mixtures ($P < 0.01$). ["]significantly lower P/V in mixtures than alone ($P < 0.05$) due to more vacuoles being produced in mixture.

Bacteria: Comparing Live versus Dead prey uptake in monocultures (Figure 3.1A) shows that *T. pyriformis* ingested a significant higher number of Live prey and these Live preys were deposited into significantly more vacuoles than Dead prey ($P < 0.01$; Figure 3.1B). Within 50:50 mixtures, significantly more Live prey were ingested compared to Dead, but the difference in ratio of each prey in the cell (56:44) was not as great as that recorded with Beads (62:38, Figure 3.1A). The number of vacuoles/cell produced in the presence of both Live and Dead prey, i.e. 3.96 vacuoles/cell, was significantly higher than with Live prey alone ($P < 0.05$) and Dead prey alone ($P < 0.01$), suggesting a synergistic interaction with regards to vacuole formation, however the resultant value most closely resembled that with Live prey alone, i.e. 3.75 vacuoles/cell (Figure 3.1B).

Data therefore show that the overall trends are that, (i) Live prey are ingested at higher rates than Dead prey or Beads and, when in mixture, Live prey controls vacuole formation with Beads and has a synergistic interaction with Dead prey and, (ii) Dead prey are ingested at higher rates than Beads and when in mixture, Dead prey control vacuole formation. This suggests that biological surface properties, in some way, influence both prey ingestion and vacuole formation. However, some of these trends did not hold for all eleven bacterial strains and these differences are highlighted in Section 3.1.3 after an examination of the level of inherent variation between experiments (Section 3.1.2).

3.1.2 Processing of Beads and inherent variation in ciliate feeding

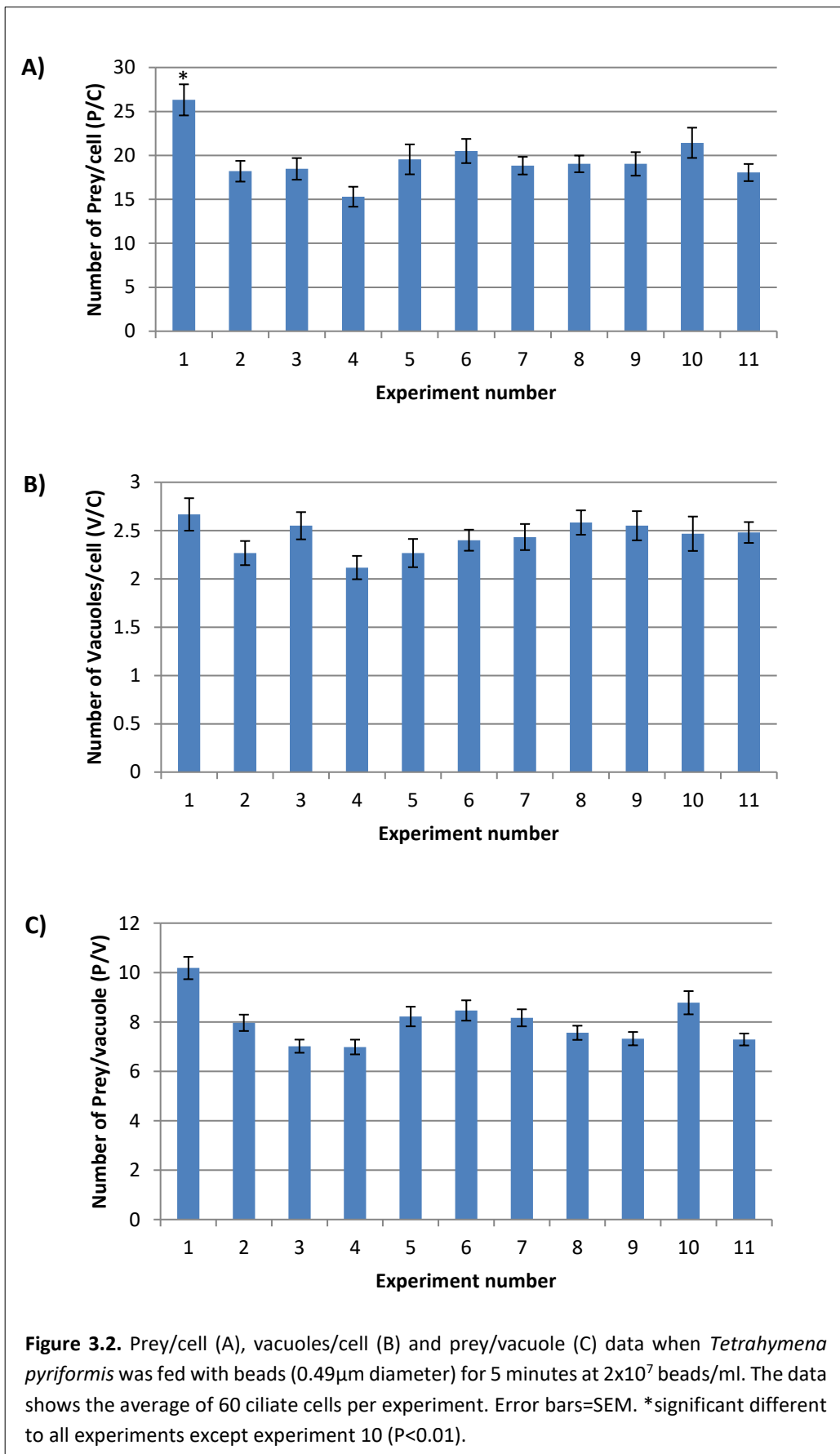
The same Beads were employed in eleven experiments (performed twice). Having the same physical and biochemical properties, their data show the level of inherent background variation in calculated ciliate feeding parameters, due to slight variations in ciliate pre-culturing (Figure 3.2).

At the extremes, Experiments 4 and 1 differed the most with P/C ranging from 15.3 to 26.32 (Figure 3.2A), V/C ranging from 2.1 to 2.6 (Figure 3.2B) and P/V ranging from 6.98 to 10.18 (Figure 3.2C). Thus the inherent variation (experiment 1 minus experiment 4) was 11.02 P/C, 0.5 V/C and 3.2 P/V. Attention should be drawn to the low level of variation with regards to V/C which

suggests a consistent basal rate of vacuole production (at 23°C) in the presence of inert particles, i.e. *ca.* 2.5 vacuoles in 5 minutes (Figure 3.2B).

When Beads were presented to *T. pyriformis* in a 50:50 mixture with either Live or Dead prey, average data (Figure 3.1A) showed that Beads were ingested to a lesser extent than the bacteria, yielding a ratio of internalized prey of *ca.* 63:47 Bacterium:Beads. This result was consistent with every single bacterial strain tested (Figures 3.3 and 3.4). The other consistent result was that Beads and bacteria occupied the same vacuoles and were not deposited into different vacuoles. The third consistent result was that Live bacteria always appeared to control the number of vacuoles formed when in mixture with Beads.

The latter trend was not consistent when Beads were mixed with Dead bacteria with only 8 of the 11 strains suggesting Dead cells control vacuole formation in mixture with Beads. For the remaining 3 strains (Pico 3, *Serratia marcescens*, *Staphylococcus aureus*), Dead cells did not upregulate vacuole formation above the basal rate obtained with Beads alone (*ca.* 2.5 V/C in 5 minutes), suggesting that these Dead strains were behaving like Beads (confirmed in Section 3.1.3.3).



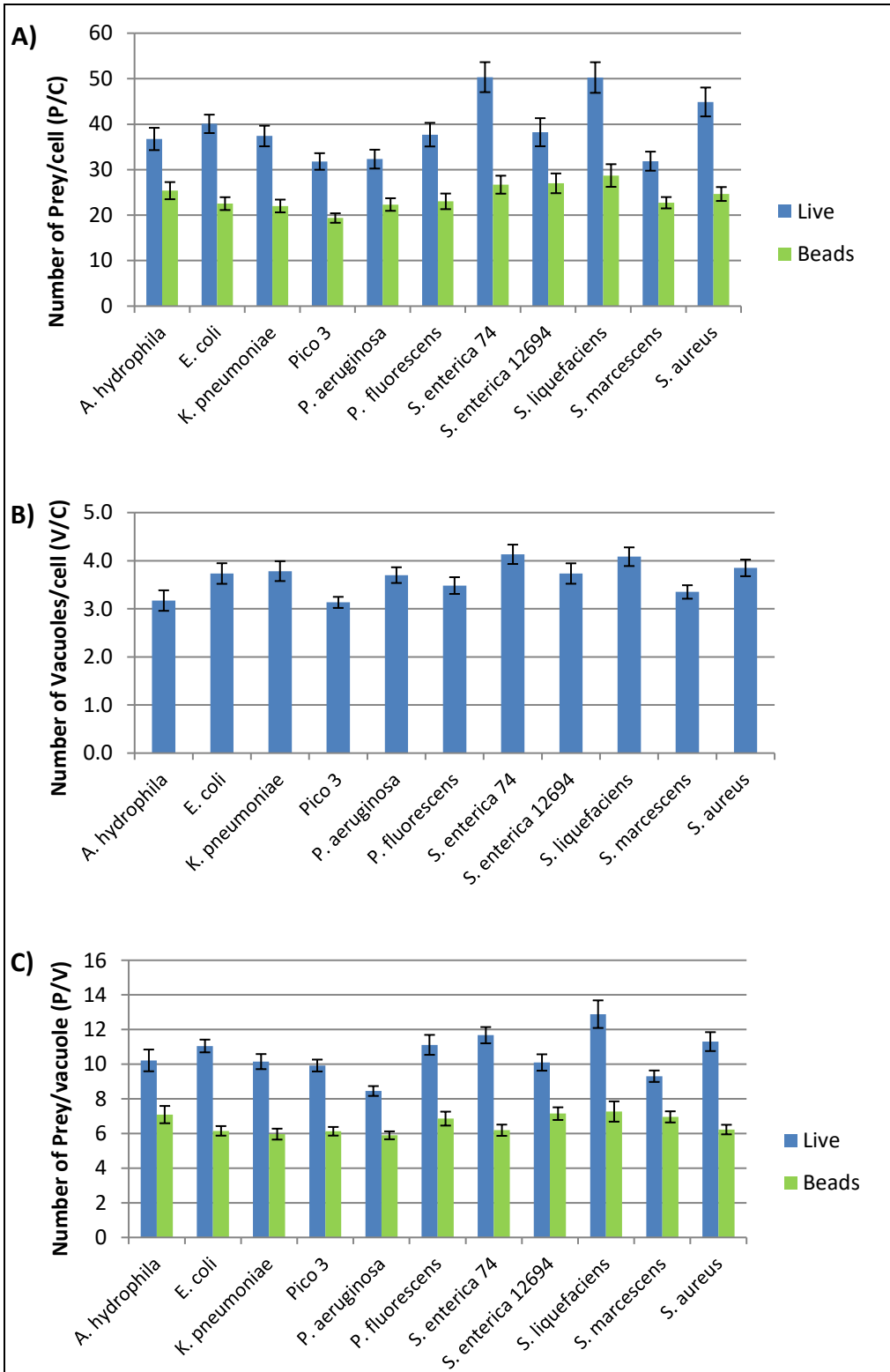
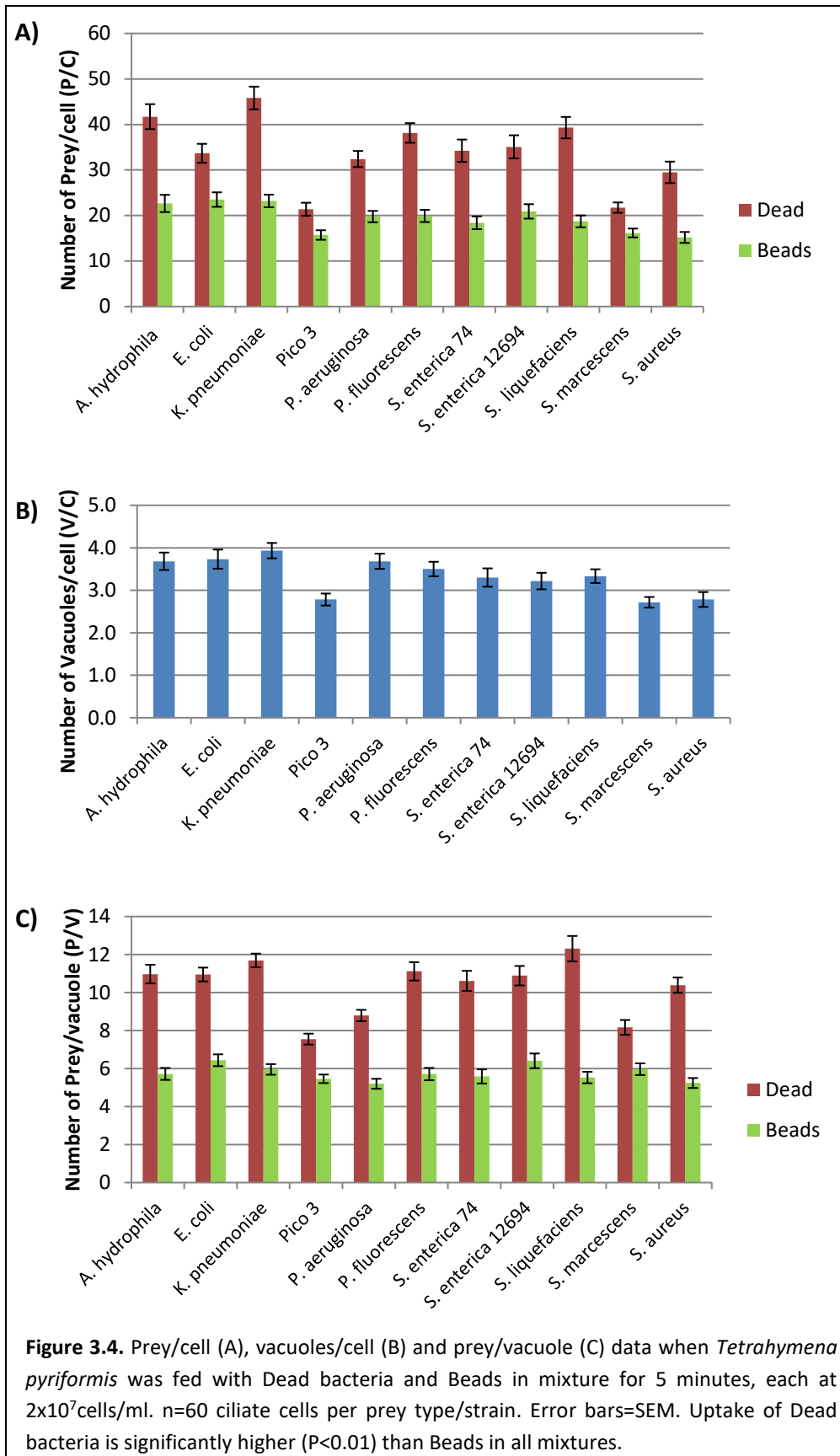


Figure 3.3. Prey/cell (A), vacuoles/cell (B) and prey/vacuole (C) data when *Tetrahymena pyriformis* was fed with Live bacteria and Beads in mixture for 5 minutes, each at 2×10^7 cells/ml. Data are the average for 60 ciliate cells per prey type from each strain. Error bars=SEM. Uptake of Live bacteria is significantly higher ($P < 0.01$) than Beads in all mixtures.



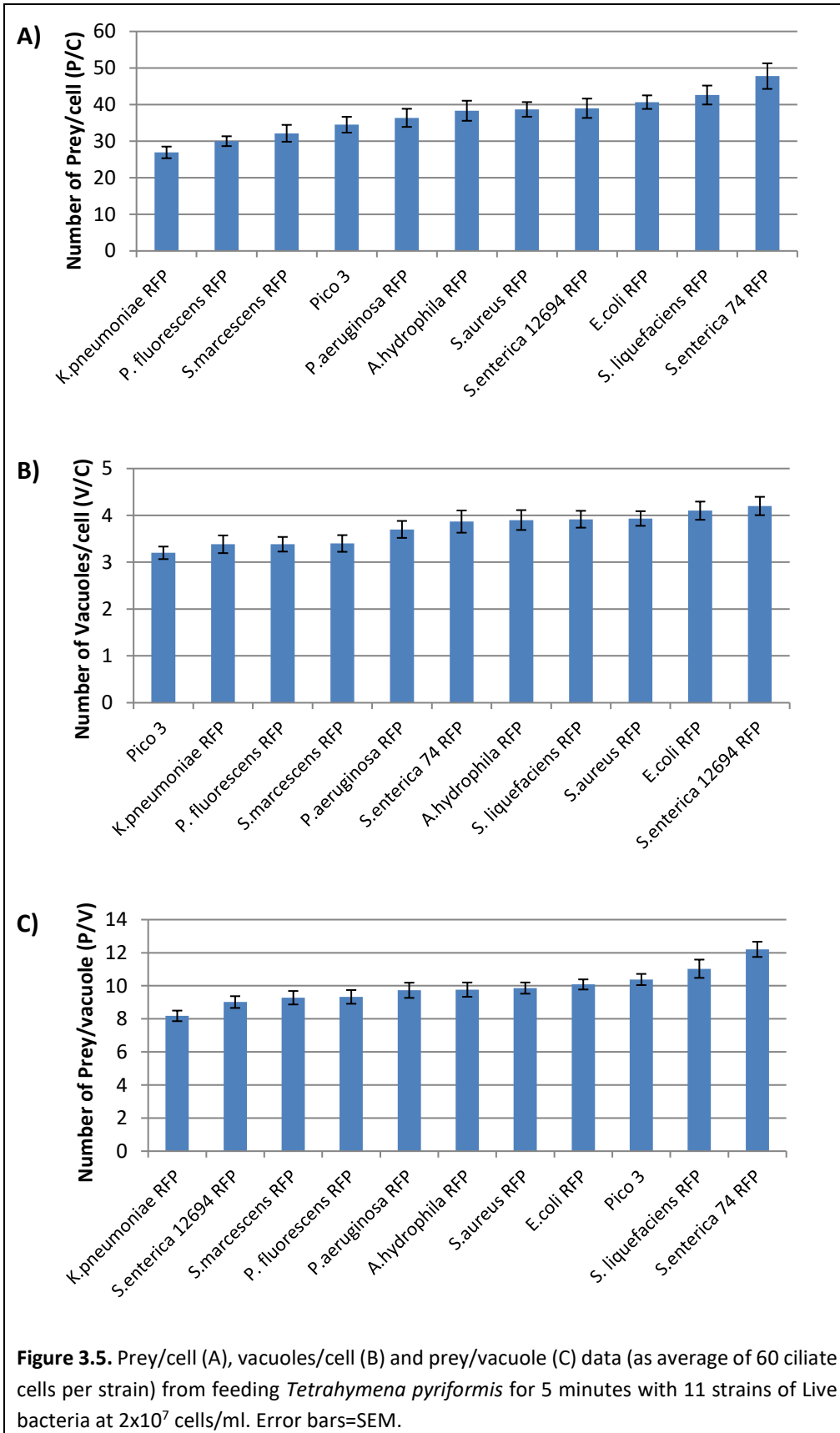
3.1.3 Processing of Bacteria

3.1.3.1 Variation between Live strains – monoculture experiments

Figure 3.5 shows data for the individual bacterial strains which contributed to the average data in Figure 3.1. At the extremes, *Klebsiella pneumoniae* and *Salmonella enterica* 74 differed the most with regards to P/C (ranging from 29.9 to 47.78 P/C, respectively [Figure 3.5A]) and P/V (ranging from 8.18 to 12.2 P/V, respectively [Figure 3.5C]), while Pico 3 and *Salmonella enterica* 12694 differed the most with regards to V/C (ranging from 3.2 to 4.2 V/C, respectively [Figure 3.5B]). The net variations (highest minus lowest values) experienced with Live prey were higher than with Beads: Live prey induced higher variation in P/C (17.88), V/C (1) and P/V (4.02) compared to Beads (11.02, 0.5 and 3.2, respectively), suggesting the involvement of some other factor(s) in addition to inherent experimental variation.

The variation observed between strains could be due to their different cell biovolumes (0.27 to $1.7\mu\text{m}^3$, Table 2.1). However, there was no significant relationship found between prey cell biovolume and any of the three feeding parameters (Figure 3.6) suggesting that prey biovolume plays no role in the instantaneous ingestion and processing of prey by *T. pyriformis*. The only significant trend ($P < 0.01$) with prey biovolume was with the volume of prey *within* a vacuole, i.e. P/V x prey biovolume. Data indicated that larger prey gave rise to larger vacuoles; not more vacuoles containing fewer prey (Figure 3.7). Therefore, it appears that the *number* of prey/vacuole remains relatively constant in monoculture (*ca.* 9 P/V, [Figure 3.7]) irrespective of the size of the prey.

Variation between strains could therefore be due to a number of other factors and those tested in this study were the presence of an S-layer and surface receptors. A crude way to disrupt both (and other factors) at the same time is to heat-kill the cells (Sections 3.1.3.2), or specific treatments can be used to evaluate the effects of S-layer removal only (Section 3.1.3.3) and surface receptor removal in isolation (Section 3.1.3.4).



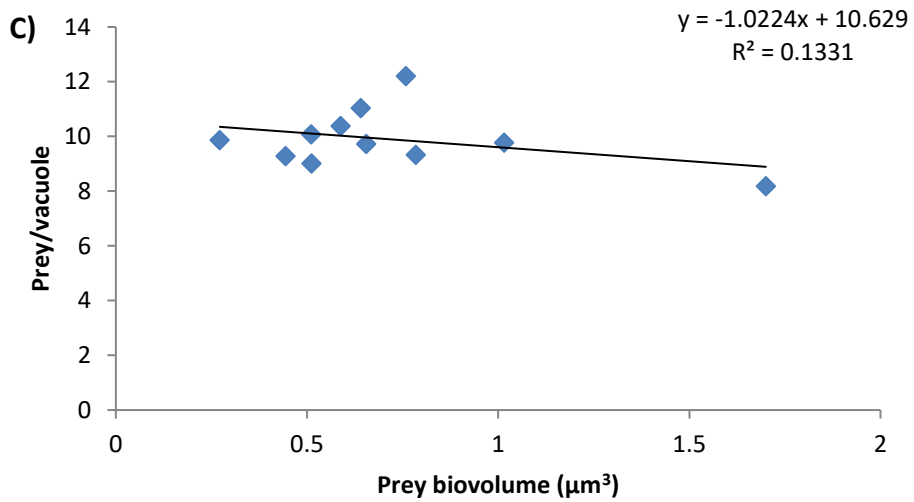
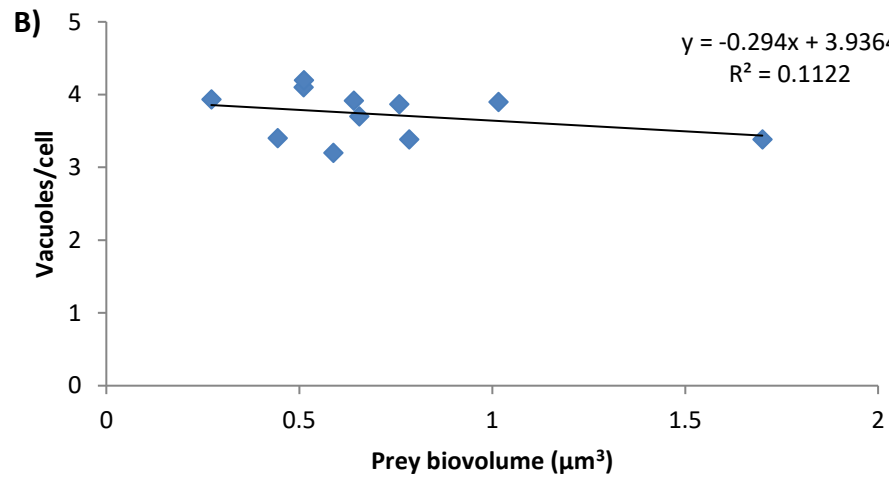
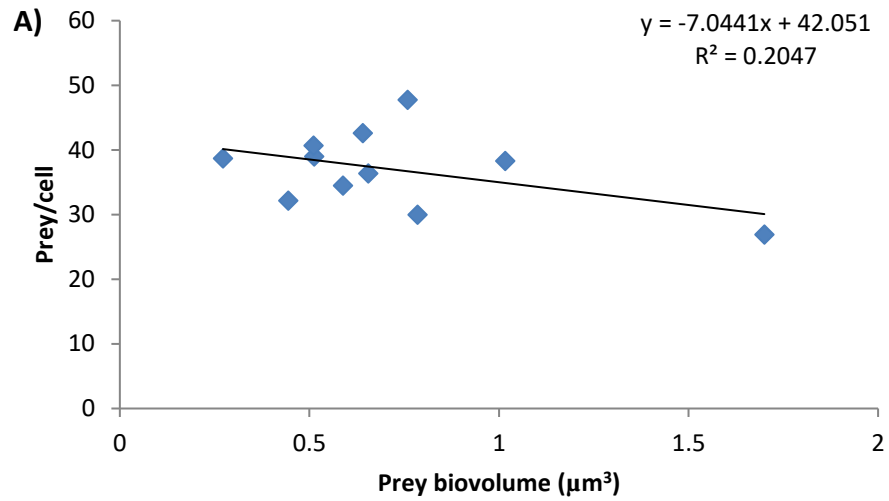
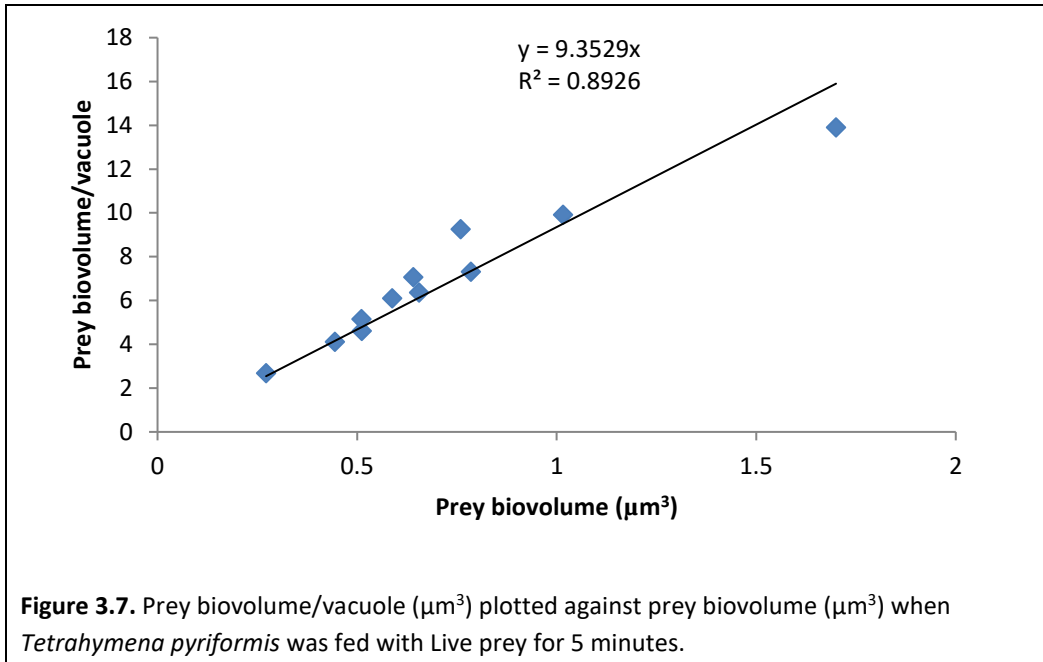


Figure 3.6. Prey/cell (A), vacuoles/cell (B) and prey/vacuole (C) data plotted against prey biovolume (μm^3) when *Tetrahymena pyriformis* was fed with Live prey for 5 minutes.



3.1.3.2 Live vs Dead (Heat-killed) cells

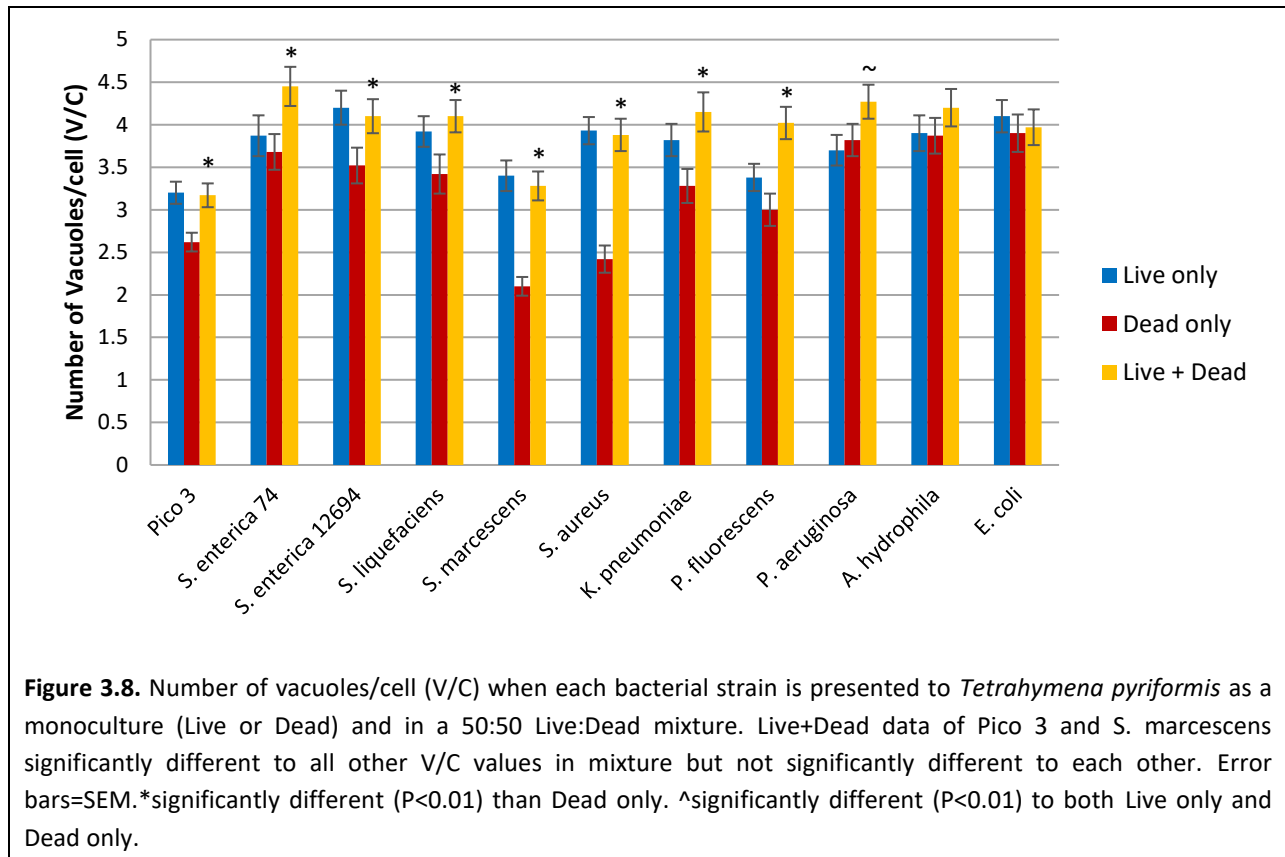
Each prey state (Live or Dead) was presented to *T. pyriformis* as (i) monocultures at 2×10^7 cells/ml and, (ii) as mixtures (50:50 Live:Dead) with each prey at 2×10^7 cells/ml; yielding an overall prey concentration of 4×10^7 cells/ml.

The *total* number of V/C with mixtures was equivalent for 9 strains (except for Pico 3 and *Serratia marcescens*) (Figure 3.8), yielding an average of 4.14 ± 0.70 V/C. The *total* number of P/C in mixture was equivalent for 8 strains (except for Pico 3, *S. marcescens* and *Pseudomonas aeruginosa*) (Figure 3.10B), yielding an average of 85.86 ± 2.09 P/C. These values were therefore considered *maximum values* for P/C and V/C in this study.

The number of vacuoles produced (in mixture), and the uptake of prey (in mixture), was compared to those values in monoculture (at 2×10^7 cells/ml) and to the proposed maximum values (Figures 3.8 and 3.10, respectively).

3.1.3.2.1 Vacuole formation factor (VFF)

Data suggest that any 'vacuole formation factor' (VFF) was completely destroyed by heat in Pico 3, *S. marcescens* and *Staphylococcus aureus* cells, with V/C (Dead cells) (Figure 3.8) being equivalent to that with Beads alone (2.46 ± 0.04 V/C, Figure 3.1B). In their Live:Dead mixtures, Live cells appeared to govern vacuole formation as V/C in mixture was only significantly different to that obtained with Dead V/C in monoculture (Figure 3.8).



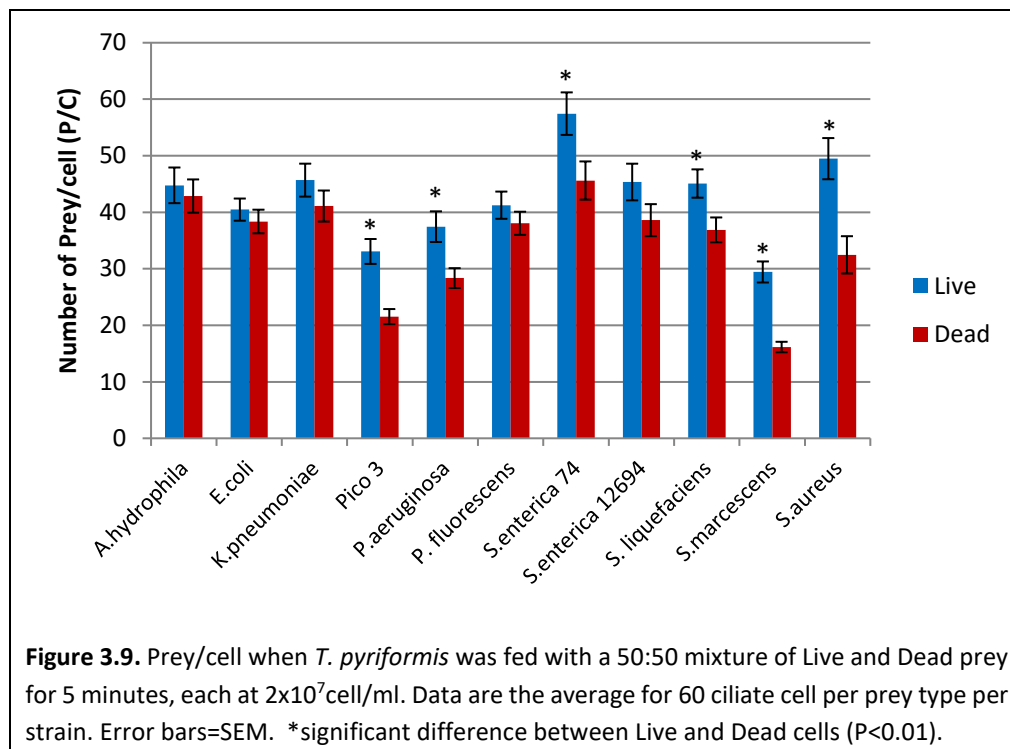
At the other extreme, data suggest that heat did not destroy the VFF in the cells of *P. aeruginosa*, *Aeromonas hydrophila* and *Escherichia coli*, with V/C in monoculture being equivalent between Live and Dead cells (Figure 3.8). When Live and Dead cells were in mixture, both prey types (with intact VFF) would be expected to significantly increase total V/C if a synergistic interaction was occurring. This was observed for *P. aeruginosa* whereby 4.27 V/C were produced in mixture compared to 3.7 and 3.82 V/C when alone (Figure 3.8). This response was not observed with *A. hydrophila* and *E. coli* but this might be due to the fact that each strain was already at their

maximum V/C with a prey concentration of 2×10^7 cells/ml (Live and Dead alone); whereas *P. aeruginosa* was not (Figure 3.8).

For the remaining 5 Dead strains (*Salmonella enterica* 74, *S. enterica* 12694, *Serratia liquefaciens*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens*), remnants of the VFF appear to have remained on the Dead cells, yielding V/C values lower than with Live cells (Figure 3.8), but this was only significant ($P < 0.05$) for *K. pneumoniae* and *S. enterica* 12694. The number of V/C in mixture was generally higher than with either Live or Dead cells alone (except for *S. enterica* 12694) suggesting a synergistic interaction; but none were significant and Live cells were deemed to govern vacuole formation (Figure 3.8).

3.1.3.2.2 Prey uptake factor(s) (PUF)

The data relating to P/C were more difficult to interpret, compared to V/C, but in all Live:Dead mixtures the uptake of Live cells was higher than Dead cells (Figure 3.9); although this was only significant for six strains ($P < 0.01$).

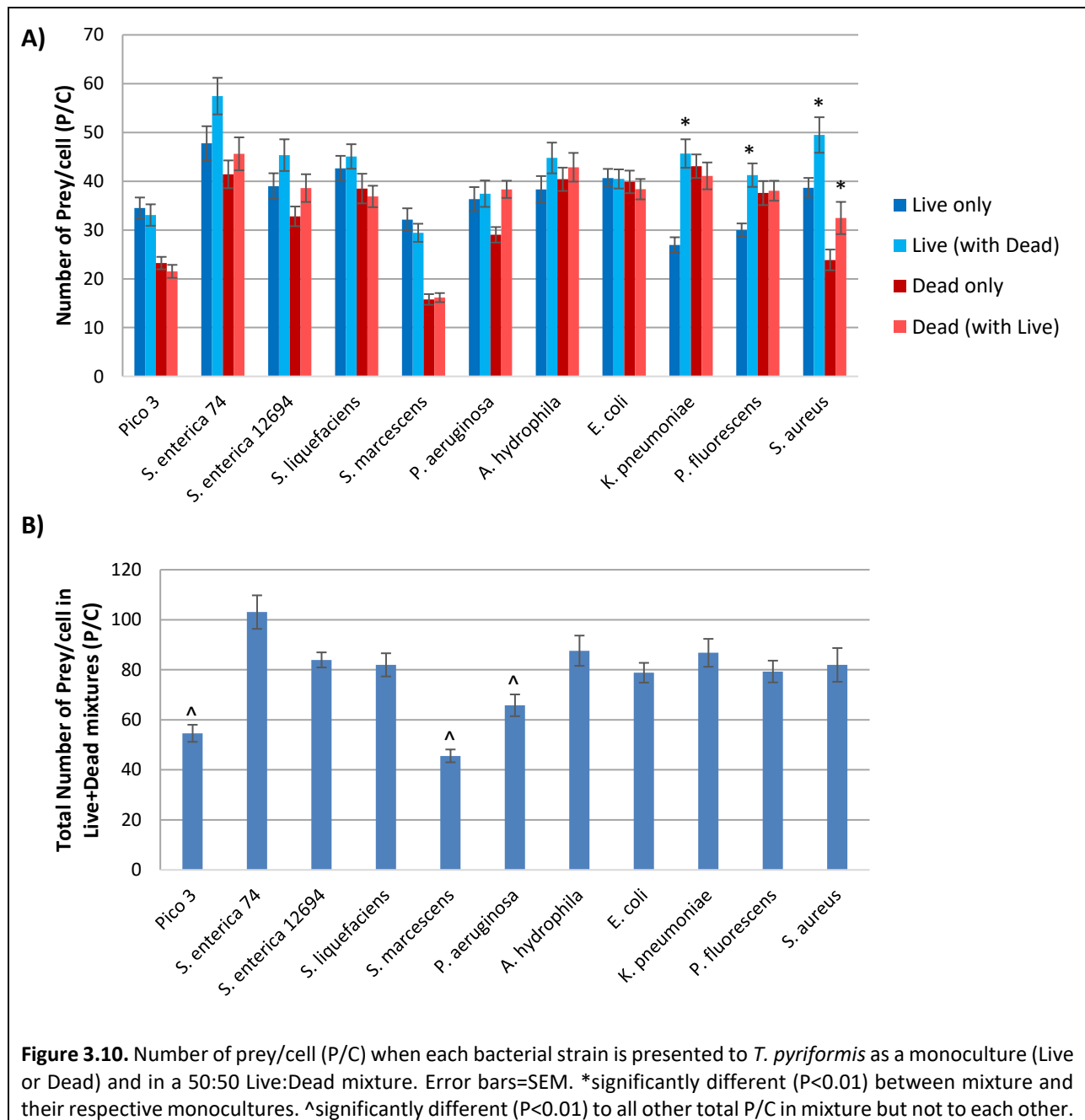


Data suggest that heat treatment completely destroyed the 'prey uptake factor' (PUF) in the cells of Pico 3, *S. marcescens* and *S. aureus* with P/C values (Dead cells) in monoculture (Figure 3.10A) being equivalent to those with Beads in monoculture (19.43 ± 0.41 P/C, Figure 3.1A). However, other data in Figure 3.10 question this conclusion for *S. aureus*.

For Pico 3 and *S. marcescens*, there was no obvious interference between the uptake of Live and Dead cells in mixture and each was consumed at equivalent rates as in monoculture (Figure 3.10A), i.e. no obvious *preference* for one over the other, just that Live cells are inherently ingested at a faster rate than Dead cells (which were 'Bead-like'). The total P/C in these mixtures was significantly lower than with any other prey strain mixture, except *P. aeruginosa*, due to the low uptake of these 'Bead-like' Dead cells (Figure 3.10B). The same cannot be said for Dead *S. aureus* cells, the only Gram-positive strain tested, which showed significant interference between Live and Dead cells in mixture. Both prey types were ingested significantly more in mixture than in monoculture (up 31% and 36% for Live and Dead, respectively) and yielded the maximum total P/C value of *ca.* 82 P/C (Figure 3.10B). Consumption of Live cells was also significantly higher than Dead cells in both monoculture ($P < 0.01$) and mixture ($P < 0.01$, Figure 3.10A).

Data suggest that heat treatment did not affect the PUF of *A. hydrophila*, *E. coli*, *S. enterica* 12694, *P. fluorescens* and *K. pneumoniae* with no significant difference between the P/C values obtained with Live and Dead cells in mixture (Figure 3.9). For the former three strains, no interference in the uptake of Live and Dead cells occurred in mixture and each was consumed at equivalent rates to each in monoculture (Figure 3.10A).

The same cannot be said for *P. fluorescens* and *K. pneumoniae* which showed interference, with Live cells (but not Dead cells) being consumed significantly more in mixture than in monoculture (Figure 3.10A). These were the only strains which, when in monoculture, the ciliate consumed significantly more Dead cells than Live cells ($P < 0.01$, Figure 3.10A) possibly suggesting the presence of a 'negative' prey uptake factor, e.g. toxin, that is destroyed by heat. However, the fact that Live cells (*with this 'negative factor'*) *can still* be ingested well *in mixture*, suggests that the PUF is intact.



Data for the three remaining three strains (*P. aeruginosa*, *S. enterica* 74 and *S. liquefaciens*) suggest that the PUF was damaged by heat but not to the extent where the Dead cells behaved like Beads, i.e. P/C values with Dead cells in monoculture were significantly lower than with Live cells (P<0.05, Figure 3.10A) but significantly higher (P<0.01) than for Beads (19.43±0.41 P/C, Figure 3.1A). Live cells were ingested more than Dead cells in mixture (P<0.01, Figure 3.9) and no interference between prey types was observed (Figure 3.10A). The only difference between

these three strains was that the total P/C obtained with Live:Dead *P. aeruginosa* was significantly lower (65.80 ± 4.35 P/C) than with *S. liquefaciens* and *S. enterica* 74 (81.97 ± 4.63 and 103.07 ± 6.70 P/C, respectively) (Figure 3.10B) possibly suggesting that the PUF in *P. aeruginosa* is more badly damaged than in the other two strains.

3.1.3.3 Summary

Table 3.1 proposes the susceptibility of VFFs and PUFs to heat-treatment for each of the 11 bacterial strains. Both VFF and PUF were unaffected by heat-treatment in two strains (*A. hydrophila* and *E. coli*), whilst both were completely destroyed in three strains (Pico 3, *S. marcescens* and possibly *S. aureus*) (Table 3.1). Both were partially destroyed in two strains (*S. enterica* 74 and *S. liquefaciens*). There were four strains which showed different heat-treatment susceptibilities: *P. aeruginosa* (VFF heat-stable, PUF heat-labile) and *S. enterica* 12694, *P. fluorescens* and *K. pneumoniae* (VFF heat-labile, PUF heat-stable).

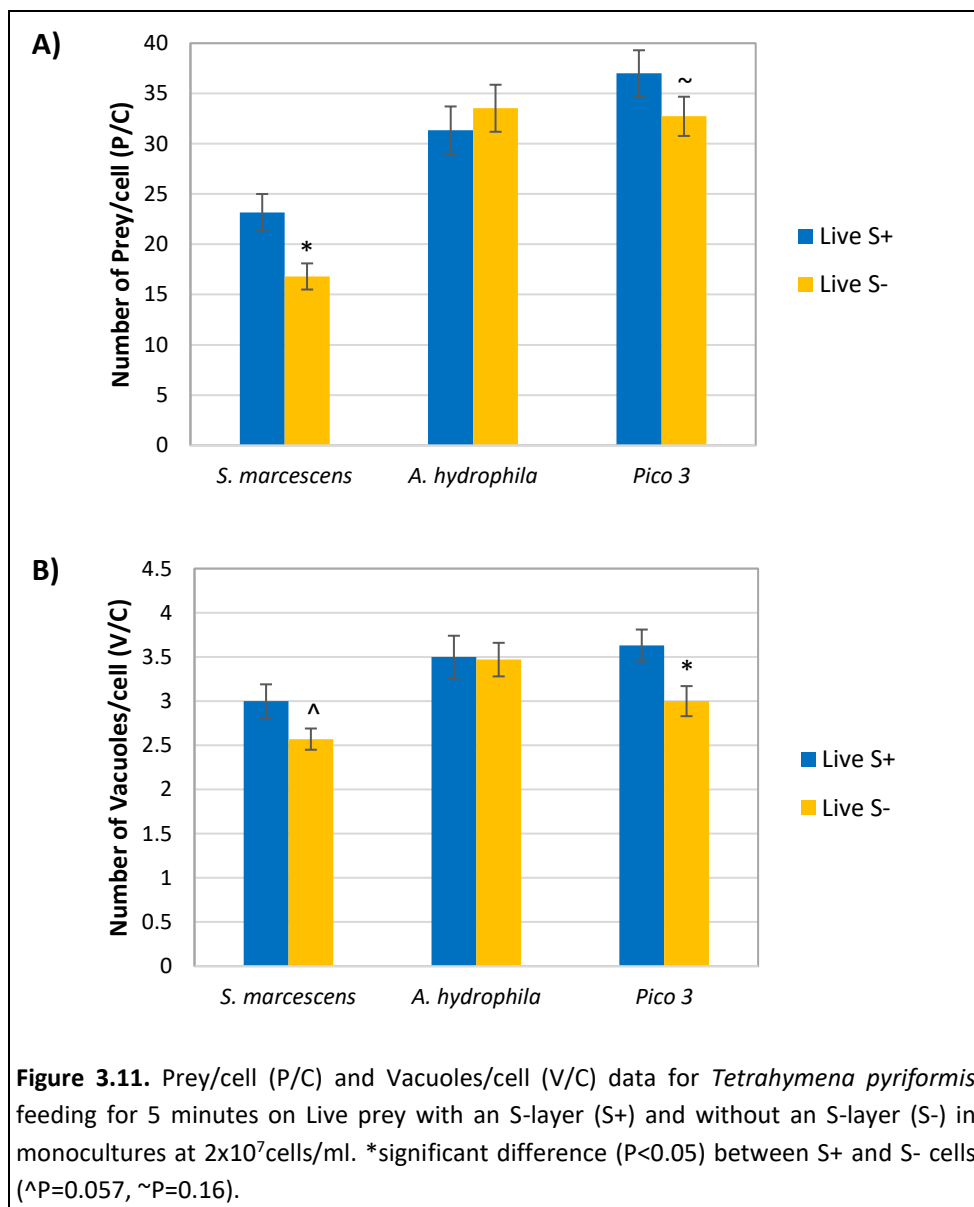
Table 3.1. Proposed stability of cell factors controlling ‘vacuole formation’ (VFF) and ‘prey uptake’ (PUF) in 11 Live bacterial strains. INTACT = no effect of heat-treatment, PARTIAL = some effect of heat-treatment, DESTROYED = complete destruction and Dead cells behave like Beads (*although unexplained interference evident in the uptake of Live and Dead cells in mixture – Figure 3.10.)

Bacterial strain	Factor	
	Vacuole formation (VFF)	Prey uptake (PUF)
<i>A. hydrophila</i>	INTACT	INTACT
<i>E. coli</i>		
<i>P. aeruginosa</i>		PARTIAL (very)
<i>S. enterica</i> 12694	PARTIAL	INTACT
<i>K. pneumoniae</i>		
<i>P. fluorescens</i>		
<i>S. enterica</i> 74		PARTIAL (slight)
<i>S. liquefaciens</i>		
Pico 3	DESTROYED	DESTROYED
<i>S. marcescens</i>		
<i>S. aureus</i> *		

3.1.4 Effect of specific treatments of Live cells on their uptake and vacuole production

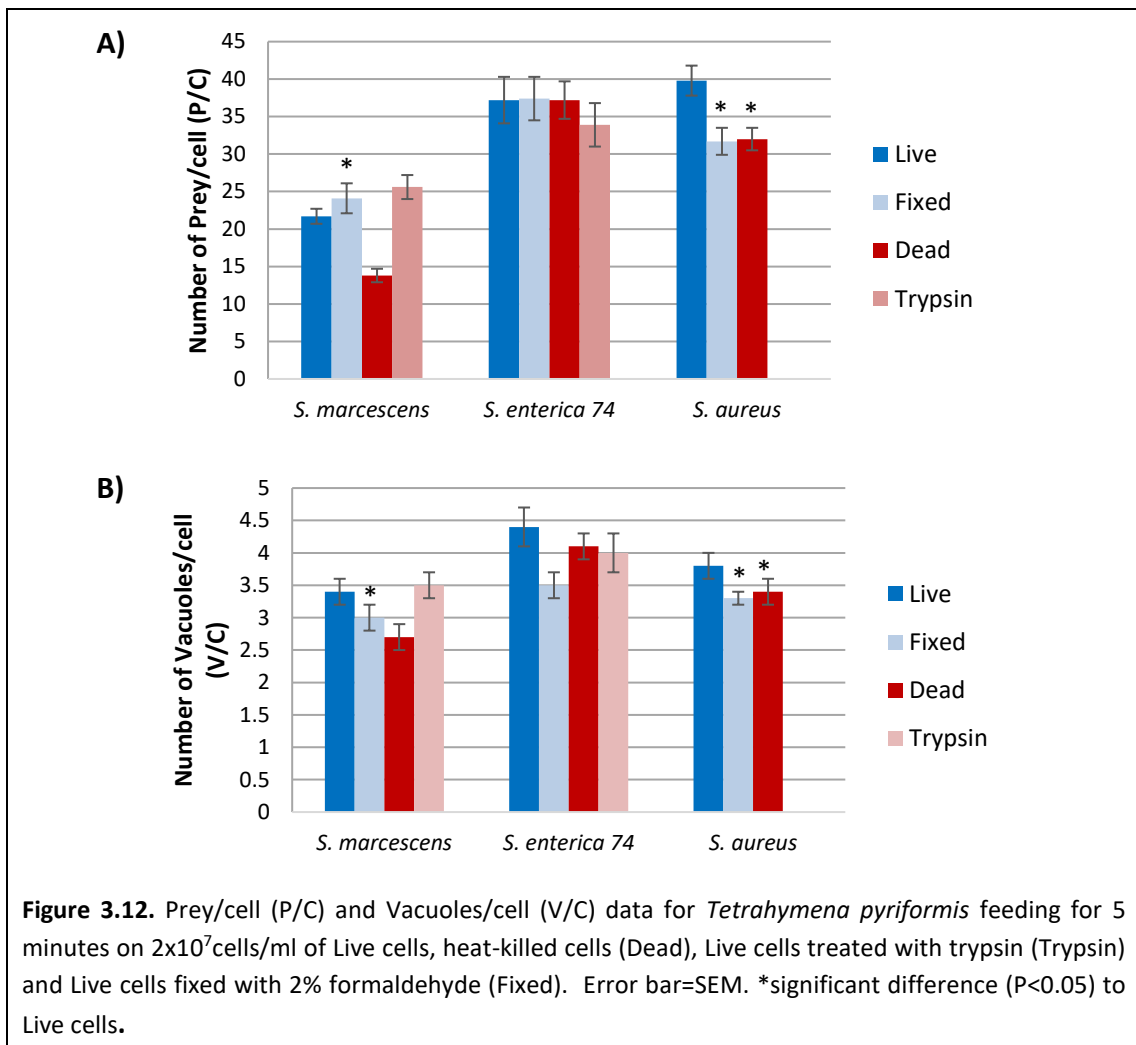
3.1.4.1 Effect of the removal of the S-layer

This was a preliminary experiment and only performed once (n=30 ciliate cells). Removal of the S-layer in *S. marcescens* and Pico 3 caused a reduction in P/C and V/C, but not all results were significant (Figure 3.11). Removal of the S-layer had no effect on the V/C and P/C values generated with *A. hydrophila* (Figure 3.11). These results mirror those obtained with heat-treatment (Table 3.1).



3.1.4.2 Effect of formaldehyde fixation and trypsin treatment

These were preliminary experiments and performed once for *S. marcescens* and *S. enterica* 74 (n=30 ciliate cells) and twice for *S. aureus* (n=60 ciliate cells). All three strains were treated with 2% formaldehyde for 30 minutes and the P/C and V/C values were compared to untreated cells (Live) and heat-killed cells (Dead) (Figure 3.12). The Gram-negative *S. marcescens* and *S. enterica* 74 were also treated with trypsin for 10 minutes to remove surface-exposed proteins.



The data shows that neither trypsin nor fixation significantly affected V/C or P/C, compared to Live cells, in the two Gram-negative strains whereas both parameters were significantly reduced in fixed *S. aureus* cells (Gram-positive) and both were equivalent to values with heat-killed (Dead) cells (Figure 3.12).

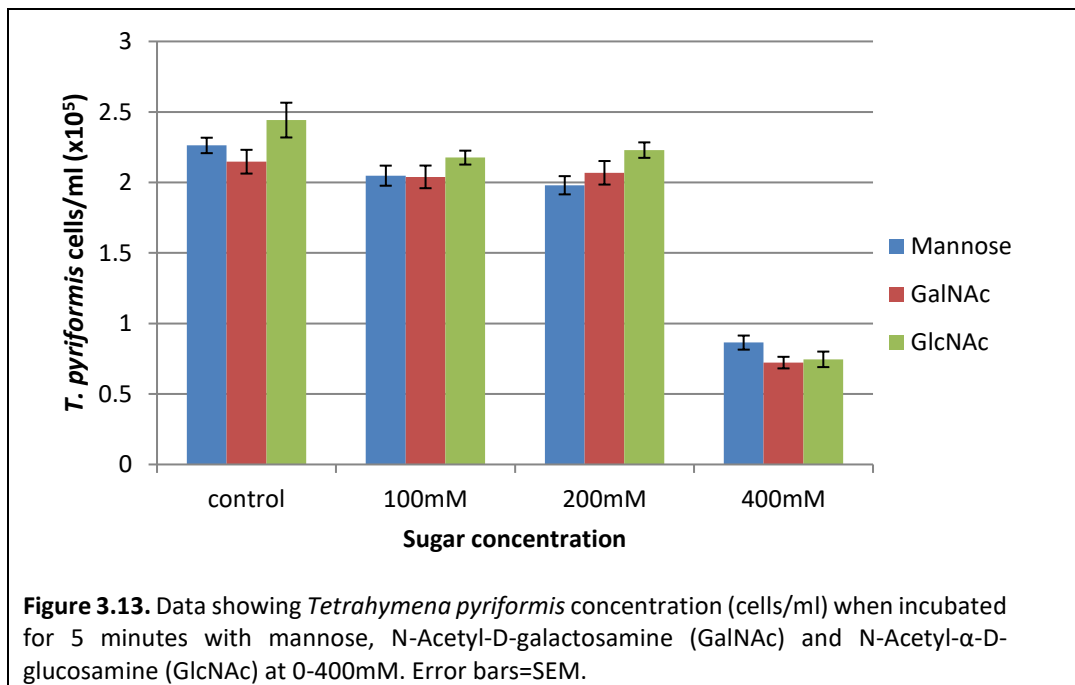
3.2 Receptor blocking experiments

3.2.1 Sugar blocking experiments

3.2.1.1 Toxicity experiment

A preliminary toxicity test was performed for each of the sugars, Mannose, N-Acetyl-D-galactosamine (GalNAc) and N-Acetyl- α -D-glucosamine (GlcNAc), whereby *T. pyriformis* was incubated with concentrations of 100, 200 and 400mM. The number of cells were determined after 5 minutes and compared to a control (0mM).

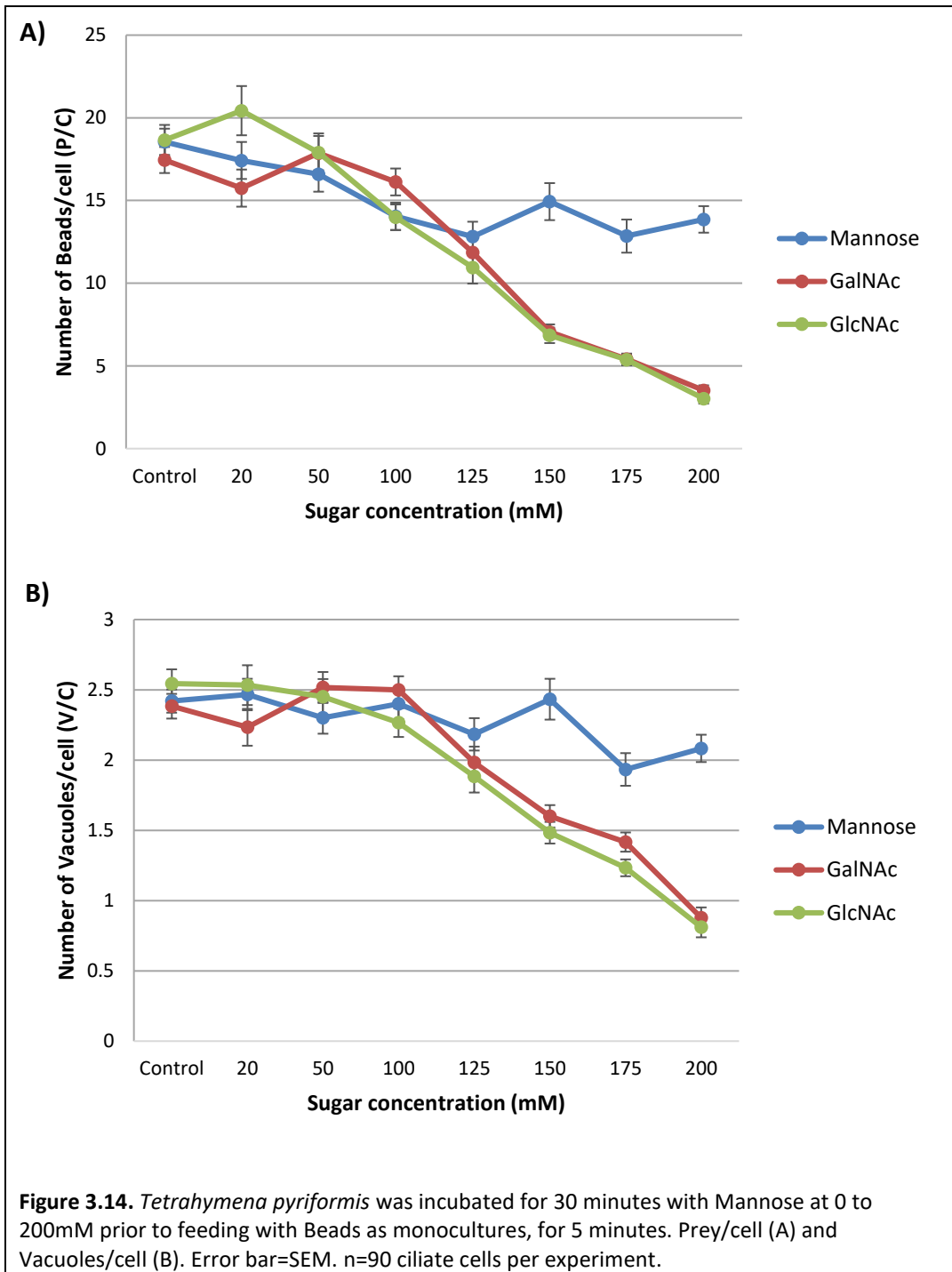
All sugars caused significant cell death at 400mM ($P < 0.01$) and only mannose caused a significant cell death at 200mM ($P < 0.01$) (Figure 3.13).



3.2.1.2 Effect of sugar blocking on bead uptake

Blocking the mannose receptor significantly reduced both uptake and vacuole formation at 100mM ($P < 0.01$) (Figure 3.14); however, no dose-response followed. Conversely, blocking with GlcNAc and GalNAc significantly reduced bead uptake at concentrations ≥ 100 mM and ≥ 125 mM ($P < 0.01$; Figure 3.14A), respectively (with a dose response), and both affected vacuole formation at 125mM (also with a dose response; Figure 3.14B).

These data suggest that the mannose receptor does not play a significant role in the uptake of, and vacuole formation with, beads whereas the receptors for GalNAc and GlcNAc do play a role with GlcNAc showing a slightly stronger response than GalNAc.



3.2.1.3 Effect of sugar blocking on bacterial uptake

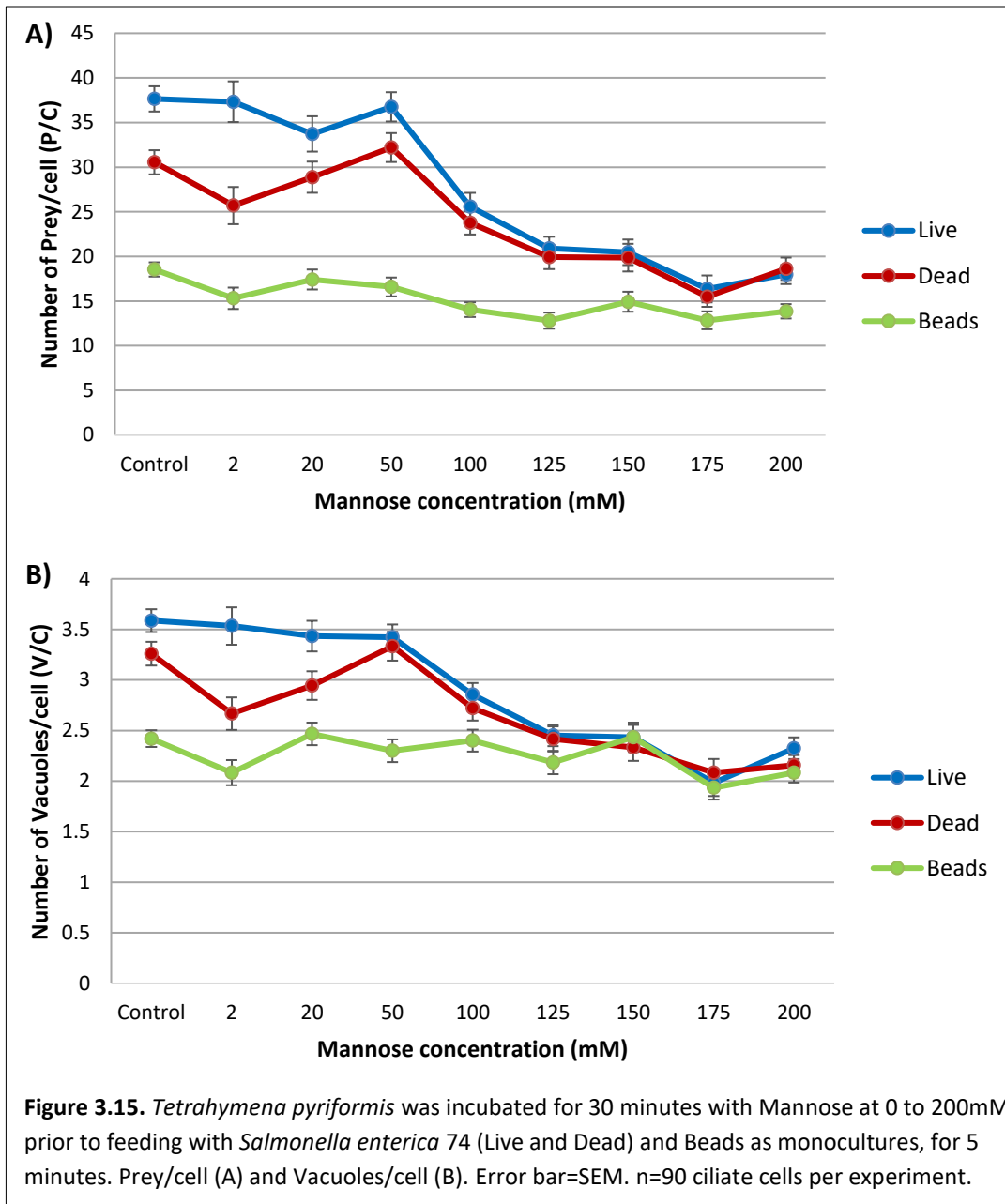
3.2.1.3.1 Blocking with Mannose

The ingestion of Live and Dead cells was significantly higher than that of Beads ($P < 0.01$) at mannose concentrations $\leq 175\text{mM}$; at which point they were equivalent ($P = 0.055$ and 0.084 , respectively). Live cells were ingested to a greater extent than Dead cells until 20mM at which point ingestion was equivalent ($P = 0.07$) (Figure 3.15A); however Dead cells showed high variation between 0 and 50mM mannose. There was then a significant reduction in prey/cell for both Live and Dead cells at 100mM ($P < 0.01$) (as with Beads) but no evidence of a further dose response (Figure 3.15A).

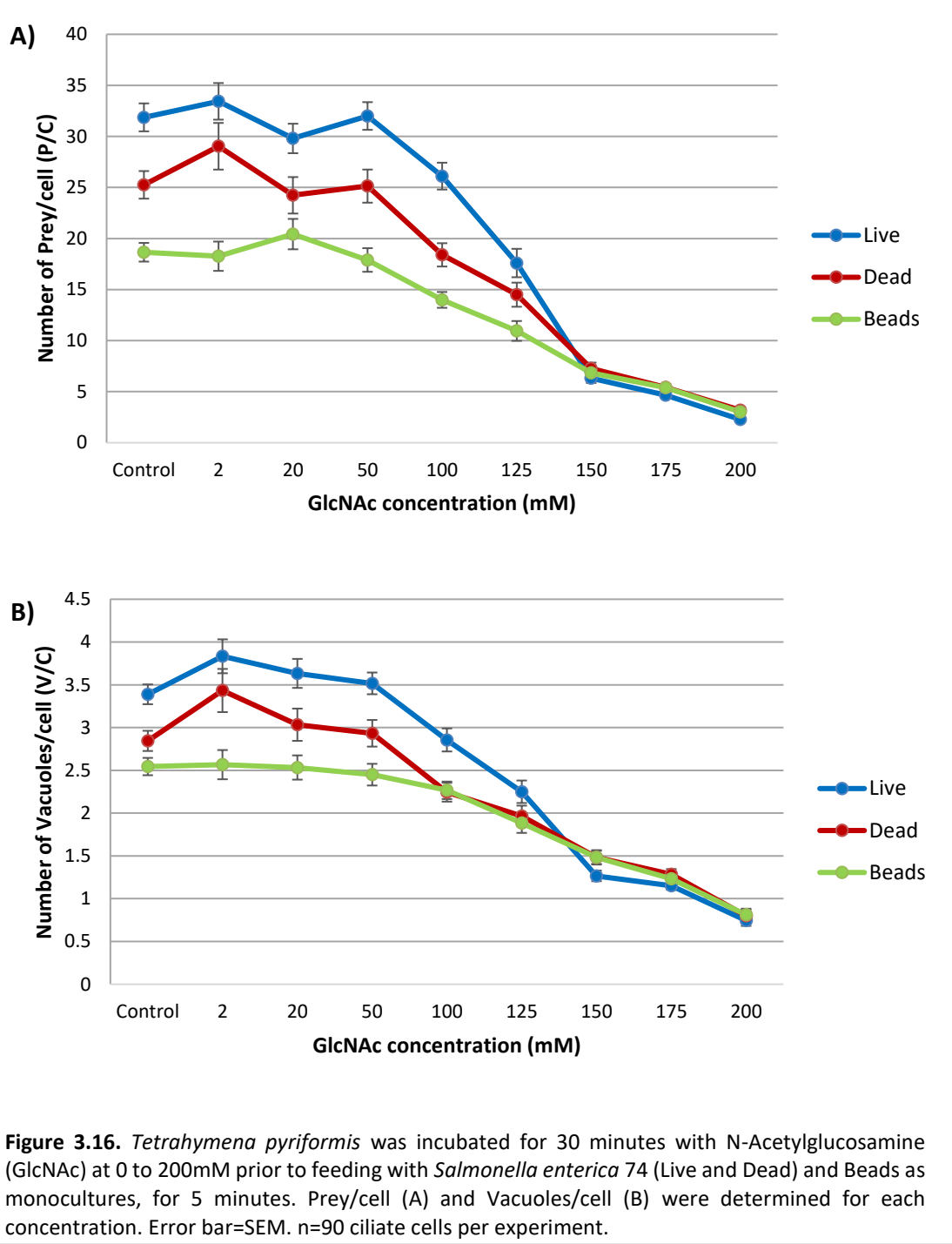
Live cells induced the formation of more vacuoles than Dead cells ($P < 0.01$) until 50mM , where they became equivalent ($P = 0.64$); however Dead cells did show high variation between 0 and 50mM mannose. There was then a significant reduction in vacuoles/cell for both Live and Dead cells at 100mM ($P < 0.01$) (as with Beads) but once again, no evidence of a dose-response (Figure 3.15B). The number of vacuoles produced was equivalent to that of Beads at 100mM ($P = 0.051$, Dead cells) and 125mM ($P = 0.09$, Live cells) (Figure 3.15B).

3.2.1.3.2 Blocking with N-Acetyl- α -D-glucosamine (GlcNAc)

Pre-incubation with GlcNAc resulted in a significant reduction in prey/cell at 100mM with all prey types ($P < 0.01$), followed by a dose-response (Figure 3.16A). The ingestion of Live and Dead cells was significantly higher than that of Beads ($P < 0.01$) at GlcNAc concentrations $\leq 150\text{mM}$; at which point they were both equivalent to Beads ($P = 0.42$ and 0.58 , respectively). Live cells were ingested to a greater extent than Dead cells until 125mM at which point ingestion was equivalent ($P = 0.90$) (Figure 3.16A).



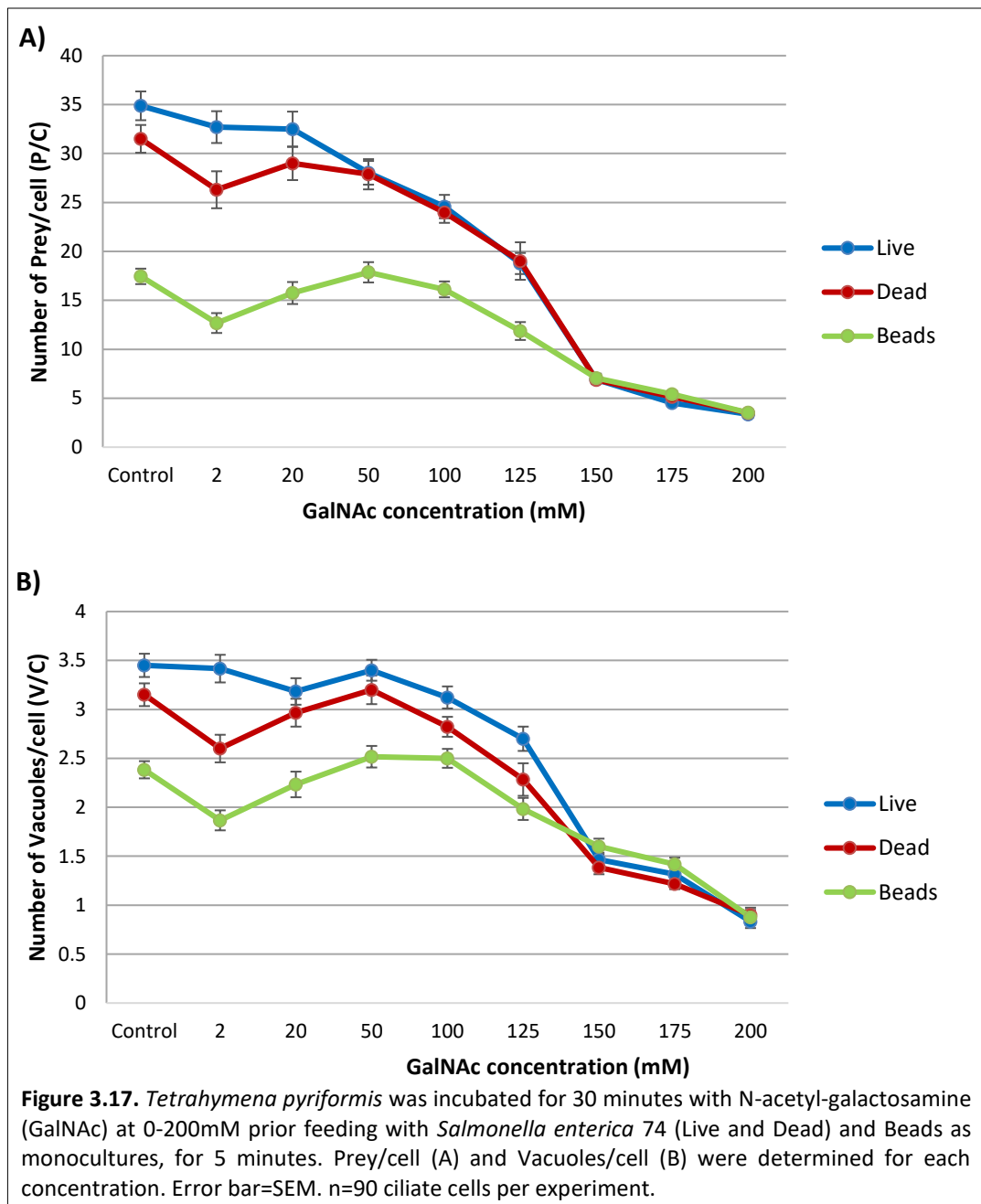
There was a significant reduction in vacuoles/cell for both Live and Dead cells at 100mM ($P < 0.01$) followed by a dose-response (Figure 3.16B). The number of vacuoles produced was equivalent to that of Beads at 100mM ($P = 0.88$, Dead cells) and 125mM ($P = 0.27$, Live cells) (Figure 3.16B). Live cells induced the formation of more vacuoles than Dead cells ($P < 0.01$) until 125mM, where they became equivalent ($P = 0.12$).



3.2.1.3.3 Blocking with N-Acetyl-D-galactosamine (GalNAc)

Pre-incubation with GalNAc caused a significant reduction in prey/cell with 50mM (Live prey) and 100mM Dead prey (P<0.01), followed by a dose-response (Figure 3.17A) (unlike Beads which

required 125mM). This is different to the requirement of only one concentration (100mM) of mannose and GlcNAC to cause such a reduction with all three prey types. The ingestion of Live and Dead cells was significantly higher than that of Beads ($P < 0.01$) at GalNAc concentrations ≤ 150 mM; at which point they were both equivalent to beads ($P = 0.79$ and 0.72 , respectively). Live cells were ingested to a greater extent than Dead cells until 50mM at which point ingestion was equivalent ($P = 0.94$) (Figure 3.17A) (similar to mannose [20mM] but lower than with GlcNAC [125mM]).



There was a significant reduction in vacuoles/cell for both Live and Dead cells at 100mM ($P < 0.01$), followed by a dose-response (Figure 3.17B). The number of vacuoles produced was equivalent to that of Beads at 125mM ($P = 0.14$, Dead cells) and 150mM ($P = 0.21$, Live cells) (Figure 3.17B). In this particular experiment, there was no significant difference in vacuole formation between Live and Dead cells at any GalNAc concentration tested, even in the control (0mM).

3.2.1.4 Summary

T. pyriformis was pre-incubated for 30 minutes with three sugars, Mannose, N-Acetyl-D-galactosamine (GalNAc) and N-Acetyl- α -D-glucosamine (GlcNAc), ranging in concentrations from 0 to 200mM. The ciliate was then fed with *S. enterica* 74 (Live and Dead) and Beads alone at 2×10^7 cells/ml. Table 3.2 summarises the sugar concentrations required to elicit a range of responses. There are some key points to note.

Table 3.2. Sugar concentrations (mM) required to cause Live cells to behave like their heat-killed counterparts (Dead) and Beads with regards to prey uptake and vacuole formation. *Tetrahymena pyriformis* was pre-incubated with each sugar at various concentrations and then fed with *Salmonella enterica* 74 for 5 minutes before determining prey/cell and vacuoles/cell. Red indicates data that is specifically referred to in the text.

Sugar concentration at which...	Sugar concentration (mM)		
	Mannose	GlcNAc	GalNAc
Live cells = Dead cells			
Prey uptake	20	125	50
Vacuole formation	50	125	0
Cell uptake is equivalent to beads			
Live cells	175	150	150
Dead cells	175	150	150
Cell vacuole formation is equivalent to beads			
Live cells	125	125	150
Dead cells	100	100	125
Significant reduction in prey uptake			
Live cells	100	100	50
Dead cells	100	100	100
Beads	100	100	125
Significant reduction vacuole formation			
Live cells	100	100	100
Dead cells	100	100	100
Beads	100	125	125

Firstly, Live cells of *S. enterica* 74 appear to be rich in GlcNAc residues, compared to mannose and GalNAc, because a higher GlcNAc concentration (125mM), compared to 20mM mannose and 50mM GalNAc, is required to cause Live cells to behave like their heat-killed counterparts. And, the receptor appears to be involved in both prey uptake (PUF) and vacuole formation (VFF) with better binding to Live cells compared to Dead cells (Table 3.2).

Secondly, a GalNAc receptor appears to be involved in prey uptake and GalNAc was the only sugar to show a differential response; a concentration of 50, 100 and 125mM was required to cause a significant reduction in the uptake of Live cells, Dead cells and Beads, respectively (Table 3.2). The receptor for GalNAc also appeared to be involved in vacuole formation, but because Live and Dead cells produced equivalent vacuoles/cell in the control ($P=0.073$), which was something not recorded in the other experiments, it prohibited any differences between Live and Dead cells from being discerned.

Finally, a receptor for mannose does appear to be involved in both prey uptake (PUF) and vacuole formation (VFF). However, results suggest that the PUF has a higher affinity for this receptor, requiring 175mM mannose in order to become equivalent to Beads (VFF only required 125mM).

3.2.2 Blocking with Dextran Sulfate and Chondroitin Sulfate

Pre-incubation with chondroitin sulfate (CS) and dextran sulfate (DS) showed no significant effect on P/C and V/C for Live, Dead or Beads suggesting that *T. pyriformis* does not possess scavenger receptors (SRs) (Figure 3.18). If SRs were involved, a significant reduction in the presence of DS (but not CS) would be expected.

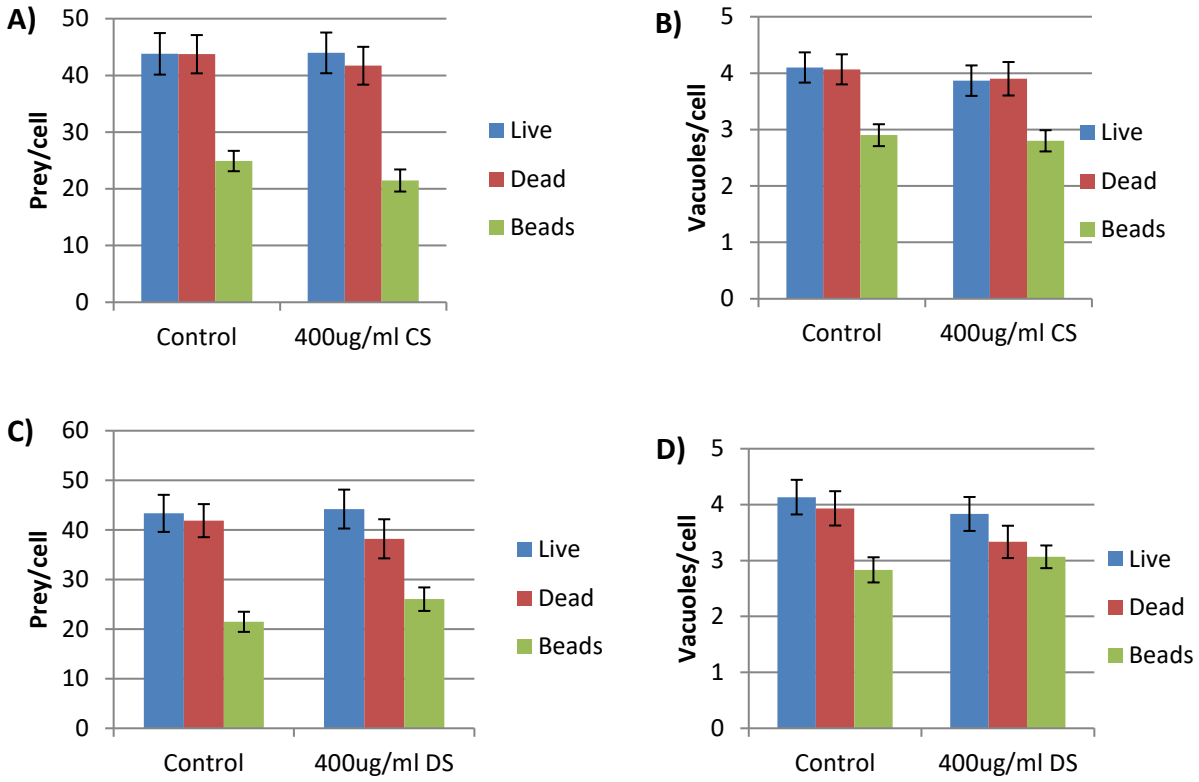


Figure 3.18. *Tetrahymena pyriformis* was incubated for 30 minutes with Chondroitin sulfate (CS) and Dextran sulfate (DS) at 400µg/ml final concentration prior feeding with *Salmonella enterica* 74 (Live and Dead) or Beads for 5 minutes. Prey/cell (A) and Vacuoles/cell (B) parameters from pre-incubation with CS. Prey/cell (C) and Vacuoles/cell (D) parameters from pre-incubation with DS. Error bars=SEM.

CHAPTER 4: DISCUSSION

4.1 Summary of major findings

This study examined the feeding of *Tetrahymena pyriformis* on 11 strains of bacteria (live and dead) and inert beads. The processing of the beads was very different to that of bacteria, with fewer beads being ingested over a 5 minutes period, in both monoculture and in mixture with bacteria. Beads also induced a relatively invariant basal rate of vacuole formation (2.1-2.6 vacuoles/cell [V/C] in 5 minutes, Figure 3.1B).

Bacterial cells upregulated vacuole formation, with live cells yielding 3.2 to 4.2 V/C and dead cells yielding 3.0 to 3.9 V/C; not including dead *Synechococcus* (Pico 3), *Serratia marcescens* and *Staphylococcus aureus* which, after heat-treatment, behaved like beads (Figure 3.8). The significant trends were that, (i) live cells were ingested at higher rates than dead cells, which were themselves ingested at higher rates than beads (except for Pico 3, *S. marcescens* and *S. aureus*), (ii) live cells controlled vacuole formation in mixture with beads, (iii) dead cells controlled vacuole formation in mixture with beads (except Pico 3, *S. marcescens* and *S. aureus*), (iv) live and dead cells together showed a synergistic effect on V/C (except Pico 3, *S. marcescens* and *S. aureus*) and, (v) the two prey types in mixture were deposited into the same food vacuoles, not into separate vacuoles.

The variation in bacterial uptake/vacuole formation between live and dead cells appeared to be governed by the integrity of the bacterial cell surface, as cell biovolume (Figure 3.6) and motility appeared not to play a significant role. Live cells would have a full complement of biological surface properties, e.g., receptors, capsule etc. while their heat-killed-DTAF-stained counterparts would presumably have disrupted biological surface properties. Beads would have no biological surface properties but would still possess a surface charge (not tested in this study).

Three bacterial strains were particularly sensitive to heat-treatment (Pico 3, *S. marcescens* and *S. aureus*), with dead cells behaving like beads with regards to the 'Vacuole Formation Factor' (VFF) and 'Prey Uptake Factor' (PUF). At the other extreme, the VFF and PUF of *Aeromonas*

hydrophila and *Escherichia coli* appeared unaffected by heat treatment, with the processing of live and dead cells being equivalent. In *Salmonella enterica* 74 and *Serratia liquefaciens*, VFF and PUF were both partially affected by heat-treatment. These data suggest that VFF and PUF might be the same. However, data for the remaining four strains question this as their VFFs and PUFs showed different sensitivities to heat-treatment: *Pseudomonas aeruginosa* (VFF heat-stable, PUF heat-labile) and *Salmonella enterica* 12694, *Pseudomonas fluorescens* and *Klebsiella pneumoniae* (VFF heat-labile, PUF heat-stable), possibly suggesting that VFF and PUF are not the same or that they differ between species/strains.

When live and dead cells were in mixture, a synergistic interaction with regards to vacuole formation was evident, with V/C being higher in mixture than with either prey type alone (although this was only deemed significant for *P. aeruginosa*) (Figure 3.8). Conversely, no synergistic interaction was evident between live and dead cells with regards to ingestion (prey/cell) as, in the majority of cases, each prey type was ingested at an equivalent rate to that in monoculture (Figure 3.10A). This further suggests that VFF and PUF might be different.

Preliminary studies, using *Salmonella enterica* 74 only, showed no evidence for *T. pyriformis* possessing Scavenger Receptors but the sugar blocking experiments suggested that the ciliate might possess receptors for mannose, N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc); all of which were involved in the processing of live and dead bacterial cells, while only GalNAc and GlcNAc appeared to be involved in the processing of beads.

All three receptors were implicated in the uptake of (PUF), and vacuole formation (VFF) with live and dead *S. enterica* 74. Uptake in the presence of live cells was higher than dead cells and beads until a particular sugar concentration led to them becoming equivalent to dead cells (Mannose at 20mM; GalNAc at 50mM; GlcNAc at 125mM), and then at an even higher sugar concentration, becoming equivalent to beads (Mannose at 175mM; GalNAc/GlcNAc at 150mM). A similar trend was observed for vacuole formation, suggesting a sequential blocking of receptors for the attachment to live cells (which would have the most ligands).

4.2 Uptake and processing of inert particles

4.2.1 Ingestion rates of inert particles in monocultures and mixtures

Numerous studies have shown that ciliates are capable of ingesting artificial prey such as Indian Ink (Elliott and Clemmons, 1966), carmine particles (Chapman-Andresen and Nilsson, 1968) and latex beads (Fenchel, 1980; Børsheim, 1984; Jonsson, 1986; Lavin et al., 1990; Jürgens and Šimek, 2000; Thurman et al., 2010a). Although *T. pyriformis* ingested latex beads in the current study, their ingestion was significantly less than that of all eleven strains of live and heat-killed bacterial cells, in both monoculture and prey mixtures.

This agrees with previously reported monoculture experiments, for example, Parry et al. (2001), showed that *T. pyriformis* ingested beads at significant lower rates than either live or heat-killed *Escherichia coli*. Sherr et al. (1987) showed that oligotrichous ciliates and a mixed population of flagellates ingested comparably more heat-killed bacteria than microspheres; however, scuticociliates ingested both at equivalent rates. The flagellates *Spumella* spp. and *Cafeteria* spp. recorded significantly higher uptake of live *Pseudomonas putida* than beads (Boenigk et al., 2001b), and Nygaard et al. (1988) showed that the flagellates *Bodo* spp. had a higher clearance rate of live *Vibrio natriegens* than beads.

A higher uptake of live prey over beads has been also demonstrated in mixed prey experiments with the ciliates *Tetrahymena vorax* feeding on *Tetrahymena thermophila* and beads (Grønlien et al., 2002), *T. pyriformis* feeding on live *Aerobacter aerogenes* and gold particles or India ink (Elliott and Clemmons, 1966) and *Cyclidium* sp. feeding on live *Pseudomonas putida* and clay particles (Boenigk and Novarino, 2004). Contrasting results have been observed with flagellates, with *Entosiphon sulcatum* and *Monosiga ovata* feeding more on live *Pseudomonas putida* than clay particles (Boenigk and Novarino, 2004), but *Ochromonas malhamensis* showing no difference in the uptake of autoclaved *Aerobacter aerogenes* and beads (Dubowski et al., 1974). However, a preference for live prey can only be inferred from these experiments, true preference requires a comparison of uptake data in mixture to those in monoculture.

Where this has been performed, a true preference for live *Synechococcus* over beads has been recorded in the ciliate *Halteria grandinella*; however, this ciliate showed no such preference for live *Chlorella minutissima* over beads (Jürgens and Šimek, 2000). Indeed, other studies have shown no true preference for live prey. For example, Boenigk et al. (2001a) showed that the flagellates *Cafeteria*, *Spumella* and *Ochromonas* had similar uptake of live *Pseudomonas putida* and beads in mixture compared to monocultures, and Dolan and Šimek (1997) showed the same for *Bodo* spp. feeding on live *Synechococcus* and beads. The present study also found no evidence for *T. pyriformis* preferentially selecting bacterial cells over beads in mixture. Each was ingested at equivalent rates to those in monoculture, and beads were just naturally ingested at a lower rate. This suggests no interference between particles and that they might be entering the cell via different routes.

Vogel et al. (1980) was the first to suggest that different prey types might enter a protist, specifically the amoeba *Dictyostelium*, via two functionally distinct routes: a non-specific receptor-based route primary governed by hydrophobicity, and a specific-receptor route based on the recognition of prey by a lectin. Since inert particles have no ligands, they are thought to enter phagocytes via the first route which is governed by hydrophobicity.

4.2.2 The involvement of surface charge/hydrophobicity in the uptake of inert particles

Studies have suggested that hydrophobicity and particle charge play an important role in the non-opsonized phagocytosis of beads by macrophages (van Oss, 1978; Absolom, 1988). To investigate this, many studies used beads coated with various substances to provide different particle charges. For example, polystyrene beads coated in hydroxyl, sulphate or carboxyl groups, yielding a more negative charge, have been shown to be preferably phagocytosed over beads coated with amine groups, which yield a more positive charge (van Oss, 1978; Absolom, 1988; Monger and Landry, 1990; Makino et al., 2003; Gu et al., 2013; Dürichen et al., 2016). Fok et al. (1988) also reported that food vacuole formation rates were 26% lower when the ciliate *Paramecium multimicronuceatum* was fed positively charged beads, compared to same-sized neutral or negatively-charged beads. It should be acknowledged however, that coating beads

with, e.g., amino acids and carboxyl groups, will not only provide the desired charge/hydrophobicity but will also add molecules to the bead's surface which could act as ligands for phagocytic receptors. Therefore, in reality, it is impossible to study the role of charge/hydrophobicity in isolation, unless non-coated beads are used.

Studies which have used non-coated beads of equal composition and size, but different surface charge, are rare and these types of beads were not included in the current study. However, using this methodology, Eisenmann et al. (2001) found no effect of hydrophobicity on the ingestion of beads by the ciliate *Epistysis* sp. and Matz et al. (2002) found that it was only extreme charges beyond -45 mV which reduced ingestion rates of the flagellate *Spumella* sp.; but such extreme charges have not been associated with natural bacterial prey.

Even though studies are conflicting, researchers seem to agree that the hydrophobicity and charge of a particle does, in some way, influence its contact probability and recognition in protists (Gurijala and Alexander, 1990; Buskey 1997; Monger et al., 1999; Matz and Jürgens, 2001), but convincing evidence to support that is lacking. Further consideration is required as to whether a 'non-specific-receptor', as suggested by Vogel et al. (1980), is indeed involved in uptake of beads.

4.2.3 Possible routes for the ingestion of inert particles

4.2.3.1 Scavenger receptors – non-specific-receptor-based route

Scavenger receptors (SRs) have been implicated in the uptake of inert particles by macrophages. In particular, MARCO (macrophage receptor with collagenous structure), which is a Class A SR, is considered the major binding receptor for un-opsonized inert particles (Arredouani et al., 2005). The exact mechanism by which MARCO binds inert particles is not known, but it has been speculated that hydrophobicity plays a role with its highly positive charged groove interacting strongly with negatively-charged particles such as latex beads (Krieger, 1994, Kobzik, 1995; Arredouani and Kobzik, 2004). COS-1 cells transfected with human MARCO showed binding of particles such as TiO₂, Fe₂O₃ and silica whilst the wild-type COS-1 cells were unable to bind these particles (Arredouani et al., 2005). Transfection of these cells with SR-A-I/II (another SR-A) conferred no binding properties (Arredouani et al., 2005). Moreover, antibodies against MARCO

inhibited the uptake of un-opsized inert particles by alveolar macrophages (Palecanda et al., 1999), while blocking human SRA-I/II did not affect macrophages binding to TiO₂ (Kzhyshkowska et al., 2004).

The author could not find any published reports of protists possessing Class A SRs, or indeed the involvement of *any* SRs in protistan phagocytosis at the time of the current study. But in late 2018 Sattler et al. showed that the amoeba *Dictyostelium discoideum* possessed homologous receptors to the mammalian class B SRs, LIMP-2 and CD36, i.e. LmpA and LmpB.

Class B scavenger receptors have been shown to bind a much more restricted range of inert particles than SR-As in macrophages: LIMP-2 binds the lysosomal hydrolase β -glucocerebrosidase (Reczek et al., 2007) and CD36 recognizes carboxylated beads (Chuang et al., 2010). Another SR-B (SR-B1) only binds silica (Tsugita et al., 2017). In *D. discoideum*, LmpA (*ca.* LIMP-2) was found in endosomes and phagolysosomes and played a role in the binding and phagocytosis of beads and bacteria, whereas LmpB (*ca.* CD36) was localized to lipid rafts of the plasma membrane and early phagosomes and was *not* involved in the uptake of beads (Sattler et al. 2018).

Tetrahymena thermophila possess a homologue of CD36/LmpB but not LIMP-2/LmpA (ciliate.org); the exact function of which has yet to be studied. However, considering LmpB is *not* involved in the uptake of beads in *D. discoideum* (Sattler et al. 2018), and in the current study, blocking *all* SRs with Dextran Sulfate showed no effect on bead uptake, it is highly unlikely that this receptor is involved in the uptake of these particles in *T. pyriformis*, but this requires confirmation. Future blocking experiments might employ sulfosuccinimidyl oleate which specifically blocks CD36 (Rozovski et al., 2018; Wraith et al., 2013), to confirm/deny the lack of involvement of the LmpB analogue in the uptake of beads by *T. pyriformis*.

4.2.3.2 Lectins for GalNAc and GlcNAc – receptor-based route

The current study found no evidence that the mannose receptor was significantly involved in the uptake of, and vacuole formation with latex beads. This agrees with Allen and Davidowicz (1990) who found that blocking with 100mM mannose did not affect their uptake by the amoeba *A.*

castellanii. However, the mannose receptor has been shown to be involved in the direct binding and phagocytosis of inert zymosan (Sung et al, 1983) and latex beads (Ichinose and Sawada, 1996) in phagocytes of the immune system.

The current study found that blocking with GlcNAc and GalNAc at 125mM caused a significant reduction in vacuole formation with beads, and concentrations of 100mM and 125mM, respectively, caused significant reduction in beads uptake. Neither of the receptors has been previously implicated in the uptake of inert particles by protists, and it is unclear how a ligand-free particle would interact with such receptors. There is only one report that suggests GlcNAc is involved in the uptake of beads by macrophages (Shinzaki et al., 2016). In this study, transgenic mice overexpressing GnT-V (N-acetylglucosaminyltransferase V) had increased GlcNAc branching on their N-glycans, which this impaired macrophage function. Specifically, these macrophages showed a reduced ability to ingest non-coated latex beads. Nonaka (2014) suggested that this was due to branched GlcNAc-glycans not interacting with their normal ligands; since, Shinzaki et al. (2016) reported that these branched glycans could not bind to the lectin *Datura stramonium* agglutinin (DSA), which binds WT glycans. The author could find no suggestion of a GalNAc receptor being involved in the uptake of beads by macrophages.

These experiments need repeating, ideally using bovine-serine-albumin (BSA)-conjugated sugars (neoglycoproteins), because even though no ciliate cell death was observed in the presence of non-BSA-GalNAc/GlcNAc (even at 200mM), the high concentration required to suggest their binding to beads (125mM) might have evoked other, undetectable, changes in the ciliate which influenced the results. Neoglycoproteins have a much higher affinity for lectins and act as much more potent inhibitors (Adler et al., 1995); concentrations used are in the μM range as opposed to the mM range. However, such neoglycoproteins are very expensive, which negated their use in the current study.

4.2.3.3 Non-receptor-based route

There is a possibility that, because there was no evidence for the involvement of SRs in beads uptake, which is the most reported mechanism of uptake in macrophages (Section 4.2.3.1), and

the observed interaction with receptors for GlcNAc and GalNAc that might be a consequence of some other metabolic feature of the ciliate, that beads are taken up *without* receptor mediation in protists. This might yield a basal ingestion rate of beads which mirrors the basal vacuole formation rate observed in the current study and by others (see Section 4.2.4). Indeed, published data on the number of beads ingested over a 5 minutes period by ciliates only varies between 14 and 30 beads/cell when concentrations of $2\text{-}5 \times 10^7$ beads/ml are used (Sherr et al., 1987; Parry et al., 2001; Fenchel et al., 1980; Wong, 2017; Eisenmann et al., 2001; Wilks and Sleigh, 2008). This is remarkably similar to the range of 15 to 26 beads/cell recorded in the current study with a bead concentration of 2×10^7 beads/ml (over 22 individual experiments). So, even though Vogel et al. (1980) suggested that different prey types might enter a protist via *two* functionally distinct routes: a non-specific receptor-based route primary governed by hydrophobicity, and a receptor route based on the recognition of prey by a lectin receptor, there might indeed be a third, i.e., a non-receptor route, which is purely 'mechanical'. This might also go some way in explaining the lack of firm evidence *for* the involvement of hydrophobicity in the uptake of prey by protists.

4.2.4 Phagosome formation in the presence of inert particles

However many routes *into* a protist there are, it appears that this only pertains to the recognition/attachment stage as all prey in the 50:50 mixtures (no matter what their type/form) were deposited into the *same* vacuoles; not separate ones. Previous reports have also found that India Ink (Elliot and Clemmons, 1966), latex beads (Grønlien et al., 2002) and clay particles (Boenigk and Novarino, 2004) have occupied the same vacuoles as the bacteria they were in mixture with. This suggests that rather than different 'routes' into a protist, there might be distinct recognition and binding mechanisms to take up the prey (see Section 4.4), but then the internalizing route is the same for all prey types.

Most work on phagosome formation has been performed on macrophages. Here, phagocytosis begins with surface receptors binding to the particle followed by a rearrangement of the actin filaments (Aderem and Underhill, 1999). The Mannose receptor, for example, recruits molecules such as Grb2 (Rajaram et al., 2017), MARKs (microtubule affinity-regulating kinases; [Allen and

Aderem, 1995]), vinculin and paxillin (Allen and Aderem, 1996a), Cdc42, Rac1 and RhoGTPases (Zhang et al., 2005; Rajaram et al., 2017) which activate actin filaments polymerization upon binding to unopsonized zymosan (Allen and Aderem, 1995, 1996b) or unopsonized live bacteria (Zhang et al., 2005; Rajaram et al., 2017).

Once the receptor binds its target, other receptors from the immediate proximity then travel laterally on the surface membrane to cluster together (Jaumouillé et al., 2014). Some of the most important kinases involved at this step are Src and Syk, that activate the SH2 domain and recruit phosphoinositide 3-kinases (PI3K), which convert phosphatidylinositol 4, 5-bisphosphate, in triphosphate form (PtdIns[3,4,5]P₃) (Cox et al., 1999; Moon et al., 2004). Within these events, a G-protein-coupled calcium-sensing receptor (CaSR) senses calcium and activates the seven-transmembrane-actin-related proteins 2 and 3 (Arp2/3) (Canton et al., 2016). This leads to the generation of pseudopod-like structures that surround the targeted particle and forms the phagocytic cup (May et al., 2000) (Figure 4.1).

Opinions have been divided with regards to exactly how the phagocytic cup takes in the particles. Non-opsonized latex beads, non-opsonized silica (Gilberti et al., 2008) and complement-opsonized zymosan (Gilberti and Knecht, 2015) were shown to be captured by pseudopod-like structures of the macrophages. However, Le Cabec et al. (2002) had suggested that while the opsonized zymosan 'sinks' into the macrophage, the non-coated zymosan is taken in by pseudopods. Lu et al. (2016) have now suggested that the 'sinking' motion is in fact *still* an extension of pseudopods, but the structure of pseudopods is different with regards to the particle opsonization, i.e., opsonized particles are taken in by 'sheet-like' pseudopodia which looks like the particle is 'sinking', while non-opsonized beads are surrounded by 'finger-like', thin pseudopods.

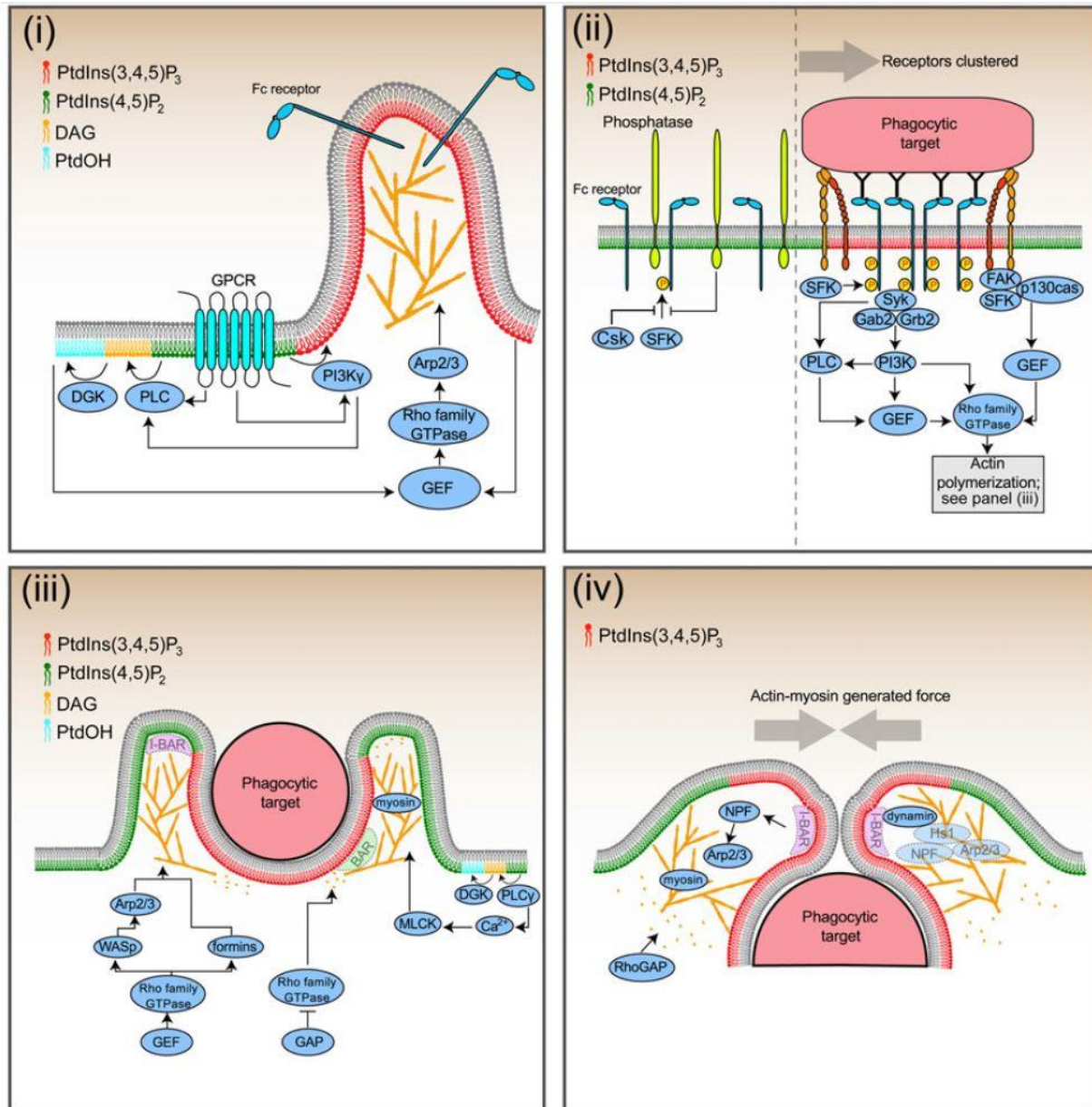


Figure 4.1. Cellular events that lead to vacuole formation. (i) The phagocyte extends pseudopod-like structures towards the target. PtdIns(3,4,5)P₃-activated GEF (guanine nucleotide-exchange factor) recruits GTPases such as Cdc42 and Rac to the site, where they bind to PtdIns(4,5)P₂ and activate the Arp2/3 complex; (ii) Phagocytic receptors cluster together and Syk (spleen tyrosine kinase) binds to the phosphorylated receptor and activates SH2 domains of Grb2 (growth factor receptor bound protein 2) and Gab2 (Grb2-associated protein 2) (see text for more details); (iii) High levels of PI3K, guanine nucleotide-exchange factor and NPF (nucleation promoting factor) recruit BAR-domain proteins to the site of membrane extension (see text for more details); (iv) Once N-BAR domains (annotated I-BAR) push the pseudopod ends closer together, dynamin and myosin are recruited at the site; dynamin binds to the BAR domains and stop polymerization of actin filaments, and myosin helps sealing the ends together. DAG- diacylglycerol; PtdOH- phosphatidic acid; DGK- diacylglycerol kinase; PLCc- phospholipase Cc; SFK- Src-family kinase; FAK- focal adhesion kinase; p130cas- cellular apoptosis susceptibility protein of 130 kDa; WASP- Wiskott–Aldrich syndrome protein; MLCK- myosin light chain kinase; GAP- GTPase activating protein; Csk- C-terminal Src kinase; Hs1- hematopoietic lineage cell-specific protein 1 (Figure taken directly from Levin et al., 2016).

During all these events, BAR (Bin/Amphiphysin/Rvs)-domain-containing proteins (Henne et al., 2007) such as FBP17 protein (Shimada et al., 2007) and N-BAR [N-terminal amphipathic helix-containing BAR] (Peter et al., 2004) are recruited to the tip of the pseudopod-like structures. Both proteins bind with their positive-charged concave side to the negatively charged membrane (Henne et al., 2007). FBP17 is recruited earlier in the phagocytic pit, where it stabilizes the phagocytic cup's membrane and activates nucleation of the Arp/3 system (Takano et al., 2008); N-BAR (greater curvature than FBP17) is recruited when the phagocytic cup is filled, in order to force the membrane to bind following their greater curvature concave shape, bringing the two pseudopod-like structures together (Blood and Voth, 2006). This leads to more Arp2/3 nucleation and recruitment of WASP protein, which further activates actin polymerization at the tip of the pseudopod-like structures (Benesch et al., 2005). A contractile force driven by myosin at the neck constriction sites help in sealing of the two ends of the pseudopods (Swanson et al., 1999). Once dynamin is recruited to the site, because of high level of Arp2/3 nucleation, the vacuole detaches from the surface and moves into the cytoplasm (Marie-Anaïs et al., 2016) (Figure 4.1).

Little is known about the molecular processes involved in the initial stages of phagosome formation in protists but it is thought to be very similar to that of macrophages. For example, *Acanthamoeba castellanii*, *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba invadens*, *Entamoeba moshkovskii*, *Naegleria fowleri* (amoebadb.org; Mullins et al., 1998), *T. thermophila* (ciliate.org), *Paramecium tetraurelia* (Sehring et al., 2007), *Pseudocohnilembus persalinus* and *Stylonychia lemnae* (uniprot.org) all possess the Arp2/3 complex.

The author could not find any published rates at which phagosomes are formed in macrophages and there are only a handful of publications which provide rates for protists; as most studies just record prey/cell with no regard for the number of vacuoles formed. In the current study, 2×10^7 beads/ml resulted in *T. pyriformis* exhibiting what appeared to be an invariant basal rate of 2.5 vacuoles produced in 5 minutes (range, 2.1-2.6 V/C). This is within the range of previously published values (1.35-2.75 V/C in 5 minutes) for *T. pyriformis* feeding at various inert/indigestible particles (at 10^4 - 10^9 particles/ml) at a similar temperature (Chapman-Andresen and Nilson, 1968; Ricketts, 1971; Nilsson, 1977; Hoffmann et al., 1974; Rasmussen, 1976). This

suggests, as did the bead uptake data (Section 4.2.3.3), that vacuole formation in *T. pyriformis* in the presence of inert beads might be a 'mechanical' response which does not involve receptors.

Vacuole formation could, however, be up-regulated in the presence of Live and Dead cells. Considering up-regulation was stronger in the presence of Live cells, it suggests that this up-regulation involves prey cell surface ligand/receptor interactions; ligands which may have been damaged by the heat-treatment used to produce Dead cells.

4.3 Effect of heat and chemical treatment on bacterial uptake

The current study concluded that, overall, live bacterial strains were ingested at higher rates than their heat-killed counterparts which agrees with many studies that demonstrate increased ingestion rates or growth rates on live versus heat-killed bacteria in flagellates (Mitchell et al., 1988; Sherr et al., 1989; Landry et al., 1991; Monger and Landry, 1992; González et al., 1993; Shannon et al., 2007), amoebae (Xinyao et al., 2006; Pickup et al., 2007a) and in ciliates (Pfister and Arndt, 1998; Parry et al., 2001). The only bacterial strains which were less attractive to *T. pyriformis* in their Live form were *Pseudomonas fluorescens* and *Klebsiella pneumoniae* (see Sections 4.3.1.2-4).

Live bacteria also induced the formation of significantly more vacuoles/cell than heat-killed cells, and when in mixture, both had a synergistic effect on vacuole formation rate. The only heat-killed strains that *did not* up-regulate vacuole formation were *Synechococcus* (Pico 3), *Serratia marcescens* and *Staphylococcus aureus*, which appeared particularly sensitive to heat-treatment and behaved like beads afterwards.

4.3.1 Effect of Heat-killing cells

Heat-killing is known to be an invasive process towards the bacteria, as treating the cells with heat results in, (i) reduced cell size (González et al., 1990; Wong, 2017) (Section 4.3.1.1), (ii) loss of motility (Monger and Landry, 1992; Matz and Jürgens, 2005) (Section 4.3.1.2), (iii) loss of cell-cell signaling (Thomas and Allsopp, 1983; Joint et al., 2000) (*applicable to bacteria in biofilms*

only), (iv) an inability to release chemical cues (Unabia and Hadfield, 1999) or toxins (Groscop and Brent, 1964; Greub and Raoult, 2004) (Section 4.3.1.3) and, (v) altered surface characterizes which are potential ligands for recognition by protozoa (Matz et al., 2002; Wilks and Sleigh, 2004) (Sections 4.3.1.4 and 4.4).

4.3.1.1 Effect on prey cell size

González et al. (1990) observed that the heat-killing process results in 'slightly smaller' sized bacterial cells compared to their live counterparts, but a direct examination of these differences was not performed. However, Wong (2017) did report a 54-84% decrease in cell biovolume (μm^3) of the same strains of *K. pneumoniae*, *S. enterica* 74 and *S. aureus* as used in the current study. No significant relationship between prey biovolume and prey uptake in *T. pyriformis* was found in the current study, similarly with some, but not all, of the published literature on 'size-selective grazing'.

A preference for larger prey over smaller prey has been documented mainly in flagellates such as *Ochromonas* (Andersson et al., 1986; González et al., 1990; Epstein and Shiaris, 1992), *Spumella* (Holen and Boraas, 1991; Boenigk and Arndt, 2000), *Paraphysomonas vestita* (Monger and Landry, 1991) and unidentified marine flagellates (Chrzanowski and Šimek, 1990; Šimek and Chrzanowski, 1992). In contrast, variations in ingestion rate in ciliates such *Tintinnopsis* sp., *Strobilidium spiralis*, *T. pyriformis*, *Paramecium* sp., *Eutintinnus pectinis*, *Favella* sp., *Balanion* sp., *Uronema* sp. have *not* been attributed to prey size (Verity, 1991; Christaki et al., 1998; Wolfe, 2000; Thurman et al., 2010b); suggesting other factors are involved.

In order to assess the *real* correlation between prey size and uptake in protozoa, inert particles with no additional ligands on their cell surface should be use. Indeed, three papers used this method to assess prey size-selection in the ciliates. Fenchel (1980) showed that the optimal prey diameter for holotrich ciliates was 0.3-1 μm , while spirotrich ciliates such as *Euplotes moebiusi* and *Blepharisma americanum* recorded the highest clearance rates with larger sized beads (4-5 μm diameter). Jonsson (1986) also found that the spirotrichs *Strombidium reticulatum* and *Lohmanniella spiralis* showed greater clearance rates with larger beads (7.9 μm and 9.7 μm

diameter, respectively), but others have recorded a preference for smaller particles, e.g., the maximum clearance rate for *Halteria grandinella* was with 2.74 μ m diameter beads (Jürgens and Šimek, 2000) and for *Strombidium vestitum*, it was with 2.1 μ m diameter beads (Jonsson, 1986). The differences between the preferred beads size was suggested to be due to physical restriction of the mouth apparatus to ingest larger particles for some ciliates (Fenchel, 1980).

4.3.1.2 Effect on prey motility

Bacteria use motility as an adaptive trait to allow them to reach nutrients and respond to chemical cues in the environment (Blackburn et al., 1998). Flagellar-driven motility enables bacteria to evade the immune system and colonize host cells (Balloy et al., 2007; Amiel et al., 2010), with some experiments indicating that flagella from some bacteria such as *P. aeruginosa* acts as ligands for macrophagic receptors such as Toll-like receptors (Smith et al., 2003; Jacchieri et al., 2003). However, there are studies that have used non-motile mutants of *E. coli*, *Vibrio cholera*, *Salmonella typhimurium* and *P. aeruginosa* to demonstrate that the loss of motility *per se*, and not the flagella alone, increases resistance to phagocytosis in professional phagocytes (Tomita and Kanegasaki, 1982; Smith et al., 2003; Amiel et al., 2010; Lovewell et al., 2011).

It is generally considered that faster swimming bacteria have a higher probability of escaping ingestion by protists compared to the less/non motile species (González et al., 1993; Buskey, 1997; Tillmann and Reckermann 2002; Matz and Jürgens, 2005). Indeed, Matz and Jürgens, (2005) used similar-sized bacterial strains with different motilities (*Pseudomonas pavonaceae*, *Pseudomonas rhodesiae*, *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Acidovorax*, *Flavobacterium columnare*) to demonstrate that highly motile prey (60 μ m/s) were ingested to a lesser extent than less motile strains (20 μ m/s) due to increased handling problems in all three flagellates tested (*Spumella*, *Ochromonas* and *Bodo saltans*). Because motility is heat-labile, these authors also found that the heat-killing of all six strains resulted in higher uptake and lower capture failure than their motile counterparts (Matz and Jürgens, 2005).

The current study only agreed to this view when *T. pyriformis* was feeding on *P. fluorescens* and *K. pneumoniae*, whereby heat-killed cells were ingested more than live cells. But this did not

appear to be governed by motility as live cells of the former were motile while those of the latter were non-motile. Also, live *Serratia marcescens* cells, which were the most motile, were ingested at significantly higher rates than dead cells; as were all the other motile strains. These observations suggest that factors, other than motility (except for possibly *P. fluorescens*), are involved in the increased uptake of live cells.

4.3.1.3 Effect on chemical cue and toxin release

As stated above, only the live cells of *K. pneumoniae* and *P. fluorescens* were ingested at significantly lower rates than their heat-killed counterparts in monoculture suggesting that *T. pyriformis* might be responding to a negative cue/toxin released by Live cells which is then destroyed by heat-treatment in Dead cells.

There are two types of chemical cues that are sensed by predators: signals that benefit the predator (kairomones), and those that benefit the prey (allomones) (Dicke and Sabelis, 1987). With regards to positive cues, chemotaxis has been reported in ciliates from the early 1900s (Jennings, 1906). Chemotaxis has been studied in *Tetrahymena* spp. where chemo-attractants such as amino acids, oligopeptides and soluble lectins (Almagor et al., 1981; Köhidai and Csaba, 1998; Köhidai et al., 2003; Köhidai et al., 2002; Leick et al., 2007; Szemes et al., 2015) have been shown to induce membrane depolarization followed by changing in swimming direction (Almagor et al., 1981; Hellung-Larsen et al., 1986; Zou and Hennessey, 2017) or increase in phagocytosis rate (Csaba and Lantos, 1973, 1976; Kovács et al., 1996, Kovács et al., 2002). Folic acid, a chemoattractant released by *K. pneumoniae* (Lima et al., 2013), and cyclic adenosine monophosphate (cAMP) are the most common chemoattractants for protists such as *Dictyostelium* (Kimmel and Parent, 2003; Manahan et al., 2004; Rifkin and Goldberg, 2006; Maeda et al., 2009; Lima et al., 2013; Kuburich et al., 2016), the ciliate *Uronema* and the dinoflagellate *Oxyrrhis marina* (Hartz et al, 2008).

Tetrahymena spp. can also be repelled by a large range of molecules, including ATP (Kim et al., 1999), lysozyme (Kuruville and Hennessey, 1998), heavy metals such as copper and zinc (Nicolau et al., 1999; Cella et al., 2006) and, benzyl acetate, furfuryl thioacetate and isobutyl propionate

(Láng et al., 2011). These chemicals either inhibit growth (Nicolau et al., 1999; Cella et al., 2006) or change the ciliate's swimming patterns (Köhidaï et al., 1998; Köhidaï et al., 2003; Szabö et al., 2002; Láng et al., 2011; Köhidaï et al., 2015; Szemes et al., 2015).

The author could find no evidence for *K. pneumoniae* or *P. fluorescens* producing negative cues towards protists; however, both strains are known to produce toxins.

K. pneumoniae can produce both heat-stable and heat-labile toxins (Schoub et al., 1977; Klipstein et al., 1983; Koo and Stein, 1986; Guarino et al., 1989). The 5kDa heat-stable toxin (referred to as ST) is not destroyed at 100°C for 10 minutes (Klipstein et al., 1983; Guarino et al., 1989) or at 60°C for 60 minutes (Guarino et al., 1989), and it is resistant to 1:1 trypsin treatment for 60 minutes (Klipstein et al., 1983). The 26kDa heat-labile toxin (referred to as K-1) is destroyed at 100°C for 15 minutes but is stable after 30 minutes at 56°C (Koo and Stein, 1986). Considering the latter conditions are similar to those used in the current study to produce heat-killed cells, both ST and K-1 could still be present in the suspension of dead cells. An aversion to *both* live and dead cells by *T. pyriformis* would then be expected, but this was not recorded.

Jousset et al. (2010) analyzed the effect of four toxic substances released by *P. fluorescens* CHA0, i.e., 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin, hydrogen cyanide and pyoluteorin, on grazing by the amoeba *Acanthamoeba castellanii* and found that the amoeba was most sensitive to DAPG and pyrrolnitrin. Both substances are heat-stable. Pyrrolnitrin is not destroyed at 100°C for 20 minutes (El-Banna et al., 1998) and DAPG is not destroyed at 60°C for 30 minutes (Souza et al., 2002). So once again, these substances would be expected to be present in suspensions of *both* Live and Dead cells, which would not explain why Dead cells were preferred by *T. pyriformis*.

It therefore appears unlikely that a negative cue or toxin is the reason for decreased uptake of Live prey by *T. pyriformis*. Another virulence factor shared by these strains is the presence of a capsule.

4.3.1.4 Effect on capsule

A capsule is a polysaccharide layer that is found on the surface of most of bacteria (Roberts, 1996). It has been shown to protect bacteria from recognition by immune cells by masking the underlying antigens (Horwitz and Silverstein, 1980; Whitfield, 2006; Waldemarsson et al., 2006), by impairing phagocytosis (Houde et al., 2011; Segura et al., 2004). Numerous studies have shown a reduced binding and ingestion of capsulated strains, compared to un-capsulated strains, e.g. with *E. coli* (Horwitz and Silverstein, 1980), *Pasteurella haemolytica* (Czuprynski et al., 1991), *Pasteurella multocida* (Poermadjaja and Frost, 2000). At the extremes, capsulated *S. aureus* (Peterson et al., 1978; Xu et al., 1992) and capsulated *Streptococcus suis* (Segura et al., 1998) are not ingested at all.

In the current study, all bacterial strains possess a capsule except *A. hydrophila* NCIMB 9240 (ATCC 7966) and *Synechococcus*, both of which possess an S-layer instead (Section 4.3.2). Of the capsulated strains, it was only with *K. pneumoniae* NCTC 5055 and *P. fluorescens* FH1 that dead cells were ingested more than live cells, suggesting a protective role of the capsule as reported in macrophage studies.

K. pneumoniae NCTC 5055 has a K2 serotype capsule which is a thick (160nm) hypermucoviscous and hypervirulent capsule (phe-culturecollections.org.uk; Clements et al., 2008). A reduced uptake of live capsulated *K. pneumoniae* cells, compared to un-capsulated cells, has been recorded with the amoeba *Dictyostelium* (Lima et al., 2013) and macrophages (Camprubí et al., 1993; Cortes et al., 2002; Pan et al., 2011; March et al., 2013; Paczosa and Meccas, 2016). This is thought to be due to the K2 capsule having a very low binding affinity to macrophages (Athamna et al., 1991), due to a lack of capsular dimannobiose ($\text{Man}\alpha 2/3\text{Man}$) and dirhamnobiose ($\text{Rha}\alpha 2/3\text{Rha}$) to which the macrophagic mannose receptor binds to; more than to mannose (Kabha et al., 1995; Keisari et al., 2001; Kostina et al., 2005). The author could find no data on the heat stability of K2 capsules but some information exists for the *K. pneumoniae* K1 capsule which is structurally and functionally similar to K2 (Lin et al., 2006). Incubation for 30 minutes at 95°C significantly reduced the capsule mucoviscosity to a very thin layer suggesting it is heat-labile;

no direct measurements were performed to determine its depth (Wu et al., 2009). This suggests that disruption of the capsule might be the reason why dead *K. pneumoniae* cells are ingested more than live cells in the current study.

Information on the capsule of *P. fluorescens* FH1 could not be found, but the capsule of *P. fluorescens* SBW25 comprises colanic acid-like polymers which resemble the colanic-acid Group 1 capsule of *E. coli* (Gallie et al., 2015). Group 1 capsules are heat-stable at 100°C (Jann and Jann, 1990) suggesting that the capsule of *P. fluorescens* FH1 is also heat-stable. Retention of the capsule after heat-killing should result in dead and live cells being ingested at equivalent rates, if the capsule was an important virulence factor, but this was not recorded. There are no published reports on the ingestion of capsulated and un-capsulated *P. fluorescens* to which these data can be compared.

Returning to the other capsulated strains used in the current study, live cells were ingested more than dead cells. This suggests that the capsule enhances ingestion; however, this is based on the assumption that heating the cells actually destroyed the capsule. If it did not, and the capsule was intact in dead cells, then other factors would be involved in the increased ingestion of live cells. Published data on the heat stability of bacterial capsules was unexpectedly sparse, although their heat-lability would be expected to differ since bacterial capsules are structurally diverse due to multiple factors including: saccharide composition, glycosidic bonds, substitution of molecules (such as *O*-acetylation), and composition of a protein anchor that links the capsule to the cell surface (Roberts, 1996).

E. coli capsules are classified into 4 groups (1-4), with more than 80 distinct serotypes of K-antigen (major surface antigen) (Whitfield, 2006). Group 1 resemble some *Klebsiella* sp. capsules; Group 2, *Neisseria meningitidis* and *Haemophilus influenzae* and Group 4, *Shigella*, *Vibrio* sp. and *Salmonella enterica*; Group 3 includes bacteria with structural similar capsules to Group 1 and 2, but with different gene clusters encoding the capsule (Whitfield, 2006; Waldor et al., 1994; Whitfield, 2009; Caboni et al., 2015). The strain used in the current study was *E. coli* JM105 (non-RFP AKN132) which has a K12 capsule (Schwarzer et al., 2012). This is a Group 2 capsule (Elsbernd

et al., 2016) of 25 nm thickness (Meno and Fujimoto, 2002). It is heat-labile at 100°C for 10 minutes (Jann and Jann, 1990). Destruction of the capsule in dead cells might therefore suggest a positive role of the capsule in ingestion. This disagrees with Horwitz and Silverstein (1980) who showed reduced binding and ingestion of capsulated *E. coli* strains, compared to un-capsulated strains, in macrophages, but it does agree with Jung et al. (2007) who recorded a 30% decrease in adherence of un-capsulated *E. coli* K1 mutants to *Acanthamoeba keratitis* compared to the wild-type.

Both virulent and avirulent *Salmonella enterica* 12694 (serovar Enteritidis) and *Salmonella enterica* 74 (serovar Typhimurium) strains, used in the current study, express a capsule (Marshall and Gunn, 2015; Snyder et al., 2006), often referred to as an *O*-antigen capsule due to its high resemblance with the *O*-polysaccharide from the LPS (Snyder et al., 2006). The capsule is similar to Group 4 capsules of *E. coli* (Waldor et al., 1994; Whitfield, 2009; Caboni et al., 2015), which are destroyed at 100°C for 2 minutes (Peleg et al., 2005). Destruction of the capsule in dead cells might therefore suggest a positive role of the capsule in ingestion, like that recorded with *E. coli*. No previous studies could be found regarding the role of the *S. enterica*'s capsule in phagocytosis and studies with other bacteria that possess a Group 4 capsule are conflicting. The capsule does not appear to influence adherence or uptake of *Vibrio cholera* O139 cells by *Acanthamoeba castellanii* (Abd et al., 2009), but it is important in adherence to intestinal cells (Yamamoto et al., 1994b) and for *Shigella* sp. cells, in invasion of epithelial cells (Caboni et al., 2015).

Eleven capsular serotypes have been described for *S. aureus* (O'Riordan and Lee, 2004), from hypervirulent, mucoid serotypes 1 and 2 attributed to strains M and Smith (Koenig, 1962; Arizono et al., 1991; Lin et al., 1994), to microcapsules of serotypes 5 and 8 (Arbeit et al., 1984; Christensson et al., 1991). The strain used in the current study (NCTC 6571) most probably has a serotype 5 or 8 capsule, which are the most prevalent amongst environmental isolates (O'Riordan and Lee, 2004) and are <80nm thick (Arizono et al., 1991). Serotype 5 is not destroyed at 100°C for 15 minutes (Karwacki et al., 2013); no data could be found for Serotype 8.

Heat-treatment of *S. aureus* NCTC 6571 resulted in the cells behaving like beads and being ingested at lower rates than live cells. This capsule would still be present on dead cells, so it is not the reason for reduced ingestion rates of these cells. This contrasts with a study by Soell et al. (1995) which showed that both serotype 5 and 8 capsules were important for the binding of *S. aureus* to tumour cells, epithelial cells and monocytes.

P. aeruginosa can possess two types of capsule; mucoid and non-mucoid (Deretic et al., 1995). The author found no information relating to capsule of the strain used in the current study, but it is most probable that it has a non-mucoid capsule, as the mucoid strains are mostly isolated from cystic fibrosis patients (Deretic et al., 1995; Martha et al., 2010). Non-mucoid capsules are composed of neutral sugars such as mannose, fucose and rhamnose, with less alginate molecules than mucoid isolates (Marty et al., 1992). Moreover, it was shown that Psl, a conserved capsular compound among the non-mucoid isolates, is a mannose-rich molecule (Byrd et al., 2009). It was suggested that this molecule interacts with the macrophagic mannose receptor in a non-opsonised environment (Speert et al., 1988). Non-mucoid capsules can be destroyed at $\geq 56^{\circ}\text{C}$ (30 minutes incubation) (Sengyee et al., 2018; Sarkar-Tyson et al., 2007) which suggests that the capsule was destroyed in dead cells and be the reason for reduced uptake of these cells.

Some strains of *Serratia marcescens* (Aucken et al., 1997) and *Serratia liquefaciens* (Olmo et al., 2010) possess capsules but there is no information in the literature on their role in phagocytosis. The author also found no information on capsule stability for the strains used in the current study, but it does appear that the capsules of all this genus are heat-labile and are destroyed at 60°C (no incubation time stated) (Aucken et al., 1997). Once again, it might therefore be that the destruction of the capsule in dead cells was the reason for their reduced uptake.

So, all the Gram-negative strains used in the study (except *P. fluorescens*) possessed a heat-labile capsule which, after heat-treatment, could have been disrupted, leading to reduced ingestion of dead cells. This suggests a positive role of the capsule in the uptake of these bacteria by protists (discussed further in Section 4.5). Conversely, the capsule of the Gram-positive *S. aureus* was heat-stable and yet heat-killed cells were ingested less than live cells, suggesting no role of the

capsule in ingestion of this bacterium by protists (see Sections 4.3.3. and 4.5.1). Only two strains, *A. hydrophila* and *Synechococcus*, did not possess a capsule but they do possess an S-layer instead and, *S. marcescens* possesses both a capsule and S-layer.

4.3.2 Effect of chemically removal of the S-layer

The S-layer is a complex of proteins and glycoproteins that self-assemble to form a two-dimensional lattice that covers the bacteria in all stages of its growth. It is found on both Gram-negative bacteria (where it adheres to the lipopolysaccharide of the outer membrane), Gram-positive bacteria (where it is attached to the peptidoglycan) but is more prevalent in the Archaea (Sleytr and Messner, 1983; Sára and Sleytr, 2000).

S-layers have been shown to contain mannose residues in *Clostridium difficile* (Bradshaw et al., 2018) and *Lactobacillus kefir* (Malamud et al., 2018; Golowczyc et al., 2009) and GlcNAc residues in *Bacillus stearothermophilus* (Messner et al., 1987). Both GlcNAc and GalNAc residues are within the S-layers of *Clostridium symbiosum* (Messner et al., 1990), and both mannose and GalNAc are in *Methanothermus fervidus* S-layers (Karcher et al., 1993). The S-layer of *Clostridium thermohydrosulfuricum* contains all three residues (Christian et al., 1988).

The S-layers of Gram-negative bacteria tend to have rhamnose in common, alongside various other sugar residues such as mannose, GlcNAc and GalNAc (Messner and Sleytr, 1991). *Serratia marcescens* has GalNAc residues in its S-layer (Oxley and Wilkinson, 1987) but no information could be found on the sugar composition of the S-layer of the other two strains in the current study, *Synechococcus* and *A. hydrophila*.

Previous studies have shown that the possession of an S-layer increases phagocytosis of the bacterium *Bdellovibrio* by *Tetrahymena thermophila*, compared to a non-S-layer counterpart (Koval, 1993) and a synergetic effect of the S-layer proteins of *Lactobacillus kefir* and the LPS from *E. coli* was found to activate a cellular response in macrophages stimulation (Malamud et al., 2018). In contrast, Tarao et al. (2009) found a 4-5-fold increase in ingestion of non-S-layered Actinobacteria by the flagellate *Poteroiochromonas* sp., compared to the S-layer counterpart.

In the current study, chemical removal of the S-layer from *S. marcescens* and *Synechococcus* cells caused a reduction in ingestion and vacuole formation in *T. pyriformis* (but not all results were significant). This is similar to the effect of capsule removal by heat with *S. marcescens*, but not as severe (Section 4.3.1.4). Similarly, Strom et al. (2012) noted that the absence of SwmA, an abundant S-layer protein in *Synechococcus* (McCarren and Brahamsha, 2005), caused a slight reduction in its uptake by the ciliates *Eutintinnus* sp. and *Salpingella* sp. but had no significant effect on predation by *Oxyrrhis marina* (Strom et al., 2017). Thus, variations in the published data regarding uptake of S-layered prey might be due to the distinct process of prey recognition and uptake in different protists.

Chemical treatment of the S-layer in *A. hydrophila* had no effect on prey uptake or vacuole formation in the current study, suggesting the treatment did not destroy it. This cannot be due to *A. hydrophila* having a thicker S-layer than *S. marcescens* or Pico 3, as for example, *A. hydrophila* (strain TF7) has an identical S-layer depth to *Synechococcus* (WH8113 and WH8102), i.e. around 12-12.5nm (reviewed in Messner et al., 2010). The difference might be explained by the differences in S-layer composition.

Thomas and Trust (1995) showed that the S-layer of all *A. hydrophila* strains is highly specific, with the S-proteins showing post-translational phosphorylation of the tyrosine residues (South et al., 1994). The S-layer of other genera/strains have proteins that are glycosylated (reviewed in Kandler and König, 1985; Mengele and Sumper, 1992); no evidence for the presence of phosphorylated tyrosine residues in the S-layer of *S. marcescens* nor *Synechococcus* could be found. This phosphorylation provides a structural role in the S-layer of *A. hydrophila* which might explain why this species was more resistant to heat and lithium chloride treatment. Indeed, while the S-layer of *S. marcescens* and *Synechococcus* can be significantly affected by EDTA and destroyed by lithium chloride (Tarao et al., 2009), that of *A. hydrophila* recorded no change in structure nor performance with EDTA treatment (Murray et al., 1988; Dooley et al., 1988), or even repeated glycine extractions that can also be used to remove the S-layer (Dooley and Trust, 1988). The author could not find any information on the role of this S-layer in phagocytosis.

It therefore appears likely that the S-layer of *A. hydrophila* was *not* removed from the cells in the current study, which would explain why there was no difference in *T. pyriformis* ingestion rate with S+ and S- cells. However, the S-layers of *S. marcescens* and Pico were probably damaged/removed, and these showed a slight reduction in ingestion rate in its absence (but all data were not significant). Even so, the removal of the capsule of *S. marcescens* via heat proved a more significant effect on ingestion than chemical removal of the S-layer alone.

It is strange though, that complete removal of the S-layer has been shown to make *S. marcescens* and *Synechococcus* cells more hydrophobic (Trust, 1983), which, if hydrophobicity is important in prey uptake (Section 4.2.2), should increase ingestion rate of treated cells rather than decrease it. It might be that not all the S-layer of these two species was removed so in future, the use of 0.05% Sodium Dodecyl Sulphate (SDS) might be considered more appropriate as it has been shown it removes significantly more S-layer proteins than even 5M of lithium chloride (ten times the concentration used in the current study) (Dooley and Trust, 1988). Even with an enhanced method of S-layer removal, it might still be that the S-layer itself is not the primarily element essential for predator recognition but that it is more important in post-ingestion protection as previously suggested (Koval, 1993; Dillon and Parry, 2009).

4.3.3 Effect of chemically disrupting cell surface proteins

The role of cell surface proteins, as opposed to carbohydrates, on prey uptake and vacuole formation rate was evaluated using two techniques. The first employed trypsin to physically remove proteins from the cell surface, while the second, formaldehyde treatment, specifically damages surface proteins but not surface carbohydrates. There is however a caveat to these experiments in that these treatments can also disrupt the thick peptidoglycan of Gram-positive cells (Ofek et al., 1975; Courtney et al. 2009) so any treatment effect must be viewed as being an effect on both/either surface proteins and/or those within peptidoglycan. However, there is no recorded effect of either treatment on the peptidoglycans of Gram-negative cells because they are protected by the outer membrane (Bunikis and Barbour, 1999; Walters and Mobley, 2009; Shewell et al., 2013, Sikora et al., 2017), and they lack lysine which is a primary target for

formaldehyde (Schleifer and Kandler, 1972). So, any treatment effects on Gram-negative cells does only relate to surface proteins.

4.3.3.1 Trypsin treatment

Previous studies have used trypsin treatment on bacteria for mainly two purposes: (i) to identify the proteins found on the bacterial cell surface ('cell shaving') and, (ii) evaluate their role in adherence to host cells (Chauvière et al., 1992; Coconier et al., 1992; Tompkins et al., 1992; Arduino et al., 1994; Tamura et al., 1994; van der Flier et al., 1995; Sarem et al., 1996; Shuter et al., 1996; Talbot et al., 1996; Xiao et al., 1998; Tuomola et al., 2000; Archimbaud et al., 2002).

In Gram-positive bacteria, trypsin treatment has been shown to reduce adhesion of *S. aureus*, *Streptococcus pneumoniae* and *Lactobacillus* sp. to a variety of host cells (Vacca-Smith et al. 1994; Tuomola et al., 2000; Chauvière et al., 1992; Coconier et al., 1992; Tompkins et al., 1992; Tamura et al., 1994; van der Flier et al., 1995; Sarem et al., 1996; Shuter et al., 1996; Tallon et al., 2007) whilst conflicting results have been obtained with *Enterococcus* (Chauvière et al., 1992; Sarem et al., 1996; Archimbaud et al., 2002; Arduino et al., 1994).

Numerous cell shaving proteomic studies have been performed on *S. aureus* (Solis et al., 2014; Monteiro et al., 2015; Bonar et al., 2015), *Streptococcus* sp. (Severin et al., 2007; Rodríguez-Ortega et al., 2005, Bensi et al., 2012), *Listeria* sp. (Zhang et al., 2013; García-del Portillo et al., 2011), *Enterococcus* sp. (Romero-Saavedra et al., 2014; Bøhle et al., 2011; Xiao et al., 1998), *Bacillus* sp. (Tjalsma et al., 2008) and *Lactococcus* (Meyrand et al., 2013). Although studies have identified a high removal of cytoplasmic proteins, lipoproteins and proteins (used in metabolism and synthesis) and, DNA transcription and cell cycle defense proteins, surprisingly few proteins have originated from the cell wall.

In most cases, these cell wall proteins were either: adhesins such as extracellular matrix protein-binding protein (Emp) in *S. aureus* (Solis et al., 2014), putative adhesion protein Spy0714 (Severin et al., 2007) or protein M (Ofek et al., 1975) in *Streptococcus*, and LPxTG anchor protein EF2224 in *Enterococcus* (Bøhle et al., 2011) or proteins that protect the bacteria against the host cell (for

example, immunoglobulin binding protein Sbi in *S. aureus*; [Monteiro et al., 2015]). This low level of cell-surface exclusive proteins identified in Gram-positive was suggested to be either due of the lack of trypsin-binding sites within the thick peptidoglycan layer, or the peptidoglycan masking the deeper protein ligands (Dreisbach et al., 2010; Zhu et al, 2016). However, Ofek et al. (1975) and Courtney et al. (2009) did show a significant loss of lipoteichoic acids (LTA) in trypsin-treated *Streptococcus* cells, which was confirmed by lack of binding with mouse monoclonal antibodies against LTA (Courtney et al., 2009). Therefore, the decrease in adherence of trypsin-treated *Streptococcus*, and indeed other Gram-positive strains, could be a result of loss of both surface bound proteins, cell wall proteins or cell wall proteins linked to other peptidoglycan components like LTA (Ofek et al., 1975; Courtney et al., 2009).

In Gram-negative bacteria such as *S. enterica* (Fagerquist and Zaragoza, 2018), *Neisseria* sp. (Shewell et al., 2013, Sikora et al., 2017), *Borellia burgdorferi* (Bunikis and Barbour, 1999) and *E. coli* (Walters and Mobley, 2009), treatment with trypsin has been shown to disrupt species specific flagellin proteins, cell invasion proteins, pathogenicity island effector proteins, and synthesis proteins attached to the outer membrane. In all studies, the most affected layer of the cell surface was the outer membrane, with no peptidoglycan or cytoplasmic proteins being found (Bunikis and Barbour, 1999; Walters and Mobley, 2009; Shewell et al., 2013, Sikora et al., 2017).

Only five studies have examined the effect of trypsin treatment on the adherence of Gram-negative bacterial cells. A significantly reduced adherence has been recorded with trypsin-treated *Mycoplasma pneumoniae* (Kannan et al., 2005) but no effect was observed with *K. pneumoniae* (Favre-Bonte et al., 1995, Meno and Amako, 1991) or *Enterobacter sakazakii* (Mange et al., 2006). And, even though trypsin-treatment reduced the adherence of *L. pneumophila*, the authors suggested that this was due to degradation of the surface heat-shock protein Hsp60 (Garduno et al., 1998), and not the expected outer membrane protein S, which is the primary adherence protein of this bacterium (Butler, 1988).

The results of the current study agree with the latter studies in that trypsin treatment of the Gram-negative *S. enterica* 74 and *S. marcescens* had no effect on their uptake by *T. pyriformis*, or

indeed vacuole formation. This suggests that any loss of proteinaceous cell surface components does not influence their interaction with this ciliate and suggests that carbohydrate moieties are more important in the interaction of protists with Gram-negative cells (discussed further in Section 4.4.2). It is unfortunate that *S. aureus* was not included in the trypsin experiments, to confirm that surface proteins are important for adhesion and uptake to phagocytic cells by Gram-positive bacteria. However, it was included in the formaldehyde experiments.

4.3.3.2 Formaldehyde treatment

Formaldehyde is a fixative that acts by forming methylene cross-links between amino acids, being most reactive when binding to amines (for example lysines) and thiols (such as cysteine) (Fraenkel-Conrat and Olcott, 1948; Metz et al., 2004). This leads to hardening of the cell surface and maintenance of the cell shape (Fraenkel-Conrat and Olcott, 1948). Lipid and fatty acids structures are conserved (Asamizu et al., 2015), together with carbohydrate residues (Hayat, 1981; Leathem and Atkins, 1983; Blanks and Johnson, 1984; Høyer and Kirkeby, 1996; Roberts et al., 2006; Uslu et al., 2019).

In contrast, surface protein structures are impaired by formaldehyde binding with amino acids in a 1:1 ratio and forming the methylene bridges between them (Puchtler and Meloan, 1985). This can also result in masking antigens such as prekeratin and vimentin on the cell surface (Puchtler and Meloan, 1985). Formaldehyde also affects the peptidoglycan of most Gram-positive cells because these contain lysine which is the third amino acid in the peptide moieties of the peptidoglycan layer, and which is a very reactive site for formaldehyde binding. The peptidoglycans of Gram-negative bacteria, and the Gram-positive *Bacillus* and *Mycobacterium*, have unreactive diaminopimelic acid (DAP) in that position, instead of lysine (Schleifer and Kandler, 1972).

Vacca-Smith et al. (1994) showed that formaldehyde-treated *Streptococcus gordonii* had a 90% reduction in the adherence to human umbilical endothelial cells (and trypsin treated cells showed 99% inhibition). In the current study, formaldehyde-fixation reduced the uptake of, and vacuole formation with, *S. aureus* but no effect was observed with the two Gram-negative strains (*S.*

enterica 74 and *S. marcescens*). This suggests, as did the trypsin experiments, that cell surface proteins are not important for the adherence and uptake of Gram-negative bacteria by phagocytes, but proteins are important in the uptake of Gram-positive bacteria; whether they be on the cell surface or within peptidoglycan.

The phagocytic scavenger receptor *Eater* in *Drosophila melanogaster* is thought to specifically bind to peptidoglycan (Chung and Kocks, 2011). Interestingly, no difference in SR binding patterns have been recorded in the presence of live and formaldehyde-treated *S. aureus* and *E. faecalis* (Chung and Kocks, 2011). This suggests that even though formaldehyde fixation alters the nature of the peptidoglycan it can still bind to this scavenger receptor, which implies that any reduction in phagocytosis of formaldehyde-treated bacteria is due to cell surface proteins as opposed to peptidoglycan.

A potential cell surface protein candidate for *S. aureus* would be the extracellular matrix protein-binding protein (Emp), which is removed by trypsin treatment (Solis et al., 2014) and is important in the adherence to mammalian cells (Geraci et al., 2017). In the current study, heat treatment of *S. aureus* caused a significant reduction in uptake and vacuole formation suggesting that PUF and VFF were completely destroyed. The author can find not heat-stability data for Emp to evaluate whether this protein could be either PUF, VFF or both.

4.4 Recognition systems for prey uptake in protists

In the current study, results suggested that the Prey Uptake factor (PUF) and Vacuole Formation Factor (VFF) in *T. pyriformis* might be different factors. For example, in the majority of Live:Dead mixtures, and in all of the Live:Bead mixtures, Live cells controlled vacuole formation. Even though vacuole formation was upregulated, coexisting Dead cells and Beads were not ingested at a greater rate; ingestion appeared to be controlled by something else. Indeed, it has been previously acknowledged that there are at least two recognition systems for prey uptake in protists; one for recognizing food particles which lead to ingestion and, a separate system inside the food vacuole deciding whether ingested material is digestible or not (Dürichen et al., 2016).

With regards to the latter, Ricketts (1971) showed that food vacuoles of *T. pyriformis* containing indigestible uncoated latex beads did not result in increased acid phosphatase activity, whereas vacuoles containing live bacteria, yeast or proteose-peptone-yeast extract medium exhibited increased acid phosphatase activity. Bowers and Olszewski (1983) showed that vacuoles within *Acanthamoeba castellanii* which contained uncoated latex beads or glutaraldehyde-fixed *Saccharomyces cerevisiae* were rapidly exocytosed when the amoeba was fed with live *Saccharomyces cerevisiae* cells. The expulsion of synthetic bovine-serum-albumin (BSA)-methacrylate microparticles upon feeding with live *E. coli* was not observed to occur in *T. pyriformis* (Dürichen et al., 2016) but, even though synthetic, this ciliate did consider these particles to be digestible. In the same study, a variety of substances were coupled to the surface of beads and food vacuole processing was monitored. For example, particles coated with isoleucine were rapidly egested and particles coated with phenylalanine were transported faster through the cytoplasm than normal, suggesting receptor-based recognition processes occurring (Dürichen et al., 2016).

With regards to the ingestion recognition system itself, which is the focus of the current study, Wilks and Sleigh (2004) suggested the presence of at least two independent sites of food selection in *Euplotes mutabilis*; one at the ciliary band (*aka* PUF) and one at the cytostome (*aka* VFF). It was also noted that particles moving down the ciliary band could still be rejected at the cytostome, suggesting that the latter (*aka* VFF) was the dominant selection site and that selection at these two sites relies upon different criteria. The first site could be visualised with Wheat Germ Agglutinin (WGA, which binds to GlcNAc residues) and it specifically bound to the basal halves of 8–9 membranelles at the left end of the frontal section (Wilks and Sleigh, 2004). The second site at the cytostome bound a wide range of lectins. The authors suggested that these binding sites might be essential for ‘holding’ potential prey within the food cup (against forces of water flow) until the cup closes and a food vacuole is formed. If this were correct, then increased ingestion of live cells over beads would be due to better retention of the former through stronger binding to receptors on the food vacuole membrane (Wilks and Sleigh, 2004).

Indeed, in the current study Live cells (with a full complement of ligands) always induced the formation of more vacuoles than Dead cells or Beads; and were ingested to a greater extent. When Live and Dead cells were in mixture, a synergistic interaction on vacuole formation was evident (but not on uptake), and when Live or Dead cells were in mixture with Beads, the Live/Dead cells always controlled vacuole formation, possibly suggesting stronger binding by Live cells, followed by Dead cells, followed by Beads (which mirrors the availability of ligands). The only exceptions to this were *Synechococcus*, *S. marcescens* and *S. aureus* where heat-treated Dead cells behaved like Beads, suggesting the absence of key ligands on the surface of these Dead cells.

4.5 Receptors involved in prey uptake by *T. pyriformis*

Of the receptors that might be involved in *T. pyriformis* phagocytosis, the current study found evidence of the involvement of receptors for mannose, N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) (Section 4.5.2-4.5.4); there was no evidence for *T. pyriformis* possessing scavenger receptors. These experiments employed one Gram-negative bacterium only (*Salmonella enterica* 74) for which it had been proposed that both PUF and VFF were partially destroyed by heat, and which should therefore provide quantitative differences between *T. pyriformis*'s response to live and dead cells.

4.5.1 Involvement of scavenger receptors in the uptake of *S. enterica* 74

The current study could find no evidence for the involvement of SRs in the uptake of the Gram-negative *S. enterica* 74. This contrasts with the high prevalence of SRs in professional phagocytes used in the uptake of beads and both Gram-positive (ligand = peptidoglycan/LTA) and Gram-negative bacteria (ligand = LPS) (Dunne et al., 1994; Platt et al., 1996; Thomas et al., 2000; Peiser et al., 2000; Plüddemann et al., 2006; Plüddemann et al., 2007; Areschoug and Gordon, 2009; Peruñ et al., 2016).

Evidence of SR involvement in protist phagocytosis at the time of the current study was non-existent, but then Sattler et al. (2018) showed that the amoeba *Dictyostelium discoideum*

possessed two SRs, LmpA and LmpB, which were homologous to mammalian class B SRs. In addition to the uptake of beads (Section 4.2.3.1), LmpA was found to play a role in binding and phagocytosis of Gram-negative and Gram-positive bacteria; although a much higher affinity was recorded for Gram-positive bacteria. LmpB, for which homologues have been found in *A. castellanii* and *T. thermophila*, was exclusively involved in the uptake, *but not binding*, of Gram-positive bacteria such as *Bacillus subtilis* and mycobacteria. Due to the lack of binding, the authors suggested LmpB might be acting as a co-receptor whereby the SR *activates* phagocytosis (possibly via molecular mechanisms within the cell) while other receptors (e.g. TLRs in mammalian phagocytes) would be the ones *recognizing* the particles (Sattler et al. 2018). Interestingly, Nasser et al. (2013) showed that *D. discoideum* activates distinct genes when it encounters Gram-positive versus Gram-negative bacteria, i.e., *gp130* and *AlyL*, respectively. The exact implications of activating distinct genes according to the Gram status of the prey is not known, but it could be that biochemical surface composition affects the intracellular response and prepares the predator for its incoming cargo (Nasser et al., 2013).

The current study found no evidence of SR involvement in the uptake of *S. enterica* 74 which agrees with the lack of involvement of LmpB in the uptake of Gram-negative bacteria by *D. discoideum* (Sattler et al. 2018). A later experiment (Parry, unpublished) has also found no evidence of SR involvement in the uptake of *P. aeruginosa* but, there was a strong suggestion they might be involved in the uptake of live *Synechococcus* cells. Blocking SRs with Dextran Sulfate significantly reduced prey/cell but it did not influence vacuoles/cell when *T. pyriformis* was feeding on this prey.

Despite their overall Gram-negative structure, there are significant differences between the cell walls of cyanobacteria compared to most other Gram-negative bacteria (in addition to the existence of an S-layer, Section 4.3.2) (Hoiczkyk and Hansel, 2000). With regards to peptidoglycan/lipoteichoic acid (LTA), which are the accepted ligands for SRs, the peptidoglycan layer found in cyanobacteria is thicker than the 2-6 nm in other Gram-negative cells (*Synechococcus* is 10 nm; Golecki, 1979), and although cyanobacteria do not possess teichoic acid*, their peptidoglycan is complexed with specific polysaccharides in a fashion very similar to

that of Gram-positive bacterial peptidoglycan (Jürgens et al., 1985; Jürgens and Weckesser, 1986). *A carbohydrate produced by *Synechococcus* WH7335 was identified in a PhD thesis (Arnosti, 1993) as a teichoic acid-like polysaccharide, similar in structure to teichoic acids found in cell walls of Gram-positive bacteria, but this has not been published in a peer reviewed journal. The peptidoglycan of *Synechococcus* also contains lysine (Jürgens et al., 1983), a reactive site for formaldehyde, which is absent in normal Gram-negative peptidoglycan (Schleifer and Kandler, 1972) (see Section 4.3.3.2).

The fact that SRs might be involved with the uptake of *Synechococcus* by *T. pyriformis* is interesting as this bacterium represents an almost 'half-way house' between Gram-negative and Gram-positive cells, in that its peptidoglycan (which is a potential ligand for SRs), is more like that of Gram-positive cells. In monoculture experiments, *Synechococcus* behaved similarly to *S. aureus*, i.e. heat-killing the cells made them behave like beads; yet *S. aureus* did not lose its capsule (Section 4.3.1.4) and *Synechococcus* only partially lost its S-layer (Section 4.3.2) suggesting that the loss of cell surface ligand was crucial for receptor-mediated uptake. Parry's unpublished blocking experiments suggest that vacuole formation does not involve SRs and might therefore involve a surface ligand binding to a different receptor family. Conversely, SRs appeared to be involved in the uptake of *Synechococcus* suggesting that PUF is an SR ligand, and could be the peptidoglycan. Further experiments are required, the results of which might help explain why live and dead *S. aureus* cells, in mixture, were ingested at significantly higher rates than in monoculture (whereas VFF was not affected and controlled by live cells); an explanation for this is currently lacking.

4.5.2 Mannose receptor

4.5.2.1 Uptake of Live and Dead *S. enterica* 74

There was no evidence that the mannose receptor played a significant role in the processing of beads (Section 4.2.3.2). However, it did appear to be involved in the uptake of, and vacuole formation with, the Gram-negative *S. enterica* 74. Ingestion and vacuole formation with live cells were higher than with dead cells, although a greater affinity of PUF for the receptor was

suggested as 175mM mannose was required to make live and dead cells have equivalent prey/cell to Beads whereas only 125mM was required to make vacuoles/cell equivalent to Beads.

For phagocytes of the immune system, the mannose receptor has been shown to be involved in the direct binding and phagocytosis of inert particles such as zymosan (Sung et al., 1983) and latex beads (Ichinose and Sawada, 1996) together with live and heat-killed bacteria (Bar-Shavit et al., 1977; Ezekowitz et al., 1990; Kruskal et al., 1992; Stahl and Ezekowitz, 1998; Zamze et al., 2002; Cambi et al., 2005; Esparza et al., 2014).

In protist studies which have employed sugar blocking experiments (normally 100mM), a mannose-like receptor has been shown to be involved in the uptake of live *E. coli*, *S. marcescens* and *Isochrysis galbana* (Bracha et al., 1982; Wootton et al., 2007), glutaraldehyde-fixed *E. coli* and *S. marcescens* (Bracha et al., 1982) and heat-killed *Saccharomyces cerevisiae* and *E. coli* (Allen and Davidowicz, 1990; Alsam et al., 2005) in the amoebae *Entamoeba histolytica*, *Acanthamoeba castellanii* and dinoflagellate *Oxyrrhis marina*. It has however, not been associated with the uptake of beads (Allen and Davidowicz, 1990). All agree with the current study.

4.5.2.2 Location of mannose in the ciliate cell

There have been many studies investigating lectin binding to carbohydrates in a broad range of protists but, most of them have fixed the cells prior to labelling which causes permeabilization of cell walls, resulting in lectins binding to internal cell structures in addition to those on the cell surface (Roberts et al., 2006). Those studies that used live cells (followed by fixation) have found different results, depending on the protist used. For those studies using dinoflagellates, Wootton et al. (2007) found FITC-labelled mannose-BSA to be concentrated at the non-permanent cytostome of *O. marina* and Roberts et al. (2006) also recorded Concanavalin A (ConA) binding at this site in *O. marina* and in *Goniomonas amphinem*. Roberts et al. (2006) found no binding of ConA to the flagellate *Paraphysomonas imperforata* but did record binding to the food vacuoles (no surface binding) in the ciliate *Euplotes vannus*. Likewise, Dürichen et al. (2016) reported that ConA only bound to food vacuole membranes of *T. pyriformis*. The latter authors concluded however, that this did not necessarily mean that neither the plasma membrane nor the

cytostome were not glycosylated, since *T. pyriformis* is covered by a slime, secreted by mucocysts, which could hide glycosylated structures (Nilsson, 1972). However, there might be another reason for the lack of ConA binding to the cytostome in ciliates.

Both Roberts et al. (2006) and Dürichen et al (2016) incubated the cells for 1 hour before visualizing, so they might have missed cytostome labelling during this period if the sequence of events in *T. pyriformis* is similar to that described by Wilks and Sleight (2004) for *Euplotes mutabilis*. Here, staining with ConA showed a distinct series of events: (i) staining of the cytostome only (in a comma shaped binding pattern), (ii) simultaneous staining of the cytostome and vacuoles, (iii) staining of the vacuoles only, (iv) no staining. At 10 seconds, 90% of cells showed staining of the cytostome, with 31% showing simultaneous staining of food vacuoles. The former dropped to 20% at 40 seconds by which time 75% of cells showed staining of food vacuoles only. By 30 minutes, 60% of the cells showed no staining (Wilks and Sleight, 2004).

Wilks and Sleight (2004) also recorded a cyclical pattern in mannose binding, which suggested it was involved in food vacuolar membrane recycling. This had been studied in *T. pyriformis* by Scott and Hufnagel (1983) who specifically showed that the mannose receptor was involved in the normal egestion of food vacuoles at the cytoproct. When blocked with ConA the vacuole membrane was cast out of the cell, along with the contents of the vacuole, instead of being recycled. Maloney (1986) also showed that binding of ConA induced delays in oral regeneration in the ciliate *Stentor coeruleus*.

The published involvement of the mannose receptor in particle uptake (at the cytostome), vacuole formation, defecation and membrane recycling in ciliates makes it as a strong candidate for being an important receptor in *T. pyriformis* that binds both PUF or VFF, particularly since blocking with mannose *only* affected uptake and vacuole formation with bacterial cells (with ligands) and not beads (no ligands).

4.5.2.3 Mannose-containing ligands in bacteria

As demonstrated previously, mannose residues are present in numerous bacterial cell structures, however Gram-positive strains such as *S. aureus* do not possess them (Mirelman et al., 1980). In Gram-negative strains, mannose is mainly associated with the *O*-antigen evidenced in *E. coli* (Jansson et al., 1985; Perry et al., 1993; Baintner et al., 2011), *A. hydrophila* (Zhang et al., 2002; Dworaczek et al., 2019), *P. fluorescens* (Veremeichenko et al., 2005), *K. pneumoniae* (Jansson et al., 1985; Zamze et al., 2002) and *S. marcescens* (Aucken et al., 1996).

Some species also have mannose residues in their core oligosaccharide such as *K. pneumoniae* (Man-Kupisinska et al., 2018) and *P. fluorescens* (Veremeichenko et al., 2005). *P. aeruginosa* (PAO1) does not express *O*-antigen but instead has a 'A-band' LPS that contains small amount of mannose (Arsenault et al., 1990). It also has mannose residues in the extracellular glycolipid component (slime glycolipoprotein) it produces (Lagoumintzis et al., 2003).

Mannose residues have also been found in the S-layer of *Clostridium difficile* (Bradshaw et al., 2018) and *Lactobacillus kefir* (Golowczyc et al., 2009; Malamud et al., 2018) but not in those of *Synechococcus* sp. (Strom et al., 2017) or *S. marcescens* (Arraes et al., 1997). They have been found in the capsules of *K. pneumoniae* (Ofek et al., 1993; Sahly et al., 2008). and *S. pneumoniae* (Zamze et al., 2002), and within mannose-capped lipoarabinomannan (ManLAM) from the *Mycobacterium tuberculosis* cell surface (Kang et al., 2005). The list appears endless, so determining a candidate ligand for the mannose receptor, and where it is on the bacterial cell, is difficult.

However, the current study used two strains of *S. enterica* which might shed some light on the possible identity of the ligand; *S. enterica* 74 serovar Typhimurium which was proposed to have heat-labile PUF and VFF and *S. enterica* 12694 serovar Enteritidis which was proposed to have a heat-labile VFF but a heat-stable PUF. Both strains contain mannose residues in the *O*-antigen (Samuel and Reeves, 2003; Son et al., 2008; Li et al., 2017), which for both serovars is heat-labile, being destroyed at 65°C for 2h (Bravo et al., 2011) suggesting that the *O*-antigen might be the VFF, but it cannot be the PUF.

There are other bacterial structures that bind mannose which *S. enterica* possesses, specifically Type I fimbriae. Type I fimbriae are one of the best-characterized fimbrial adhesins and are found in many bacteria in the family Enterobacteriaceae (Glegg and Gerlach, 1987; Old et al., 1989; Ponniah et al., 1991; Althouse et al., 2003). In the current study only *A. hydrophila* (Kirov et al., 1999), *P. aeruginosa* (Murray et al., 2007), *P. fluorescens* (Sun et al., 2016), *S. aureus* (Pollitt et al., 2015) and *Synechococcus* sp. (Nagar et al., 2017) would *not* possess Type I fimbriae; possessing Type IV fimbriae instead.

Type I fimbriae are also known as ‘mannose-sensitive’ fimbriae since exogenous mannose blocks their binding to receptors on red blood cells (Hibberd et al., 1990; Ghosh et al., 1994). They have been shown to mediate *adherence only* (PUF) of bacteria to mannose-containing glycoconjugates on phagocytic cells (Ofek et al., 1995) such as neutrophils (Tewari et al., 1993), mast cells (Malaviya et al., 1994a; Malaviya et al., 1994b), macrophages (Baorto et al., 1997), enterocytes (Hamrick et al., 2000) and other leukocytes (Ponniah et al., 1992). The major component of the Type I fimbrial appendage is made up of repeating FimA subunits with an adhesin molecule (FimH) at the tip of the fimbriae (Weissman et al., 2006; Tchesnokova et al., 2011) that confers adherence to mannose-containing glycoconjugates on host cells (Hamrick et al., 2000; Le Trong et al., 2010).

Interestingly, even though both *S. enterica* serovars express Type I fimbriae (Duguid et al., 1976; Firon et al., 1984), they possess distinct amino acids sequences, which leads to slightly different recognition and phagocytic responses (Kuźmińska-Bajor et al., 2012). These serovars also have different stabilities to heat-treatment, with serovar Enteritidis (*S. enterica* 12694) being more resistant to heat than serovar Typhimurium (*S. enterica* 74) (Doyle and Mazzotta, 1999). This correlates with the proposed heat-stability of PUF in the current study, whereby that of *S. enterica* 12694 is heat-stable while that of *S. enterica* 74 is heat-labile. A potential candidate for the PUF in *S. enterica* could therefore be Type I fimbriae (whilst VFF could be the *O*-antigen).

4.5.3 The GlcNAc receptor

4.5.3.1 Uptake of Live and Dead *S. enterica* 74

There was evidence for the involvement of a GlcNAc receptor in the processing of beads (Section 4.2.3.2) whereby 100mM GlcNAc caused a significant reduction in both vacuole formation and bead uptake. Both live and dead *S. enterica* 74 cells also gave this same response. In addition, the general trend for uptake and vacuole formation in the presence of GlcNAc was that live cells were ingested to a greater extent than dead cells until 125mM, at which point they were equivalent. This was higher than the 20mM required by mannose and 50mM required by GalNAc suggesting that *S. enterica* 74 was richer in GlcNAc residues compared to mannose and GalNAc. However, prey uptake receptors for GlcNAc appeared to be blocked by 150mM GlcNAc and vacuole formation receptors by 125mM, when both cells types started to behave like beads.

There is one study which examined the role of a GlcNAc receptor in phagocytosis in protists. Wootton et al. (2007) showed that sugar blocking with GlcNAc-BSA (20 μ M) did not affect the phagocytosis of *Isochrysis galbana* by *Oxyrrhis marina*, which differs from the results of the current study. However, sugar blocking studies with macrophages have shown that GlcNAc (100mM) inhibits the phagocytosis on live Gram-negative *Neisseria* by macrophages, even when the bacterium is coated with the mannose binding-protein; a common opsonin found in the human body (Jack et al., 2004). Also, 50mM of GlcNAc can significantly reduce the phagocytosis of apoptotic cells by macrophages (Hall et al., 1994; Garcia-Aguilar et al., 2016).

4.5.3.2 GlcNAc-containing ligands in bacteria

Unlike mannose residues, which are components of many different bacterial structures (see Section 4.5.3.3), GlcNAc is more localized. It is one of the main cell surface carbohydrates which, together with N-acetylmuramic acid, forms the peptidoglycan layer of bacterial cells (Rogers et al., 1980). GlcNAc residues are also present in the core polysaccharide of *E. coli*, *S. enterica* serovar Typhimurium (*S. enterica* 74), *Neisseria gonorrhoeae* and *Haemophilus ducreyi* (van Kooyk and Geijtenbeek, 2003; Maeda et al., 2003; Klena et al., 2005; Zhang et al., 2006a; Hedlund et al., 2010); none of these bacteria possess mannose in their core

polysaccharide (Zhang et al., 2006b). Possessing GlcNAc residues in both the peptidoglycan and core polysaccharide (which are relatively large structures) might explain why *S. enterica* 74 was considered to be rich in GlcNAc residues (compared to mannose and GalNAc residues).

4.5.3.3 Potential receptors of GlcNAc

There is no 'GlcNAc receptor' as such, but there are three receptors in professional phagocytes that recognize GlcNAc residues in bacterial cells: Mannose Receptor, Peptidoglycan Recognition Proteins (PGRPs) and DC-SIGN.

The Mannose receptor is known to bind GlcNAc residues with a much lower affinity than mannose (East et al., 2002; Allavena et al., 2004). As no dose-response was found with mannose in the current study, but one was observed with GlcNAc, it is unlikely that GlcNAc was binding to the mannose receptor. Also, a receptor for GlcNAc was implicated in the uptake of beads, whereas the mannose receptor was not.

Peptidoglycan Recognition Proteins (PGRPs) are highly conserved between phyla and can bind the peptidoglycan layer of bacteria with a high affinity (reviewed in Liu et al., 2001; Dziarski, 2004). Even though their role in bacterial recognition has been acknowledged, their exact role in phagocytosis has not yet been defined, as PGRPs activation leads predominantly to the intracellular killing of bacteria within neutrophils (Kaneko et al., 2004; Cho et al., 2005). There are no reports of protists possessing PGRPs.

DC-SIGN (DC-specific ICAM-grabbing nonintegrin) from dendritic cells is involved in phagocytosis of Gram-negative bacteria such as *E. coli*, *S. enterica* serovar Typhimurium (*S. enterica* 74), *Neisseria gonorrhoeae* and *Haemophilus ducreyi*, by binding directly to the GlcNAc sugar residues from the core-lipopolysaccharide layer (van Kooyk and Geijtenbeek, 2003; Maeda et al., 2003; Klena et al., 2005; Zhang et al., 2006a; Hedlund et al., 2010).

No DC-SIGN homologous have been found in protists; instead reports show that GlcNAc from peptidoglycan can be recognized, with high specificity, by a protein motif named LysM, which is widely distributed amongst organisms except the Archaea (Buist et al., 2008; Gust, 2015). In

the plant *Arabidopsis thaliana*, receptors containing LysM motifs bind both Gram-negative and Gram-positive bacteria and, together with the transmembrane receptor kinase CERK1, builds a transmembrane receptor domain that activates the plant immune system (Miya et al., 2007; Willmann et al., 2011). The parasitic protist *Plasmodium falciparum* has also been shown to express the LysM domain on its cell surface and subsequently used it to bind to purified cell walls of Gram-positive bacteria (Bosma et al., 2006). *T. thermophila* possesses a LysM domain (ciliate.org) but its role in phagocytosis is yet to be explored and is an exciting avenue of future work.

4.5.3.4 Location of GlcNAc in the ciliate cell

Wilks and Sleight (2004) observed rapid binding of WGA to GlcNAc residues in the 8–9 oral membranelles and cytostome of *Euplotes mutabilis*, followed by binding to food vacuoles. No binding was observed after 60 minutes. Roberts et al. (2006) observed no binding of WGA to *Euplotes vannus* but they may have missed it as cells were observed after a 1h incubation. The most recent lectin binding study (Dürichen et al., 2016) showed that WGA binds specifically to patches of the vacuole membrane of *T. pyriformis*, unlike ConA (for mannose) which labels the whole food vacuole. And, whereas ConA bound to all food vacuoles, WGA only bound to young vacuoles near the cytostome. The authors did not observe any WGA surface binding at the cytostome, again, possibly due to the 1h incubation time employed.

The published involvement of a ‘GlcNAc receptor’ in particle uptake (at the cytostome) and vacuole formation in ciliates makes it as a strong candidate for being a receptor in *T. pyriformis* that binds both PUF and/or VFF. The current study used two strains of *S. enterica* which might shed some light on which process dominates over the other; *S. enterica* 74 serovar Typhimurium which was proposed to have heat-labile PUF and VFF and *S. enterica* 12694 serovar Enteritidis which was proposed to have a heat-labile VFF but a heat-stable PUF. Considering it is likely that peptidoglycan is the predominant bacterial ligand for a ‘GlcNAc receptor’, and both serovars have the same structure to their peptidoglycan, one might assume that this would also possess the same heat-lability. Both serovars were proposed to have an equivalent heat response (heat-

labile) for their VFF possibly suggesting a more dominant role of peptidoglycan in vacuole formation rather than cell uptake. Both serovars also possess GlcNAc residues in their core polysaccharide which, being surface-bound, has more potential to show differences in heat lability between strains, and might therefore be the PUF.

4.5.4 The GalNAc receptor

4.5.4.1 Uptake of Live and Dead *S. enterica* 74

There was evidence for the involvement of a GalNAc receptor in the processing of beads (Section 4.2.3.2) whereby 125mM GalNAc caused a significant reduction in both vacuole formation and bead uptake. A lower concentration (100mM) was required to significantly reduce both parameters with dead *S. enterica* 74, and live cells require only 50mM to significantly reduced prey/cell (100mM for vacuoles/cell). With regards to vacuole formation, both live and dead cells yielded equivalent vacuoles/cell at 0mM GalNAc (the control), which was not recorded in the other sugar blocking experiments. Therefore, no differences between live and dead cells, with regards to VFF, could be discerned in the presence of this sugar.

The general trend for prey uptake (PUF) in the presence of GalNAc was that live cells were ingested to a greater extent than dead cells until 50mM, at which point they were equivalent (similar to mannose [20mM] but lower than GlcNAc [125mM]). All receptors for GalNAc appeared to be blocked by 150mM GalNAc and cells behaved like beads with regards to both prey uptake and vacuole formation.

There is little information of the role of a GalNAc receptor with phagocytes of the immune system but it has been implicated in the binding of *Fusobacterium nucleatum* to polymorphonuclear leukocytes, lymphocytes, gingival fibroblasts and HeLa line cells, with 100mM galactose and GalNAc reducing adhesion by 75-87% and 78-85%, respectively (Weiss et al., 2000; Metzger et al., 2009); mannose, fucose, glucose showed no effect.

Compared to the other two sugars, and mammalian systems, there is more information in the literature regarding to the role of GalNAc residues in the uptake of bacteria by protists. This is

principally because this residue is involved in the uptake of the pathogenic bacterium *Legionella pneumophila* by amoebae, which harbor it. Studies have shown that the addition of 100mM galactose/GalNAc (but not mannose, glucose or lactose) can block phagocytosis of this bacterium by *Vermamoeba (Hartmannella) vermiformis* (Venkataraman et al., 1997a; Cao et al., 1998; Harb et al., 1998; Medina et al., 2014) and *Naegleria lovaniensis* (Declerck et al., 2007). However, galactose/GalNAc does not appear to affect the uptake of this bacterium by *A. castellanii* (Declerck et al., 2005). Little effect of galactose addition has also been recorded for *A. castellanii* feeding on *E. coli* (Alsam et al., 2005) and no effect has been recorded for *A. polyphaga* feeding on *Listeria monocytogenes* (Akyu et al., 2009). Wootton et al. (2007) also reported that GalNAc residues were not involved in the phagocytosis of *I. galbana* by *O. marina*.

4.5.4.2 GalNAc-containing ligands in bacteria

GalNAc residues are building blocks in the cell walls of both Gram-positive and Gram-negative bacteria where they are part of the glycosaminoglycans, teichoic acids and lipopolysaccharides (Abeygunawardana et al., 1989; Michael et al., 2002; Fox et al., 2003; Freymond et al., 2006; Rush et al., 2010; Liu et al., 2014; Whitworth and Imperiali, 2015; Leker et al., 2017). The O-antigen of *S. enterica* serovar Typhimurium (*S. enterica* 74) is a component of LPS and has a trisaccharide backbone of mannose-rhamnose-galactose (Bogomolnaya et al. 2008). *P. aeruginosa* has surface-exposed mannose residues in its slime-GLP (Lagoumintzis et al., 2003), but addition of 10 µg/ml galactosidase for 30 minutes impairs phagocytosis in macrophages, suggesting the presence of GalNAc residues in this slime also (Speert et al., 1988).

4.5.4.3 Potential receptors of GalNAc

GalNAc residues are recognized by two main C-type lectin receptors in macrophages: (i) the Asialoglycoprotein receptor (ASGR) (Spiess and Lodish, 1985; Porat et al., 1995; Rensen et al., 2001; Tanowitz et al., 2017) and, (ii) the macrophage galactose receptor (MGL) (Kawasaki et al., 1986; Oda et al., 1989; Sato et al., 1992; van Sorge et al., 2009); neither of which have homologues in protists.

A 170 kDa Gal/GalNAc has been described in both *Entamoeba histolytica* and is a transmembrane lectin, with carbohydrate binding domains located extracellularly (Petri et al., 1987). *E. histolytica* uses this Gal/GalNAc lectin to bind to sugar moieties on bacteria such as live *E. coli* serotype O55; a bacterial strain rich in Gal and GalNAc residues on its cell surface (Bär et al., 2015). *Vermamoeba (Hartmannella) vermiformis* has been shown to use a similar 170kDa transmembrane lectin to bind and internalize gentamicin-killed *Legionella pneumophila* (Venkataraman et al., 1997; Harb et al., 1998). Interestingly, addition of antibodies against the Gal/GalNAc lectin found in *E. histolytica* also inhibited the uptake of *L. pneumophila* by *V. vermiformis*, indicating that the lectins possess similar epitopes for the carbohydrate residues.

4.5.4.4 Location of GalNAc on the ciliate cell

Wilks and Sleight (2004) showed that the GalNAc-binding lectin SBA (*Glycine max* [Soy Bean]) bound to the cytostome and food vacuoles of 25% of *Euplotes mutabilis* cells after a one-minute incubation. However, they found no clear binding pattern with another GalNAc-binding lectin, BPA (*Bauhinia purpurea*). Roberts et al. (2006) found that the binding of PNA (Peanut agglutinin) and MAA (*Maackia amurensis* agglutinin) to GalNAc residues occurred in food vacuoles only (after a 1h incubation though); and like Wilks and Sleight (2004) they found no binding with another GalNAc-binding lectin (*Sambucus nigra* agglutinin, SNA); highlighting the importance of pre-screening numerous lectins before use in experiments.

The published involvement of a 'GalNAc receptor' in particle uptake (at the cytostome) and vacuole formation in ciliates makes it as a strong candidate for being a receptor in *T. pyriformis* that binds both PUF and/or VFF. Considering the O-antigen of *S. enterica* serovar Typhimurium (*S. enterica* 74) has a trisaccharide backbone of mannose-rhamnose-galactose (Bogomolnaya et al., 2008), and has been previously proposed as a potential ligand for the mannose receptor (Section 4.5.2.3), it could equally be proposed as a ligand for the Gal/GalNAc receptor although currently, a prediction as to whether it is VFF or PUF cannot be made.

4.6 Conclusions

This study examined the feeding of *T. pyriformis* on 11 strains of bacteria to evaluate the importance of receptor-ligand interactions in prey uptake and food vacuole formation. Three prey ‘types’ were employed; live cells with a full complement of receptor ligands, heat-killed (dead) cells with disrupted receptor ligands and, latex beads with no ligands. Five specific aims were addressed and conclusions for each are presented below:

Aim 1: Compare the ingestion rates and vacuole formation rates when *T. pyriformis* feeds on the three prey types in monocultures, and evaluate whether there was any significant trend with regards to prey surface ligand availability.

Prey uptake, and vacuole formation, in *T. pyriformis* was highest with live cells, followed by dead cells and then beads; except with the remarkably heat-sensitive Pico 3, *S. marcescens* and *S. aureus* which behaved like beads after heat-treatment. This general trend mirrored the proposed availability of surface ligands on each of the prey types. The processing of beads, devoid of ligands, was very different to that of bacteria, and they induced what appeared to be basal, almost mechanical, rate of vacuole formation and ingestion. However, these appeared to be upregulated in the presence of live cells, and to a lesser extent, dead cells; suggesting they are receptor-mediated.

Aim 2: Determine whether *T. pyriformis* could actively select a specific prey type within a prey mixture, and evaluate whether there was any significant trend with regards to prey surface ligand availability.

There was no evidence for *T. pyriformis* actively selecting one prey type over another in any of the 50:50 mixtures. In most cases, this was demonstrated by each prey being ingested at the same rate as in monoculture. Three cases were more complicated. Live cells of *S. aureus* were ingested to a greater extent in mixture compared to monoculture, but so too were the dead cells in this mixture, suggesting that neither was ‘selected’, but another unknown factor resulted in this synergistic uptake. In the Live:Dead mixtures of *K. pneumoniae* and *P. fluorescens*, live cells

were ingested at higher rates in mixture, compared to monoculture whereas dead cells were ingested at the same rate as in monoculture. This might imply 'selection' of live cells over dead cells but why would a ciliate actively select a prey that, in monoculture, was less attractive than its dead counterpart? This particular interaction is complex and involves some currently unknown mechanism by which a negative factor associated with live cells is ameliorated by being in mixture with dead cells.

Significant trends regarding vacuole formation were observed in prey mixtures (discounting the three remarkably heat-sensitive strains) in that, (i) live cells always controlled vacuole formation in mixture with beads, (ii) dead cells always controlled vacuole formation in mixture with beads and, (iii) live and dead cells together showed a synergistic effect on vacuoles/cell (but did not show synergy with regards to prey uptake). The latter observation led the author to propose that different receptor-ligand interactions might be in place for vacuole formation (involving a vacuole formation factor, VFF) and prey uptake (involving a prey uptake factor, PUF). Yet, no matter whether the prey enters a cell via different mechanisms/routes there appears to be only one joint route into the food vacuole, as demonstrated by the fact that in *all* prey mixtures, both prey types were deposited into the same food vacuole, not separate vacuoles.

Aim 3: Compare the effects of heat and chemical treatments of bacteria on prey uptake by *T. pyriformis*; specifically, via removing the bacterial S-layer, removing bacterial surface proteins and fixing bacterial cells with formaldehyde.

The effect of heat-killing cells on prey uptake (employing PUF) and vacuole formation (employing VFF) was evaluated. In most cases, both factors might be the same ligand; in three bacterial strains (*Synechococcus* sp., *S. marcescens* and *S. aureus*) both VFF and PUF appeared to be destroyed by heat (with dead cells behaving like beads), in two strains (*A. hydrophila* and *E. coli*) neither factor appeared to be affected by heat, while in another two strains (*S. enterica* 74 and *S. liquefaciens*) both factors were partially affected by heat-treatment. However, data for the remaining four strains questioned whether VFF and PUF was the same factor: *P. aeruginosa* (VFF

heat-stable, PUF heat-labile) and, *S. enterica* 12694, *P. fluorescens* and *K. pneumoniae* (VFF heat-labile, PUF heat-stable).

All the Gram-negative strains used in the study (except *P. fluorescens* and *A. hydrophila*) possessed either a heat-labile capsule or S-layer which, after heat-treatment, could have been disrupted, leading to a reduced uptake of dead cells. This suggests a positive role for these layers in the uptake of these bacteria by protists. The capsule of the Gram-positive *S. aureus* was heat-stable and yet dead cells were ingested less than live cells, suggesting no role of the capsule in ingestion of this bacterium by protists.

Experiments involving trypsin and formaldehyde fixation, which damage cell-associated proteins but not carbohydrates, suggested that the uptake of Gram-negative bacteria is unaffected by these treatments and that their phagocytic ligands are more likely to be carbohydrates. Conversely, the uptake of the Gram-positive *S. aureus* was significantly affected suggesting that proteins might be important ligands for the uptake of Gram-positive cells by protists. Based on heat-lability, and unpublished data from Parry, it was proposed that a cell surface protein might be involved with vacuole formation (receptor - unknown) and that the peptidoglycan might be involved in prey uptake (receptor - scavenger protein).

Aim 4: Using *Salmonella enterica* 74 only (live and dead), together with beads, evaluate whether *T. pyriformis* possesses scavenger receptors.

There was no evidence for the involvement of scavenger receptors in the uptake of beads or live and dead Gram-negative *S. enterica* 74. However, a publication (Sattler et al., 2018) after the experimental work showed that *D. discoideum* possessed LmpB which is a homologue of a mammalian Class-B SR and which was shown to be exclusively involved in the uptake of Gram-positive bacteria. Such a homologue exists in *T. thermophila* so it could be that, SRs in *T. pyriformis* would have shown involvement in the uptake of *S. aureus* if this strain had been included in these experiments.

Aim 5: Using *Salmonella enterica* 74 only (live and Dead), together with beads, determine whether blocking three C-type lectins, with their specific sugar targets, affects the processing of any of the three prey types and evaluate whether there was any significant trend with regards to prey surface ligand availability.

Sugar blocking experiments suggested that the ciliate possessed receptors for mannose, N-acetylgalactosamine (GalNAc) and N-acetylglucosamine (GlcNAc). Only the latter two appeared to be involved in the processing of beads but the mechanisms by which these receptors 'bind' a particle devoid of ligands is currently unknown.

All three receptors were implicated in the uptake of (PUF), and vacuole formation with (VFF), live and dead *S. enterica* 74. Uptake in the presence of live cells was higher than dead cells and beads until a particular sugar concentration led them to become equivalent to dead cells (Mannose at 20mM; GalNAc at 50mM; GlcNAc at 125mM) and then at an even higher sugar concentration, becoming equivalent to beads (Mannose at 175mM; GalNAc/GlcNAc at 150mM). A similar trend was observed for vacuole formation, suggesting a sequential blocking of receptors for the attachment to live cells (which would have the most ligands). As for the ligands, by comparing the heat-lability of PUF and VFF between two strains for *S. enterica*, it was suggested that: Type 1 fimbriae (PUF) and the O-antigen (VFF) are potential ligands for the mannose receptor, peptidoglycan (PUF) and the core polysaccharide (VFF) are potential ligands for a GlcNAc receptor (possibly LysM), while the O-antigen is a potential ligand for a Gal/GalNAc receptor, but no further predictions could be made at this time.

APPENDIX

BG11 BROTH

Stock solutions:

(A) In 1L distilled water:

NaNO₃ – 15 g
K₂HPO₄ – 4 g
MgSO₄·7H₂O – 7.5 g
CaCl₂·2H₂O – 3.6 g
Citric acid - 0.6 g
Ammonium ferric citrate green - 0.6 g
EDTANa₂ - 0.1 g
Na₂CO₃ – 2 g

(B) In 1L distilled water:

Trace metal solution:

H₃BO₃ - 2.86 g
MnCl₂·4H₂O - 1.81 g
ZnSO₄·7H₂O - 0.22 g
Na₂MoO₄·2H₂O - 0.39 g
CuSO₄·5H₂O - 0.08 g
Co(NO₃)₂·6H₂O - 0.05 g

Make 100ml of Solution A.

Use 10mL of stock solutions (A) and 1 mL of stock solution (B) for 1L BG11 broth.

Make up to 1 liter with distilled water. Adjust pH to 7.1 with 1M NaOH or HCl.

Autoclave at 121°C for 15 minutes.

CHALKLEY'S MEDIUM

Stock solution: in 100ml distilled water:

NaCl - 2 g
KCl - 0.08g
CaCl₂ - 0.12g

Add 5mL of stock solution to 1L distilled water. Autoclave at 121°C for 15 minutes.

LYSOGENY BROTH (LB) AGAR (with and without antibiotics)

NaCl – 10 g

Typtone – 10 g

Yeast extract - 5 g

Agar No. 2 – 15 g

Add to 1 litre distilled water. Autoclave at 121°C for 15 minutes. Cool to 47°C before pouring aseptically.

Add 1 mL of filter sterilized antibiotic stock when necessary after the LB got autoclaved:

Ampicillin stock - 100 µg/mL

Chloramphenicol stock - 6 µg/mL

Gentamycin stock - 60 µg/mL

Kanamycin stock - 50 µg/mL

5-([4,6-Dichlorotriazin-2-yl]amino) fluorescein (DTAF) staining

Solution 1

In 100ml distilled water:

Na₂HPO₄ - 0.71 g

NaCl - 0.85 g

Autoclave at 121°C for 15 minutes.

Solution 2

In 100ml distilled water:

tetrasodium pyrophosphate - 0.89 g

NaCl - 0.85 g

Autoclave at 121°C for 15 minutes.

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