

Comparative Pathogenesis of *Yersinia enterocolitica* Biotypes

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A thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

March 2015

DECLARATION

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Abstract

Yersinia enterocolitica is a well known food-borne bacterium which belongs to the *Enterobacteriaceae* family. *Y. enterocolitica* is pathogenic for humans and it causes a wide spectrum of diseases ranging from self-limiting gastrointestinal diseases to fatal sepsis depending on the age and the immunity of the infected person. Oral ingestion of undercooked raw meat products, unpasteurised milk and contaminated water are the main sources of acquisition of this bacterium. *Y. enterocolitica* strains are categorized into six biotypes (1A, 1B, 2, 3, 4 &5). These six biotypes are further classified into high pathogenic, low pathogenic and non-pathogenic biotypes depending on their pathogenicity in the mouse infection model. This project compared the pathogenic potential of *Yersinia enterocolitica* high pathogenic, low pathogenic and non-pathogenic biotypes. The HEp-2 laryngitic human epithelial cell line and the alternative infection model *Galleria mellonella* greater wax moth larvae are the main infection models used to investigate the pathogenic potential of *Y. enterocolitica* biotypes. A diverse collection of *Y. enterocolitica* strains was used by which this strains collection is encompassing all six biotypes and all major serotypes. These strains were isolated from human and animal origins. Here novel results are presented showing new infection phenotypes of the *Y. enterocolitica* strains. All *Y. enterocolitica* biotypes strains were invasive to the HEp-2 epithelial cell line and pathogenic to the *Galleria* insect model. The non-pathogenic biotype was the most lethal in the *Galleria* model while the high pathogenic biotype shows little to no pathogenicity. The low pathogenic biotypes were moderately pathogenic to the *Galleria* insect model. The pYV plasmid showed a minor role in modulating the virulence in the *Y. enterocolitica* pathogenic biotypes. Investigating the *in vivo Y. enterocolitica-Galleria* interaction showed that *Y. enterocolitica* pathogenic biotypes bacterial cells were growing inside the *Galleria* larvae while the non-pathogenic biotype bacterial cells were dying. The heat-killed *Y. enterocolitica* strains were completely avirulent to the *Galleria* larvae.

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ACKNOWLEDGMENTS

I would like to thank my leader of study Doctor Alan McNally for giving me the opportunity to carry out this PhD project under his supervision and for his excellent support, kind guidance and encouragement throughout the duration of my studies at NTU.

Many thanks to my second supervisor Professor Graham Balls for his helpful guidance, assistance and support during this project. Many thanks to Doctor Georgina Manning and I am very grateful for her assistance during my project approval period and her advice during the lab meetings.

I am thankful and very grateful for the lab help and assistance provided by the great staff of School of Science and Technology namely Pam, Mike, Barbara, Andy, Nick, Steven, Iain, Aaron and all the rest of staff who provided their kind help behind the scenes.

My sincere thanks to my fantastic colleagues in the pathogen group Fahad Alhashash, Abdulaziz Alqasim, Hall Miquette and Alya Redwan who I am so proud of their friendship and very grateful for their help and assistance.

My thanks are extended to my friends and laboratory fellows Faisal Almajed, Jade Gouriet, Sandra, Abiyad, Gemma, Aldukali and Naqash for their nice company.

A special thanks to my family. Words cannot express how grateful I am to them. Without their kind support, patience and encouragement I wouldn't be able to finish this work. Many thanks to my sweet little daughter Rahaf and I wish her the best future.

Abbreviations

| Abbreviation | Description |
|-------------------------|---|
| Ail | attachment-invasion locus |
| Bla-A | β -lactamase A enzyme |
| Bla-B | β -lactamase B enzyme |
| BT | Biotype |
| C.F.U. | colony forming unit formula |
| CaCl₂ | Calcium Chloride |
| CDs | Coding Sequences |
| CHO | Chinese Hamster Ovary cell line |
| CIN | Cefsulodin irgasan novobiocin agar |
| cm | centimetre |
| CO₂ | Carbon dioxide |
| CRMOX agar | congo red-magnesium oxalate agar medium |
| DAG | diacylglycerol |
| DMSO | Dimethyl Sulfoxide solvent |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxyribonucleotide triphosphate |
| DPBS | Dulbecco's Phosphate Buffered Saline |
| FAE | Follicle associated epithelium |
| Fops | flagellar outer proteins |
| F-Primer | Forward primer |
| g | gram |
| HeLa | human epithelial cell line |
| HEp-2 | human laryngitic epithelial cell line |
| HKY | heat killed <i>Y. enterocolitica</i> |
| HPI | High Pathogenicity Island |
| inv | gene encoding for Invasin protein |
| Inv | Invasin protein |
| Kbp | Kilo base pair |
| kDa | Kilo Dalton |

| | |
|---|--------------------------------|
| L | Litre |
| LB Agar | Luria-Bertani Agar |
| LB Broth | Luria-Bertani Broth |
| Lcr | low calcium response |
| LD₅₀ | The median lethal doses |
| Log | Logarithm |
| LPS | Lipopolysaccharides |
| M | Molar |
| M.O.I. | Multiplicity of infection |
| Mb | Mega base |
| M-cells | Microfold cell |
| MEM | Minimum Essential Medium Eagle |
| Mg²⁺ | Magnesium Cation |
| MgCl₂ | Magnesium Chloride |
| ml | millilitre |
| mm | millimetre |
| mm | millimetre |
| mM | millimolar |
| Myf | Mucoid <i>Yersinia</i> factor |
| Na₂C₂O₄ | Sodium Oxalate |
| NaCl | Sodium chloride |
| ng | nanogram |
| nm | nanometre |
| O-antigen | Outer antigen |
| OD | optical density |
| p.i. | post infection |
| PAI | Pathogenicity islands |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PG | phylogroups |
| PLCs | Phospholipases |
| PMNs | polymorphonuclear leukocytes |
| Pmol | picomole |

| | |
|----------------------------|--|
| PP | Peyer's Patches |
| pYV | Plasmid of Yersinia Virulence |
| rpm | rounds per minute |
| R-Primer | Reverse Primer |
| rRNA | ribosomal ribonucleic acid |
| SC | species complexes |
| sdH₂O | sterile distilled water |
| sec | seconds |
| Subsp | subspecies |
| Syc | <u>S</u> pecific <u>y</u> op <u>c</u> haperone |
| T2SS | type II secretion system |
| T3SS | type III secretion system |
| tc | Insecticidal toxin genes |
| Tc | Toxin complex |
| tc-PAI^{Ye} | <i>tc</i> pathogenicity island of <i>Y. enterocolitica</i> |
| TSA | Tryptone Soy Agar |
| UV light | ultraviolet light |
| VirF | Virulence regulon transcriptional activator |
| YadA | <i>Yersinia</i> adhesin A |
| Ybt | Yersiniabactin |
| YGT | <i>Yersinia</i> genus type three secretion system |
| Yop | <i>Yersinia</i> outer membrane proteins |
| YplA | The phospholipase protein |
| Ysa | <i>Yersinia</i> secretion apparatus |
| YSA PI | <i>Yersinia</i> secretion apparatus pathogenicity island |
| Ysc | Yop secretion |
| Ysps | <i>Yersinia</i> secreted proteins |
| YST | <i>Yersinia</i> stable toxins |
| Yts1 | <i>Yersinia</i> type II secretion 1 |
| Yts2 | <i>Yersinia</i> type II secretion 2 |
| α | alpha |
| β | beta |
| γ-proteobacteria | Gammaproteobacteria |

| | |
|--------------------|------------------|
| λ | lambda |
| μg | microgram |
| μl | microliter |
| μM | micromolar |
| -ve control | negative control |
| +ve control | positive control |

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Chapter 1
Introduction

1.1 The Genus *Yersinia* Classification and Characteristics:

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and includes Gram-negative, rod shaped bacteria (Carniel *et al.*, 2006). Species of the genus *Yersinia* are non spore-forming bacteria with a shape ranging from rod to coccobacilli (Fredriksson-Ahomaa, 2007a). Biochemical identification tests and DNA-DNA hybridization are the main techniques used to subtype within the *Yersinia* genus species (Sulakvelidze *et al.*, 2000). All the *Yersinia* species are oxidase-negative, catalase-positive and facultative anaerobic bacteria (Fredriksson-Ahomaa, 2007a). Currently, the *Yersinia* genus includes 17 species (Savin *et al.*, 2012) of which only three species are pathogenic to humans and animals, *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* (Fuchs *et al.*, 2011). *Y. pestis* is the aetiological agent of bubonic and pneumonic plague (or the Black Death) which is transmitted by flea vectors (Perry *et al.*, 1997), while *Y. pseudotuberculosis* and *Y. enterocolitica* are enteropathogens acquired by the consumption of spoiled food and contaminated water (Zhang *et al.*, 2008). The remaining 14 species are considered avirulent to humans and these species are *Y. aldovae*, *Y. bercovieri*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. rohdei*, *Y. mollaretii*, *Y. ruckeri*, *Y. aleksiciae*, *Y. entomophaga*, *Y. massiliensis*, *Y. nurmii*, *Y. pekkanenii* and *Y. similis* (Fuchs *et al.*, 2011). The final six species were recently added to the genus *Yersinia* (Fernandez *et al.*, 2007). The species *Y. ruckeri* is characterised as a fish pathogen causing enteric red mouth disease (Fernandez *et al.*, 2007) and the species *Y. entomophaga* is an insect pathogen (von Tils *et al.*, 2012).

1.2 *Y. enterocolitica* Biotypes:

Y. enterocolitica is a zoonotic pathogen that has been identified as a major cause of bacterial gastrointestinal disease in many developed and developing countries around the world (Rahman *et al.*, 2011) and is the third most common cause of food-borne gastrointestinal disease in Europe (Fredriksson-Ahomaa *et al.*,2007b). *Y. enterocolitica* is a well known food-borne pathogen (Fredriksson-Ahomaa and Korkeala,2003) and is ubiquitous in domestic and wild animals (Kot *et al.*,2005). Pigs are considered to be the major source of *Y. enterocolitica* human infections (Laukkanen-Ninios *et al.*,2014). *Y. enterocolitica* is a gram-negative, short rod or coccobacilli bacteria with a length of 0.5–1-2µm. *Y. enterocolitica* are non-spore forming, facultative anaerobes which can survive in a wide range of temperatures from 0°C to 45°C. The optimal growth temperature is from 22 to 29°C (Zadernowska *et al.*, 2013). *Y. enterocolitica* is a highly heterogeneous species (Tennant *et al.*, 2003; Viridi & Sachdeva, 2005) and is classified into six biotypes on the basis of biochemical characteristics (Singhal *et al.*, 2014) described in table 1.1. These biotypes are 1A, 1B, 2, 3, 4 & 5. These six biotypes are further classified into three groups (non-pathogenic biotype 1A, the high pathogenic biotype 1B and the low pathogenic biotypes 2, 3, 4 and 5) depending on their pathogenicity towards the mouse animal infection model (Bottone, 1999; Lepka and Wilharm, 2010). Recent phylogenomic analysis of *Y. enterocolitica* revealed that species is also genetically heterogeneous and diverse (Reuter *et al.*, 2014). *Y. enterocolitica* biotypes were phylogenetically classified into two species complexes (SC) according to a phylogenetic tree that was constructed depending on core genes common to the whole *Yersinia* genus. This approach classified the *Y. enterocolitica* biotypes into two separate SCs which are SC6 and SC7. SC6 complex contains the high pathogenic biotype 1B and the non-pathogenic biotype 1A, while the SC7 complex includes the four low pathogenic biotypes 2, 3, 4 & 5. Another phylogenetic tree based on core

genome SNPs of the *Y. enterocolitica* species revealed that *Y. enterocolitica* forms six distinctive lineages called phylogroups (PG) 1-6. PG1 contains biotype 1A, PG2 contains biotype 1B, PG3 contains bioserotype 4/O:3, PG4 includes bioserotypes 2/O:5,27 and 3/O:5,27, PG5 includes bioserotypes 2/O:9 and 3/O:9 while PG6 contains biotype 5 (Reuter *et al.*, 2014). The first complete *Y. enterocolitica* genome was published in 2006 of the high pathogenic biotype 1B/O:8 strain 8081 (Thomson *et al.*, 2006). Later, other genome drafts were sequenced from other strains representing a number of *Y. enterocolitica* low pathogenic biotypes (Batzilla *et al.*, 2011a; Fuchs *et al.*, 2011; Wang *et al.*, 2011). The genome size of *Y. enterocolitica* biotypes ranges from 4.5Mb to 4.9Mb. The genome size of all low pathogenic biotypes is ~4.5Mb and ~4.6Mb for the high-pathogenic biotype while the genome size of the non-pathogenic biotype is around 4.9Mb. The low pathogenic biotype 4 genome has the lowest number of coding sequences (CDSs) while the non-pathogenic biotype 1A include the largest number of CDSs (Reuter *et al.*, 2012). *Y. enterocolitica* pathogenicity is strongly related to the bioserotypes of this bacterium (Rahman *et al.*, 2011).

Table 1.1: Table from Bialas *et al.*(2012) of biotyping scheme for *Y. enterocolitica* (modified from Bottone, 1999)

| Biochemical Reaction | Reactions in different <i>Y. enterocolitica</i> biotypes | | | | | |
|----------------------------------|--|----|---|---|---|---|
| | 1A | 1B | 2 | 3 | 4 | 5 |
| Inositol fermentation | + | + | + | + | + | + |
| Salicin fermentation | + | - | - | - | - | - |
| Sorbose fermentation | + | + | + | + | + | - |
| Trehalose fermentation | + | + | + | + | + | - |
| Ornithine decarboxylase activity | + | + | + | + | + | + |
| Lipase activity | + | + | - | - | - | - |
| Pyrazinamidase activity | + | - | - | - | - | - |
| Nitrate reduction | + | + | + | + | + | - |
| Esculin hydrolysis | +/- | - | - | - | - | - |

+: (activity present) ; -: (activity absent)

1.3 *Y. enterocolitica* Serotypes:

The *Y. enterocolitica* species includes about 70 serotypes (Garzetti *et al.*, 2012; Pajunen *et al.*, 2001). The serotypes are characterised according to the variable structure of the outer membrane O-antigen (Simonova *et al.*, 2007) using serological tests (Saleh *et al.*, 2012). Among those 70 serotypes, only serotypes O:3, O:9, O:8 and O:5,27 are found to be pathogenic to humans (Chart & Cheasty, 2006; Saleh *et al.*, 2012). However, the precise detection of pathogenic *Y. enterocolitica* isolates requires the characterization of both the biotype and serotype of the isolated strain. Most *Y. enterocolitica* strains found in human clinical cases belong to 4/O:3, 1B/O:8, 2/O:5,27, 2/O:9 and 3/O:3 (Rahman *et al.*, 2011; Schaake *et al.*, 2013). Serotypes O:3, O:9 and O:5,27 were found to be the most dominant in Europe (Chart & Cheasty, 2006) while serotype O:8 is the most predominant in North America (Schaake *et al.*, 2013). Table 1.2 illustrates major serotypes of *Y. enterocolitica*.

Table 1.2 Adapted from Tennant *et al.* (2003) Table shows relationship between biotype, O serotype, and pYV carriage of *Y. enterocolitica*

| Biotype | Serotypes |
|----------------|--|
| 1A | O:4; O:5; O:6,30; O6,31; O:7,8; O:7,13; O:10; O:14; O:16; O:21; O:22; O:25; O:37; O:41,42; O:46; O:47; O:57; NT ^a |
| 1B | O:4,32 ^b ; O:8 ^b ; O:13a,13b ^b ; O:16; O:18 ^b ; O:20 ^b ; O:21 ^b ; O:25; O:41,42; NT ^a |
| 2 | O:5,27 ^b ; O:9 ^b ; O:27 |
| 3 | O:1,2,3 ^b ; O:3 ^b ; O:5,27 ^b |
| 4 | O:3 ^b |
| 5 | O:2,3 ^b |

^aNT: not typable.

^bSerotypes which include strains that carry pYV.

1.4 *Y. enterocolitica* virulence factors

Y. enterocolitica virulence factors are encoded by both the chromosome and the pYV plasmid (Paixão *et al.*, 2013). The pathogenic biotypes (1B, 2, 3, 4 and 5) are considered to be pathogenic due to their acquisition of major virulence factors such as the *Yersinia* virulence 70kb sized pYV plasmid (Cornelis *et al.*, 1998) which is completely absent from the non-pathogenic biotype 1A strains (Sabina *et al.*, 2011). The pYV plasmid encodes for major virulence factors such as the *Yersinia* type three secretion system (Ysc), the *Yersinia* outer proteins (Yops) and the *Yersinia* adhesion protein A (YadA). *Y. enterocolitica* high pathogenic biotype 1B strains exclusively harbour additional chromosomally encoded virulence determinants such as the High Pathogenicity Island (HPI) and the Ysa T3SS (Bhagat & Viridi, 2007; Sabina *et al.*, 2011). These are discussed in more details below:

1.4.1 pYV plasmid encoded virulence factors

1.4.1.1 The Ysc Type Three Secretion System Injectisome:

The Ysc (for Yop secretion) is a type three secretion system (T3SS) found in all human *Yersinia* species and encoded by the *Yersinia* virulence pYV plasmid (Cornelis *et al.*, 1998). Ysc is considered the major virulence factor responsible for *Y. enterocolitica* pathogenesis. The Ysc injectisome is a bacterial organelle which is synthesised and assembled when *Y. enterocolitica* encounters temperatures of 37°C. The Ysc secretion system is composed of two main parts which are the basal body, spanning both the inner and outer bacterial membranes in addition to the periplasmic peptidoglycan layer, and the needle-like structure located on the top of the basal body and extends to outside of the cell. (Broz *et al.*, 2007; Mueller *et al.*, 2008). These parts of the Ysc injectisome are made from number of structural proteins encoded by the pYV plasmid. Proteins YscC, YscD, YscJ YscI YscN, YscK, YscL,

YscQ, YscR, YscS, YscT, YscU and YscV form the basal body part while proteins YscF, YscO, YscP, YscU, YscV, YscX, YscY and YopR build up the injectisome needle (Dewoody *et al.*, 2013; Cornelis, 2010).

1.4.1.2 The Secreted Yop proteins:

The Ysc injectisome acts as a protein pump to secrete a number of proteins called Yop proteins (for *Yersinia* outer proteins) (Cornelis, 2002). At least six of the secreted Yops called effector Yop proteins which are YopH, YopE (Grosdent *et al.*, 2002; Trosky *et al.*, 2008), YopM (Viboud and Bliska, 2005), YopT (Trulzsch *et al.*, 2004), YopO (also called YpkA in *Y. pestis* and *Y. pseudotuberculosis*) (Grosdent *et al.*, 2002; Juris *et al.*, 2000) and YopP (also called YopJ in *Y. pseudotuberculosis* and *Y. pestis*); (Denecker *et al.*, 2002). These effector Yop proteins are toxic proteins injected into the cytosol of the target eukaryotic cell causing the disruption of signal transduction pathways (Lee *et al.*, 2000) and actin cytoskeleton dynamics (Cornelis, 2002) leading to cellular apoptosis (Lee *et al.*, 2000). These cellular alterations inhibit the phagocytosis ability of many phagocytes including macrophages, polymorphonuclear leukocytes (Cornelis, 2002) and dendritic cells (Koberle *et al.*, 2009). This enables the *Y. enterocolitica* pathogenic strains to escape killing by the immune system (Koberle *et al.*, 2009). The secretion of the Yop proteins to the external milieu is controlled by a protein complex called the “Calcium Block” made of the proteins YopN-TyeA-YscB-SycN. This YopN-TyeA-YscB-SycN plug blocks the passage of the synthesized Yop proteins through the injectisome secretion channel in the presence of a high Calcium (Ca^{2+}) concentration and the absence of contact with the eukaryotic cells. Yop secretion occurs once the *Y. enterocolitica* cells induce contact with eukaryotic cells and Ca^{2+} levels drop (Dewoody *et al.*, 2013). The Ysc injectisome secretes the pore forming

translocator proteins YopB, YopD and LcrV which are required to translocate the secreted effector proteins into the cytosol of the target eukaryotic cell by forming a pore on the target host cell (Broz *et al.*, 2007; Mueller *et al.*, 2008).

1.4.1.3 Syc Chaperones

Yop protein secretion is assisted by another group of proteins called Syc chaperones (Syc for Specific yop chaperone); (Schmid *et al.*, 2006). These chaperones are classified into Class I chaperones, class II chaperones and class III chaperones. Chaperones of Class I interact with the effector proteins, chaperones in class II interact with the translocator proteins and chaperones of class III interact with T3SS needle constituents (Schreiner and Niemann, 2012). Each chaperone interacts with its specific partner of the Yop proteins (Fàbrega and Vila, 2012). The Syc proteins discovered up to date are SycD chaperone which binds to both of the YopB and YopD translocator proteins, SycE chaperone which binds to the YopE effector protein, SycH chaperone binds to the YopH effector protein, the SycT chaperone which is specific for the YopT protein and the SycN chaperone which specifically bind to the YopN protein (Cornelis *et al.*, 1998; Fàbrega and Vila., 2012).

1.4.1.4 *Yersinia* adhesin A (YadA)

The *yadA* gene is located on the pYV plasmid of the pathogenic species *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* and it encodes the *Yersinia* adhesin A (YadA) protein. YadA is an adhesion protein and it is characterised as a significant virulence factor of *Y. enterocolitica* (Schutz *et al.*, 2010) while in *Y. pseudotuberculosis* YadA protein is not essential in virulence. YadA is not expressed in *Y. pestis* due to a frameshift mutation

silencing the *yadA* gene (El Tahir and Skurnik, 2001; Mikula *et al.*, 2013) turning it into a pseudogene (Mikula *et al.*, 2013). YadA protein is expressed at 37°C via the action of the transcription regulator protein VirF (Biedzka-Sarek *et al.*, 2008; El Tahir and Skurnik, 2001). The main function of YadA is to bind to extracellular matrix molecules such as collagen, laminin and fibronectin. YadA binds very efficiently to collagen but with less affinity to laminin and fibronectin (El Tahir and Skurnik, 2001). YadA possess other functions like mediating autoagglutination (Mikula *et al.*, 2013) and serum resistance (Mikula *et al.*, 2013; Schutz *et al.*, 2010).

1.4.2 *Y. enterocolitica* chromosomally encoded virulence factor

1.4.2.1 *Yersinia* secretion apparatus (Ysa)

The *Y. enterocolitica* high pathogenic 1B/O:8 strains exclusively possess an extra secretion system called the *Yersinia* secretion apparatus (Ysa); (Venecia and Young, 2005). Ysa secretion system is a type three secretion system (T3SS); (Young and Young, 2002a) which is distinct from the well-studied Ysc T3SS and works independently from it, but the role of the Ysa T3SS in pathogenesis is still ambiguous (Venecia and Young, 2005). The Ysa T3SS system is encoded by a unique chromosomal locus called the *Yersinia* secretion apparatus pathogenicity island (YSA PI) which is only present in the chromosome of the high pathogenic biotype 1B *Y. enterocolitica* strains. The Ysa T3SS secretes extracellular effector proteins called *Yersinia* secreted proteins (for Ysps) into the cytosol of eukaryotic cells encoded by the *ysa* locus genes (Walker and Miller, 2009). The Ysa secretion apparatus have been found, so far, to secrete about 16 Ysp proteins which are YspB, YspC, YspD, YspG, YspH, YspJ, YspA, YspE, YspF, YspI, YspK, YspL, YspM, YspN, YspP and YspY (Fàbrega and Vila, 2012). Part of these proteins build up the tranlocon apparatus and the

others act as effectors in the host cells (Fàbrega and Vila, 2012; Matsumoto and Young, 2005).

1.4.2.2 *Y. enterocolitica* Type 2 Secretion System (T2SS)

The T2SS is not a very well characterized secretion system in the Genus *Yersinia*, compared to the well-studied *Yersinia* type three secretion systems (von Tils *et al.*, 2012). Two distinct types of T2SS have been so far identified in *Yersinia* species which are Yts1 (for *Yersinia* type II secretion 1) and Yts2 (for *Yersinia* type II secretion 1) (Iwobi *et al.*, 2003). Yts1 is found in the *Y. enterocolitica* high pathogenic biotype 1B/O:8 strains and Yts2 is present in all *Y. enterocolitica* biotypes (Shutinoski *et al.*, 2010). In *Y. enterocolitica*, the Yts1 and Yts2 T2SSs are encoded on the chromosome and their genes occur in clusters. Yts1 was the first characterised T2SS by Iwobi *et al.* (2003) in the high pathogenic biotype 1B/O:8 strain WA-314 (von Tils *et al.*, 2012) and located in the chromosomal plasticity zone (Iwobi *et al.*, 2003; von Tils *et al.*, 2012) while the structure and function of Yts2 T2SS is still not very well characterized (von Tils *et al.*, 2012).

1.4.2.3 *Y. enterocolitica* Flagella and motility

Y. enterocolitica is a motile bacterium which exhibits peritrichously distributed flagella for swimming and swarming motility (Young *et al.*, 1999). *Y. enterocolitica*, along with *Y. pseudotuberculosis*, are the only human pathogenic motile members of the Genus *Yersinia* by which *Y. pestis* is non-motile due to a frameshift mutation in the *flhD* master regulator protein (Kim *et al.*, 2008). *Y. enterocolitica* is only motile at temperatures below 30°C (Young *et al.*, 1999). Like some other *Enterobacteriaceae* members, the *Y. enterocolitica* flagella biosynthesis is controlled by chromosomally encoded genes expressed in a complex hierarchy order (Atkinson *et al.*, 2006; Bleves *et al.*, 2002). Besides motility, the

Y. enterocolitica flagella works as a secretion apparatus that secretes a non-flagellar proteins called 'Fops'. Fops stand for flagellar outer proteins (Atkinson *et al.*, 2011; Young and Young, 2002b). The phospholipase (YplA) protein is an example of a Fop protein secreted by the flagellar type III secretion apparatus prior the formation of the flagellar filament (Atkinson *et al.*, 2011). Phospholipase (YplA) is a virulence factor that enables *Y. enterocolitica* to colonize host tissues (Schmiel *et al.*, 2000). Although, the pathogenic role of *Y. enterocolitica* flagella towards the host is still enigmatic (Lepka and Wilharm, 2010), it is suggested that the flagella pathogenic role occurs at the early stages of infection (Raczkowska *et al.*, 2011).

1.4.2.4 Lipopolysaccharides (LPS) O-antigen of *Y. enterocolitica*

In *Y. enterocolitica*, the LPS O-antigen has been implicated in virulence and in colonisation of the murine infection model intestinal tissues (Bengoechea *et al.*, 2004). The O-antigen is an elongated sugar polymer by which each polymer is composed of 2–8 sugar residues (Sirisena and Skurnik, 2003). The *Y. enterocolitica* O-antigen biosynthetic genes are chromosomally encoded by the *wb*-clusters genes (Bengoechea *et al.*, 2002). Similar to *Y. pseudotuberculosis*, the *Y. enterocolitica* LPS O-antigen are temperature regulated (Bengoechea *et al.*, 2004). *Y. enterocolitica* LPS O-antigen is highly expressed when bacterial cells are grown in room temperature (22-25°C) and trace amounts of O-antigen are expressed at 37°C (Bengoechea *et al.*, 2002; Bengoechea *et al.*, 2004). On the other hand, *Y. pestis* does not express the O-antigen due to the presence of five pseudogenes in the O-antigen biosynthesis *wb*-cluster genes (Bengoechea *et al.*, 2002).

1.4.2.5 *Y. enterocolitica* HPI

Y. enterocolitica high pathogenic biotype 1B is the only biotype that contains high pathogenicity island (HPI); (Carniel, 1999). The HPI Island so-called because it differentiates the high pathogenic strains from the low pathogenic ones in *Y. enterocolitica* (Bach *et al.*, 1999). The *Y. enterocolitica* HPI is 45kbp and carries virulence genes called the yersiniabactin locus responsible for iron uptake (Bach *et al.*, 1999; Carniel *et al.*, 1996). Yersiniabactin (Ybt) is a siderophore and enables the bacteria to gain iron molecules required for its *in vivo* growth and dissemination (Bach *et al.*, 1999). The yersiniabactin locus genes encode for yersiniabactin biosynthesis, transport and regulation. As a result the *Yersinia* HPI is also called the “iron-capture island” (Carniel, 1999).

1.4.2.6 Invasin protein (Inv)

Invasin (Inv) is an adhesin protein expressed by the enteric *Yersiniae*, *Y. enterocolitica* and *Y. pseudotuberculosis*. *Y. enterocolitica* Invasin is an outer-membrane protein with a 92 kDa weight (Revell and Miller, 2000) and encoded by the chromosomal *invA* gene (Mikula *et al.*, 2013). Invasin acts in the early stages of infection (Mikula *et al.*, 2013) by promoting an early stage of intestinal infection (Schmid *et al.*, 2004). The *Y. enterocolitica* Invasin attaches to the Peyer’s Patches of the small intestine by binding to the β 1-Integrins of the M-cells followed by translocation to the underlying lymphoid tissues (Grassl *et al.*, 2003). Invasin binds to different β 1-Integrins such as α 3 β 1, α 4 β 1, α 5 β 1, α 6 β 1, and α v β 1. *Y. enterocolitica* Invasin binding to β 1-Integrins causes *Y. enterocolitica* internalization through the epithelial cells by an invasion mechanism called the “zipper” mechanism (Mikula *et al.*, 2013).

1.4.2.7 Ail protein

Ail refers to the “attachment-invasion locus” and it was first described in *Y. enterocolitica* species. Ail is an outer membrane protein with a ~ 17 kDa molecular weight (Hinnebusch *et al.*, 2011) and encoded by the *ail* locus gene (Miller and Falkow, 1988; Miller *et al.*, 1990). Ail is expressed at 37°C (Białas *et al.*, 2012) and enables *Y. enterocolitica* strains to invade the intestinal epithelium and to play an important role in serum resistant (Huang *et al.*, 2010). Unlike the *inv* gene which is present in all pathogenic and non-pathogenic *Y. enterocolitica* strains, *ail* is only found in *Y. enterocolitica* virulent strains (Huang *et al.*, 2010). Recent work based on whole genome sequence data of the entire genus *Yersinia* has shown that the *ail* gene was a main virulence factor, along with the pYV plasmid, that was gained during the evolution of *Yersinia* pathogenic lineage. This acquisition of *ail* gene separates the non-pathogenic *Yersinia* lineages from the pathogenic ones (Reuter *et al.*, 2014).

1.4.3 Auxiliary *Y. enterocolitica* virulence factors

1.4.3.1 Mucoïd *Yersinia* factor (Myf)

The *Y. enterocolitica* chromosomal *myf* locus encodes for the biosynthesis of the Myf antigen (Białas *et al.*, 2012; Fàbrega and Vila, 2012). The *myf* locus contains 5 genes which are the *myfA*, *myfB*, *myfC*, *myfE* and *myfF* which encodes for parts that build the Myf antigen. MyfA forms a fibrous layer which covers the extracellular side of bacterial cells and expands two microns from the bacterial surface (Mikula *et al.*, 2013). Despite of the unknown function of the Myf antigen, some experimental work shows that the *Y. enterocolitica* Myf antigen can interact with the host intestinal epithelium and may protect *Y. enterocolitica* cells from the bactericidal activity of phagocytes (Białas *et al.*, 2012).

1.4.3.2 YST (*Yersinia* stable toxins)

YST (*Yersinia* stable toxins) is a chromosomally encoded, 7494 Da, thermostable enterotoxin produced by *Y. enterocolitica* strains (Bancerz-Kisiel *et al.*, 2012; Platt-Samoraj *et al.*, 2006). YST is a virulence factor that enables the *Y. enterocolitica* strains to invade and damage the intestinal epithelium tissues (Bancerz-Kisiel *et al.*, 2012) causing diarrhoea (Platt-Samoraj *et al.*, 2006). *Y. enterocolitica* produces three variants of the Yst toxin which are the YstA, YstB and YstC encoded by *ystA*, *ystB* and *ystC* genes respectively. The YstA is produced by pathogenic *Y. enterocolitica* strains while YstB and YstC variants are encoded only by the non-pathogenic biotype 1A strains. *ystB* has been found to be present in almost all clinical isolates of non-pathogenic biotype 1A strains (Bancerz-Kisiel *et al.*, 2012). As a result, *ystB* have been applied as a prominent PCR marker for the detection of the clinical isolates of non-pathogenic biotype 1A strains (Ramamurthy *et al.*, 1997).

1.4.4 *Y. enterocolitica* Route of Infection:

Y. enterocolitica is facultative intracellular enteropathogen (Beuscher *et al.*, 1995) characterised as both a food-borne and water-borne pathogen (Belgin Siriken, 2004) by which it is believed to be transmitted through the fecal-oral route (Bari *et al.*, 2011; Guinet *et al.*, 2011). When orally ingested, *Y. enterocolitica* crosses and survives the stomach using urease activity, to tolerate the high acidic nature of the stomach, and enters the small intestine (Handley *et al.*, 2004). Once reaching the small intestine *Y. enterocolitica* replicates (Oellerich *et al.*, 2007) and rapidly colonizes the lumen of the small intestine (Trulzsch *et al.*, 2004). Then *Y. enterocolitica* strains invade and translocate through the M cells that overlie the follicle associated epithelium (FAE) (Oellerich *et al.*, 2007) of the Peyer's Patches (PP) located in the distal part of the intestine (Jung *et al.*, 2010). Once translocated, *Y. enterocolitica* colonizes the Peyer's Patches by subverting the immune system through

stopping the phagocytosis action of macrophages and polymorphonuclear leukocytes (PMNs) and inhibiting several immune inflammatory cascade components (Handley *et al.*, 2006). Finally, *Y. enterocolitica* proceeds to disseminate to other body organs (Trcek *et al.*, 2010).

1.4.5 *Y. enterocolitica* clinical manifestations

1.4.5.1 *Y. enterocolitica* gastrointestinal infections

Y. enterocolitica primarily causes gastrointestinal diseases with the ability to cause extraintestinal complications in patients with compromised immune systems (Bottone, 1997; Bottone, 1999). *Y. enterocolitica* gastrointestinal infections include acute enteritis accompanied with fever and watery diarrhoea, and rarely bloody diarrhoea in children. Acute terminal ileitis and mesenteric lymphadenitis mimicking appendicitis are more common in young adults. In infants, *Y. enterocolitica* can also cause prolonged severe gastrointestinal diseases such as fatal necrotizing enterocolitis and “pseudo-tumorigenic” form of suppurative mesenteric adenitis. The infection period in infants may proceed for 3 to 28 days while in adults it lasts for 1 to 2 weeks. The potency of the gastrointestinal disease is dependent on the *Y. enterocolitica* strain serotype. *Y. enterocolitica* serotype O:8 strains can cause severe complications such as gastrointestinal tract ulceration and death. On the other hand, *Y. enterocolitica* strains of serotypes O:9 and O:3 are clinically found to be less virulent to the gastrointestinal tract (Bottone, 1997).

1.4.5.2 *Y. enterocolitica* extra intestinal infections:

Y. enterocolitica also causes extra intestinal infections such as Septicaemia mostly in patients with immune complications or with iron overload complications. Septicaemia may be accompanied with metastatic spread of the invading *Y. enterocolitica* strains to different body

organs (Bottone, 1999). Metastatic diseases following *Y. enterocolitica* septicaemia are diverse and they include focal abscess formation in the kidneys, lungs, spleen, and liver. It also includes cutaneous infections such as pyomyositis, cellulitis, pustules, and bullous lesions. Metastatic diseases may also involve meningitis, osteomyelitis, panophthalmitis, endocarditis, pneumonia and cavitary pneumonia (Bottone, 1997; Bottone, 1999). *Y. enterocolitica* extra intestinal infections also include post-infection, long-term sequelae resulting from acute bacteraemia (Fàbrega and Vila, 2012). Sequelae manifestations include reactive arthritis, erythema nodosum, glomerulonephritis and myocarditis where reactive arthritis is strongly associated with HLA-B27-antigen positive individuals (Bottone, 1999). However, reactive arthritis and erythema nodosum are the most common sequelae diseases associated with *Y. enterocolitica* infection. Sequelae infections are mainly observed in young adults and in rare clinical conditions they were found to be specifically linked to serotype O:3 infections (Fàbrega and Vila, 2012).

1.4.6 Project Background

Y. enterocolitica consists of six biotypes differentiated by their biochemical characteristics into 1A, 1B, 2, 3, 4 and 5. These six biotypes are arranged in three lineages depending on their pathogenicity in the mouse infection model; the high pathogenic biotype 1B, the low pathogenic biotypes 2-5 and the non-pathogenic biotype 1A. The *Y. enterocolitica* high pathogenic biotype 1B is lethal in a mouse infection model while the low pathogenic biotypes cause only a mild disease in mice without any death. The *Y. enterocolitica* non-pathogenic biotype 1A doesn't cause any disease in the mouse infection model. However, the high pathogenic biotype is rarely isolated from human clinical cases, whilst the low pathogenic biotypes are mostly associated with human diseases and they cause

the same disease. The non-pathogenic biotype is completely avirulent in mouse infection model but their pathogenicity in humans is controversial (Tennant *et al.*, 2003). A number of studies support the implication of the non-pathogenic biotype in human diseases while others deny it. Non-pathogenic biotype 1A are frequently isolated strain types from healthy persons and from patients suffering from gastrointestinal diseases humans (Batzilla *et al.*, 2011b). Although the non-pathogenic biotype strains lack all virulence factors present in the pathogenic biotypes such as the pYV plasmid, ysa T3SS, HPI, Ail, YstA and MyfA, they still repeatedly cause symptoms similar to those caused by the other pathogenic *Y. enterocolitica* (Batzilla *et al.*, 2011b; Bhagat & Viridi, 2007; Stephan *et al.*, 2013). This confusing pathogenic potential of *Y. enterocolitica* biotypes is the key reason of establishing this project. In this project, the pathogenic potential of *Y. enterocolitica* biotypes will be investigated in the *in vitro* model HEp-2 human epithelial cell line and in the emerging alternative insect infection model *Galleria mellonella* (*G. mellonella*).

1.4.7 Project General Aims:

- 1) Comparing the pathogenic potential of *Y. enterocolitica* biotypes using the HEp-2 cell line model and *G. mellonella* insect model.

- 2) Investigating the *in vivo* interaction of *Y. enterocolitica* biotypes with the alternative infection model *Galleria* larvae.

- 3) Investigating the role of the *Y. enterocolitica* major virulence factor pYV plasmid in modulating the virulence of the pathogenic biotypes in *Galleria*.

Chapter 2
Materials & Methods

2.1 Materials and Reagents

2.1.1 Bacterial strains and growth conditions: All strains used in this project are listed in table 2.1 and were grown from (-80°C) freezer stocks prior to use. All *Y. enterocolitica* were grown at either 25°C or 37°C according to the experiment conditions. Strains other than *Y. enterocolitica* were grown at 37°C. All *Y. enterocolitica* strains were grown aerobically on LB agar plates and in LB broth medium at both 25°C and 37°C. LB broth cultures were incubated overnight in a shaking incubator at 200 rpm for 18 hours.

Table 2.1: Bacterial strains used in this study

| No. | Strain Name | Bio/Serotype | Biological Origin | Source |
|-----|-------------|--------------|-------------------|---|
| 1 | 8081 | 1B/O:8 | Human | Thomson <i>et al.</i> (2006) |
| 2 | 0902 | 1A/O?* | Sheep | McNally, UK |
| 3 | 0903 | 1A/O:6,30 | Human | McNally, UK |
| 4 | 3403 | 1A/O:5 | Human | McNally, UK |
| 5 | 3503 | 1A/O?* | Pig | McNally, UK |
| 6 | 5303 | 1A/O:5 | Human | McNally, UK |
| 7 | NZ3 | 1A/O?* | Sheep | Dr. Muriel Dufour, ESR NCBID, Australia |
| 8 | 119/02 | 2/O:9 | Sheep | McNally, UK |
| 9 | 21202 | 2/O:9 | Pig | McNally, UK |
| 10 | 215/02 | 3/O:9 | Pig | McNally, UK |
| 11 | 218/02 | 3/O:9 | Pig | McNally, UK |
| 12 | 2403 | 3/O:9 | Human | McNally, UK |
| 13 | 5603 | 3/O:9 | Human | McNally, UK |
| 14 | 5803 | 3/O:9 | Human | McNally, UK |
| 15 | 111/02 | 3/O:5,27 | Sheep | McNally, UK |
| 16 | 14902 | 3/O:5,27 | Sheep | McNally, UK |
| 17 | 153/02 | 3/O:5,27 | Cattle | McNally, UK |
| 18 | 226/02 | 3/O:5,27 | Pig | McNally, UK |
| 19 | 231/02 | 3/O:5,27 | Pig | McNally, UK |
| 20 | 232/02 | 3/O:5,27 | Pig | McNally, UK |
| 21 | 01/2012 | 4/O:3 | Human | Claire Jenkins HPA,2012 |
| 22 | 02/2012 | 4/O:3 | Human | Claire Jenkins HPA,2012 |
| 23 | 03/03 | 4/O:3 | Human | McNally, UK |

| | | | | |
|----|---------------------------------------|---------------|---------|---|
| 24 | 1203 | 4/O:3 | Human | McNally, UK |
| 25 | 201/02 | 4/O:3 | Pig | McNally, UK |
| 26 | 204/02 | 4/O:3 | Human | McNally, UK |
| 27 | 213/02 | 4/O:3 | Pig | McNally, UK |
| 28 | NZ15 | 4/O:3 | Unknown | Dr. Muriel Dufour, ESR NCBID, Australia |
| 29 | Y1 | 4/O:3 | Human | Petra Dersch, 2009 |
| 30 | 3094/96 | 5/O:2a,2b,3 | Animal | Fuchs lab |
| 31 | 14902/pAJD434 (Transformed Strain) | 3/O:5,27 | Sheep | |
| 32 | 1203/pAJD434 (Transformed Strain) | 4/O:3 | Human | This Study |
| 33 | 5303/pAJD434 (Transformed Strain) | 1A/O:5 | Human | This Study |
| 34 | 21202/pAJD434 (Transformed Strain) | 2/O:9 | Pig | This Study |
| 35 | 8081/pAJD434 (Transformed Strain) | 1B/O:8 | Human | This Study |
| 36 | 8081c (pYV-cured derivative) | 1B/O:8 | Human | This Study |
| 37 | 14902c (pYV-cured derivative) | 3/O:5,27 | Sheep | This Study |
| 38 | 1203c (pYV-cured derivative) | 4/O:3 | Human | This Study |
| 39 | <i>E. coli</i> DH5 α | (-ve) control | Unknown | Invitrogen |

*?: Non-typable serotype

2.1.2 Luria-Bertani Agar (LB) agar: LB Agar Miller (Fisher Scientific) was used to grow all strains used in this project. LB agar is manufactured from the following components (per L): Tryptone, 10g; Yeast extract, 5g; Sodium chloride, 10g; Agar 15g, pH7.0 \pm 0.2 at 25°C and was prepared according to the manufacturer instruction by dissolving LB agar powder in distilled water. The molten agar suspension was then sterilised by autoclaving at 121°C for 15 minutes.

2.1.3 Luria-Bertani Broth (LB) Broth: LB Broth (Lennox, SIGMA-ALDRICH) was used to grow all strains used in this project. The LB broth is pre made from the following components (per L): Tryptone, 10g; Yeast extract, 5g; Sodium chloride (NaCl), 5g. The LB broth was prepared according to the manufacturer instruction by dissolving LB broth powder in distilled water. The broth suspension was then sterilised by autoclaving at 121°C for 15 minutes. The sterilized LB broth was incubated in room temperature and used within 4 weeks or stored in 4°C fridge for long period usage.

2.1.4 Yersinia Selective Agar (CIN) (Schiemann's CIN agar) Formula and Preparation:

Yersinia selective agar was prepared from *Yersinia* selective agar base powder (Fisher Scientific) and the *Yersinia* Selective supplement (Fisher Scientific). The CIN agar base formula is manufactured from the following chemicals in (gram/litre): Special peptone, 20g/L; Yeast extract, 2g/L; Mannitol, 20g/L; Sodium pyruvate, 2g/L; Sodium Chloride, 1g/L; Magnesium Sulphate, 0.01g/L; Sodium desoxycholate, 0.5g/L; Neutral red, 0.03g/L; Crystal Violet, 0.001g/L; Agar, 12.5g/L, pH 7.4 ± 0.2 @ 25°C. The *Yersinia* selective supplement is composed of Cefsulodin (7.5mg), Irgasan (2mg) and Novobiocin (1.25mg). The content of one vial is enough to supplement 500 ml of the *Yersinia* Selective Agar Base. CIN agar was prepared according to the manufacturer's instructions. Briefly, 29g of the *Yersinia* Selective Agar Base was dissolved in 500ml of distilled water and the suspension was then autoclaved at 121°C for 15 minutes. The autoclaved agar was then cooled down to approximately 55°C. The *Yersinia* Selective supplement was prepared by dissolving the whole powder amount in 1ml of pure ethanol (95%) + 2ml of sterile distilled water. The dissolved selective supplement was added to the cooled 500ml *Yersinia* Selective Agar Base and the mixture was mixed thoroughly. The CIN agar was poured into 90mm sterile Petri Dishes and stored according to the manufacturer instructions.

2.1.5 CRMOX Agar Preparation: The CRMOX agar was prepared according to the protocol described in the handbook of microbiological media book (3rd edition); (Atlas, 2004): The CRMOX agar was prepared in the lab using multiple components including chemicals and dehydrated media. Each of the CRMOX agar components was prepared separately as a solution with a fixed concentration. All the prepared solutions were finally mixed together to obtain 1 litre of CRMOX agar. The CRMOX agar preparation procedure was carried out as follows:

Table 2.2: CRMOX reagents and preparation procedure

| Chemical/Reagent Name | Method of Preparation | Final Volume per litre |
|--|---|------------------------|
| Magnesium Chloride (MgCl ₂) (SIGMA-ALDRICH). | 0.25M of MgCl ₂ was prepared by dissolving 50.8g of MgCl ₂ in 1L of distilled water. The mixture was sterilised by autoclaving at 121°C for 15 minutes. The sterile MgCl ₂ stock was stored aseptically in room temperature and volumes of 80ml were used to prepare 1L of CRMOX agar. | 80ml |
| Sodium Oxalate (Na ₂ C ₂ O ₄) (SIGMA-ALDRICH) | 0.25M of Sodium Oxalate was prepared by dissolving 33.2g of Sodium Oxalate in 1L of distilled water. The mixture was sterilised by autoclaving at 121°C for 15 minutes. The sterile Sodium Oxalate stock was stored aseptically in room temperature and volumes of 80ml were used to prepare 1L of CRMOX agar. | 80ml |
| D-galactose (SIGMA-ALDRICH). | 20% of D-galactose was prepared by dissolving 2g of D-galactose in 10ml of distilled water. The mixture was filter sterilized and aseptically stored in room temperature. | 10ml |
| Congo Red (Formula: C ₃₂ H ₂₂ N ₆ Na ₂ O ₆ S ₂) (SIGMA-ALDRICH) | 1% of Congo Red was prepared by dissolving 0.05g of Congo Red powder in 5ml of distilled water. The mixture was sterilised by autoclaving at 121°C for 15 minutes. | 5ml |
| Tryptone soy agar (TSA) | 40g of TSA have been dissolved in 825ml of distilled water and autoclaved at 121°C for 15 minutes. The molten autoclaved TSA agar was allowed to cool down to approximately 55°C then the last prepared chemicals were aseptically added to the warm TSA. | 825ml |
| | CRMOX agar final volume | 1L |

2.1.6 Tryptone Soy Agar (TSA);(Fisher Scientific): Pancreatic digest of casein 15.0g/L; Enzymatic digest of soya bean, 5.0g/L; Sodium chloride, 5.0g/L; Agar, 15.0g/L.

2.1.7 1X TAE buffer preparation: 1X Aqueous Tris Acetate EDTA (TAE) buffer was prepared by diluting 20ml of the 50X concentrated TAE buffer (Fisher) in 980ml of sterile distilled water (sdH₂O). The 50X TAE buffer (Fisher) is composed of:

- 242g Tris base

- 100ml 0.5M Na₂EDTA

- 57.1ml Glacial acetic acid

2.1.8 Agarose Powder (Fisher Scientific): Agarose DNase and RNase free, for electrophoresis.

2.1.9 Phosphate Buffered Saline (PBS);(SIGMA-ALDRICH): PBS was prepared by dissolving 1 PBS tablet in 200mL of deionised water. The dissolved tablet yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, with pH 7.4. The mixture was then sterilized by autoclaving at 121°C for 15 minutes.

2.1.10 Glycerol freezing medium preparation: Glycerol freezing medium is used to store bacterial cells for long term storage in extremely low temperatures. 80% Glycerol solution

was prepared by mixing 80% of pure Glycerol reagent with 20% distilled water. The mixture then autoclaved at 121°C for 15 minutes and allowed to cool down in room temperature and finally stored in 4°C fridge. The freezing medium was prepared in 1ml aliquots and loaded in 2ml sterile cryogenic plastic tubes. Every 1ml contains 20% of the pre prepared Glycerol (0.2ml) with 80% of the appropriate broth medium (0.8ml).

2.1.11 Trimethoprim antibiotic preparation: The Trimethoprim antibiotic was used in a 100µg/ml concentration. To prepare 100µg/ml Trimethoprim solution, 0.05g of Trimethoprim powder (SIGMA ALDRICH) was dissolved in 1ml of pure DMSO (Fisher Scientific) to produce 50µg/ml Trimethoprim stock solution. The Trimethoprim stock solution was then sterilised by filtration using a sterile syringe and membrane filter size 0.22µm. The sterilized 50µg/ml Trimethoprim stock solution was added to 500ml media to obtain 100µg/ml concentration.

2.1.12 TrypLE Express Enzyme (1X) + phenol red: TrypLE (Gibco) is a recombinant enzyme alternative to porcine or bovine Trypsin. TrypLE was used to dissociate the attached cell lines on the plastic tissue culture growing flasks. Once received it was stored in 4°C.

2.1.13 Dulbecco's Phosphate Buffered Saline (DPBS): Dulbecco's Phosphate Buffered Saline (SIGMA-ALDRICH UK) is a liquid buffer which is supplemented with MgCl₂ and CaCl₂ salts and it is sterile-filtered by the manufacturer. Once received it was stored and used for all cell culture assays.

2.1.14 Minimum Essential Medium Eagle (MEM): Minimum Essential Medium Eagle (SIGMA-ALDRICH UK) is sterile-filtered liquid medium supplemented with Earle's salts, L-glutamine and sodium bicarbonate. The Minimum Essential Medium Eagle has been used primarily to grow the HEp-2 cell line in addition the other tissue culture assays.

2.1.15 Penicillin-Streptomycin Antibiotics (Pen-Strep): Penicillin-Streptomycin antibiotics (SIGMA-ALDRICH UK) are purchased as a sterile liquid bio reagent manufactured by mixing 10,000 units of penicillin with 10 mg streptomycin/ml. Penicillin-Streptomycin antibiotics were used to maintain the HEp-2 cell line and prevent bacterial and fungal contamination. A specific amount of Penicillin-Streptomycin antibiotics was added to the Minimum Essential Medium to create a growth media for the HEp-2 cell line. Penicillin-Streptomycin antibiotics were stored at 4°C.

2.1.16 Gentamicin Antibiotic Solution: Gentamicin (SIGMA ALDRICH UK) was stored at 4°C. Gentamicin inhibits the growth of many Gram-positive and Gram-negative bacteria. Gentamicin was used in the invasion assay to kill the extracellular bacteria with a concentration of 100µg/ml.

2.1.17 Triton X: Triton X-100 (SIGMA ALDRICH UK) was used to lyse HEp-2 cells. 1% of Triton X-100 diluted stock solution was prepared by mixing 1ml of the 100X concentrated solution in 99ml distilled water. The diluted mixture was then autoclaved at 121°C for 15

minutes. The autoclaved mixture was left to cool down in room temperature and then stored in 4°C fridge for long term usage.

2.1.18 HEp-2 cell line: HEp-2 human laryngitic epithelial was used in all tissue culture work. HEp-2 cell line was obtained from the liquid nitrogen storage chamber and prepared for tissue culture work.

2.1.19 Hamilton Syringe Preparation: The 50 μ L volume Hamilton GASTIGHT Syringe with a removable 22s ga. sized bevel tip needle was used for all the *Galleria* injection procedures. The Hamilton syringe was cleaned and sterilised prior to each injection procedure using 95% pure ethanol, sterile distilled water and the special metal string supplied with the syringe. Syringe sterilization procedure is done by drawing up the ethanol into the syringe barrel and then thrust it out of the syringe and repeating this procedure three or more times followed by washing with sterile distilled water using the same last mechanical procedure to remove the excess ethanol.

2.1.20 *Galleria mellonella* larvae storage and usage: All *Galleria* larvae batches in the final 6 instars stage were purchased from (Livefood UK Ltd, Somerset). The *Galleria* larvae were stored in the dark on wood chips inside plastic boxes at room temperature and were used within 5 days from the day of delivery. Only healthy larvae without dark spots and exhibiting a light creamy colour and active movement were selected for experimental work. Moreover, larvae with similar sizes ranging from 2cm-2.5cm were selected in order to obtain

similar results from different experiments. This selection was performed immediately prior to each individual experiment.

2.1.21 Genomic DNA extraction:

Genomic DNA was extracted from *Y. enterocolitica* strains pre-grown in LB agar cultures. Whole *Y. enterocolitica* colony content of each LB agar culture was collected by sterile cotton swabs and was dissolved in 1.5 ml of sterile PBS. The genomic DNA was extracted from the PBS *Y. enterocolitica* cells suspension using the GenElute™ Bacterial Genomic DNA kit (SIGMA) according to the procedure described in the manufacturer's user guide booklet.

2.1.22 1kbp ladder (Promega)

2.1.23 QIAprep Spin Miniprep Kit (50); (QIAGEN): For 50 high-purity plasmid minipreps extraction.

2.1.24 SOC Medium: The SOC medium (SIGMA-ALDRICH) is composed of: (20 g/L) of Tryptone, (5 g/L) of Yeast Extract, (4.8 g/L) of MgSO₄, (3.603) g/L of dextrose, (0.5g/L) of NaCl & (0.186 g/L) of KCl.

2.1.25 pAJD434 plasmid: pAJD434 plasmid contains a trimethoprim resistance gene. pAJD434 resulted from cloning the p34s-Tp trimethoprim resistant gene in the PstI site of the 'λ red recombinase expression plasmid pKD46' (Maxson and Darwin, 2004).

2.1.26 GenePulser Xcell electroporator system (Bio-Rad)

2.1.27 Electroporation cuvette (2ml); (Flowgen, UK)

2.2 Methods and Procedures

2.2.1 Inoculum (Initial dose) and dose Preparation using the plate count technique: The plate count technique has been used to approximately count and standardize inoculum numbers. In this method, the optical density (O.D) of each strain is measured and linked to the number of bacteria. This helps to initially estimate that the overnight broth culture is reaching the desired number of the bacterial cells. In this procedure, the O.D of the bacterial overnight cultures were measured by spectrophotometer at 600nm wave length. Then number of cells from the overnight broth cultures were enumerated using the Miles & Misra method (Hedges, 2002; Miles and Misra, 1938) followed by the colony forming unit formula (c.f.u.).

2.2.2 Cell Line Growth and Maintenance: The HEp-2 human laryngitic epithelial cell line (Draganov *et al.*, 2008-2009) was used in all tissue culture assays. The HEp-2 cell line was obtained from long term Liquid Nitrogen Storage and cultured in Minimum Essential Medium Eagle with L-glutamine (MEM; Sigma Aldrich UK) in specific 75cm³ tissue culture flasks. The MEM medium was supplemented with 10% of Fetal Bovine Serum (Sigma Aldrich UK), 1% of MEM Non-essential Amino Acid Solution (Sigma Aldrich UK) and 1% of Penicillin-Streptomycin antibiotics (Sigma Aldrich UK). The HEp-2 cells were incubated at 37°C in the tissue culture humidified incubator with 5% CO₂ atmosphere. The cells were monitored daily and were sub-cultured either every four days or once every week. The growth medium was routinely changed every four days. The cultured HEp-2 cells were left until they became a fully confluent monolayer. The HEp-2 cells were used on passage 6 for all association and invasion assays. At 48 hours before the association and invasion assays, the monolayer cell lines were detached from the tissue culture flask surface using

TrypLE enzyme (Life Technologies). The detached cells were centrifuged on 1500 rpm for 3 minutes and the pellet was resuspended with 4 ml of growth medium. Twenty microliters of the suspension was taken and loaded in the haemocytometer to determine the cell count. Depending on the haemocytometer count, approximately 2×10^5 cells were seeded in 24-well plates. Sterile 24-well plates (nunc) were used to seed the HEp-2 cells for the association and invasion assays. Each strain was seeded in triplicate for each assay. The seeded 24-well plates were incubated in the tissue culture incubator at 5% atmospheric CO₂ and humid conditions for approximately 48 hours till the seeded HEp-2 cells became almost or completely confluent.

2.2.3 Association and Invasion assays: Overnight bacterial broth cultures were pelleted with centrifugation at 10,000 rpm for 5 minutes. The pellets were suspended with MEM tissue culture infection media supplemented with only 1% of MEM Non-essential Amino Acid Solution (Sigma Aldrich UK). The suspended bacterial cells were diluted in the media to reach approximately 2×10^7 cells as determined by the plate count method and these dilutions were used as inoculum. Confluence of the 24-well plate seeded cells was checked under the inverted light microscope. After that, the tissue culture growth medium was removed and all wells were washed twice with Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich UK) and in each wash all wells were cleaned three times. 0.5 ml of each bacterial suspension was added in triplicate in the pre-seeded wells (M.O.I. 100). Plates were incubated for 3 hours at 37°C at 5% atmospheric CO₂ and humid conditions. After 3 hours, the bacterial suspensions were emptied from all wells and washed thrice with Dulbecco's Saline. After washing, the 24 well plates specified for the invasion assay were filled with 0.5 ml of tissue culture media supplemented with 100µg/ml Gentamicin antibiotic and were further incubated for 2 hours in

the same last conditions. At the same time, the association assay 24 well plates were loaded with 0.1ml of 1% of Triton X-100 in order to lyse the infected HEp-2 cells and release the bacterial cells. The bacterial cells were serially diluted and counted using Miles & Misra method (Hedges, 2002; Miles and Misra, 1938). After 2 hours of incubation, the invasion assay 24 well plates were washed from the Gentamicin twice with the Dulbecco's Saline and also lysed with 0.1ml of 1% of Triton X-100. The bacterial cells were also diluted and counted using Miles & Misra method (Hedges, 2002; Miles and Misra, 1938).

2.2.4 *Galleria* infection assay: Larvae were inverted on their backs over a 6mm diameter wide plastic tube attached to the lid of a 90mm Petri dish using the thumb and index fingers. All *Galleria* larvae were micro-injected in the second right leg from the head side using Hamilton syringe. *Galleria* larvae were infected with a series of nine, 10-fold serial dilutions containing 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 & 10^9 cfu/ml of each *Y. enterocolitica* strain. Each dose was injected in 10 μ l-aliquots into a group of 10 *G. mellonella* larvae. After injection, each group was placed on a separate 90 mm sterile Petri Dish containing a 90mm diameter Whatman filter paper in order to observe any leakage caused by a Trauma. The injected *Galleria* groups were then incubated in the dark statically at 25°C or 37°C. In addition to the bacterial doses that were injected, 10 larvae were injected with a sterile PBS, and 10 were incubated without any form of injection or treatment. All experiments were repeated in triplicate independently.

2.2.5 Monitoring of infected *Galleria* larvae: Reduction in movement and changes in larvae cuticle colour were checked to distinguish dead larvae from living ones (Larvae mortality). Each larva was gently agitated with a sterile plastic pipette tip and inverted on its back to check for the roll response as an indicative of larval life. *Galleria* larvae darkening

and recorded as a sign of the larvae morbidity. Once the *Galleria* larvae failed to display any movement they were recorded as dead. The dead larvae were instantly removed and aseptically disposed.

2.2.6 Calculating the LD₅₀: median lethal doses were calculated using the following Spearman Karber formula: $\text{Log}_{10} \text{Median Dose} = m - \Delta(\sum p - 0.5)$; (Wardlaw, 1985, p.105).

2.2.7 Time To Death (Survival Curves): Survival curves were designed in order to predict the time of death by comparing the time post infection (x-axis) against the percentage of live *Galleria* (y-axis).

2.2.8 PCR Reaction Preparation

All PCR amplification reactions were performed in 50µl final volumes in ice. The 50µl PCR reaction components and volumes are prepared as described in Table 2.3:

Table 2.3 PCR reagents and their volumes and concentrations:

| PCR Reagent | PCR Reagent Concentration | PCR Reagent Volume |
|----------------------------------|---------------------------|--------------------|
| Green Flexi PCR Buffer (Promega) | 5X | 5µl |
| MgCl ₂ (Promega) | 1.5mM | 5µl |
| dNTPs Mix (Promega) | 10µM | 2µl |
| sdH ₂ O | - | 33.7µl |
| F-Primer | 10 pmol | 1µl |
| R-Primer | 10 pmol | 1µl |
| GoTaq DNA Polymerase (Promega) | 5U/µM | 0.3µl |
| DNA Template | ~10 ng | 2µl |
| Total Volume | | 50µl |

The same reaction was carried out for each type of template. PCR negative control reactions were carried out without adding template DNA and compensate it with sdH₂O.

2.2.9 PCR Reaction

PCR amplification reactions were carried out in Thermo cycler machine. Each 50µl PCR reaction mix was added in separate PCR sterile plastic tubes and then was placed in the thermo cycler machine. The PCR thermo cycler was programmed using the following PCR condition: initial denaturation for 5 min at 94°C, followed by 30 denaturation cycles at 94°C for 30 sec for each cycle, annealing temperature for 30 sec by which the temperature was dependent on the primer's annealing temperature, extension at 72°C for 90 sec and a final extension at 72°C for 10 min. The final PCR products were held at 4°C at the end of the PCR amplification cycles.

2.2.10 Amplified DNA analysis using agarose gel electrophoresis

All PCR amplified products were visualised on 2% agarose gel. The 2% agarose gel was prepared by adding 2g of the agarose powder to 100 ml of 1XTAE buffer. The mixture was dissolved completely by boiling in microwave to produce a homogenised solution. The boiling agarose solution was left to cool down and was stained by adding 10µl of 0.01% SYBR® Safe™ gel stain (Invitrogen, UK). Then the ready gel solution was poured in gel tray containing a comb and the gel left to solidify in room temperature. The comb was used to create wells in the gel. The ready gel was then placed in electrophoresis tank by which the wells side was located towards the negative electrical anode. The electrophoresis tank was filled with 1XTAE buffer till the gel was completely covered with the TAE buffer. 5µl aliquots of each PCR product were loaded in the appropriate wells. In addition, 5µl of 100bp DNA ladder (Promega) were loaded in the first well to be used as DNA marker. The loaded DNA samples were separated by the effect of electric field by running the agarose gel for 45minutes on 90V. Finally, the fragmented DNA bands on the gel were visualised by the InGenius® gel system under the ultraviolet (UV) light (Syngene; UK).

Chapter 3

Studying the Pathogenic Potential of *Y. enterocolitica* Biotypes in HEp-2 Cell Line and *G. mellonella* Insect Model

3.1 Introduction

3.1.1 *In vitro* infection models for *Y. enterocolitica* Pathogenesis

Tissue culture cell lines have been widely used to study *Y. enterocolitica* pathogenesis *in vitro* (Portnoy *et al.*, 1981). These cell lines have been derived from different human and animal tissue origins. HEp-2 (human epithelial cell line), HeLa (human epithelial cell line) and CHO (Chinese Hamster Ovary cell line) are types of human and animal epithelial cell lines frequently used to study the association and invasion potential of *Y. enterocolitica* strains (Boyd *et al.*, 2000; Finlay and Falkow, 1988). *Y. enterocolitica* has been shown to infect HEp-2 human epithelial cell line to higher levels than other epithelial cell lines commonly used to assay bacterial virulence (Finlay and Falkow, 1988). HEp-2 human epithelial cells are derived from human larynx carcinoma (Nottet *et al.*, 1993). They were first established in 1952 by inducing tumours in young laboratory rats inoculated with a human epidermoid carcinoma tissue (Draganov *et al.*, 2008-2009). The HEp-2 cell line has been used to study many viruses and bacteria including *Y. enterocolitica* (Draganov *et al.*, 2008-2009). In addition to epithelial cell lines models used to study *Y. enterocolitica* association and invasion potential, phagocytic cell lines have been used to study the *Y. enterocolitica*-Macrophage interaction *in vitro*. Examples of phagocytic cell lines include the murine macrophage cell line J774 (Hoffmann *et al.*, 2004) and the human macrophage cell line U937 (Boyd *et al.*, 2000). J774 and U937 macrophage cell lines are widely used to study *Y. enterocolitica* interaction with and persistence within macrophages *in vitro* (Hoffmann *et al.*, 2004; Grant *et al.*, 1999). The HeLa cell line was used to study the intracellular behaviour of *Y. enterocolitica* and revealed that *Y. enterocolitica* have the ability to invade HeLa cells but without showing intracellular multiplication (Devenish and Schiemann, 1981). *In vitro* U937 macrophage tissue culture models also showed the ability of

the *Y. enterocolitica* biotypes to persist within macrophages as well as avoid the phagocytic effect of macrophages (McNally *et al.*, 2006). *In vitro* animal cell lines have also been used to investigate the pathogenic role of *Y. enterocolitica* flagella and motility in pathogenesis of *Y. enterocolitica* non-pathogenic biotype (McNally *et al.*, 2007). Other studies have used tissue culture models to study the pathogenic role of many *Y. enterocolitica* adhesins and invasins *in vitro* including Ail protein (Miller & Falkow, 1988), YadA and invasin proteins (Uliczka *et al.*, 2011; Young *et al.*, 1992). Tissue culture models have suggested that all *Y. enterocolitica* biotypes show human pathogenic potential by their ability to invade HEp-2 epithelial cell lines *in vitro* (McNally *et al.*, 2006) including the non-pathogenic biotype strains (Grant *et al.*, 1999; McNally *et al.*, 2006). *In vitro* U937 macrophage tissue culture models have shown that *Y. enterocolitica* pathogenic and non-pathogenic biotypes were able to persist in macrophages (McNally *et al.*, 2006). Although tissue culture cell line models are considered as a powerful tool to study bacterial pathogenesis *in vitro* (Bhunia, 2008), they cannot be used alone to study the complex disease patterns caused by human pathogens because they lack the complex structure of the host organs which makes the need for live infection models important (Harrison *et al.*, 2014).

3.1.2 Mammalian Infection Models

Many mammalian infection models have been used to study the pathogenesis of *Y. enterocolitica* including guinea pigs, gerbils, rabbits and mice (Portnoy *et al.*, 1981). Attempts to study *Y. enterocolitica* pathogenicity using guinea pigs and rabbits as an animal infection model have failed because these animals require a very high dose of bacterial inoculum to kill them and also death occurs very rapidly (within 48 hours). Further investigation has shown that guinea pigs and rabbits die from an enterotoxin effect and do not

resemble any similarities to the human infection caused by *Y. enterocolitica*. Carter and Collins successfully developed a suitable mouse infection model to investigate *Y. enterocolitica* pathogenesis (Carter and Collins, 1975). They used the CD-1 mouse strain to study the pathogenic potential of *Y. enterocolitica* WA strain (Carter and Collins, 1975) which belongs to bioserotype 2/O:8 and was isolated from blood of a human patient (Carter *et al.*, 1973). They concluded from their study that the pathogenic phenotype induced by *Y. enterocolitica* in mice was similar to the natural pathogenic conditions observed in humans infected with the same *Y. enterocolitica* WA strain (Carter and Collins, 1975). Consequently, the mouse model was widely applied to study the *Y. enterocolitica* infection process (Handley *et al.*, 2004). *Y. enterocolitica* doses can be injected intravenously or fed orally to the mouse infection model (Handley *et al.*, 2004). Different inbred mice strains have been used to study the pathogenicity of *Y. enterocolitica* clinical strains (Schippers *et al.*, 2008). C3H/HeN, BALB/C, BALB/B, DBA/2, SWISS, SWR and C57BL/6 are different mice strains used to study *Y. enterocolitica* virulence (Autenrieth *et al.*, 1994; Wang *et al.*, 2013). C3H/HeN, BALB/C, BALB/B, DBA/2, SWISS and SWR mice strains are highly susceptible to infection with *Y. enterocolitica* with a median lethal dose (LD₅₀) of 10². On the other hand, C57BL/6 mouse strain was found to be resistant to *Y. enterocolitica* infection with a median LD₅₀ of 10⁵ (Wang *et al.*, 2013). Although the mouse infection model shows similar gastrointestinal disease patterns to those observed in humans (Carter, 1975a), the mouse infection model responded differently to infection with different *Y. enterocolitica* biotypes. This variance in response classified *Y. enterocolitica* biotypes into three pathogenic groups by which biotype 1B was able to kill the mouse infection model and as a result it was called the high pathogenic biotype. Biotypes 2, 3, 4 and 5 caused mild disease by which they were called the low pathogenic biotypes and finally biotype 1A was called the non-pathogenic biotype because it was not able to cause any disease in the mouse infection model (Carter and

Collins, 1975; Howard *et al.*, 2006). However, the *Y. enterocolitica* pathogenic potential in the mouse infection model doesn't reflect exactly the pathogenic potential of *Y. enterocolitica* biotypes observed in cell culture models as these models show that all *Y. enterocolitica* biotypes display a pathogenic potential towards the epithelial and Macrophage cell lines (Grant *et al.*, 1999; McNally *et al.*, 2006; McNally *et al.*, 2007). As a result, it is important to test a new alternative infection model to investigate the pathogenic potential of *Y. enterocolitica* biotypes using the insect alternative infection model the lepidopteran *Galleria mellonella* wax worms larvae.

3.1.3 Insect Infection Model

Insects have emerged as a non-mammalian infection model to study bacterial pathogens and the virulence potential of human pathogenic bacteria (Champion *et al.*, 2009). The ethical and financial problems of using mammals as an infection model are the main reasons to choose an alternative insect infection model to study human pathogens (Garcia-Lara *et al.*, 2005). The *Caenorhabditis elegans* (*C. elegans*) nematode and the tobacco hornworm *Manduca sexta* (*M. sexta*) are two types of insect host models that have been used to study *Y. enterocolitica* insecticidal toxin genes (*tc*) and their expression at lower temperatures of 10°C -15°C (Fuchs *et al.*, 2008; Spanier *et al.*, 2010). However, *C. elegans* and *M. sexta* have not been successfully used to study *Y. enterocolitica* infection as they don't live well at temperatures over 25°C (Fuchs *et al.*, 2008; Spanier *et al.*, 2010). As such, *Y. enterocolitica* virulence factors which are mainly expressed at the optimal mammalian body temperature (37°C) will not be expressed, and the relevance of pathogenesis data is therefore questionable (Champion *et al.*, 2009). To counter this, the greater Wax Worm moth *Galleria mellonella* caterpillars, which can live in a range of 15°C - 37°C, have emerged as a successful

alternative infection model to study the pathogenesis of numerous bacterial pathogens (Bender *et al.*, 2013; Harding *et al.*, 2012; Harding *et al.*, 2013; Kavanagh & Reeves, 2004) including the virulence of *Yersinia pestis* and *Yersinia pseudotuberculosis* (Champion *et al.*, 2009; Erickson *et al.*, 2011). This is because of the *Galleria* larvae ability to survive at 37°C incubation temperature in the lab (Joyce *et al.*, 2010). Incubating *Galleria* at 37°C enables the investigation of many human pathogenic virulence mechanisms (Desbois and Coote, 2011; Jander *et al.*, 2000; Vogel *et al.*, 2011). The main advantage of *G. mellonella* over the other insect models is that it can survive at wide range of incubation temperatures ranging from 15°C to 37°C (Aperis *et al.*, 2007) and even in higher temperatures as when it was used to investigate specific *C. jejuni* virulence traits at 42°C (Champion *et al.*, 2010; Senior *et al.*, 2011). Technically, *Galleria* larvae are large in size, with an approximate weight of 250mg, which helps to infect them rapidly with precise different bacterial doses (Champion *et al.*, 2010; Jander *et al.*, 2000). *Galleria* larvae can be easily handled and they are able to survive for almost three weeks before going into the pupal stage without the need for feeding (Champion *et al.*, 2010). In addition, *Galleria* doesn't require ethical permissions for experimental work (Seed *et al.*, 2008). Other unique advantages of *Galleria* insect model is their ability to be treated with antibiotic which helps to assess antimicrobial agents efficiency towards the microbial pathogens (Peleg *et al.*, 2009; Vogel *et al.*, 2011).

Additionally, *Galleria* possess an innate immune system similar to the human immune system which is able to respond to microbial infections making *Galleria* an important insect infection model to study many pathogens (Fallon *et al.*, 2011; Mukherjee *et al.*, 2013). This insect immune response is believed to be developed from *Galleria* interaction with several microbes in their natural habitat in beehives (Fuchs *et al.*, 2010). The *Galleria* immune system include cellular and humoral components similar to the mammalian immune system

(Harding *et al.*, 2013; Kavanagh and Reeves, 2004). The *Galleria* insect cuticle and antimicrobial peptides are the first line of the *Galleria* innate immune defence (Brennan *et al.*, 2002). The *G. mellonella* cuticle is a part of the innate immune system which acts as an efficient physical barrier as the mammalian skin against the invading pathogens. In the case of *Galleria* cuticle penetration, the *G. mellonella* produces antimicrobial peptides as an innate humoral response (Brennan *et al.*, 2002; Kavanagh and Reeves, 2004; Senior *et al.*, 2011). In addition, the *Galleria* innate immune system includes phagocytic cells called ‘haemocytes’. There are six types of Haemocytes identified in *G. mellonella* (Brennan *et al.*, 2002; Fuchs *et al.*, 2010; Mylonakis *et al.*, 2007). The six identified types of haemocytes in *Galleria* lepidopteran insect are granulocytes, spherulocytes, prohemocytes, oenocytoids, coagulocytes and plasmatocytes (Fuchs *et al.*, 2010; Salem *et al.*, 2014). Haemocytes are capable of phagocytosis and eliminate invading pathogens similarly to the human innate immune system neutrophils (Fallon *et al.*, 2011).

3.2 Aim and Objectives

3.2.1 Aim

To study the pathogenic potential of *Y. enterocolitica* biotypes in HEp-2 cell line and *G. mellonella* insect model.

3.2.2 Objectives

A) To study the interaction of all *Y. enterocolitica* 1A, 1B, 2, 3, 4 and 5 biotypes with the HEp-2 human epithelial cell line *in vitro* by evaluating the association and invasion levels of all biotypes.

B) To investigate the pathogenic potential of all *Y. enterocolitica* 1A, 1B, 2, 3, 4 and 5 biotypes in the insect alternative infection model *Galleria mellonella* (*G. mellonella*) by calculating the medial lethal dose (LD₅₀) and survival curves on different incubation temperatures.

3.3 Materials & Methods

3.3.1 *Y. enterocolitica* strains growth conditions: All tested bacterial strains in this chapter are listed in table 3.1. All tested *Y. enterocolitica* strains were pre-grown on LB agar plates and in LB broth medium at both 25°C or 37°C temperatures. LB broth was incubated in shaker incubator at 200 rpm for 18 hours as described in chapter two section 2.1.

Table 3.1 Bacterial strains used in chapter 3

| No. | Strain Name | Bio/Serotype | Biological Origin | Source |
|-----|-----------------------------|---------------|-------------------|------------------------------|
| 1 | 8081 | 1B/O:8 | Human | Thomson <i>et al.</i> (2006) |
| 2 | 5303 | 1A/O:5 | Human | McNally, UK |
| 3 | 21202 | 2/O:9 | Pig | McNally, UK |
| 4 | 5603 | 3/O:9 | Human | McNally, UK |
| 5 | 14902 | 3/O:5,27 | Sheep | McNally, UK |
| 6 | 01/2012 | 4/O:3 | Human | Claire Jenkins HPA,2012 |
| 7 | 02/2012 | 4/O:3 | Human | Claire Jenkins HPA,2012 |
| 8 | 1203 | 4/O:3 | Human | McNally, UK |
| 9 | Y1 | 4/O:3 | Human | Petra Dersch, 2009 |
| 10 | 3094/96 | 5/O:2a,2b,3 | Animal | Fuchs lab |
| 11 | <i>E. coli</i> DH5 α | (-ve) control | Unknown | Invitrogen |

3.3.2 Statistical analysis

The significance of difference between the number of associated and invasive bacterial cells for each tested strain and that of the negative control strain *E. coli* DH5 α strain was determined using the one-way ANOVA test available in Excel (2010). The significance of difference between the LD₅₀ values of *Y. enterocolitica* strains were determined using the one sample T-test . The significance of difference in virulence between the *Y. enterocolitica* pre-growing temperatures and between the *Galleria* incubation temperatures were determined using the T-test of two independent samples. All the above statistical analysis was done with a statistical significance with a *P* value of ≤ 0.05 .

3.4 Results

3.4.1 Association and Invasion

The HEP-2 human epithelial cell line was used to screen the ability of 10 *Y. enterocolitica* strains to adhere and invade the cultured cell line monolayer *in vitro*. These 10 strains represent all the 6 biotypes of *Y. enterocolitica* species and they were isolated from different human and animal origins. All the 10 strains were pre-grown at 2 different temperatures, 25°C and the 37°C prior to infection of cultured HEP-2 cell line. All tissue culture results are represented in figures 3.1 and 3.2. Figure 3.1 represents the results of the Association and Invasion assays of the *Y. enterocolitica* strains pre-grown on 25°C, while figure 3.2 shows the Association and Invasion assays of the *Y. enterocolitica* strains pre-grown at 37°C. Association and Invasion values were represented in Log₁₀ c.f.u/ml.

3.4.1.1 Association and Invasion results of the HEP-2 cell line infected with *Y. enterocolitica* strains pre-grown at 25°C:

The results show that all biotypes are able to adhere and invade the HEP-2 epithelial human cell line, with the high pathogenic biotype 1B and the low pathogenic biotypes 2, 3 & 4 showing increased ability to attach and invade the HEP-2 cell line. The average value of association was ~ 6.2 Log₁₀ c.f.u/ml while the average invasion value was ~ 5 Log c.f.u/ml. These data show that 10% of the bacterial inoculum was able to attach to the cultured HEP-2 epithelial monolayers and 1% of the original inoculum invaded the cultured HEP-2 cell line monolayers. The statistical analysis shows that the difference between all the tested strains and the negative control in terms of association ability was statistically insignificant (p -value= 0.255). In addition, the statistical analysis shows that the difference between all the tested strains and the negative control in terms of invasion ability was statistically significant (p -value ≤ 0.05). In contrast, the low pathogenic biotype 5, strain 3094/96 shows an attenuated ability to invade the cultured HEP-2 cell line compared to the other high and low

pathogenic biotypes. The invasion value was 3.44 Log_{10} c.f.u/ml which was statistically significant with a p -value = 0.000141 compared to the invasion levels of the other pathogenic biotypes. Similarly, the non-pathogenic biotype 1A strain 5303 was showing a low invasion levels (invasion = 3 Log_{10} c.f.u/ml) and the 5303 invasion levels were statistically significant with a p -value = 0.001569 compared to all the other pathogenic biotypes. The negative control *E. coli* DH5 α strain was completely non-invasive. Moreover, there was no statistical significance in levels of invasion between the non-pathogenic biotype 1A strain 5303 and the low pathogenic biotype 5 strain 3094 (p -value = 0.0513).

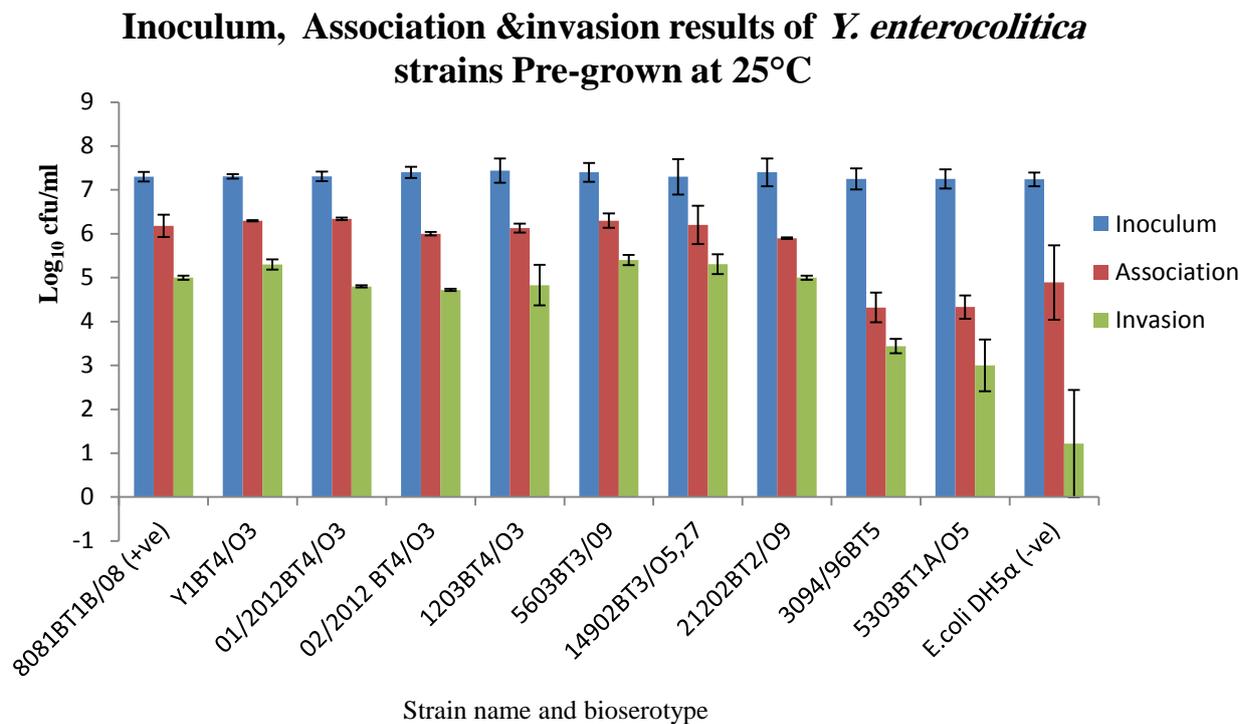


Figure 3.1: Association and Invasion of the HEp-2 cell line infected with *Y. enterocolitica* strains pre-grown at 25°C. Graph represents the mean value of three independent tissue culture experiments including Association and Invasion assays at 25°C pre-grown *Y. enterocolitica* representative strains. Error bars were plotted after calculating the standard deviation of the mean. 10 different *Y. enterocolitica* strains representing all the 6 biotypes were used to infect the HEp-2 human epithelial cell line. The high pathogenic strain 8081 bio/serotype 1B/O:8 used as a positive control and the *E. coli* DH5 α strain used as a negative control. All the bacterial numbers were calculated using the Log_{10} c.f.u/ml.

3.4.1.2 Association and Invasion results of the HEp-2 cell line infected with *Y. enterocolitica* strains pre-grown at 37°C:

The same 10 *Y. enterocolitica* representative strains were pre-grown at 37°C and were able to attach and invade the cultured HEp-2 epithelial human cell line. The high pathogenic biotype 1B and the low pathogenic biotypes 2, 3 and 4 strains again showing high attachment and invasion levels to the cultured HEp-2 cell line *in vitro*. The statistical analysis shows that difference between all the tested strains and the negative control in terms of association ability was statistically insignificant (p -value= 0.2). In addition, the statistical analysis shows that difference between all the tested strains and the negative control in terms of invasion ability was statistically significant (p -value ≤ 0.01). The low pathogenic strain 3094/96 biotype 5 remained the least invasive pathogenic strain between all the other pathogenic biotypes with a statistical significance of p -value = 0.000167. The non-pathogenic 5303 strain remained the least invasive strain between the all biotypes with a statistical significance of a p -value = 0.000157. There was no statistical significance in levels of invasion between the non-pathogenic biotype 1A strain 5303 and the low pathogenic biotype 5 strain 3094 (p -value = 0.057). Interestingly, the high pathogenic biotype 1B and the low pathogenic biotypes 2, 3 & 4 strains showed higher levels of association and invasion exceeding the number of microorganisms added in the inoculum (average inoculum $\approx 7.2 \text{ Log}_{10} \text{ c.f.u./ml}$, average association value $\approx 7.8 \text{ Log}_{10} \text{ c.f.u./ml}$ and average invasion value $\approx 7.2 \text{ Log}_{10} \text{ c.f.u./ml}$). The negative control *E. coli* DH5 α strain was completely non-invasive.

Data suggests that all *Yersinia enterocolitica* strains pre-grown at both 25°C and 37°C temperatures were able to associate with and invade HEp-2 cell line monolayers *in vitro*. Strains belonging to biotypes 1B, 2, 3 and 4 showed a potent ability to attach and invade HEp-2 cell line. The biotype 5 strain revealed weak virulence potential to the tissue culture model while the non-pathogenic BT 1A strain 5303 was the weakest strain among all the

biotypes. *Y. enterocolitica* biotypes strains were more virulent to the HEp-2 cell line when pre-grown at 37°C. This high virulence was unexpected since the *Y. enterocolitica* pre-grown at 37°C has been suggested to not be able to adhere (Lassen and Kapperud, 1986; Okamoto *et al.*, 1980) and not be able to invade the *in vitro* cell line models (Cornelis *et al.*, 1987). The interesting result here is that the BT1A strain was still invasive at the 37°C pre-growing temperature.

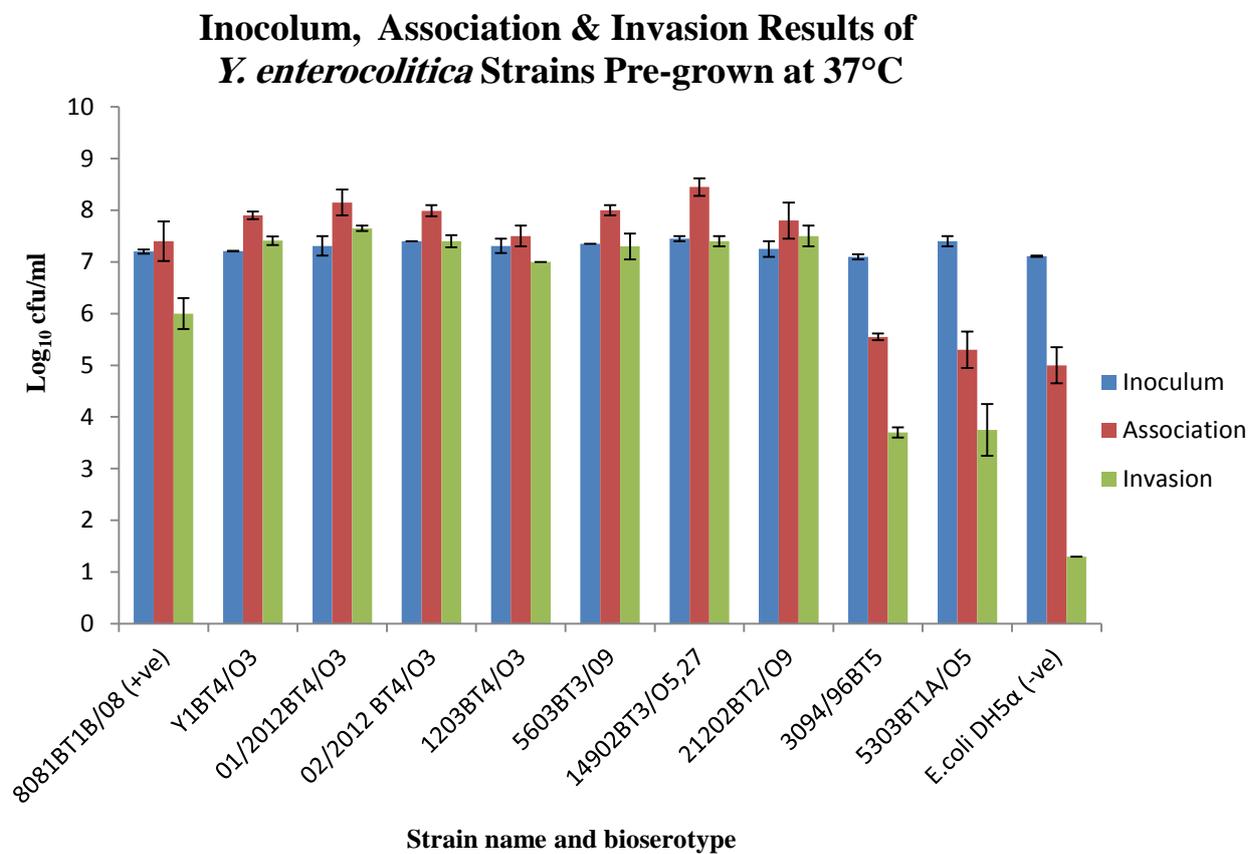


Figure 3.2: Association and Invasion of the HEp-2 cell line infected with *Y. enterocolitica* strains pre-grown at 37°C. The graph represents the mean value of three independent tissue culture experiments including Association and Invasion assays of *Y. enterocolitica* representative strains pre-grown at 37°C. Error bars were plotted after calculating the standard deviation of the mean. 10 different *Y. enterocolitica* strains representing the all 6 Biotypes were used to infect the HEp-2 human epithelial cell line. The high pathogenic strain 8081 Bio/serotype 1B/O8 was used as a positive control and the *E.coli* DH5a strain used as a negative control. All the bacterial numbers were calculated using the Log₁₀ c.f.u/ml.

3.4.2 *Galleria mellonella* Alternative Infection Model Results

3.4.2.1 Median Lethal Dose (LD₅₀) Results:

The median lethal dose (LD₅₀) values are presented in figure 3.3 and figure 3.4. *Galleria* larvae were infected with the same 10 *Y. enterocolitica* strains used to infect the HEp-2 cell line. Figure 3 shows the LD₅₀ values of the *Y. enterocolitica* strains when pre-grown at 25°C while figure 4 shows the LD₅₀ results of the *Y. enterocolitica* strains pre-grown at 37°C. In both figures, the blue bars represent the LD₅₀ results required to kill the infected *Galleria* larvae incubated at 37°C temperature post infection (p.i.) while the red bars represent the LD₅₀ results required to kill the *Galleria* larvae incubated at 25°C temperatures post infection (p.i.).

3.4.2.1.1 LD₅₀ results of *Y. enterocolitica* strains pre-grown at 25°C:

LD₅₀ values of the 10 *Y. enterocolitica* representative strains pre-grown at 25°C are shown in figure 3.3. *Galleria mellonella* larvae were infected with these strains and the infected larvae were incubated at two different temperatures, 25°C and 37°C. The blue bars represent the mean results of three independent experiments of incubating the infected *Galleria* larvae at 37°C while the red bars shows the mean results of three independent experiments of incubating the infected *Galleria* larvae at 25°C. All the LD₅₀ values were calculated using the Spearman-Kärber method. High LD₅₀ numbers indicate low pathogenicity in the model while small values represent high virulence of the strains used. The observed data shows that the non-pathogenic 5303 strain has the lowest LD₅₀ at both incubation temperatures. The non-pathogenic 5303 strain has a LD₅₀ of only 10 bacteria when infected *Galleria* are incubated at 25°C while the LD₅₀ at 37°C was 6.6 Log₁₀c.f.u/ml. Conversely, the high pathogenic 8081 strain displays the highest LD₅₀ result at 37°C incubation compared to all the other biotypes. The LD₅₀ dose was 9.6 Log₁₀ c.f.u/ml which means that the highest injected dose 2.33x10⁹ (Log₁₀ c.f.u/ml = 9.36) was not able to kill any of the injected larvae

at 37°C incubation. Additionally, the LD₅₀ of the high pathogenic 8081 strain incubated at 25°C was 7.6 Log₁₀ c.f.u/ml. This LD₅₀ value is in the same range as the low-pathogenic biotypes incubated at 25°C. The low pathogenic 14902 strain which belongs to the Bio/serotype 3/O:5,27 displayed the lowest LD₅₀ values at 25°C incubation compared to the other low pathogenic and the high pathogenic biotypes while the LD₅₀ results at 37°C incubation remain similar to the other low and high pathogenic biotypes. Finally, the low pathogenic strain 3094/96 belonging to biotype 5 has the highest LD₅₀ value at 25°C incubation, while the 37°C incubation results showing a similar LD₅₀ result with the others.

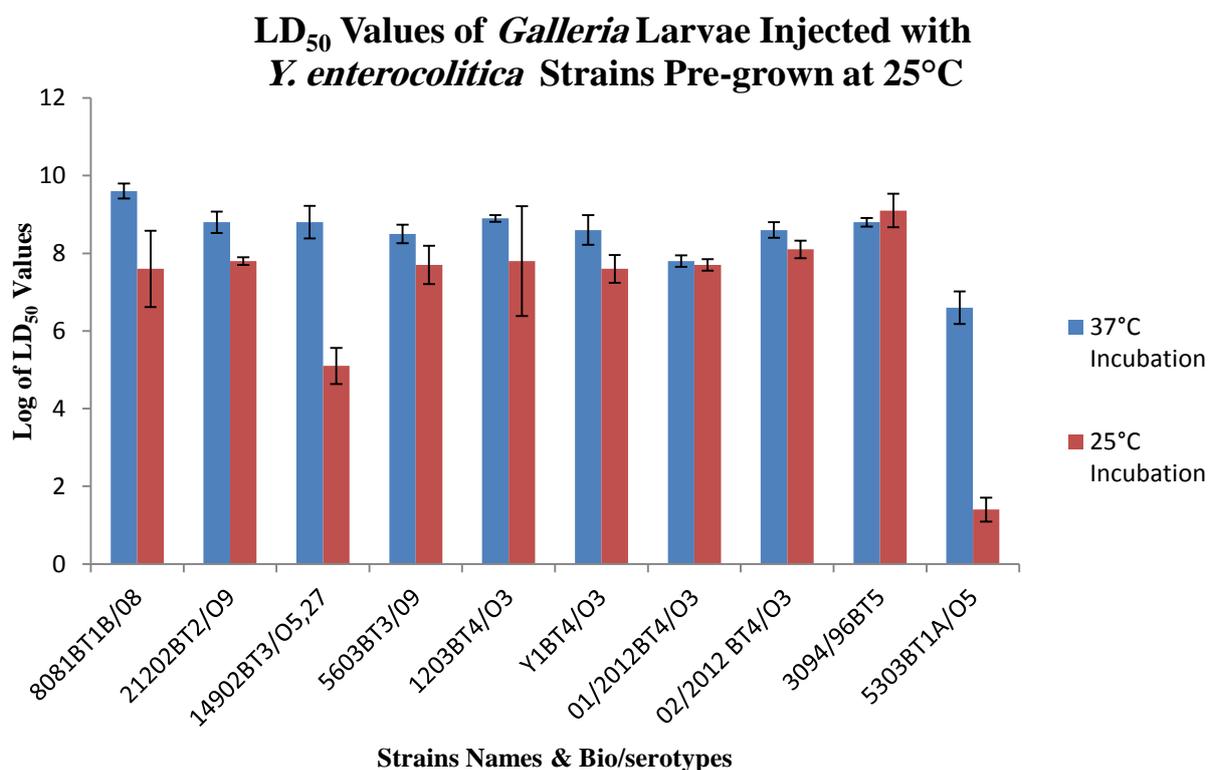


Figure 3.3 LD₅₀ Results of *Y. enterocolitica* strains pre-grown at 25°C with the mean of three independent repeats of the LD₅₀ experiments of infecting *Galleria mellonella* larvae with 10 *Y. enterocolitica* representative strains that were pre-grown at 25°C. The infected *Galleria* larvae were incubated on 2 different temperatures; the 25°C & the 37°C temperatures. The blue bars represent the mean of three different LD₅₀ values for the *Galleria* larvae incubated at 37°C post infection. The red bars represent the mean of three different LD₅₀ values for the *Galleria* larvae incubated at 25°C post infection. The LD₅₀ values were calculated using the Spearman-Kärber method. Error bars were plotted after calculating the standard deviation of the mean.

3.4.2.1.2 LD₅₀ results of *Y. enterocolitica* strains pre-grown at 37°C:

LD₅₀ values of the *Y. enterocolitica* strains pre-grown at 37°C are shown in figure 3.4. *Galleria mellonella* larvae were infected with these strains and the infected larvae were then incubated at two different temperatures; 25°C and 37°C. Similar to figure 3.3, the blue bars represent the mean results of three independent experiments incubating the *Galleria* larvae at 37°C while the red bars show the mean results of three independent experiments incubating the *Galleria* larvae at 25°C. All the LD₅₀ values were calculated using the Spearman-Kärber method. The high LD₅₀ numbers indicate the low virulence effect while the small LD₅₀ values represent the high virulence of the strains used. From the graph it can be noticed that the non-pathogenic 5303 strain again has the lowest LD₅₀ at both incubation temperatures compared to the other biotypes with 1.4 and 1 Log₁₀ c.f.u/ml LD₅₀ value (≈10 bacteria) at 25°C incubation temperature and 6.6 + 6.5 Log₁₀ c.f.u/ml LD₅₀ dose at 37°C incubation. To the contrary, both of the high pathogenic 8081 strain and the low pathogenic strain 3094/96 (biotype 5) share the highest LD₅₀ values at both incubation temperatures between all the other biotypes. The low pathogenic 14902 (Bio/serotype 3/O:5,27) again showed the lowest LD₅₀ result at 25°C incubation temperature, in comparison with the other low- and the high pathogenic biotypes, whereas the LD₅₀ result at 37°C incubation condition continue to be in the same range with the other low and high pathogenic biotypes.

The LD₅₀ values obtained when infected larvae were incubated at 25°C were always lower than the LD₅₀ values at 37°C except in one condition when the larvae were infected with strain 3094/96, pre-grown at 25°C, and incubated at 25°C. Eight of the ten *Y. enterocolitica* representative strains show a difference in LD₅₀ values not exceeding 2 logarithmic units between the two infected *Galleria* incubation temperatures. However, the difference in LD₅₀ between both incubation temperatures was the highest in the non-pathogenic strain 5303 with approximately 5.4 logarithmic units while it is around 3.8 logarithmic units in the low

pathogenic strain 14902. Moreover, the LD₅₀ values of each *Y. enterocolitica* representative strain were closely similar when these strains were pre-grown either at 25°C or 37°C temperatures except for the Y1 strain of bioserotype 4/O:3. The Y1 strain pre-grown at 37°C was more virulent to the *Galleria* larvae when pre-grown at 25°C. The LD₅₀ values of the Y1 strain were 8.6 and 7.6 at 37°C and 25°C incubation temperatures respectively while pre-growing Y1 strain at 25°C show reduced LD₅₀ values at 6.7 and 6.5 for the 37°C and the 25°C incubation temperatures respectively.

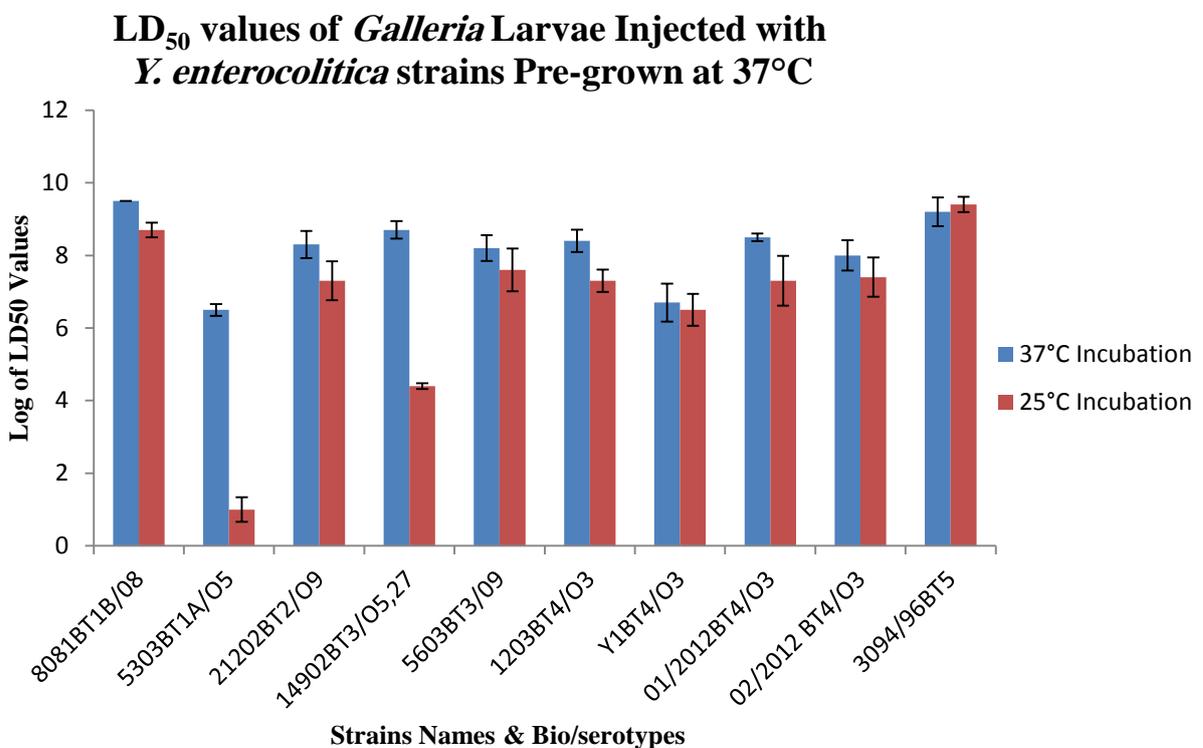


Figure 3.4 LD₅₀ results of *Y. enterocolitica* strains pre-grown at 37°C with the mean of three independent repeats of the LD₅₀ experiments of infecting *Galleria mellonella* larvae with 10 *Y. enterocolitica* representative strains that were pre-grown at 37°C. These strains represent all the 6 Biotypes of *Y. enterocolitica* species. The infected *Galleria* larvae were incubated at 2 different temperatures, 25°C & 37°C temperatures. The blue bars represent the mean of three different LD₅₀ values for the *Galleria* larvae incubated at 37°C post infection. The red bars represent the mean of three different LD₅₀ values for the *Galleria* larvae incubated at 25°C post infection. The LD₅₀ values were calculated using the Spearman-Kärber method. Error bars were plotted after calculating the standard deviation of the mean.

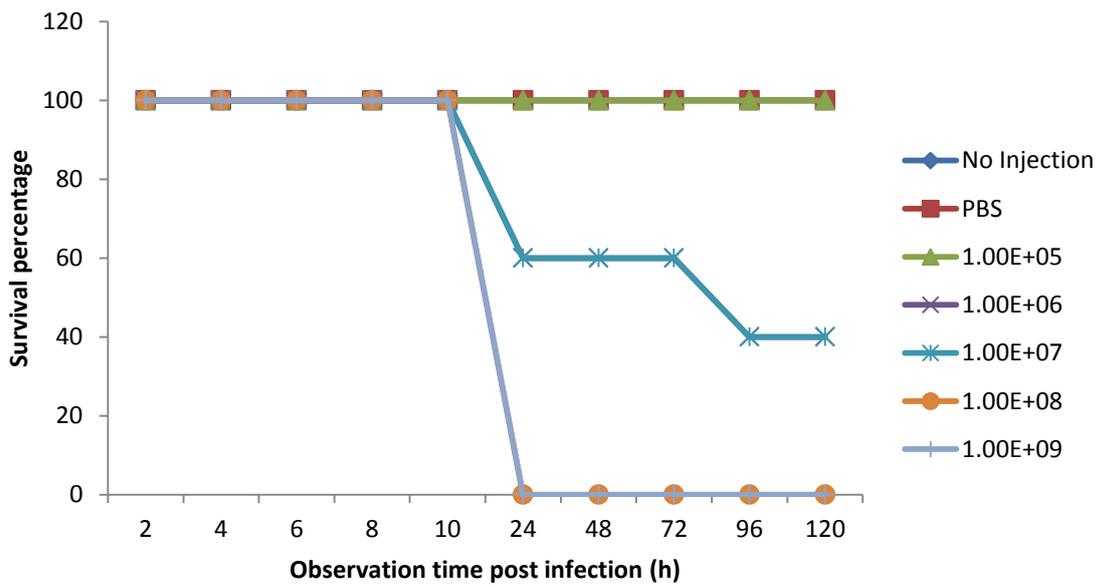
3.4.2.2 Survival Curves

3.4.2.2.1 Survival curves of *Galleria* infected with strain YE5303 (BT 1A/O:5)

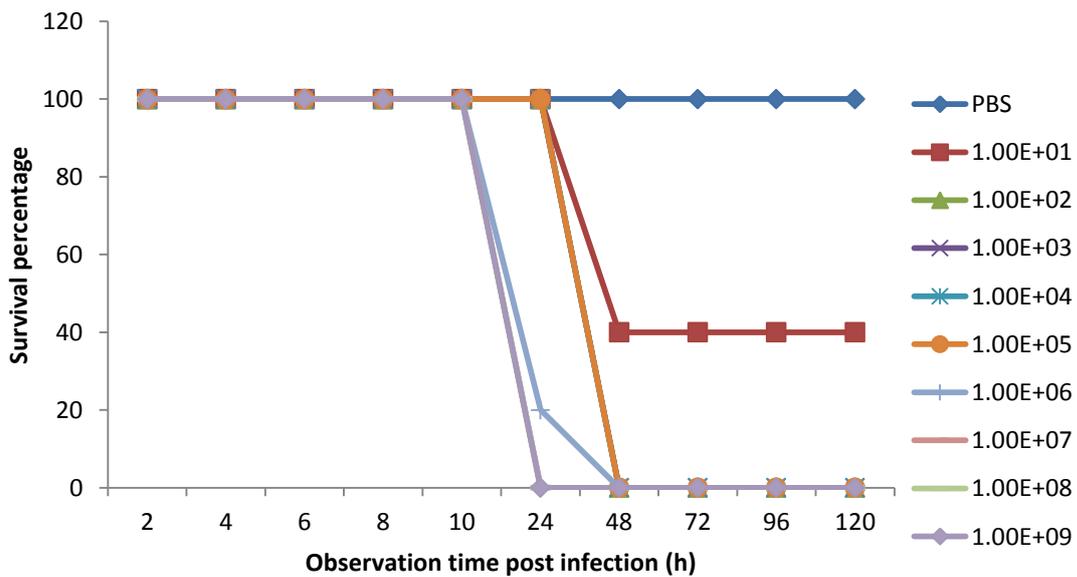
Strain 5303 was pre-grown at 25°C and 37°C. Both temperatures led to the 5303 strain being virulent to *Galleria* larvae. 5303 strain pre-grown at 25°C caused death to the *Galleria* larvae when incubated at 25°C and 37°C (fig. 5.3a & 5.3b). At the 37°C incubation temperature (fig. 5.3a), 4 doses including $10^9, 10^8, 10^7$ and 10^6 doses were virulent to the *Galleria* larvae. Both the 10^9 and 10^8 doses caused death for all larvae within 24h p.i. In addition, doses of 10^7 and 10^6 caused the death of 3 larvae (60% survival) in each of these doses within the same time point of 96h p.i. At the 25°C incubation temperature (fig 5.3b), all 9 used doses caused death of *Galleria* larvae including doses from 10^1 - 10^9 . Doses from 10^2 - 10^9 killed all the larvae at varying time points. Doses $10^9, 10^8$ and 10^7 killed all the larvae in each dose after 24h p.i. while doses $10^6, 10^5, 10^4, 10^3$ and 10^2 killed all the larvae after 48h p.i. In addition 10^1 dose killed 3 larvae (40% survival) after 48h p.i. Furthermore, 5303 strain pre-grown at 37°C had a very similar survival-curve profile to that observed when the strain was pre-grown at 25°C (Fig. 5.3c & 5.3d). At the 37°C incubation condition (Fig. 5.3c), 4 doses including $10^9, 10^8, 10^7$ and 10^6 caused mortality to the *Galleria* larvae. 10^9 and 10^8 doses killed all the *Galleria* larvae during 24h p.i. in both doses. The 10^7 dose killed 3 larvae (40% survival) during 72h p.i. while the 10^6 dose killed 2 larvae (60% survival) after 48h p.i. In the 25°C incubation condition (Fig 5.3d), all the 9 used doses killed the *Galleria* larvae in doses from 10^1 - 10^9 . Doses from 10^2 - 10^9 killed all the *Galleria* larvae with a different time points. Doses $10^9, 10^8, 10^7$ and 10^6 killed all *Galleria* larvae in each dose after 24h p.i. while doses $10^5, 10^4, 10^3$ and 10^2 killed all the larvae after 48h p.i. Finally, 10^1 dose killed 4 larvae (20% survival) after 48h p.i. These results show that the non-pathogenic 5303 strain was lethal to *Galleria* larvae regardless of the temperature it is pre-grown at. The

infected *Galleria* larvae incubated at 25°C were susceptible to killing with all the doses used. However, when infected larvae were incubated at 37°C they were susceptible only to the $10^9, 10^8, 10^7$ and 10^6 doses. This is a 5 log reduction in lethal dose for the larvae incubated at 25°C compared to the 37°C incubation temperature.

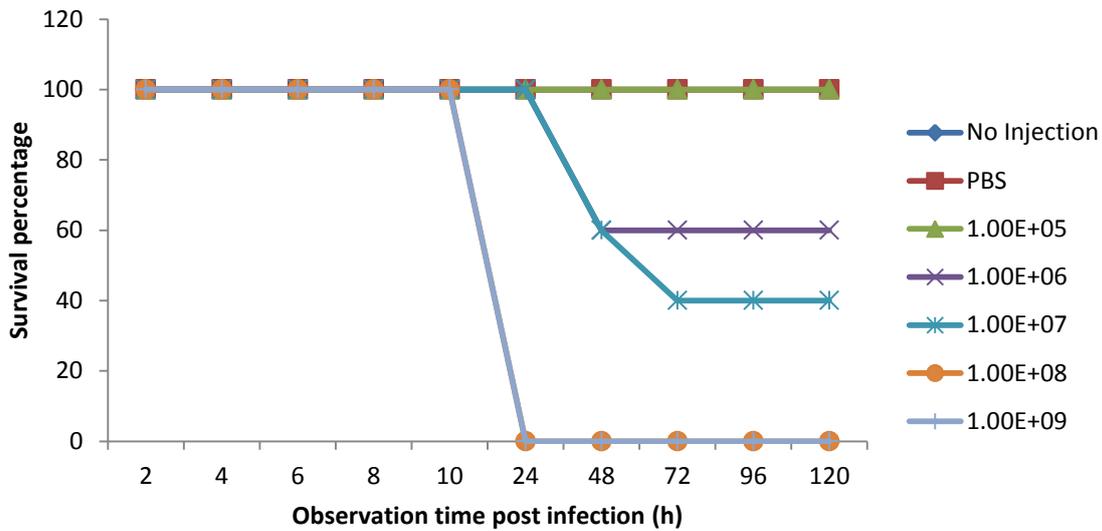
a-Survival curve of *Galleria* incubated at 37°C and infected with strain YE5303 pre-grown at 25°C



b- Survival curve of *Galleria* incubated at 25°C and infected with strain YE8081 pre-grown at 25°C



c- Survival curve of *Galleria* incubated at 37°C and infected with strain YE8081 pre-grown at 37°C



d- Survival curve of *Galleria* incubated at 25°C and infected with strain YE8081 pre-grown at 37°C

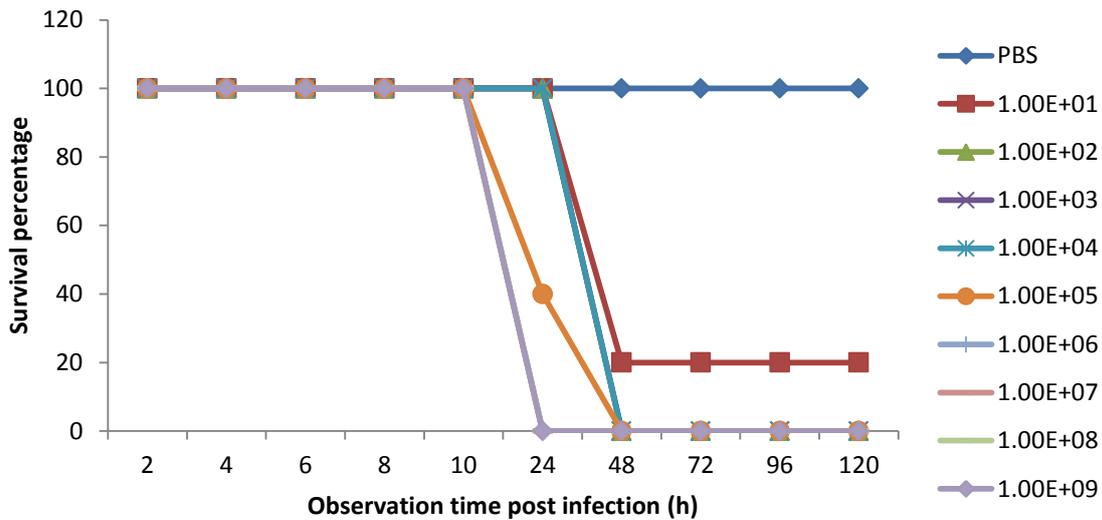
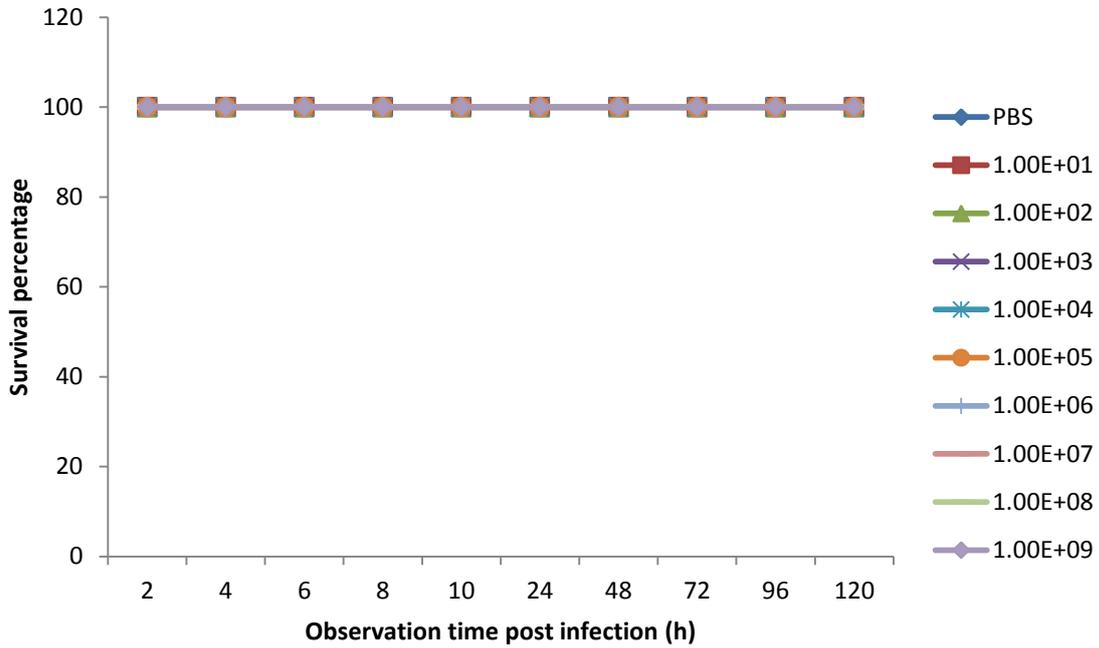


Figure 3.5: Survival curves of *Galleria* infected with the non-pathogenic strain YE5303 (BT 1A/O:5). Figure 3.5 includes four sub figures (3.5a, 3.5b, 3.5c & 3.5d) representing the percentage of survival rates of the *Galleria mellonella* larvae infected strain 5303. A number of 5 larvae used for each dose. Figures 3.5a & 3.5b represent the *Galleria* survival percentage graphs injected with strain 5303 strain pre-grown at 25°C; Figure 3.5a shows the survival results of incubating the *Galleria* larvae at 37°C post infection while figure 3.5b shows the survival results of incubating *Galleria* larvae at 25°C post infection. Figures 3.5c & 3.5d represent the *Galleria* survival percentage graphs injected with strain 5303 pre-grown at 37°C; Figure 3.5c shows the survival of *Galleria* at 37°C post infection while figure 3.5d shows the survival of *Galleria* at 25°C incubation. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

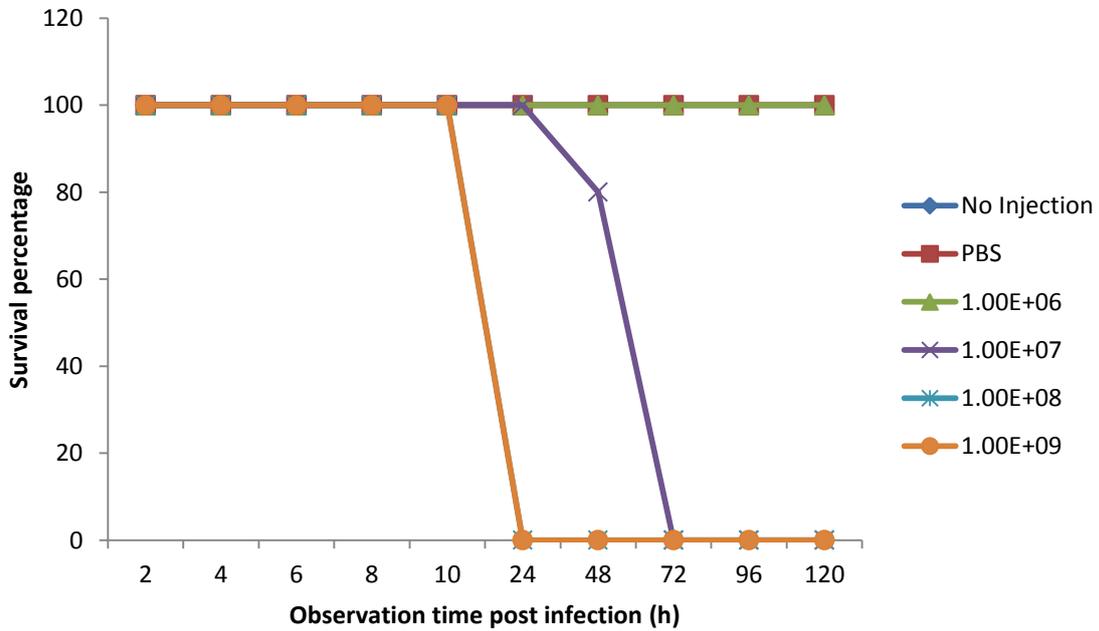
3.4.2.2.2 Survival curves of *Galleria* infected with strain YE8081 (BT 1B/O:8)

Strain 8081 Bioserotype 1B was pre-grown at 25°C and 37°C and doses from each pre-grown temperature were injected into *Galleria* larvae which were incubated at 25°C and 37°C. The 8081 strain didn't cause any death to the infected larvae when the larvae were incubated at 37°C (Fig. 3.6a & 3.6c). However when larvae were incubated at 25°C, doses from 8081 strain pre-grown at both 25°C and 37°C caused killing to the *Galleria* larvae (Fig. 3.6b & 3.6d). Doses 10^9 , 10^8 and 10^7 of the 8081 pre-grown at 25°C caused death to the injected *Galleria* larvae (Fig. 3.6b). Doses 10^9 and 10^8 killed all the larvae groups after 24h p.i. while 10^7 dose killed all the *Galleria* larvae after 72h p.i. The 10^9 and 10^8 doses of 8081 pre-grown at 37°C caused death to *Galleria* larvae with the 10^9 dose killing 4 larvae (20% survival) at 24h p.i. while the 10^8 dose killed one larva (80% survival) after 72h p.i (Fig. 3.6d). In summary, the injected larvae with the 8081 strain pre-grown at 25°C didn't die at the 37°C incubation temperature. Contrary, 8081 strain caused death to the infected *Galleria* larvae incubated at 25°C temperature. At the 25°C incubation condition, 8081 strain pre-grown on 25°C caused more death to *Galleria* compared to the infection with doses from the 37°C pre-grown 8081 strain.

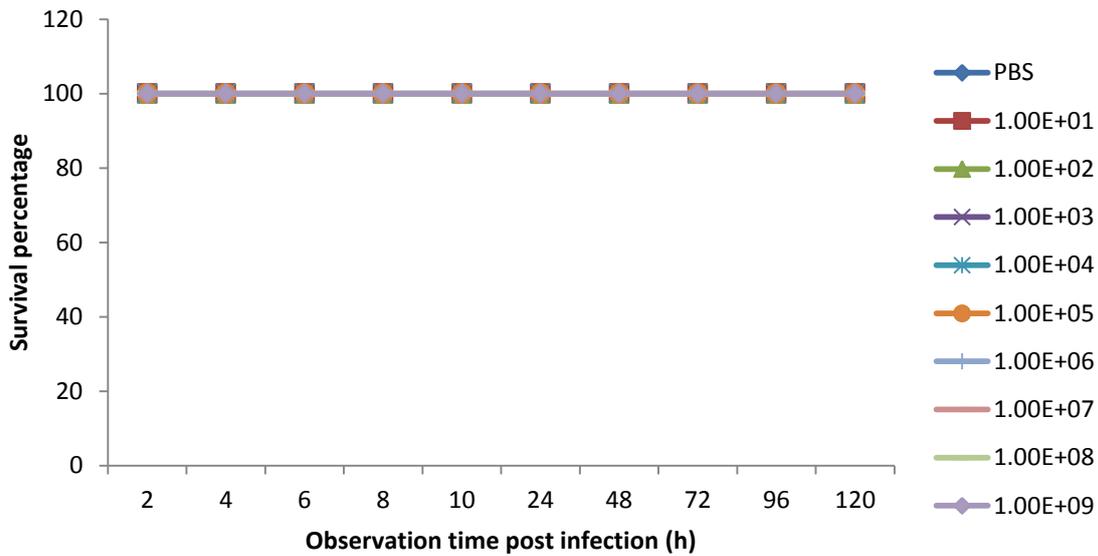
a- Survival curve of *Galleria* incubated at 37°C and infected with strain YE8081 pre-grown at 25°C



b- Survival curve of *Galleria* incubated at 25°C and infected with strain YE8081 pre-grown at 25°C



c- Survival curve of *Galleria* incubated at 37°C and infected with strain YE8081 pre-grown at 37°C



d- Survival curve of *Galleria* incubated at 25°C and infected with strain YE8081 pre-grown at 37°C

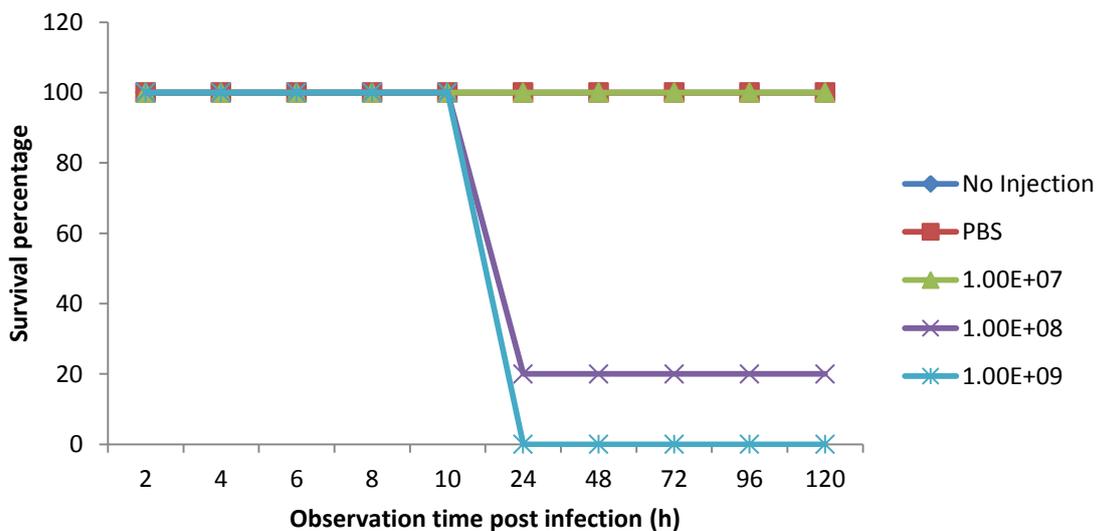


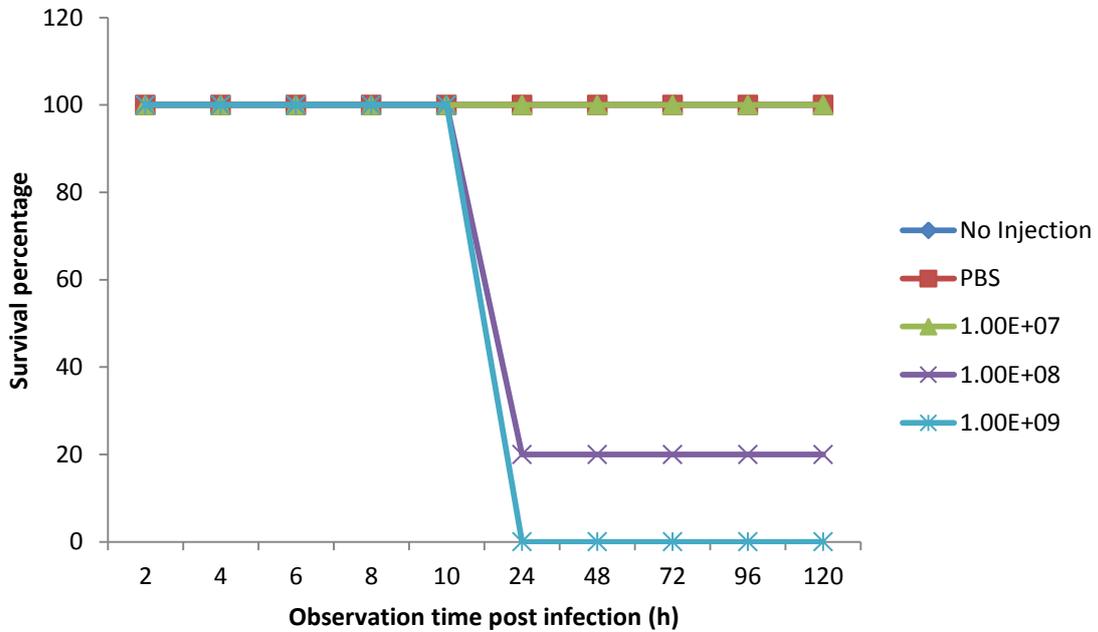
Figure 3.6: Survival curves of *Galleria* infected with the high pathogenic strain YE8081 (BT 1B/O:8). Figure 3.6 includes four sub figures (3.6a, 3.6b, 3.6c & 3.6d) representing the percentage of survival rates of the *Galleria* larvae infected strain 8081. A number of 5 larvae were used for each dose. Figures 3.6a & 3.6b represent the *Galleria* survival injected with strain 8081 pre-grown at 25°C; Figure 3.6a shows the survival of incubating *Galleria* at 37°C while figure 3.6b shows the survival of incubating *Galleria* at 25°C post infection. Figures 3.6c & 3.6d represent the *Galleria* survival percentage graphs injected with the strain 8081 pre-grown at 37°C; Figure 3.6c shows the survival results of incubating the *Galleria* larvae at 37°C post infection while figure 3.6d shows the survival of *Galleria* larvae at 25°C incubation. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

3.4.2.2.3 Survival curves of *Galleria* infected with strain YE14902 (BT 3/O:5,27)

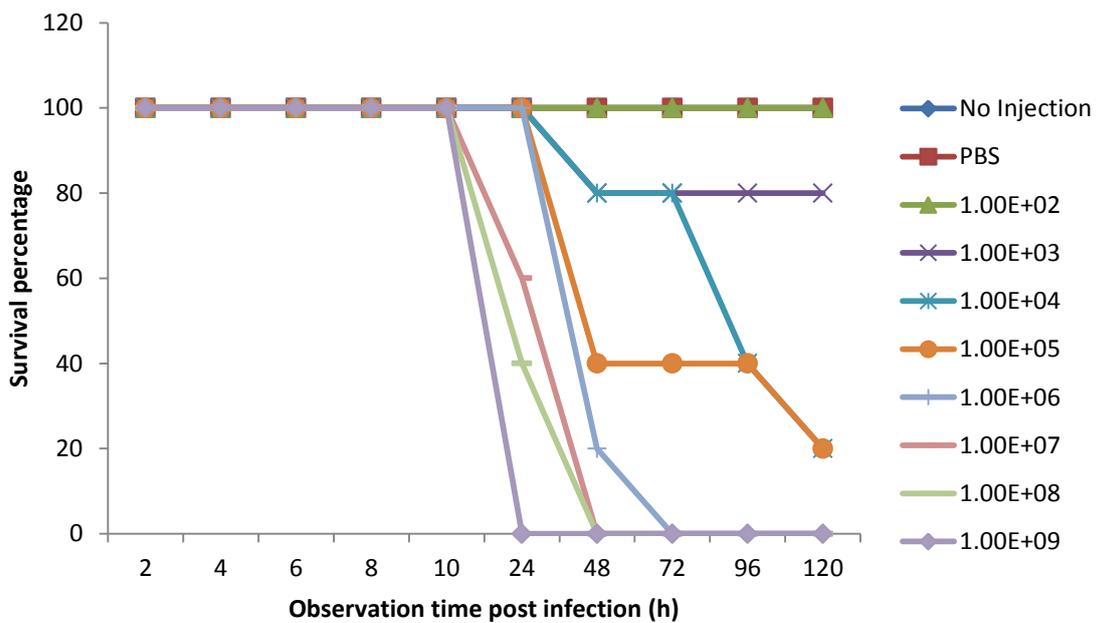
Strain 14902 was pre-grown at both 25°C and 37°C and both growing temperature induced virulence into the infected *Galleria* larvae. Strain 14902 pre-grown at 25°C induced death in the infected larvae incubated at both 25°C and 37°C incubation temperatures (Fig. 3.7a & 3.7b). At the 37°C incubation temperature, dose 10^9 and dose 10^8 caused mortality in the infected *Galleria* larvae by which dose 10^9 killed all the *Galleria* larvae during 24h p.i. while the 10^8 dose killed 4 larvae (80% survival) after 24h p.i (Fig. 3.7a). At the 25°C incubation condition, 7 different doses killed the infected larvae and these doses are $10^3, 10^4, 10^5, 10^6, 10^7, 10^8$ and 10^9 (Fig. 3.7b). All the *Galleria* larvae groups infected with doses $10^9, 10^8, 10^7$ and 10^6 were killed. Doses $10^9, 10^8$ and 10^7 killed all the infected larvae at 24h p.i. while 10^6 killed all the infected larvae at 72h p.i. In addition, each of the 10^4 and 10^5 doses killed 4 larvae (20% survival) after 120h p.i. while the 10^3 dose killed only 1 larva (80% survival) after 24h p.i. Pre-growing the 14902 strain at 37°C lead to death of the infected larvae at both incubation temperatures (25°C and 37°C); (Fig. 3.7c & 3.7d). In the 37°C incubation temperature, 10^9 and 10^8 doses caused death to the *Galleria* larvae where the 10^9 dose killed all the larvae after 24h p.i. while the 10^8 dose killed 2 larvae (60% survival) after 24h p.i (Fig. 3.7c). At the 25°C incubation temperature, doses from 10^3 - 10^9 caused death to the infected *Galleria* larvae (Fig. 3.7d). Doses 10^9 and 10^8 killed all the *Galleria* groups at 24h p.i. and 48h p.i. respectively for each dose. Dose 10^7 killed 4 larvae (80% survival) after 48h p.i. Doses 10^6 and 10^5 killed 3 larvae (40% survival) of the infected larvae groups at the same 72h p.i. time point for both doses. In addition, dose 10^4 killed 2 larvae (60% survival) during 72h p.i. while dose 10^3 killed 1 larva (80% survival) after 72h p.i. Strain 14902 was more lethal at 25°C incubation condition compared to the 37°C incubation. At the 37°C incubation temperatures the first 3 high doses 10^7 - 10^9 caused death to the injected *Galleria* larvae while at 25°C incubation temperature a wider range of doses

10^3 - 10^9 caused death to the infected larvae. However, pre-growing 14902 strain at the 25°C temperature shows a small increase in virulence towards the infected larvae.

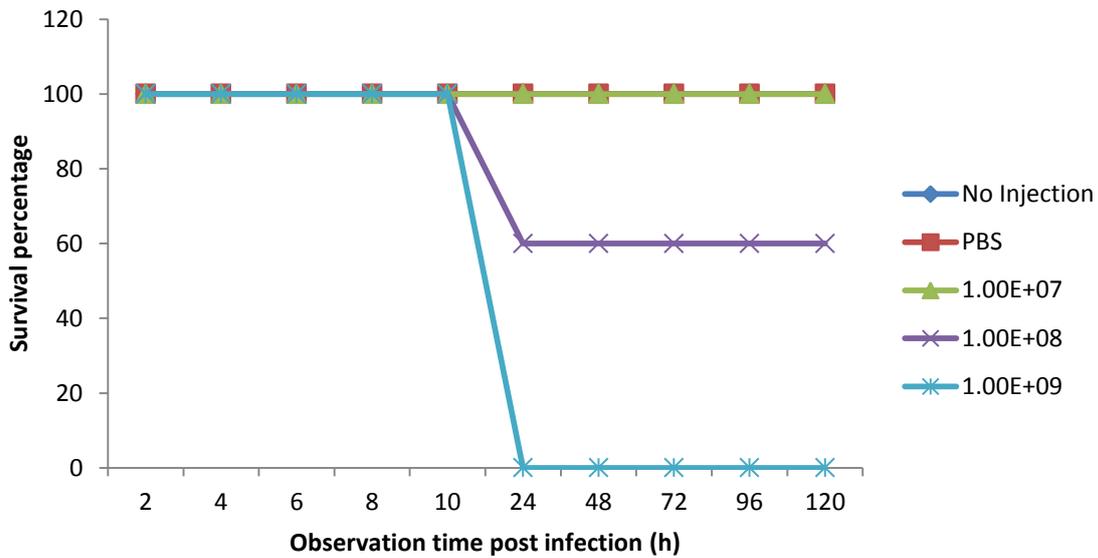
a- Survival curve of *Galleria* incubated at 37°C and infected with strain YE14902 pre-grown at 25°C



b- Survival curve of *Galleria* incubated at 25°C and infected with strain YE14902 pre-grown at 25°C



c- Survival curve of *Galleria* incubated at 37°C and infected with strain YE14902 pre-grown at 37°C



d- Survival curve of *Galleria* incubated at 25°C and infected with strain YE14902 pre-grown at 37°C

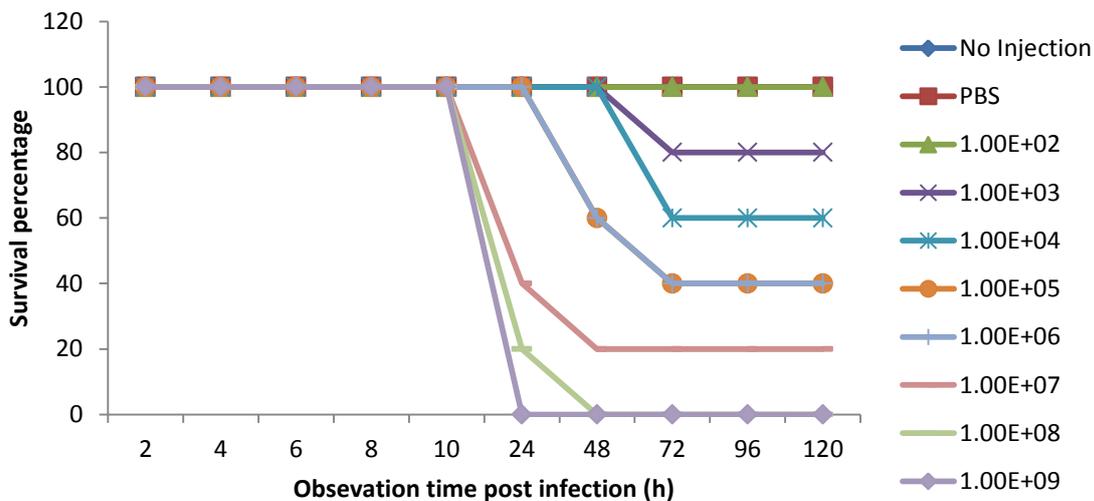
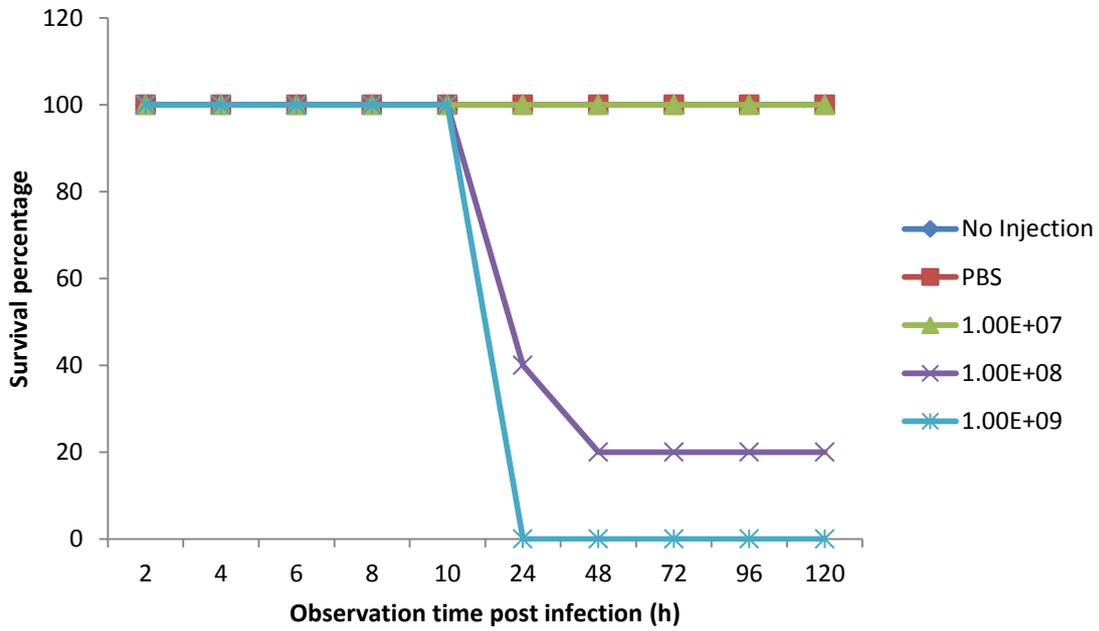


Figure 3.7: Survival curves of *Galleria* infected with the low pathogenic strain YE14902 (BT 3/O:5,27). Figure 3.7 includes four sub figures (3.7a, 3.7b, 3.7c & 3.7d) representing the survival of *Galleria* infected with the strain 14902. Figures 3.7a & 3.7b represent the *Galleria* survival graphs injected with strain 14902 pre-grown at 25°C; Figure 3.7a shows the survival of *Galleria* at the 37°C incubation while figure 3.7b shows the survival of incubating *Galleria* larvae at 25°C post infection. Figures 3.7c & 3.7d represent the *Galleria* survival injected with strain 14902 pre-grown at 37°C; Figure 3.7c shows the survival of *Galleria* at 37°C while figure 3.7d shows the survival *Galleria* at 25°C incubation. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

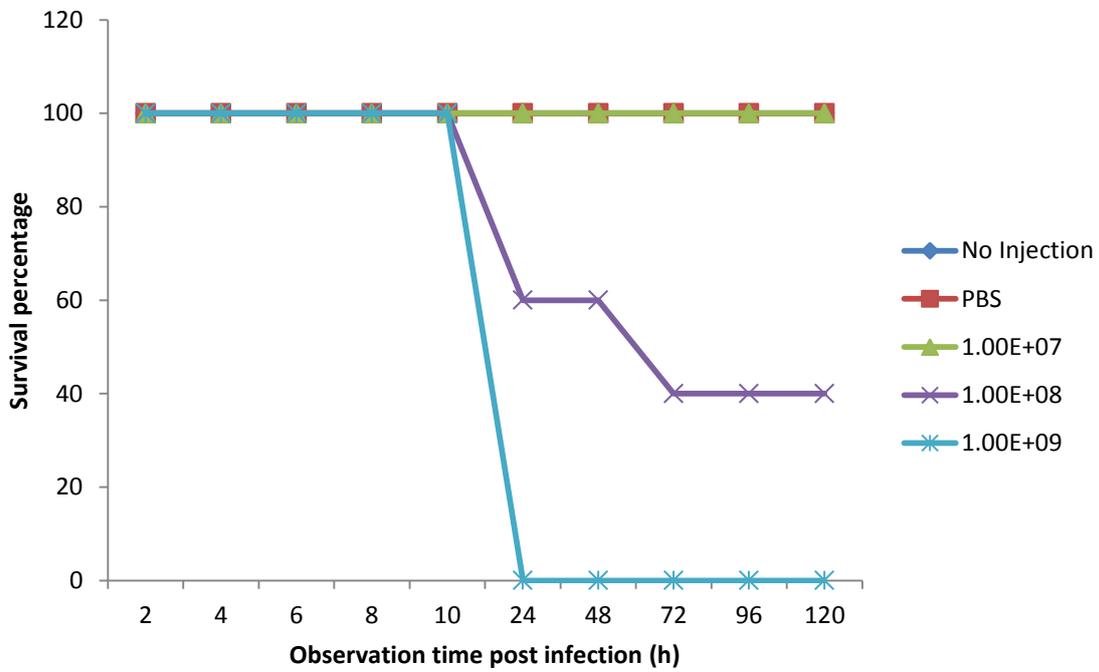
3.4.2.2.4 Survival Curves *Galleria* infected with Strain YE5603 (BT 3/O:9):

Low pathogenic strain 5603 which belongs to bioserotype 3/O:9 was able to kill *Galleria* larvae when pre-grown at both 25°C and 37°C temperatures. At 25°C and 37°C incubation of the infected larvae, the 25°C and 37°C pre-grown cultures were lethal to *Galleria* at only the highest doses 10^8 and 10^9 . 5603 strain pre-grown at 25°C killed all the injected larvae groups after 24 h p.i with the highest dose 10^9 in both of the larvae incubation temperatures 25°C and 37°C (Fig. 3.8a & 3.8b). The 10^8 dose killed 4 larvae (20% survival) within 48 hours p.i. in the 37°C incubation condition (Fig. 3.8a) while the same dose killed 3 larvae (40% survival) and in a longer period of 72h at the 25°C incubation temperature (Fig. 3.8b). The virulence effect of strain 5603 was similar when it was pre-grown at 37°C. The 10^9 and 10^8 doses of 5603 strain pre-grown at 37°C was able to kill *Galleria* larvae. The 10^9 dose killed all larvae within 24 h p.i. The 10^8 dose killed 3 larvae (60%) after 72h at the 37°C incubation (Fig. 3.8c) while at the 25°C incubation temperature all larvae were killed with in 72h p.i (Fig. 3.8d).

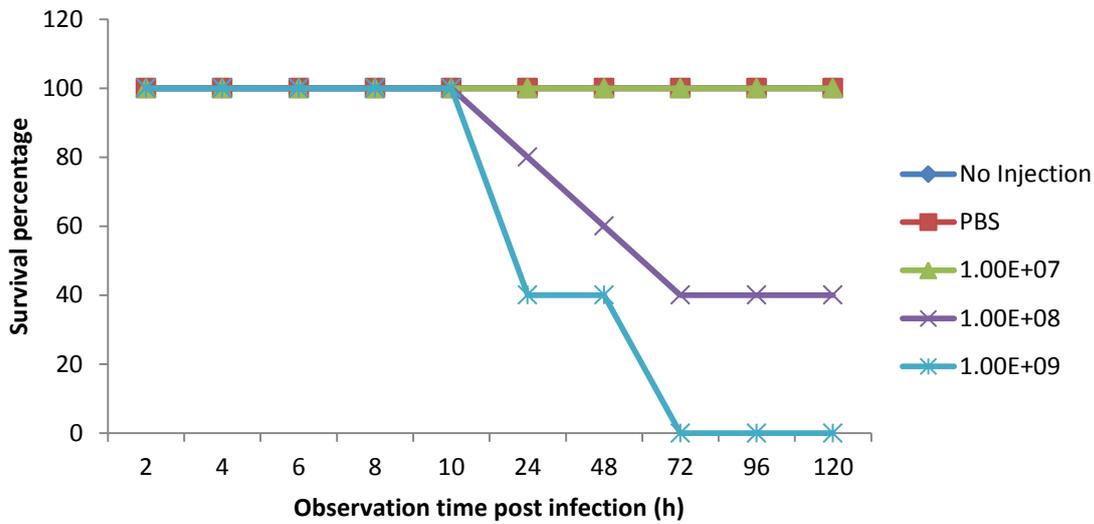
a- Survival curve of *Galleria* incubated at 37°C and infected with strain YE5603 pre-grown at 25°C



b- Survival curve of *Galleria* incubated at 25°C and infected with strain YE5603 pre-grown at 25°C



c- Survival curve of *Galleria* incubated at 37°C and infected with strain YE5603 pre-grown at 37°C



d- Survival curve of *Galleria* incubated at 25°C and infected with strain YE5603 pre-grown at 37°C

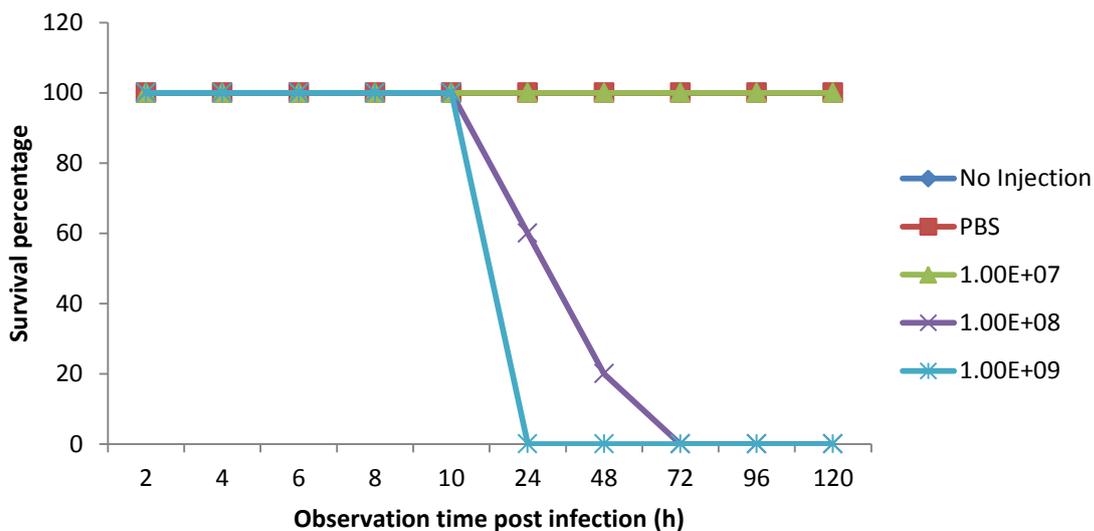
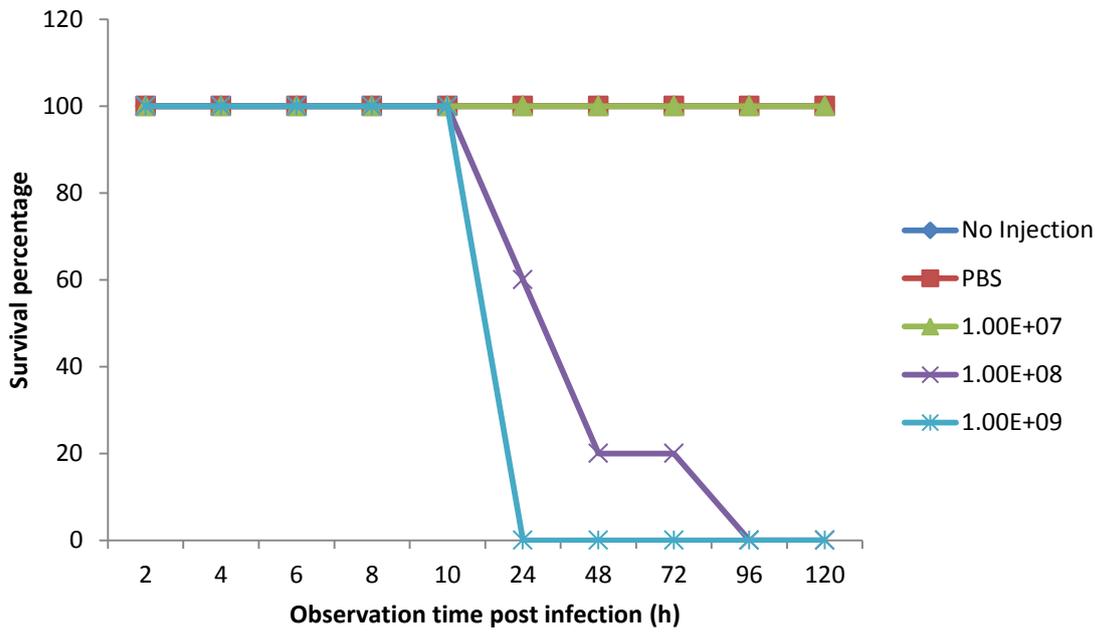


Figure 3.8: Survival curves of *Galleria* infected with the low pathogenic strain YE5603 (BT 3/O:9). Figure 3.8 includes four sub figures (3.8a, 3.8b, 3.8c & 3.8d) representing the percentage of survival rates of the *Galleria* larvae infected with the strain 5603. A number of 5 larvae were used for each dose. Figures 3.8a & 3.8b represent the *Galleria* survival percentage injected with strain 5603 pre-grown at 25°C; Figure 3.8a shows the survival results of incubating *Galleria* at 37°C post infection while figure 3.8b shows the survival results of incubating *Galleria* at 25°C post infection. Figures 3.8c & 3.8d represent the *Galleria* survival percentage graphs injected with strain 5603 pre-grown at 37°C; Figure 3.8c shows the survival results of incubating the *Galleria* larvae at 37°C post infection while figure 3.8d shows the survival results of incubating *Galleria* larvae at 25°C post infection. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

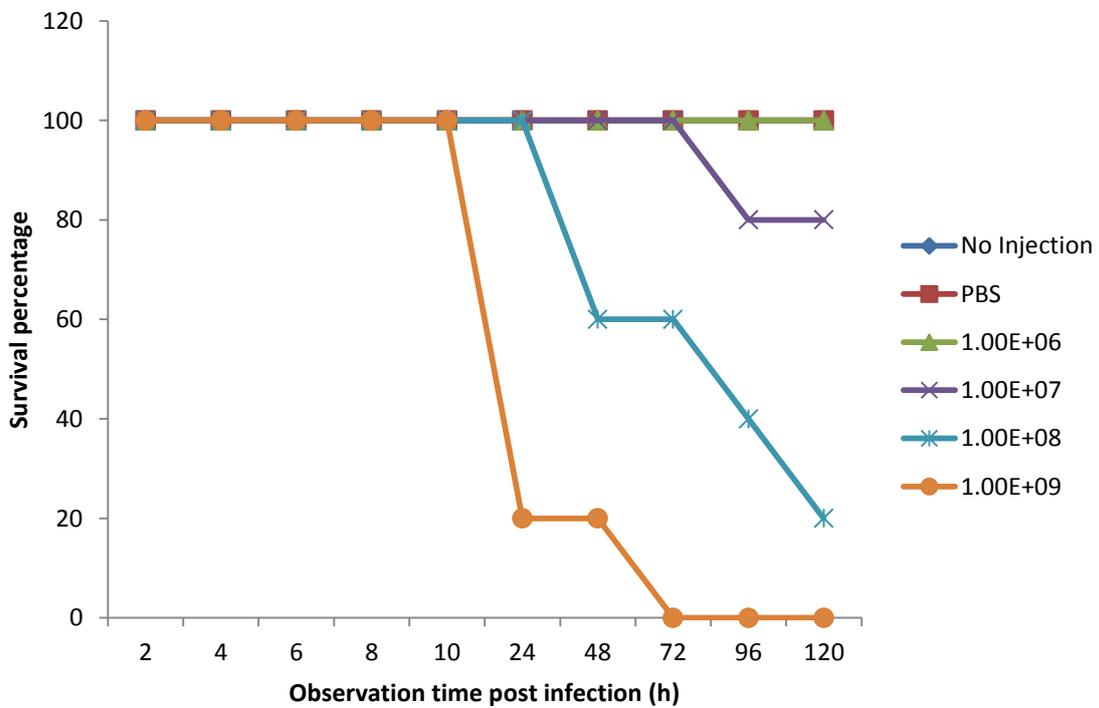
3.4.2.2.5 Survival curves of *Galleria* infected with strain YE21202 (BT 2/O:9):

Low pathogenic strain 21202 (bioserotype 2/O:9) was also virulent to *Galleria* larvae at both pre-growing temperatures (25°C and 37°C). The larvae injected with the 25°C pre-grown 21202 strain were killed with doses of 10^8 and 10^9 at the 37°C incubation temperature (Fig. 3.9a) while at the 25°C incubation temperature the lethal doses were 10^7 , 10^8 and 10^9 (Fig. 3.9b). At 37°C incubation temperature, the 10^9 highest dose killed all the 5 larvae during 24 h post infection and the 10^8 dose killed all the 5 larvae after 96h p.i. At the 25°C incubation temperature, 10^9 dose killed all the larvae within 24 hours p.i., 10^8 dose killed 4 larvae (20% survival) after 120 hours post infection while 10^7 dose killed only 1 larva (80% survival) after 96h p.i. Moreover, pre-growing strain 21202 at 37°C temperature lead to death of the *Galleria* larvae in both incubation temperatures. At the 37°C incubation temperature (Fig. 3.9c), 10^9 , 10^8 doses were virulent to the *Galleria* larvae while on the 25°C incubation temperature (Fig. 3.9d), 4 doses managed to cause mortality to *Galleria* (10^6 - 10^9 doses). Dose 10^9 killed all the *Galleria* larvae after 24h and the 10^8 dose killed 4 larvae (20% survival) after 48h. In the 25°C incubation temperature, the 10^9 dose managed to kill all the larvae within 24 h p.i. The 10^8 dose was lethal to 3 larvae (40% survival) at 48h p.i, 10^7 dose killed 2 larvae (60% survival) and 10^6 dose killed 1 larva (80% survival) at 48 hours post infection.

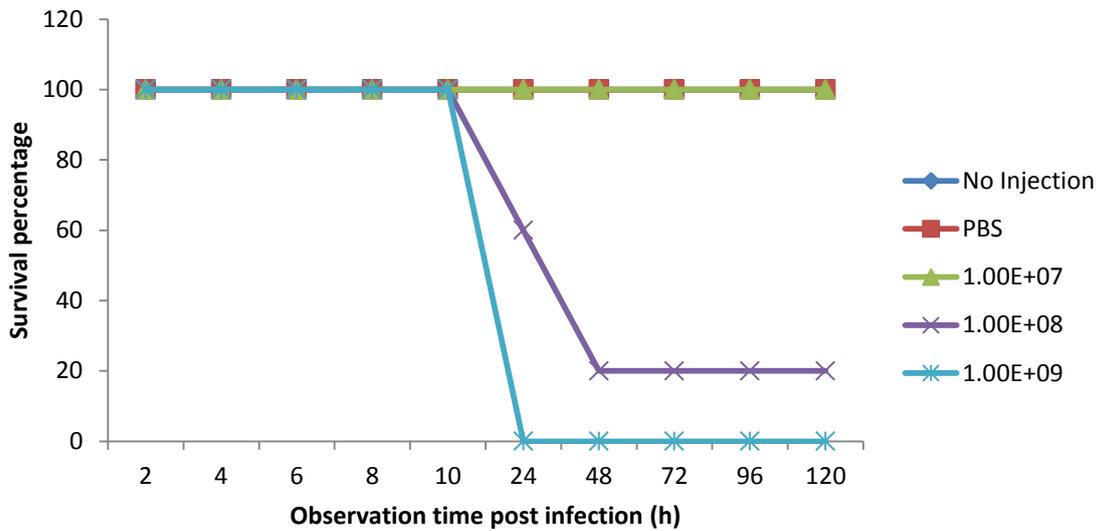
a- Survival curve of *Galleria* incubated at 37°C and infected with strain YE21202 pre-grown at 25°C



b- Survival curve of *Galleria* incubated at 25°C and infected with strain YE21202 pre-grown at 25°C



c- Survival curve of *Galleria* incubated at 37°C and infected with strain YE21202 pre-grown at 37°C



d- Survival curve of *Galleria* incubated at 25°C and infected with strain YE21202 pre-grown at 25°C

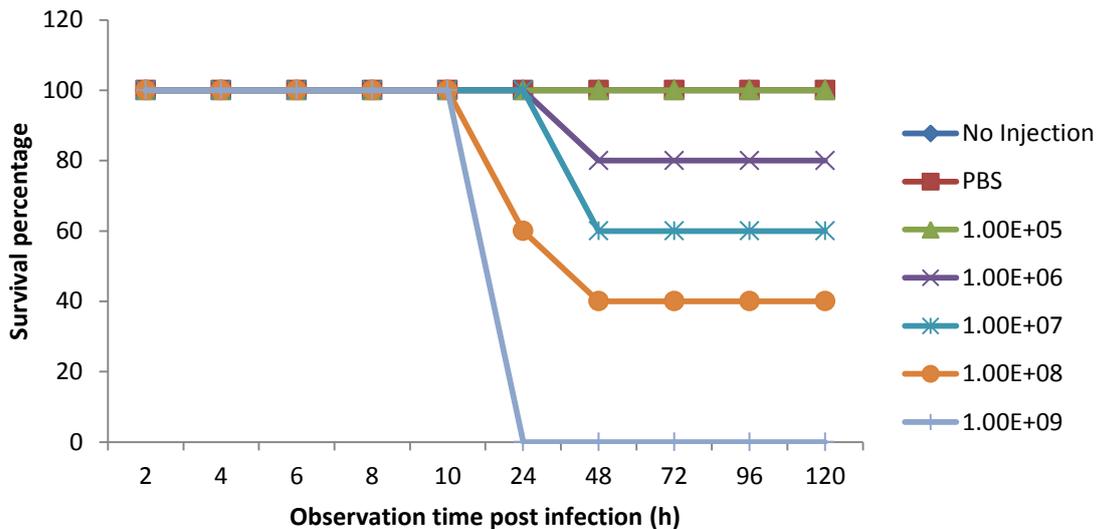
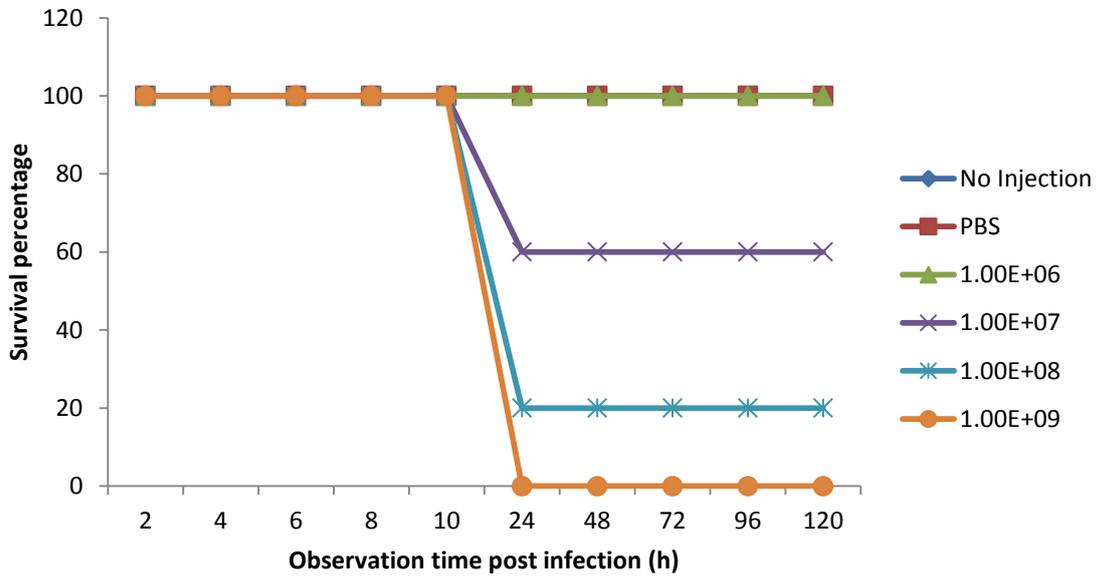


Figure 3.9: Survival curves of *Galleria* infected with the low pathogenic strain YE21202 (BT 2/O:9). Figure 3.9 includes four sub figures (3.9a, 3.9b, 3.9c & 3.9d) representing the percentage of survival rates of the *Galleria* infected with strain 21202. A number of 5 larvae were used for each dose. Figures 3.9a & 3.9b represent the *Galleria* survival injected with strain 21202 strain pre-grown at 25°C; Figure 3.9a shows the survival of *Galleria* incubated at 37°C post infection while figure 3.9b shows the survival of incubating *Galleria* at 25°C. Figures 3.9c & 3.9d represent the *Galleria* survival injected with strain 21202 pre-grown at 37°C; Figure 3.9c shows the survival results of incubating the *Galleria* larvae at 37°C post infection while figure 3.9d shows the survival results of incubating *Galleria* larvae at 25°C post infection. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

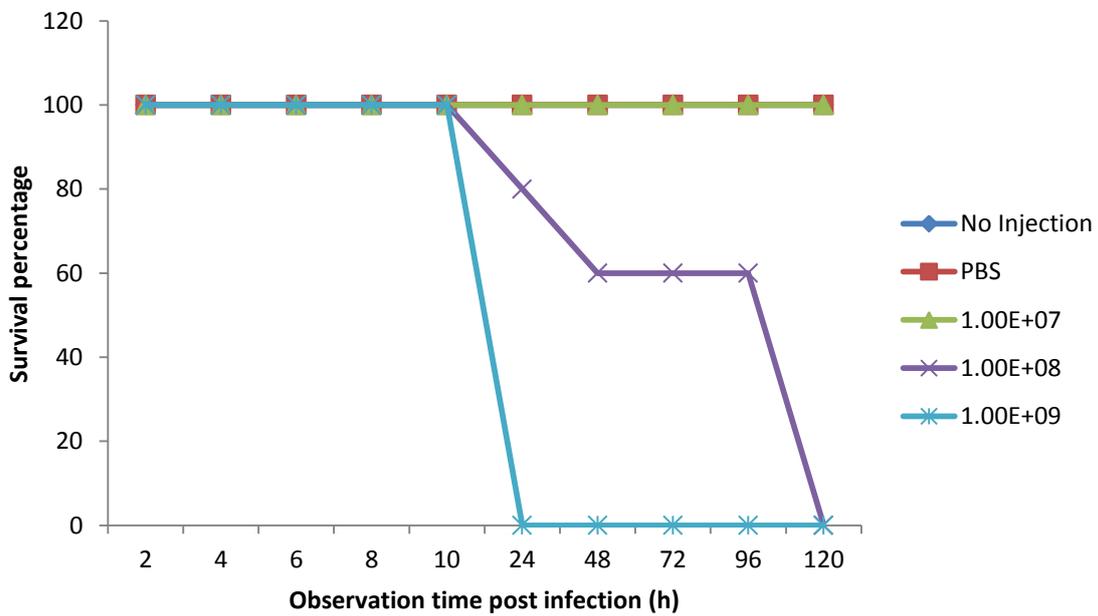
3.4.2.2.6 Survival curves of *Galleria* infected with strain YE1203 (BT 4/O:3):

Low pathogenic strain 1203, of bioserotype 4/O:3, was able to show mortality to *Galleria* larvae with both 25°C and 37°C pre-grown temperatures. Strain 1203 is the representative strains of the bioserotype 4/O:3 used to infect the *Galleria* larvae. With the 1203 strain 25°C pre-grown temperature, the infected larvae were killed with the 3 highest doses 10^9 , 10^8 and 10^7 at the 37°C incubation temperature (Fig. 3.10a) while at the 25°C incubation temperature only the highest doses 10^8 and 10^9 were lethal to the infected larvae (Fig. 3.10b). At the 37°C incubation temperature (Fig. 3.10a), 10^9 dose killed all the infected larvae after 24 hours. 10^8 dose killed 4 larvae (20% survival) and the 10^7 dose killed 2 larvae (40% survival) and both killing times were observed after 24 hours. At the larval 25°C incubation temperature (Fig. 3.10b), 10^8 and 10^9 doses killed all the infected larvae by which 10^9 dose killed all larvae within 24h while the 10^8 dose killing effect proceeded to the 120h p.i. time point. When the 1203 strain was pre-grown at 37°C, only the highest doses 10^9 and 10^8 caused death to the infected larvae. The 10^9 dose killed all the infected larvae after 24 h p.i. and the 10^8 dose killed 3 larvae (40% survival) within 96h at both 25°C and 37°C incubation temperatures (Fig. 3.10c & 3.10d). These result show that pre-growing the 1203 strain on 25°C temperature shows a higher virulence to the infected *Galleria* at the 37°C incubation temperature compared to a less virulence at the 25°C larval incubation temperature. However, pre-growing the 1203 strain on the 37°C temperature was more virulent to the infected *Galleria* larvae when the larvae incubated at the 25°C temperature from the larvae incubated at 37°C.

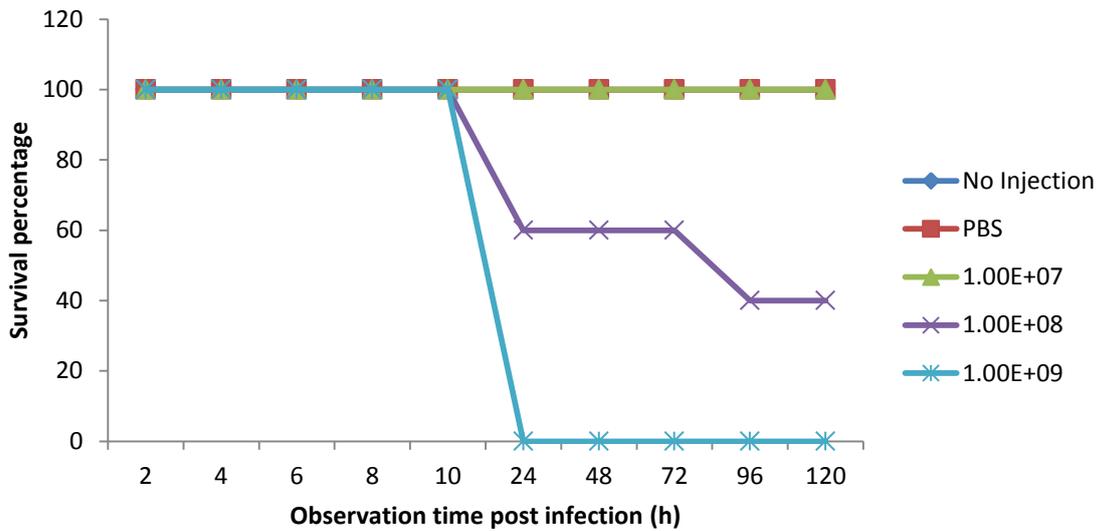
a-Survival curve of *Galleria* incubated at 37°C and infected with strain YE1203 pre-grown at 25°C



b- Survival curve of *Galleria* incubated at 25°C and infected with strain YE1203 pre-grown at 25°C



c- Survival curve of *Galleria* incubated at 37°C and infected with strain YE1203 pre-grown at 37°C



d- Survival curve of *Galleria* incubated at 25°C and infected with strain YE1203 pre-grown at 37°C

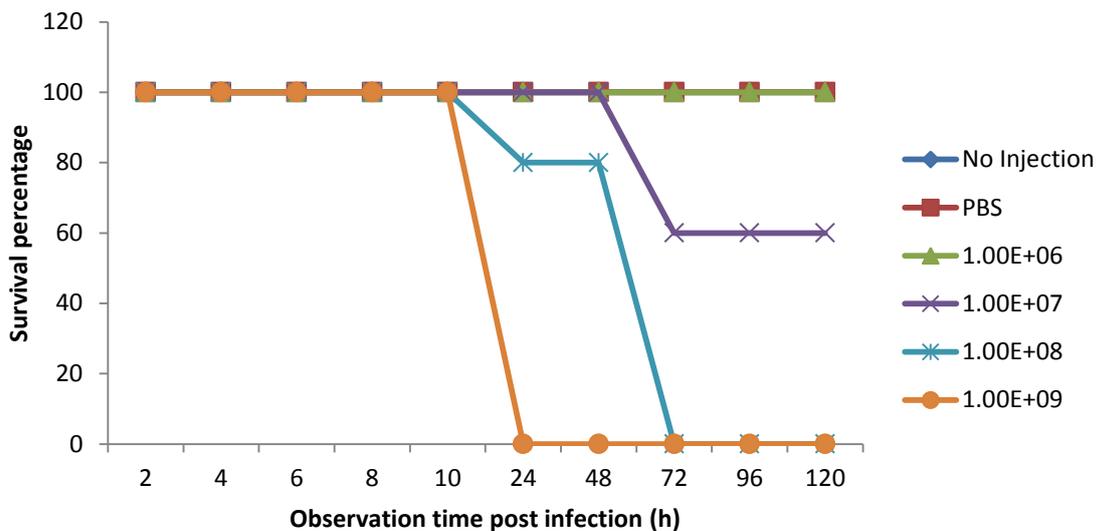
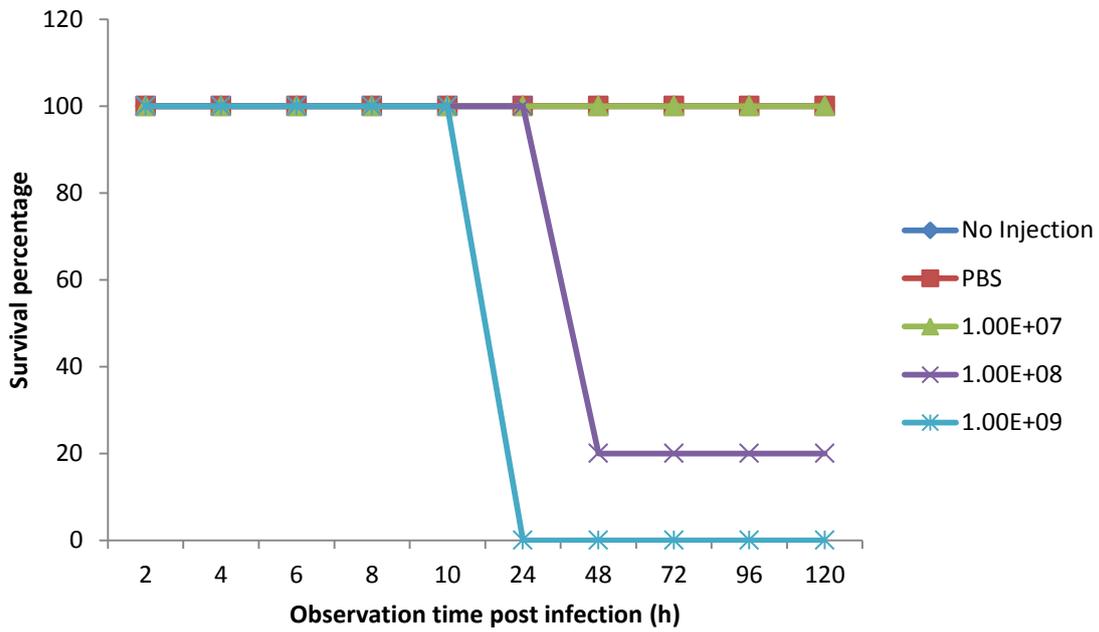


Figure 10: Survival curves of *Galleria* infected with the low pathogenic strain 1203 (BT 4/O:3). Figure 3.10 includes four sub figures (3.10a, 3.10b, 3.10c & 3.10d) representing the percentage of survival rates of the *Galleria* larvae infected with strain 1203. A number of 5 larvae were used for each dose. Figures 3.10a & 3.10b represent the *Galleria* survival injected with strain 1203 pre-grown at 25°C; Figure 3.10a shows the survival of *Galleria* incubated at 37°C while figure 3.10b shows the survival results of incubating *Galleria* at 25°C post infection. Figures 3.10c & 3.10d represent the *Galleria* survival injected with strain 1203 pre-grown at 37°C; Figure 3.10c shows the survival results of incubating the *Galleria* larvae at 37°C post infection while figure 3.10d shows the survival results of incubating *Galleria* larvae at 25°C post infection. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

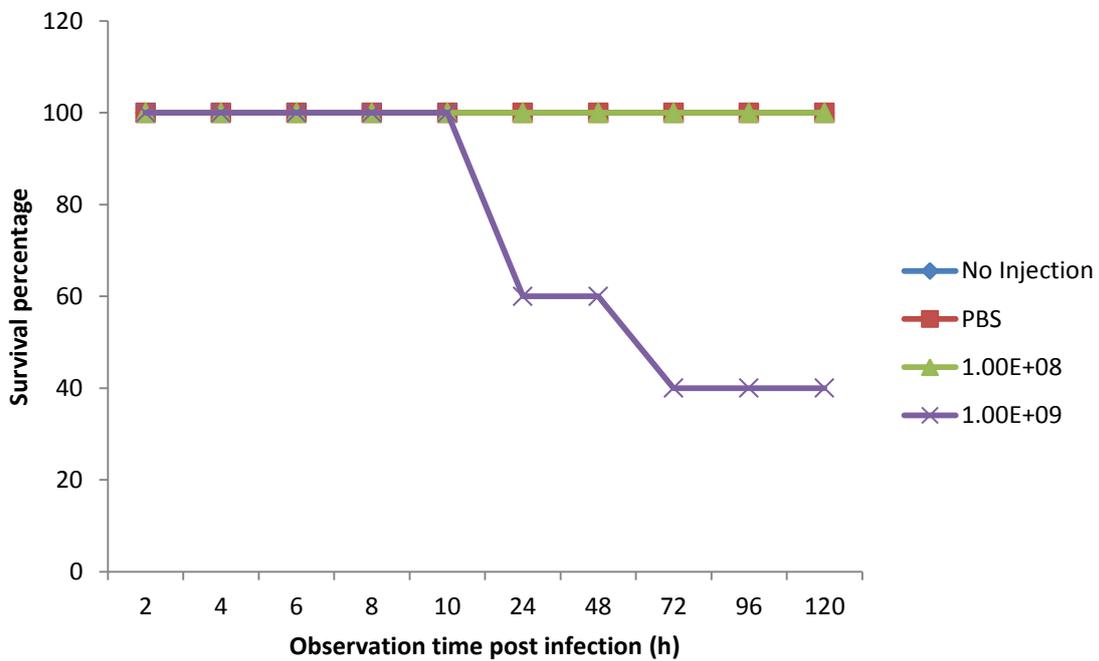
3.2.2.2.7 Survival curves of *Galleria* infected with strain YE3094/96 (BT 5/O: 2a,2b,3)

Low pathogenic strain 3094/96 (Bioserotype 5/O: 2a,2b,3) was pre-grown at 25°C and 37°C and doses from each pre-growing temperature caused death in *Galleria* larvae. Injecting *Galleria* larvae with the 25°C pre-grown 3094/96 strain caused death in larvae incubated at 25°C and 37°C (Fig. 3.11a & 3.11b). At the 37°C incubation temperature (Fig. 3.11a), dose 10^9 and dose 10^8 was able to kill *Galleria* larvae. The 10^9 dose killed all the larvae after 24h p.i. and the 10^8 dose killed 4 larvae (20% survival) within a period of 48h p.i. At the 25°C incubation temperature (Fig. 3.11b), only the 10^9 dose caused killing to the injected larvae by which 3 larvae (40% survival) killed within 72h p.i. Furthermore, the infected *Galleria* larvae with different doses from growing the 3094/96 strain at 37°C caused death in both pre-growing conditions. At both larval incubating conditions, the only mortal dose was 10^9 . Dose 10^9 killed 4 larvae (80% survival) within 24h p.i. at the 37°C incubation temperature (Fig. 3.11c) while this dose killed only 2 larvae (60% survival) at 24h p.i. in the 25°C larval incubation condition (Fig. 3.11d). There was a difference in virulence between pre-growing the 3094/96 strain at 25°C from 37°C. The 25°C pre-growing temperature of the 3094/96 strain caused more death to *Galleria* larvae compared to the 37°C pre-growing temperature. However, the infected *Galleria* larvae incubated at 37°C were more susceptible for killing from ones incubated at 25°C. In addition, it has been observed that the effective lethal dose for the 3094/96 strain was only the 10^9 dose in most of the larval incubating conditions. This reflects the low pathogenic potential of the 3094/96 strain.

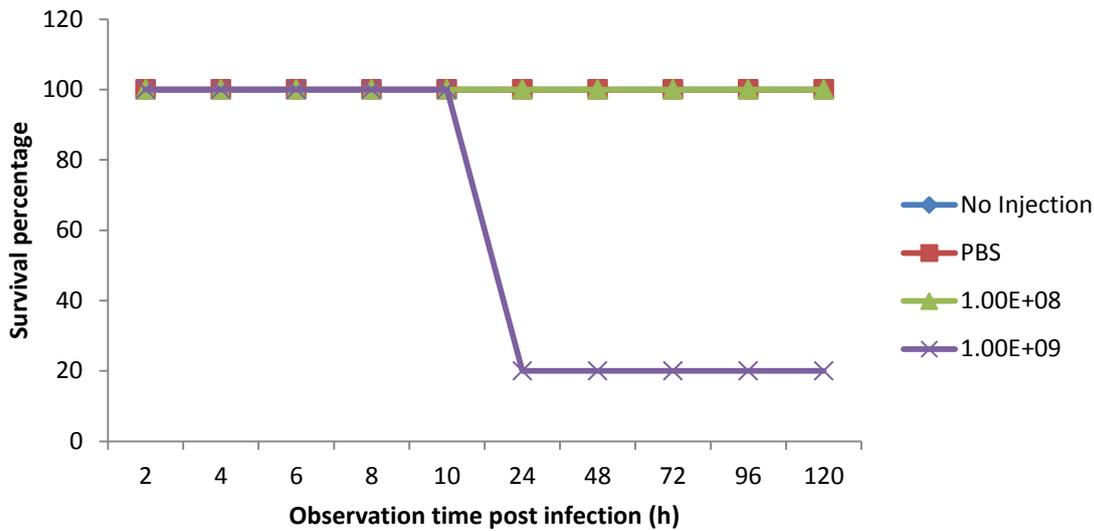
a- Survival curve of *Galleria* incubated at 37°C and infected with strain YE3094/96 pre-grown at 25°C



b- Survival curve of *Galleria* incubated at 25°C and infected with strain YE3094/96 pre-grown at 25°C



c- Survival curve of *Galleria* incubated at 37°C and infected with strain YE3094/96 pre-grown at 37°C



d- Survival curve of *Galleria* incubated at 25°C and infected with strain YE3094/96 pre-grown at 37°C

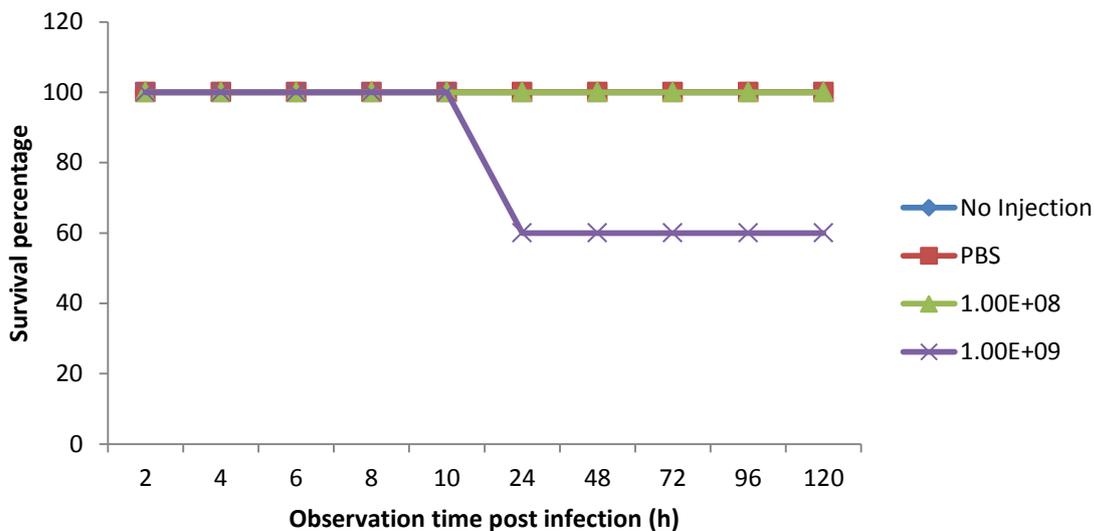


Figure 3.11: Survival curves of *Galleria* infected with the low pathogenic strain YE3094/96 (BT 5/O:2a,2b,3). Figure 3.11 includes four sub figures (3.11a, 3.11b, 3.11c & 3.11d) representing the survival of *Galleria* infected with strain 3094/96. A number of 5 larvae were used for each dose. Figures 3.11a & 3.11b represent the *Galleria* survival injected with strain 3094/96 pre-grown at 25°C; Figure 3.11a shows the survival *Galleria* larvae incubated at 37°C post infection while figure 3.11b shows the survival results of incubating *Galleria* at 25°C post infection. Figures 3.11c & 3.11d represent the *Galleria* survival injected with strain 3094/96 pre-grown at 37°C; Figure 3.11c shows the survival results of incubating the *Galleria* larvae on 37°C post infection while figure 3.11d shows the survival results of incubating *Galleria* larvae at 25°C post infection. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

In summary, all *Y. enterocolitica* strains start killing the *Galleria* larvae within 24h. However, the low-pathogenic and high-pathogenic strains were killing fewer numbers of *Galleria* larvae and in longer time periods. All injected doses of high pathogenic biotype 1B/O:8 strain 8081 were avirulent to *Galleria* larvae at 37°C incubation condition. On the other hand, all injected doses of the non-pathogenic biotype biotype 1A/O:5 strain 5303 were able to kill the injected *Galleria* larvae. *Y. enterocolitica* strains were killing higher numbers of *Galleria* larvae at 25°C incubation temperature and fewer numbers at the 37°C incubation condition.

3.5 Discussion

Y. enterocolitica have two life stages including living freely in the environment followed by a stage of living inside a specific host. The free-living period includes the growth of *Y. enterocolitica* strains in nature in temperatures below 28°C while the host-dependent stage requires the growth of *Y. enterocolitica* in temperatures above 35°C (Young *et al.*, 1999). *Y. enterocolitica* adapts its physiological characteristics including its virulence factors as a result of changes in the environmental conditions (Bottone, 1997). Most *Y. enterocolitica* virulence factors are thermoregulated and their expression or repression is regulated by temperature (Fàbrega and Vila., 2012). For this reason, the pathogenicity of *Y. enterocolitica* biotypes at two different temperatures; 25°C and the 37°C was studied. The 25°C temperature is known to be the optimal temperature to grow *Y. enterocolitica* strains (Gu *et al.*, 2012) because *Y. enterocolitica* shows higher metabolism levels and more physiological activities at this temperature (Grassl *et al.*, 2003; Gu *et al.*, 2012; Kawaoka *et al.*, 1983). In contrast, at 37°C temperature, *Y. enterocolitica* strains show lower metabolic levels and most of the physiological activities are abolished (Kawaoka *et al.*, 1983). In addition, *Y. enterocolitica* pathogenic biotypes undergo virulence gene induction upon temperature shifting from environmental temperatures (25°C-28°C) to the 37°C human optimal body temperature (Fàbrega and Vila., 2012). Since *Y. enterocolitica* is environmentally ubiquitous (Rahman *et al.*, 2011), this shift in temperature is essential in allowing the organism to move from the free living state and adapt to the mammalian host (Fàbrega and Vila., 2012). *Y. enterocolitica* pathogenic strains have been found to be more efficient at invading mammalian cells after this temperature upshift from 25°C to 37°C (Fàbrega and Vila., 2012 & Kawaoka *et al.*, 1983). The most significant thermoregulated virulence factor in *Y. enterocolitica* pathogenic strains is the VirF transcription regulator protein. VirF is encoded by the pYV plasmid and is highly expressed and activated after shifting to the 37°C

temperature (Fàbrega and Vila., 2012 & Rohde *et al.*, 1999). When it is expressed, VirF up regulates the secretion of the Yop proteins and the assembly of Ysc secretion system subunits to start the infection of the intestine epithelial tissue and to overcome the immune protection of the host (Fàbrega and Vila., 2012). The high pathogenic biotype 1B is considered the most virulent of all the *Y. enterocolitica* biotypes since it is lethal in the mouse infection model in addition to its acquisition of extra virulence factors such as the high pathogenic island (HPI) and the Ysa chromosomal type three secretion system (Garzetti *et al.*, 2012). The high pathogenic strain 8081 shows a high invasion potential toward the HEP-2 cell line on both pre-growing temperatures (25°C and 37°C). However, the 8081 strain gave unexpected results with the *Galleria* larvae infection model, and was completely avirulent to the *Galleria* larvae incubated at 37°C post infection. However 8081 strain was able to kill *Galleria* larvae injected with doses of 10^9 , 10^8 and 10^7 cfu/ml incubated at 25°C after 24 hours post infection. Indeed at the 37°C incubation temperature, the high pathogenic biotype was the least virulent of all the biotypes. At the *Galleria* 37°C incubation, the 25°C pre-grown 8081 strain and the 37°C pre-grown 8081 strain were the weakest strains with a statistical significance of p -value = 0.000979 and p -value = 0.001232 respectively for each strain compared to all other biotypes. At the 25°C incubation condition, the high pathogenic biotype lethal dose (LD₅₀) was similar to the effect of the other low pathogenic biotypes which logically should be higher (LD₅₀=7.6 and 8.7 Log₁₀c.f.u/ml).

The non-pathogenic strain 5303 also showed unexpected results and was the most lethal of all the biotypes used to infect the *Galleria* host model. Although the 5303 strain was the strain least able to invade the tissue culture cell line, it was the most virulent strain towards the *Galleria* larvae. The extraordinarily high lethal effect was detected (when the strain was pre-grown at both 25°C and 37°C) and also at both of the incubation temperatures used (25°C and 37°C) post-infection. The LD₅₀ values of strain 5303 at the *Galleria* 37°C incubation

were the lowest compared to all the LD₅₀ values of the other tested biotypes and showing a statistical significant of p -value < 0.00001 (when the 5303 strain was pre-grown at 25°C) and p -value = 0.000107 (when the 5303 strain was pre-grown at 37°C). Moreover, The LD₅₀ values of strain 5303 at the *Galleria* 25°C incubation also were the lowest compared to all the LD₅₀ values of the other tested biotypes and showing a statistical significant of p -value < 0.00001 for both 25°C and 37°C pre-growing temperatures of strain 5303. In addition, LD₅₀ results were always lower at the 25°C incubation conditions from the LD₅₀ values at 37°C incubation and the difference was statistically significant with a p -value = 0.0015. At 25°C incubation temperature, the lethal dose equalled about 1.4 Log₁₀ c.f.u/ml (or = 10^{1.1} c.f.u.) while at the 37°C incubation temperatures the lethal dose was 6.6 Log₁₀ c.f.u/ml (or = 10⁶ c.f.u) at both pre-growing temperatures. In addition, strain 5303 was the only strain that caused killing of the *Galleria* larvae in all the nine doses used at the 25°C incubation temperature by which the maximum number of the *Galleria* larvae was killed in a lethal time between 24 hours, for the first four highest doses, and 48 hours for the rest doses. In the same time and at the 37°C incubation temperature, the 5303 lethal time used to kill the maximum number of larvae was ranging from 24 hours to 96 hours post infection. These results generate a large debate about the pathogenicity of the non-pathogenic biotype 1A. The biotype 1A is classified as a non-pathogenic biotype since it doesn't cause any symptomatic disease in the mouse infection model in addition to the fact that it does not harbour any of the *Y. enterocolitica* virulence factors such as the pYV virulence plasmid (Tennant *et al.*, 2003). However, there is growing evidence that the non-pathogenic 1A biotype may be associated with human disease (McNally *et al.*, 2007; Tennant *et al.*, 2003). In addition, the non-pathogenic 1A biotype strains were the most commonly isolated strains from clinical cases in the United Kingdom (McNally *et al.*, 2004) and in other parts of Europe such as Finland (Huovinen *et al.*, 2010). Previous studies by McNally's group had

suggested that the non-pathogenic biotype 1A strains were able to infect and invade epithelial cells and macrophages *in vitro* causing cellular physiological changes in them (McNally *et al.*, 2006). The present results of the high virulence of the non-pathogenic biotype 1A towards the infection model *Galleria* further support potential virulence of these strains certainly in insect hosts, and maybe in other untested models.

Insecticidal toxin genes, called *tc* genes, which encode for an insect Toxin complex (Tc), have been found in all *Y. enterocolitica* low pathogenic biotypes strains but absent from the high pathogenic biotype 1B strains and the majority of the non-pathogenic known biotype 1A strains tested. The conservative clusters of these genes are carried on a specific chromosomal locus called the '*tc* pathogenicity island (*tc*-PAI^{Ye}) of *Y. enterocolitica*' (Fuchs *et al.*, 2008) which is present in all the low pathogenic biotypes 2-5 strains (Fuchs *et al.*, 2008). Among the 1A non-pathogenic biotype strains, only the previously studied T83 strain was found to include remnants of the *tc* genes with homology to the well described conservative *tc* genes in the *tc*-PAI^{Ye} (Bresolin *et al.*, 2005; Tennant *et al.*, 2005). The *tc* genes of the non-pathogenic T83 strain were highly expressed at low temperatures from 10-20°C and down-regulated at 37°C. However, these *tc* genes are completely absent from the non-pathogenic biotype BT1A lineage (Reuter *et al.*, 2014), so their role in strain 5303 virulence observed phenotype is impossible.

Bioserotype 4/O:3 is becoming the most predominant biotype of *Y. enterocolitica* species and it is the most isolated biotype from *Y. enterocolitica* disease cases in Europe and worldwide (Batzilla *et al.*, 2011a; Rosner *et al.*, 2010). Due to the high prevalence of the bioserotype 4/O:3, four strains including the German Y1 strain (Uliczka *et al.*, 2011) have been studied since infections with bioserotype 4/O:3 strains are very high in Germany (Rosner *et al.*, 2010). All strains of the bioserotype 4/O:3, that were pre-grown on both temperatures the 25°C and 37°C, were able to invade the HEp-2 human cell line in high numbers and in

constant ratios between the two pre-growing temperature. Interestingly, the 25°C pre-grown Y1 strain was a slightly more invasive than the other BT4 strains used. This highly invasive ability of the 4/O:3 biotype strains may support other studies which show the high incidence of this bioserotype in clinical isolates worldwide. In addition, 4/O:3 bioserotype have acquired a number of unique presumed virulence determinants including RtxA-like toxin, an insecticidal toxin, novel beta-lactamase -encoding GIYep-01 genomic island and the genetically active PhiYep-3 prophage. These newly characterized virulence determinants are believed to enhance the pathogenic potential of *Y. enterocolitica* 4/O:3 bioserotype making it more adaptable in hosts and consequently emerging as a worldwide spread isolate (Batzilla *et al.*, 2011a). Similarly, all the strains of the bioserotype 4/O:3 were lethal to the *Galleria* larvae. The bioserotype 4/O:3 strains killed the injected *Galleria* larvae at the highest $10^9, 10^8$ & 10^7 doses at all the pre-growing and incubating temperatures. Most of the 4/O:3 strains, which were pre-grown on both 25°C and 37°C temperatures, didn't show large differences in the LD₅₀ lethal doses between both incubation temperatures of the *Galleria* larvae and were statistically insignificant (*p* value = 0.174688). Exceptionally, the Y1 strain pre-grown at 37°C has shown more lethality among the 4/O:3 strains with a lower lethal dose values (LD₅₀) at the both incubation temperatures in comparison with its lethality when it was pre-grown at 25°C temperature but this difference is statically insignificant (*p*-value = 0.098). Moreover, although there was little difference in LD₅₀ results of the 4/O:3 biotype strains, they have displayed different survival results. In general, the 4/O:3 biotype strains has shown a time to death ranging from 24 hours to 72 hours at all pre-growing and incubation temperatures.

Moreover, two representative strains of the biotype 3, one strain belongs to serotype O:9 and one strain belongs to serotype O:5,27 were studied. Strain 5603 is a representative of the bioserotype 3/O:9 which is associated with human diseases (McNally *et al.*, 2004; Wang *et*

al, 2011). Strain 14902 represents the bioserotype 3/O:5,27 which is only isolated from animals in the UK and is never associated with human disease (McNally *et al.*, 2011). In tissue culture experiments, both strains were the highest invasive strains between all the biotypes used on both of the two pre-growing temperatures and in particular the human 5603 strain was the most invasive.

More Interestingly, the 14902 bioserotype 3/O:5,27, pre-grown on both 25°C and 37°C temperatures, was the most lethal strain to the *Galleria* larvae at the 25°C incubation temperatures among all the low pathogenic and high pathogenic biotypes and showing a statistical significance of a p -value = 0.0145. This is an unexpected result from an animal restricted strain since it managed to show a pathogenic potential towards an insect host model. The ability of the animal restricted bioserotype to infect other types of organisms may support the ubiquitous nature of *Y. enterocolitica*. The LD₅₀ at the 37°C incubation temperature was in the same range with the other low biotypes LD₅₀ results incubated at the same 37°C incubation temperatures. The high virulence of the 3/O:5,27 bioserotype strain at the 25°C temperatures may be linked to a virulence factors expressed at these low temperatures. The time of death caused by 3/O:9 strain was between 24 hours and 72 hours post infection at all the incubation temperatures. The time of death for the 14902 bioserotype 3/O:5,27 strain was shorter time between 24 hours and 48 hours post infection. This ability of rapid killing positively correlates with the low LD₅₀ results of this 14902 strain.

Biotype 2 strains are also implicated in causing human disease in particular strains belonging to the O:9 serotype (Rahman *et al.*, 2011). The bioserotype 2/O:9 strains are frequently isolated in many parts of the world (Rahman *et al.*, 2011) but they are less commonly isolated in the UK (McNally *et al.*, 2011). The pig isolate strain 21202 was used as a representative strain to the bioserotype 2/O:9. The low pathogenic bioserotype 2/O:9 21202 strain was highly invasive to the human epithelial cell line and the invasion levels were more close to

the invasion levels of the 4/O:3 bioserotype strains. Using the *Galleria* larvae infection, the 21202 strain pre-grown on 25°C and 37°C temperatures was more lethal to the *Galleria* larvae at the 25°C incubation temperatures of the infected larvae with a statistical significance of p -value = 0.47. In addition, the biotype 2 strain 21202 has shown more prolonged lethal time periods compared to the strains of the low pathogenic biotypes 3 and 4. The lethal time of killing the *Galleria* larvae with the 21202 strain was between 24 hours and 96 hours at both incubation temperatures. In addition, the lethal dose values (LD₅₀) of the bioserotype 2/O:9 strain were slightly higher than the biotypes 3 and 4 strains.

Although biotype 5 is classified as a low pathogenic biotype, it is extremely rare and has only ever been isolated from Hares (Iteman *et al.*, 1996). Strain 3094/96 was used as a representative to the low pathogenic biotype 5. Strain 3094/96 was the weakest strain to invade the HEp-2 cell line among the low and high pathogenic biotypes. In addition, the 3094/96 strain was also the weakest virulent strain among the low pathogenic and the non-pathogenic biotypes and it was showing a high LD₅₀ values with a statistical significance difference of p -value = 0.000761. As a result the 3094/96 strain can be considered as the second weakest strain towards *Galleria* larvae after the high pathogenic strain which was the weakest among all the biotypes used. The 3094/96 strain managed to kill a small number of larvae at the 37°C incubation temperatures by which the high pathogenic biotype strain 8081 didn't manage to kill any at this incubation temperature. In comparison, at the 25°C incubation temperature the high pathogenic 8081 strain was more lethal than the low pathogenic biotype 5 strain, but the biotype 5 strain 3094/96 could be considered to be more virulent since it managed to kill in both incubating temperatures.

Moreover, the T-test statistical analysis shows that there was no statistical significance difference between the LD₅₀ values of *Y. enterocolitica* strains at the 25°C and 37°C pre-growing temperatures (p -value = 0.22389 and p -value = 0.369193 respectively). This

indicates that the *Y. enterocolitica* pre-growing temperature doesn't have any effect on the *Y. enterocolitica* strains virulence in *Galleria*. On the other hand, there was a statistical significance difference between the LD₅₀ values of *Y. enterocolitica* strains at the 25°C and the 37°C incubation temperatures (p -value = 0.030845 and p -value = 0.038294 respectively) at both pre-growing temperatures. This indicates that the *Galleria* incubation temperature have an effect on the virulence of *Y. enterocolitica* strains in *Galleria*. The LD₅₀ values of *Y. enterocolitica* strains at 25°C incubation temperatures are lower from the LD₅₀ values of the same strains at 37°C incubation temperature which indicates that at the 25°C incubation temperatures *Y. enterocolitica* strains are more virulent in *Galleria*.

3.6 Conclusion

The HEp-2 epithelial cell line was used to study the pathogenicity of *Y. enterocolitica* biotypes. HEp-2 cell line is applied to study the virulence of many bacterial pathogens (Draganov *et al.*, 2008-2009). It has been observed from tissue culture results (Fig 3.1 & 3.2) that all *Y. enterocolitica* biotypes were capable to invade the HEp-2 cell line including the non-pathogenic biotype 1A representative strain 5303. However, the non-pathogenic 5303 representative strain was the least invasive to HEp-2 cell line (Fig 3.1 & 3.2).

The high pathogenic biotype which is historically known to be lethal in the mouse infection model was completely avirulent to *Galleria* larvae at 37°C incubation temperature which mimics the human normal physiological temperature. On the other hand, the non-pathogenic biotype 1A, which lacks all the classical virulence factors of the pathogenic biotypes and does not cause any symptomatic disease in animals or humans, was the most lethal biotype in the alternative infection model. All low pathogenic biotype representative strains caused death to *Galleria* host model. The low biotype representative strains display a diverse pathogenic potential to the infected *Galleria* larvae by which the low pathogenic biotype 5 representative strain 3094/96 was the less virulent towards *Galleria* host model. The High pathogenic biotype 1B/O:8 strain 8081 and the non-pathogenic biotype 1A/O:5 strain 5303 have shown opposing results between tissue culture and *Galleria* infections. The non-pathogenic strain 5303 was the least invasive strain to the HEp-2 cell line but it was the most pathogenic to *Galleria* alternative infection model. On the other hand, the high pathogenic strain 8081 was highly invasive to the HEp-2 cell line but was completely avirulent to the *Galleria* infection model at the 37°C incubation temperature.

Chapter 4

Diverse Pathogenic Potential and *in vivo* Interaction of *Y. enterocolitica* Biotypes in *Galleria*

4.1 Introduction

The previous chapter provided new results on the pathogenic potential of *Y. enterocolitica* biotypes in *Galleria*. The high pathogenic representative strain 8081 was completely avirulent to moderately pathogenic in *Galleria* depending on the incubation temperature used. The non-pathogenic representative strain 5303 was the most lethal in *Galleria* between all the *Y. enterocolitica* biotypes at all incubation conditions. On the other hand, the low pathogenic biotypes were all mildly virulent to *Galleria*. These results show that both the non-pathogenic biotype and the high pathogenic biotype show a distinct pathogenic profile from the low pathogenic biotypes. This distinctive pathogenic profile can be linked to the phylogenetic distribution of *Y. enterocolitica* biotypes when both of the high pathogenic BT 1B and the non-pathogenic BT 1A were clustered in a separate species cluster (SC6) while all the low pathogenic biotypes were clustered in a the SC7 cluster (Reuter *et al.*, 2014). The results obtained in the previous chapter were observed from a limited number of *Y. enterocolitica* representative strains. Here the data were tested against a wider set of strains to validate the previous results. In addition, the *in vivo* behaviour of *Y. enterocolitica* strains inside the *Galleria* larvae was investigated. Moreover, the virulence effect of heat killed *Y. enterocolitica* strains in *Galleria* larvae was also investigated. Heat-killed *Y. enterocolitica* have been found to stimulate an immune response in the murine infection model and this response was believed to be due to the presence of outer membrane proteins and mainly LPS (Ruiz-Bravo *et al.*, 2003). The *Galleria* response between live and killed *Y. enterocolitica* cells is going to be tested here. Here, an evidence showing that *Galleria* larvae are dying from an active process or from an outer membrane antigen is needed to be provided.

4.2 Aims:

- a) To study the pathogenic potential of diverse *Y. enterocolitica* strains, from different human and animal origins and belonging to a diverse group of biotypes and serotypes, in the *G. mellonella* alternative infection model.
- b) To investigate the virulence potential of heat killed *Y. enterocolitica* wild type strains in the *G. mellonella* insect infection model.
- c) To study the *in vivo* infection dynamics of the *Y. enterocolitica* High- Low- and Non-pathogenic biotypes in the *G. mellonella* alternative infection model.

4.3 Materials and Methods

4.3.1 *Y. enterocolitica* culture and *Galleria* infection procedures:

All *Y. enterocolitica* strains studied in this chapter are listed in table 4.1. The strains used to infect *Galleria* belong to the low pathogenic bioserotype 4/O:3, the low pathogenic bioserotype 3/O:5,27, the non-pathogenic biotype 1A and to serotype O:9. Five *Y. enterocolitica* strains were tested for each of the bioserotype 3/O:5,27, biotype 1A and serotype O:9. Eight strains of the bioserotype 4/O:3, half of human origin and half of non-human origin were tested. All *Y. enterocolitica* strains were pre-grown at 25°C temperature in LB agar and LB broth media as described in chapter two section 2.1. The *Galleria* larvae were injected with the tested *Y. enterocolitica* strains following the same injection procedure described in chapter two section 2.2.4. The injected *Galleria* larvae were incubated and monitored using the same incubation and monitoring protocols described in chapter two section 2.2.5. Median lethal doses and survival curves were calculated following procedures described in chapter two sections 2.2.6 & 2.2.7.

Table 4.1: Bacterial strains used in chapter 4

| No. | Strain Name | Bio/Serotype | Biological Origin | Source |
|-----|-------------|--------------|-------------------|---|
| 1 | 0902 | 1A/O?* | Sheep | McNally, UK |
| 2 | 0903 | 1A/O:6,30 | Human | McNally, UK |
| 3 | 3403 | 1A/O:5 | Human | McNally, UK |
| 4 | 3503 | 1A/O?* | Pig | McNally, UK |
| 5 | NZ3 | 1A/O?* | Sheep | Dr. Muriel Dufour, ESR NCBID, Australia |
| 6 | 119/02 | 2/O:9 | Sheep | McNally, UK |
| 7 | 215/02 | 3/O:9 | Pig | McNally, UK |
| 8 | 218/02 | 3/O:9 | Pig | McNally, UK |
| 9 | 2403 | 3/O:9 | Human | McNally, UK |
| 10 | 5803 | 3/O:9 | Human | McNally, UK |
| 11 | 111/02 | 3/O:5,27 | Sheep | McNally, UK |
| 12 | 153/02 | 3/O:5,27 | Cattle | McNally, UK |

| | | | | |
|----|---|----------|---------|--|
| 13 | 226/02 | 3/O:5,27 | Pig | McNally, UK |
| 14 | 231/02 | 3/O:5,27 | Pig | McNally, UK |
| 15 | 232/02 | 3/O:5,27 | Pig | McNally, UK |
| 16 | 01/2012 | 4/O:3 | Human | Claire Jenkins HPA,2012 |
| 17 | 02/2012 | 4/O:3 | Human | Claire Jenkins HPA,2012 |
| 18 | 03/03 | 4/O:3 | Human | McNally, UK |
| 19 | 201/02 | 4/O:3 | Pig | McNally, UK |
| 20 | 204/02 | 4/O:3 | Human | McNally, UK |
| 21 | 213/02 | 4/O:3 | Pig | McNally, UK |
| 22 | NZ15 | 4/O:3 | Unknown | Dr. Muriel Dufour, ESR NCBID, Australia |
| 23 | Y1 | 4/O:3 | Human | Petra Dersch, 2009 |
| 24 | <i>E. coli</i> DH5 α | | Unknown | Invitrogen |
| 25 | 14902/pAJD434 | 3/O:5,27 | Sheep | This Study |
| 26 | 1203/pAJD434 | 4/O:3 | Human | This Study |
| 27 | 5303/pAJD434 | 1A/O:5 | Human | This Study |
| 28 | 21202/pAJD434 | 2/O:9 | Pig | This Study |
| 29 | 8081/pAJD434 | 1B/O:8 | Human | This Study |
| 30 | <i>E. coli</i> DH5 α /pAJD434 | | Unknown | This Study |

*?: Non-typable serotype

4.3.2 Heat Killed *Y. enterocolitica*:

The *Y. enterocolitica* representative strains 8081, 1203, 14902, 5603 & 5303 were pre-grown on LB agar and in LB broth medium at 25°C as shown in chapter two (section 2.1). The overnight LB broth cultures were heat killed in water bath preheated at 60°C for 1hour (Autenrieth *et al.*, 1994). The O.D values were measured in a spectrophotometer at 600nm absorption. The O.D₆₀₀ values for each strain were measured in triplicate before and after the heat killing procedure to compare the concentration of the cellular material. The heat killed *Y. enterocolitica* strains were serially diluted and plated on LB agar plates using the Miles and Misra method to confirm complete cell killing.

4.3.3 Infection Dynamics

4.3.3.1 Preparation of *Y. enterocolitica* electro-competent cells

1 ml of overnight bacterial broth culture was used to inoculate 49 ml fresh LB broth culture in 1:50 dilution. The diluted bacterial suspension was then incubated at 25°C with shaking at 200 rpm until the OD₆₀₀ value reaches a range between 0.6 and 0.9. Bacterial suspension was then aseptically transferred to a 50ml pre-chilled falcon tube and left in ice for 5 minutes. Then bacterial cells were pelleted by centrifugation at 4,000 rpm for 10 minutes at 4°C. The supernatant was discarded and pellet was then gently suspended in 25ml of ice cold 10% glycerol. Bacterial cells were then harvested as above and re-suspended in 12.5 ml of ice cold 10% glycerol. The Bacterial suspension was then statically incubated in ice for 1 hour. Lastly, the *Y. enterocolitica* bacterial suspension was harvested again and re-suspended in 2ml of 10% glycerol. 50µl aliquots of bacterial suspension were then transferred into 1.5ml microcentrifuge tubes and were reserved in -80°C freezer for future work. *E. coli* DH5α strain was used as a control for the electro-competent cells preparation procedure.

4.3.3.2 Transformation of *Y. enterocolitica* electro-competent cells

1µl of pAJD434 plasmid was added into 50µl aliquot of *Y. enterocolitica* competent cells and the *E. coli* DH5α competent cells, mixed by gentle pipetting, and incubated on ice for 10 minutes. The mixture was then aseptically transferred to a 2ml chilled electroporation cuvette (Flowgen, UK). The electroporation was done using the GenePulser Xcell electroporator system (Bio-Rad) and the electroporation programme was set out at 2.5kV, 2.5µF, 200Ω. Immediately after electroporation 950µl of sterile SOC medium was added to the electro-transformed bacterial cells, mixed gently, and the suspension was then transferred to a 1.5ml microcentrifuge tube. The reaction was incubated for 2 hours at 25°C with shaking at 200rpm. 400µl of the electro-transformed bacterial cells were plated on pre-warmed LB agar

plate with 100µg/ml Trimethoprim antibiotic. Plates were then incubated overnight at 25°C. Transformation of the pAJD434 plasmid was confirmed by plasmid purification and by PCR. The *E. coli* DH5α strain was used as a control for the efficiency of electroporation.

4.3.3.3 pAJD434 plasmid extraction:

1-5 colonies were picked from the overnight agar cultures of each of the electro-transformed *Y. enterocolitica* strains including the *E. coli* DH5α control strain and were used to inoculate 5 ml of LB broth containing 100 µg/ml of Trimethoprim. The broth culture was incubated aerobically in shaker incubator on 200rpm at 25°C for overnight and not exceeding 18 hours incubation time. Next day, 1.5 ml aliquotes of overnight broth culture were used for plasmid extraction. Plasmid extraction was done by the manufacturer protocol supplied with the Qiagen miniprep (QIAGEN). The extracted plasmid was then visualised on agarose gel.

4.3.3.4 PCR procedure: The pAJD434 plasmid was further confirmed by PCR amplification targeting the trimethoprim gene located on the pAJD434 plasmid. The trimethoprim gene was amplified using primers listed in Table 4.1. PCR reaction and condition were carried out as described in parts 2.2.8 and 2.2.9 described in chapter 2 except the annealing temperature which was adjusted depending on the primers annealing temperature described in Table 4.1. PCR products were visualized on agarose gel electrophoresis as described in part 2.2.10 in chapter 2.

Table 4.2: PCR primers

| Primer Name | Nucleotide Sequence | Primer Function | Annealing Temperature (°C) | Replication size (bp) |
|-------------|-------------------------------|---|----------------------------|-----------------------|
| pAJD5' | 5'-CTG CAC TCG CAA CGC TG-3' | Checking for the presence of the pAJD434 by targeting the Trimethoprim gene | 54 | ~300 |
| pAJD3' | 5'-GGC ATG TAC AGG ATT CAT-3' | | | |

4.3.3.5 Strain preparation and growth conditions: *Galleria* larvae were infected with 5 *Y. enterocolitica* strains containing pAJD434 plasmid. Strains YE8081/pAJD434, YE1203/pAJD434, YE14902/pAJD434, YE5303/pAJD434 and YE21202/pAJD434 are Trimethoprim resistance due to the presence of Trimethoprim gene harboured on the pAJD434 plasmid. Trimethoprim *Y. enterocolitica* resistance strains were grown on LB agar plates and LB broth media containing 100µg/ml Trimethoprim using the same procedure described in chapter two (section 2.1). The cultured YE8081/pAJD434, YE1203/pAJD434, YE14902/pAJD434 and YE21202/pAJD434 strains were incubated at 25°C while YE5303/pAJD434 strain was incubated at 37°C. Cells from 1ml overnight broth cultures for each strain were harvested by centrifugation and the pellets were suspended in 1ml sterile PBS. The cells were serially diluted in sterile PBS and counted using Miles & Misra (Hedges, 2002; Miles and Misra, 1938) and CFU/ml methods to determine the initial dose for each strain.

4.3.3.6 Infection and bacterial cells recovery: Bacterial lethal and non-lethal doses were selected from each of the reference strains listed in table 4.2. A number of 60 *Galleria* larvae

were injected with each selected lethal and non-lethal dose (total of 120 injected larvae per strain). The selected lethal and non-lethal doses were injected in *Galleria* larvae in 10µl microinjected doses using the same injection procedure described in chapter two section (2.2.4) and all the injected larvae were incubated using the same procedure described in chapter two part (2.2.5). Bacterial cells were recovered from the infected larvae for a total period of 5 days at the following time points; 2h,4h,6h,8h,24h,48h,72h,96h & 120h. At each time point, bacterial cells were isolated from a pool of 5 larvae for each selected lethal and non-lethal dose (total of 10 larvae for each strain). Bacterial cells were recovered from each *Galleria* larva by dissecting the bottom section of the larva. Prior to dissection, all the infected larvae were placed in ice for 20 minutes. After incubation on ice, each larva was surface sterilised with 70% ethanol. Approximately 2mm from the tail side of each *Galleria* larva was removed using sterile scissors and each larva was placed inside sterile eppendorf tube containing 0.5ml of sterile PBS. The total internal content of each larva was drained inside the eppendorf tube using sterile forceps. The scissors and forceps were sterilised between each larva dissecting procedure with 70% ethanol and sterile distilled water (sdH₂O). In addition, after each time point the scissors and forceps were sterilised with 70% ethanol and sterile distilled water (sdH₂O) followed by flame sterilization using a Bunsen burner. The drained contents were serially diluted and the recovered bacterial cells were enumerated on LB agar plates containing 100µg/ml Trimethoprim using the Miles & Misra (Hedges, 2002; Miles and Misra, 1938) and CFU counting methods. All experiments were repeated independently in triplicate. PBS and non-injection controls were used in each separate experiment.

4.3.3.7 Statistical analysis

The statistical work done in this chapter was done using the one sample T-test and the two independent samples T-test . All the statistical analysis was done by a statistical significance with a P value of ≤ 0.05 .

4.4 Results

4.4.1 Results of the pathogenic effect of the *Y. enterocolitica* diverse strains

Twenty three *Y. enterocolitica* strains were tested for their pathogenesis in the *Galleria* alternative infection model. All the 23 strains were pre-grown at 25°C and were injected in *Galleria* larvae followed by incubating the infected larvae at 37°C. Twenty one of the tested *Y. enterocolitica* strains were able to kill the infected *Galleria* larvae with the remaining two strains unable to induce any sort of virulence in *Galleria*. The results of the median lethal dose and survival time curve of each strain are represented below.

4.4.1.1 Median Lethal Dose (LD₅₀) Results:

All median lethal doses (LD₅₀) of the 23 *Y. enterocolitica* strains are presented in figure 4.2. These strains belong to biotype 1A, bioserotype 3/O:5,27, bioserotype 4/O:3 and serotype O:9. Twenty one strains managed to kill the infected *Galleria* larvae. Only 2 strains were avirulent to the infected larvae which are the animal origin strain 119/02 (bioserotype 2/O:9) and the non-human origin strain NZ15 (bioserotype 4/O:3). The LD₅₀ values of biotype 1A strains are ranging from 7.09 to 8.99 with an average LD₅₀ of 7.83. When comparing the LD₅₀ values of the biotype 1A diverse strains they show higher LD₅₀ results than the reference strain 5303 of bioserotype 1A/O:5 which has a LD₅₀=6.6 and showing a statistical significance of p -value = 0.022. However, The *Y. enterocolitica* non-pathogenic biotype 1A

strains group is showing a significance difference from all the other *Y. enterocolitica* biotypes strains with a p -value of 0.003148. This result shows that the non-pathogenic biotype 1A strains are the most virulent in *Galleria* compared to all the other *Y. enterocolitica* biotypes strains. Moreover, LD₅₀ values of bioserotype 3/O:5,27 strains are ranging from 8.1 to 8.9 and the average LD₅₀ of this bioserotype is 8.6. The reference strain LD₅₀ value is 8.8 and it exists in the same range of the bioserotype 3/O:5,27 diverse strains and was statistically insignificant with a p -value = 0.877173. Furthermore, strains of serotype O:9 belong to biotypes 2 and 3. Strain 204/02 was the only strain belongs to biotype 2 serotype O:9. The remaining strains belongs to biotype 3 serotype O:9 and these strains are 5803, 24/03, 215/02 and 218/02. The LD₅₀ values of serotype O:9 ranges from 8.55 to 9.82 and the average LD₅₀ value is 8.934. The range of the LD₅₀ values of serotype O:9 diverse strains is similar to the LD₅₀ values of serotype O:9 reference strains which are strain 5603 (bioserotype 3/O:9) and strain 21202 (bioserotype 2/O:9) by which these two reference strains have LD₅₀ values of 8.5 and 8.8 respectively. Statistical analysis shows that there is no statistical significant difference between the LD₅₀ values of serotype O:9 diverse strains and the LD₅₀ value of the reference strain 5603 (bioserotype 3/O:9) giving a p -value = 0.167. Statistical analysis also shows that there is no statistical significant difference between the LD₅₀ values of serotype O:9 diverse strains and the LD₅₀ value of the reference strain 21202 (bioserotype 2/O:9) and giving a p -value = 0.179. Finally, strains of bioserotype 4/O:3 were divided into human origin and non-human origin strains. The human origin strains are Y1, 01/2012, 02/2012 and 03/03. On the other hand, the non-human bioserotype 4/O:3 strains are 204/02, NZ15, 213/02 and 201/02. The LD₅₀ values of bioserotype 4/O:3 human origin strains are ranging from 7.8 to 8.6 with an average LD₅₀ value of 8.4. On the other hand, LD₅₀ values of bioserotype 4/O:3 non-human origin strains are ranging from 8.48 to 9.85 with an average LD₅₀ value of 9.06. When comparing the LD₅₀ values of the human origin bioserotype 4/O:3

strains with LD₅₀ values from the non-human origin strains, the difference between them was statistically insignificant (p -value = 0.181006). This indicates that both of the human origin and non-human origin bioserotype 4/O:3 strains are behaving to some extent similarly and may be causing a similar virulence levels in *Galleria*. The representative strain 1203 (bioserotype 4/O:3) has LD₅₀ value of 8.9 which is higher than most of the bioserotype 4/O:3 strains. However, when statistically comparing the LD₅₀ value of the representative strain 1203 (bioserotype 4/O:3) with LD₅₀ values of bioserotype 4/O:3 human origin strains the result was statistically insignificant with a p -value = 0.106. In contrast, the LD₅₀ value of the representative strain 1203 (bioserotype 4/O:3) was statistically compared to the LD₅₀ values of bioserotype 4/O:3 non-human origin strains and shown statistical insignificance with a p -value = 0.474. This statistical measurement indicates that all the 4/O:3 strains are behaving similarly to the reference strain 1203. Although there was no significance difference between the reference strain 1203 LD₅₀ value and the LD₅₀ values of both the human origin and non-human origin bioserotype 4/O:3 strains the p -value of comparing the reference strain 1203 to the human origin strains was lower (p -value = 0.106) compared to the non-human origin bioserotype 4/O:3 (p -value = 0.474). This indicates that the bioserotype 4/O:3 human origin strains are statistically behaving more similarly to the reference strain 1203 in *Galleria* by which the reference strain 1203 is a human origin strain as well.

- LD₅₀ results of infecting *Galleria mellonella* larvae with 23 *Y. enterocolitica* diverse strains

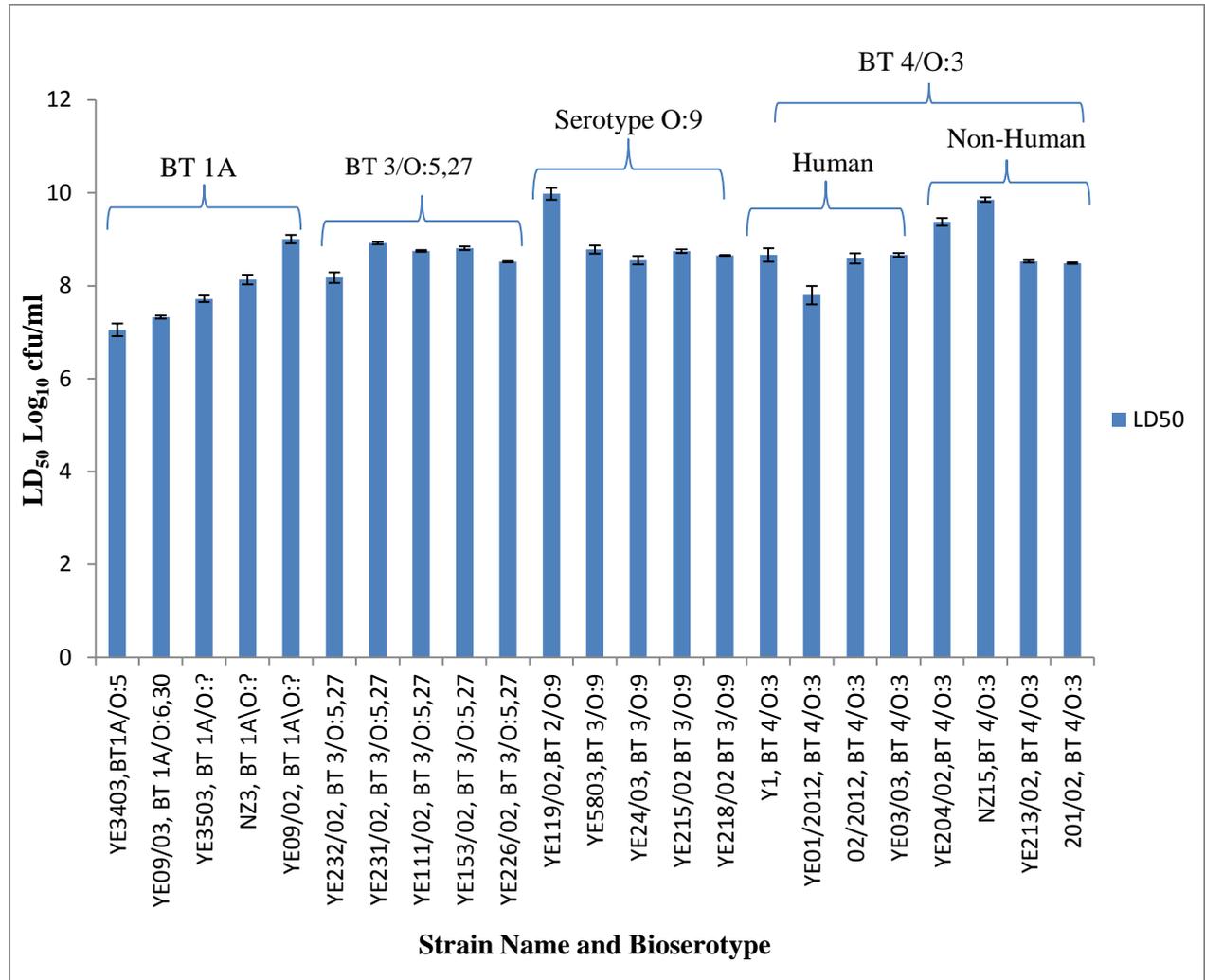


Figure 4.1: LD₅₀ results of infecting *Galleria mellonella* larvae with 23 *Y. enterocolitica* strains pre-grown at 25°C. These strains belong to biotype 1A, bioserotype 3/O:5,27, serotype O:9 and bioserotype 4/O:3. Bioserotype 4/O:3 strains are sub divided into human origin and non-human origin strains. *Y. enterocolitica* strains were injected into *Galleria* larvae and the infected larvae were incubated at 37°C temperatures. Values in the graph shows the mean of 3 independent experiments. The LD₅₀ values were calculated using the Spearman-Kärber method. All LD₅₀ numbers were calculated using Log₁₀ c.f.u/ml. The x-axis shows strains names and the y-axis shows the LD₅₀ values in Log₁₀ c.f.u/ml. Error bars were plotted after calculating the standard deviation of the mean.

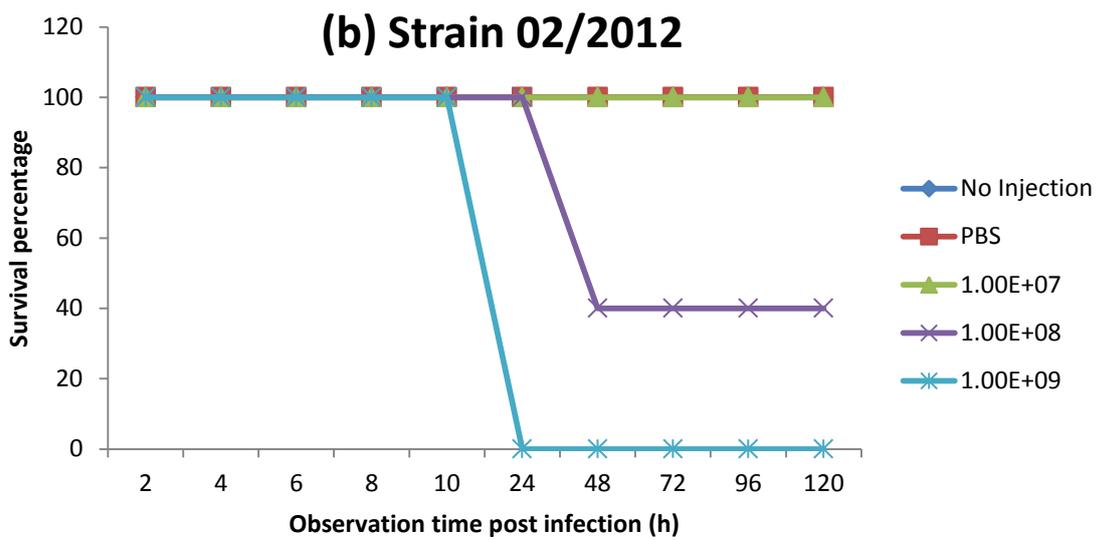
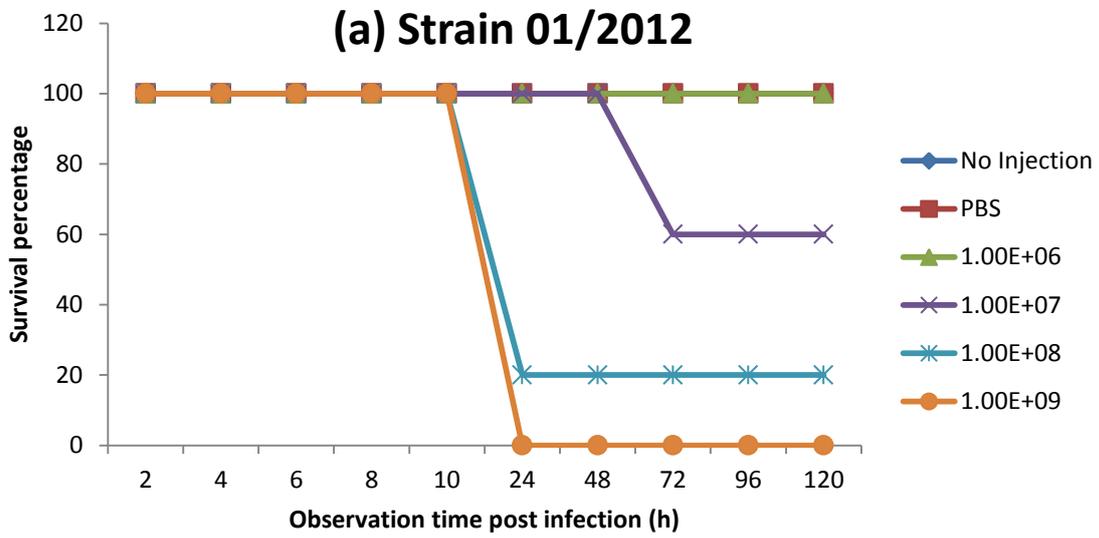
4.4.1.2 Survival Curves

4.4.1.2.1 Bioserotype 4/O:3 Strains

A) Strains of Human Origin:

Section 4.4.1.2.1 (A) Includes the survival results of four strains of the low pathogenic bioserotype 4/O:3 which were isolated from human origin 01/2012,02/2012,Y1 & 03/03. All four strains caused death to the infected *Galleria* larvae. Strain 01/2012 caused death to *Galleria* larvae with doses 10^9 , 10^8 & 10^7 (Fig. 4.2a). Dose 10^9 killed all injected larvae within 24h post infection while the dose 10^8 killed 4 of the infected *Galleria* larvae at 24h post infection (20% survival). The 10^7 dose was lethal for only 2 larvae (60% survival) at the 72h time of observation post infection. Moreover, strain 02/2012 caused killing in the infected *Galleria* larvae with 10^9 and 10^8 doses (Fig. 4.2b). Dose 10^9 killed all the injected *Galleria* larvae after 24h post infection and the 10^8 dose killed 3 out of 5 larvae (40% survival) after 48h post infection. Strain Y1 strain is showing mortality to *Galleria* larvae by which only the highest doses 10^9 and 10^8 were lethal to the *Galleria* larvae (Fig. 4.2c). Dose 10^9 killed all the infected larvae within 24h post infection while the 10^8 dose was lethal to only one larva (80% survival) at 48h post infection. The last strain in this section is strain 03/03. Strain 03/03 caused death to the infected *Galleria* larvae with only the highest 10^9 dose (Fig. 4.2d) by which this dose killed all the injected larvae within 24h post infection.

- Survival Curves of *Galleria* infected with Bioserotype 4/O:3 Strains from Human Origin



- Survival Curves of *Galleria* infected with Bioserotype 4/O:3 Strains from Human Origin

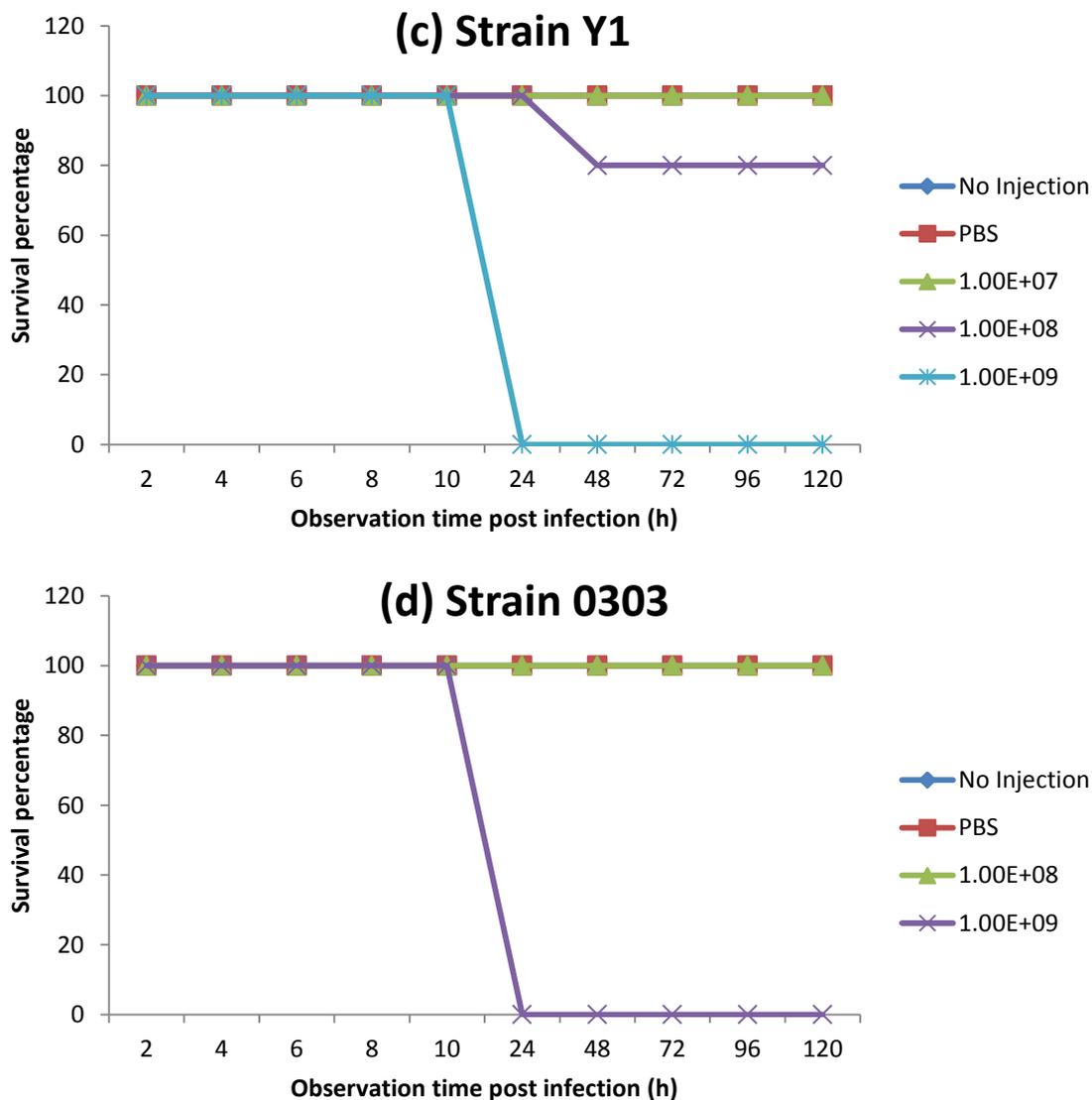
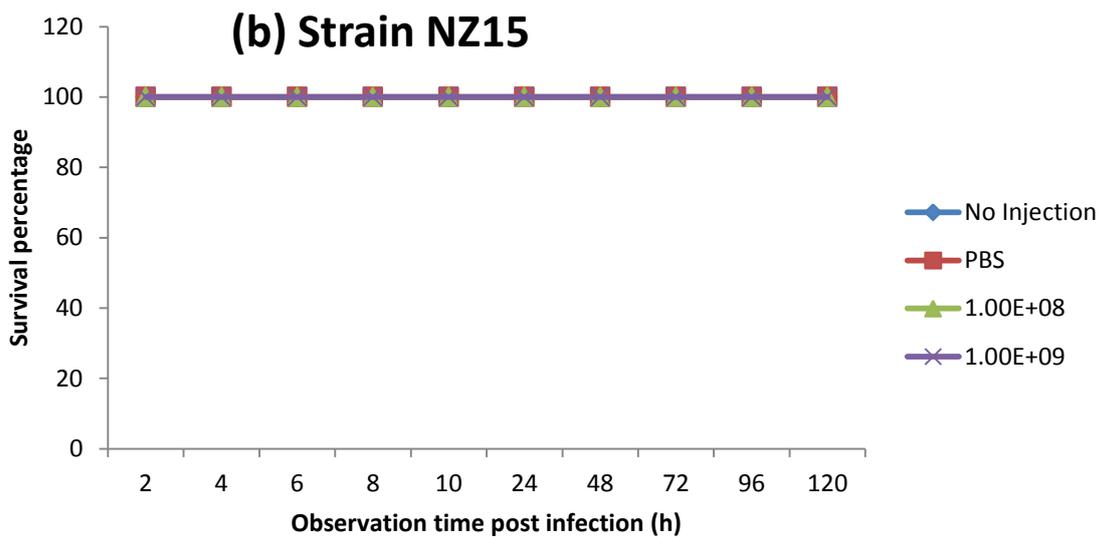
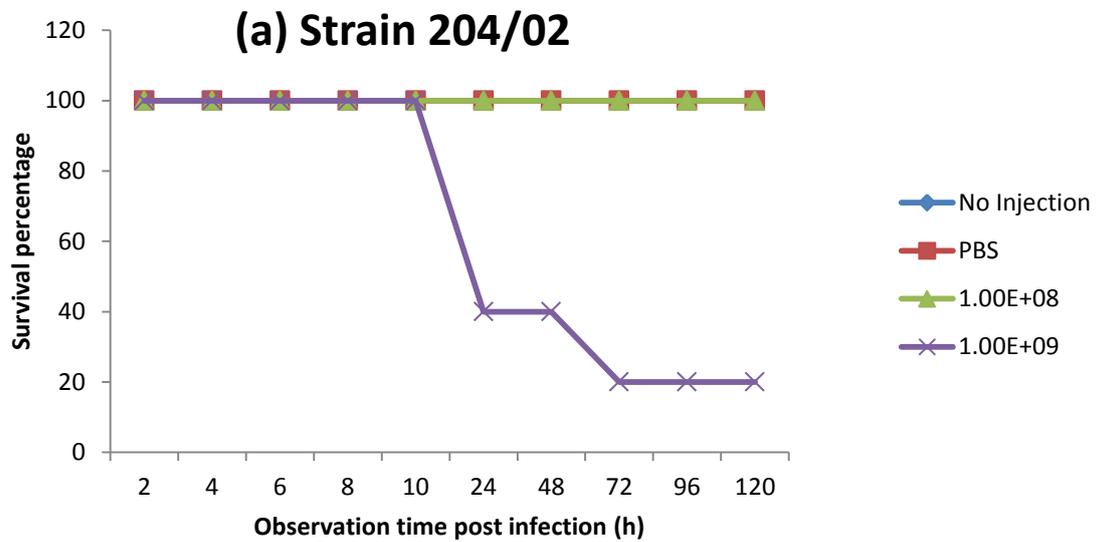


Figure 4.2: Survival curves of *Galleria* infected with BT 4/O:3 human origin strains pre-grown at 25°C or 37°C incubated *Galleria*. Figure 4.2 includes four sub figures (4.2a, 4.2b, 4.2c & 4.2d) representing the survival of *Galleria* larvae at 37°C incubation temperature infected with the 25°C pre-grown *Y. enterocolitica* human origin strains belonging to the low pathogenic bio/serotype 4/O:3. A number of 5 larvae were used for each dose. Figure 4.2a shows representative survival data of *Galleria* infected with strain 01/2012. Figure 4.2b shows representative survival data of *Galleria* infected with strain 02/2012. Figure 4.2c shows representative survival data of *Galleria* infected with strain Y1. Figure 4.2d shows representative survival data of *Galleria* infected with strain 03/03. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

B) Non-Human Origin 4/O:3 Strains:

Section 4.4.1.2.1 (B) includes the survival results of the low pathogenic bioserotype 4/O:3 strains belong to origins other than the human origin. This section includes strains from an animal origin or with an unknown origin. Strain 204/02 isolated from pigs caused death in *Galleria* larvae with only the highest dose 10^9 by which a maximum number of 4 *Galleria* killed at 72 h post infection (20% survival) (Fig. 4.3a). On the other hand, strain NZ15 was completely avirulent to the *Galleria* injected larvae (Fig. 4.3b). This strain is from unknown origin and all doses injected in the *Galleria* larvae didn't cause any death. Furthermore, strains 213/02 (Fig. 4.3c) and 201/02 (Fig. 4.3d) are both isolated from pigs and they caused similar killing patterns in the infected *Galleria* larvae. Doses 10^9 and 10^8 from both 213/02 and 201/02 strains caused death to *Galleria* larvae. 10^9 killed all the *Galleria* larvae after 24h post infection in both strains. Dose 10^8 killed 2 larvae out of 5 (60% survival) after 24h post infection also in both strains. This indicates that the highest doses 10^9 and 10^8 of strains 213/02 and 201/02 killed the same numbers of *Galleria* larvae with the same time of death. Compared to the 1203 reference strain (bioserotype 4/O:3) only the human origin strain 01/2012 killed the *Galleria* larvae with a similar doses similar to the 1203 reference strain and these doses are 10^9 , 10^8 and 10^7 . Four of the strains were able to kill the *Galleria* larvae with the 10^9 and 10^8 doses and two of them were killing the *Galleria* larvae with only the 10^9 compared to the 1203 reference strain. Only the strain NZ15 was avirulent to the injected *Galleria* larvae.

-Survival Curves of *Galleria* infected with Bioserotype 4/O:3 Strains from Non-Human Origin



- Survival Curves of *Galleria* infected with Bioserotype 4/O:3 Strains from Non-Human Origin

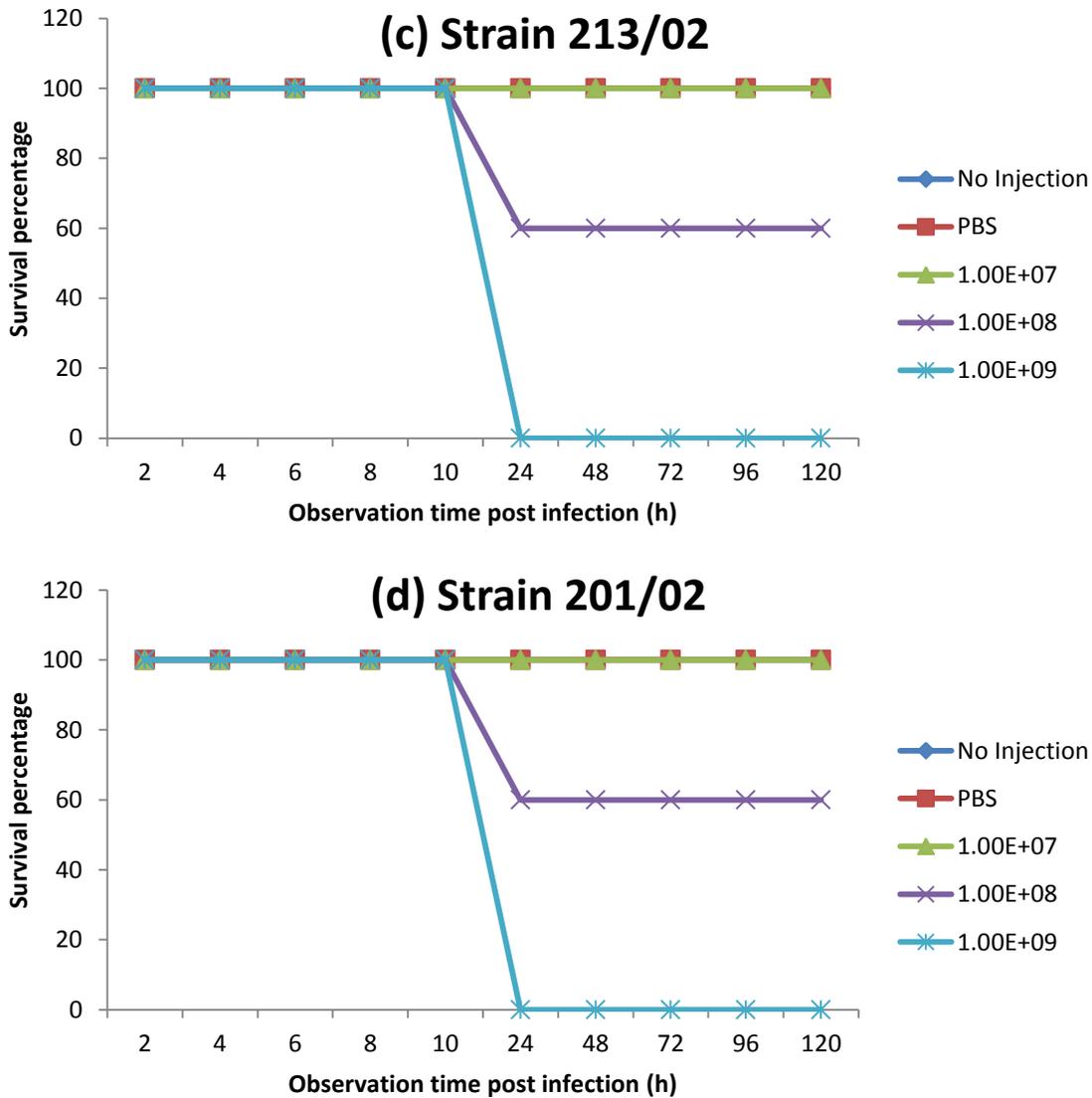
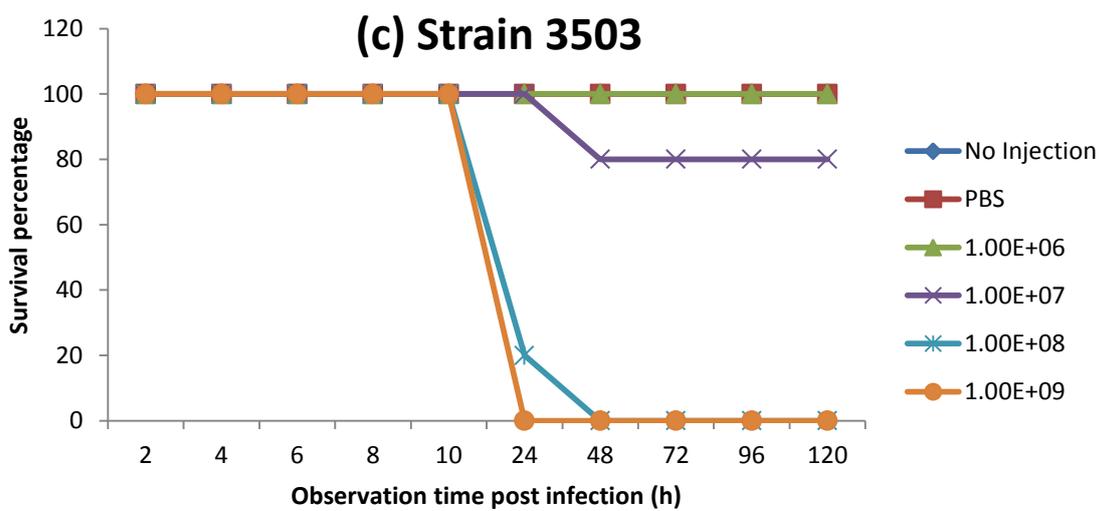
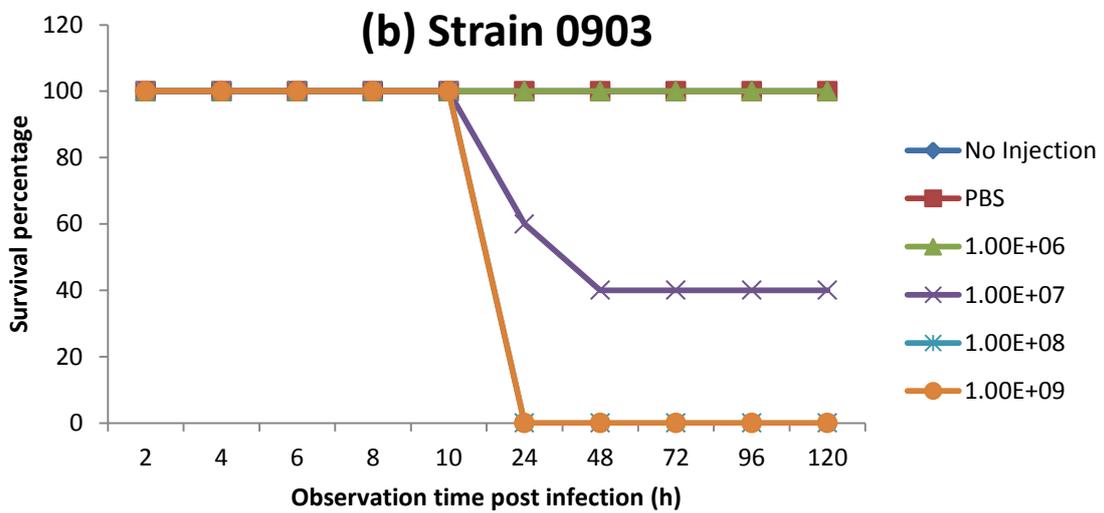
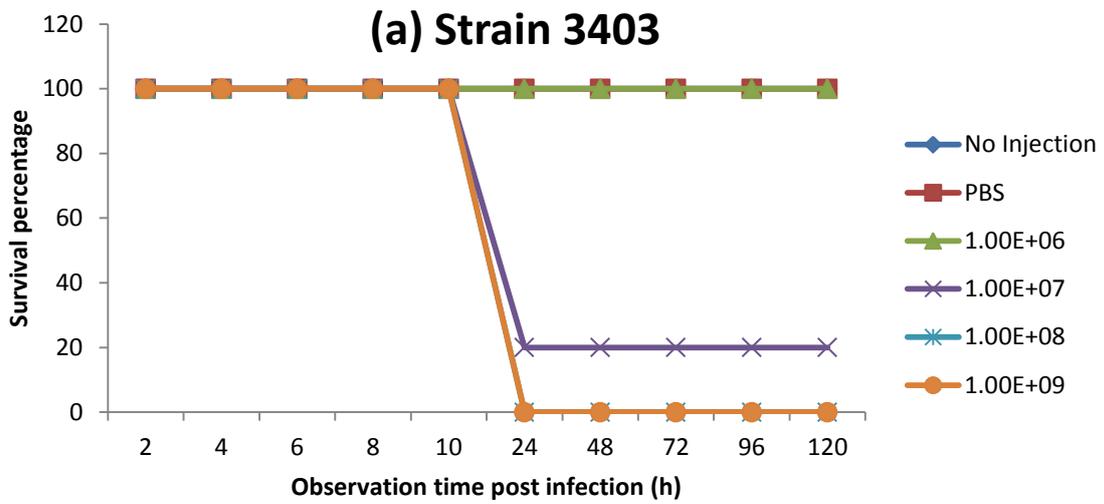


Figure 4.3: Survival curves of *Galleria* infected with BT 4/O:3 human origin strains pre-grown at 25°C or 37°C incubated *Galleria*. Figure 4.3 includes four sub figures (4.3a, 4.3b, 4.3c & 4.3d) representing the survival of *Galleria* at 37°C incubation temperature infected with the 25°C pre-grown non-human origin strains belonging to bio/serotype 4/O:3. A number of 5 larvae were used for each dose. Figure 4.3a shows representative survival of *Galleria* infected with strain 204/02. Figure 4.3b shows representative survival of *Galleria* infected with strain NZ15. Figure 4.3c shows representative survival of *Galleria* infected with strain 213/02. Figure 4.3d shows representative survival of *Galleria* infected with strain 201/02. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

4.4.1.2.2 Survival curves of *Galleria* infected with biotype 1A strains

Section 4.4.1.2.2 describes the survival results of strains belonging to the non-pathogenic biotype 1A. The survival of five strains in was studied in this section, strain 3403 (Fig. 4.4a), strain 0903 (Fig. 4.4b), strain 3503 (Fig. 4.4c), strain NZ3 (Fig. 4.4d) and strain 0902 (Fig. 4.4e). These strains were isolated from different origins and they belong to different serotypes and all of them caused death in the infected *Galleria*. Strain 3403 is isolated from humans and belongs to serotype O:5. Doses 10^9 , 10^8 and 10^7 from this strain caused death to the infected *Galleria* larvae. Both 10^9 and 10^8 doses killed all the infected larvae at 24h post infection (in both doses) while dose 10^7 killed 4 larvae (20% survival) within 24h post infection (Fig. 4.4a). Strain 0903 is a human isolate and it belongs to bioserotype O:6,30. 0903 strain killed the *Galleria* larvae with doses 10^9 , 10^8 & 10^7 . Doses 10^9 and 10^8 killed all the infected *Galleria* larvae at 24h post infection while dose 10^7 killed 3 larvae (40% survival) within 48h post infection (Fig. 4.4b). Strain 3503 is isolated from pigs but the serotype of this strain is unknown (O:?). Strain 3503 caused death to the infected *Galleria* larvae with doses 10^9 , 10^8 & 10^7 . Both 10^9 and 10^8 doses killed all the infected *Galleria* at 24h and 48h post infection respectively for each dose. The 10^7 dose killed only one *Galleria* larva at 48h post infection (Fig. 4.4c). In addition, strain NZ3 was isolated from sheep and it belongs to an unknown serotype. Strain NZ3 caused death to *Galleria* with doses 10^9 and 10^8 . 10^9 killed all the *Galleria* larvae within 24h post infection while the 10^8 dose killed 3 out of 5 larvae (40% survival) after 96h post infection (Fig. 4.4e). Strain 0902 is the last strain studied from the biotype 1A strains. Similar to strain NZ3, strain 0902 is also isolated from sheep and belongs to unknown serotype. Doses 10^9 and 10^8 from strain NZ3 was able to kill the *Galleria* larvae. Dose 10^9 killed 4 larvae out of 5 (20% survival) post infection while the dose 10^8 killed only 1 larvae (80% survival) post infection (Fig. 4.4e). The last results are similar to what previously observed in the 5303 reference strain.

- Survival curves of *Galleria* infected with biotype 1A strains



- Survival curves of *Galleria* infected with biotype 1A strains

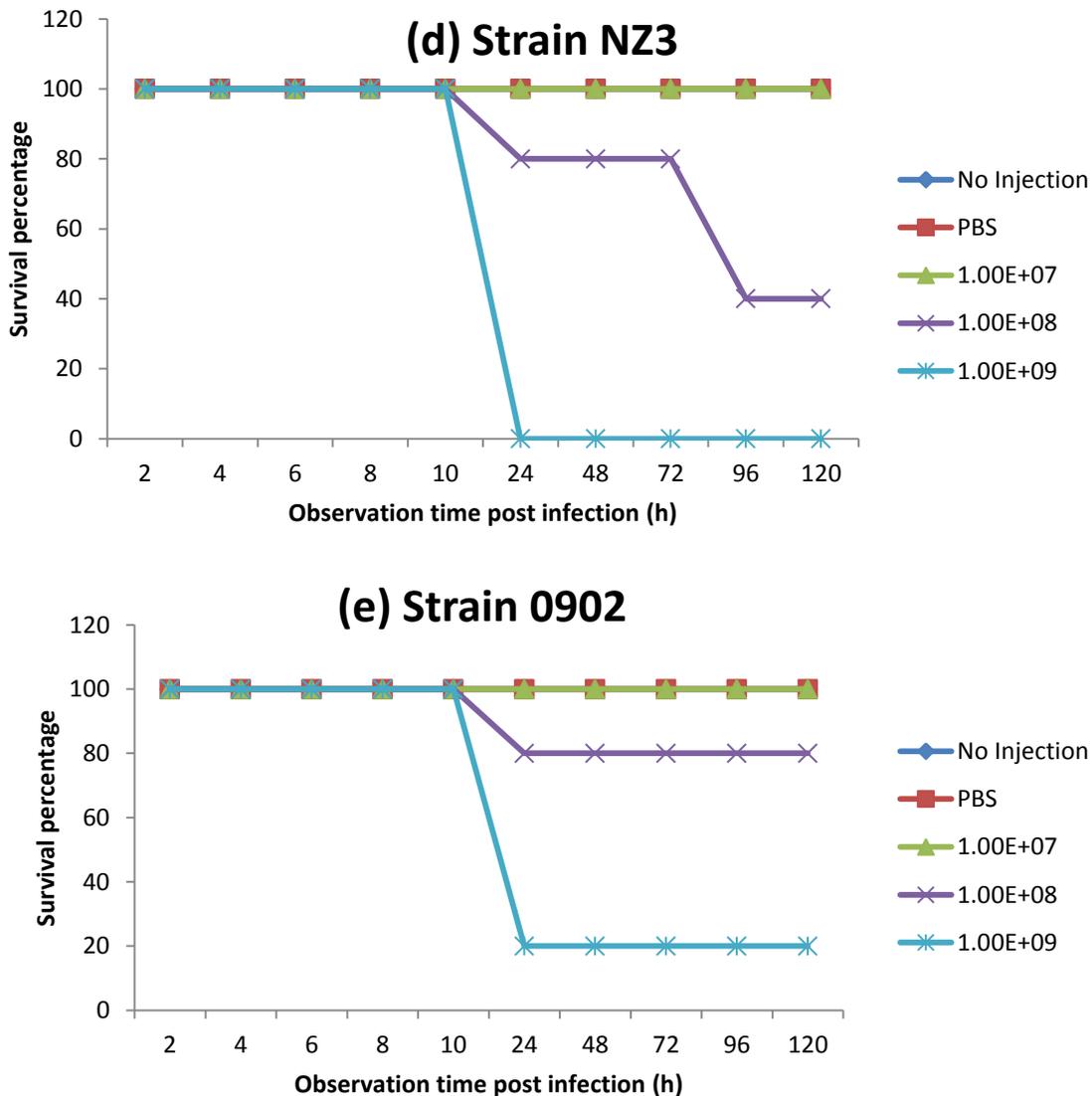
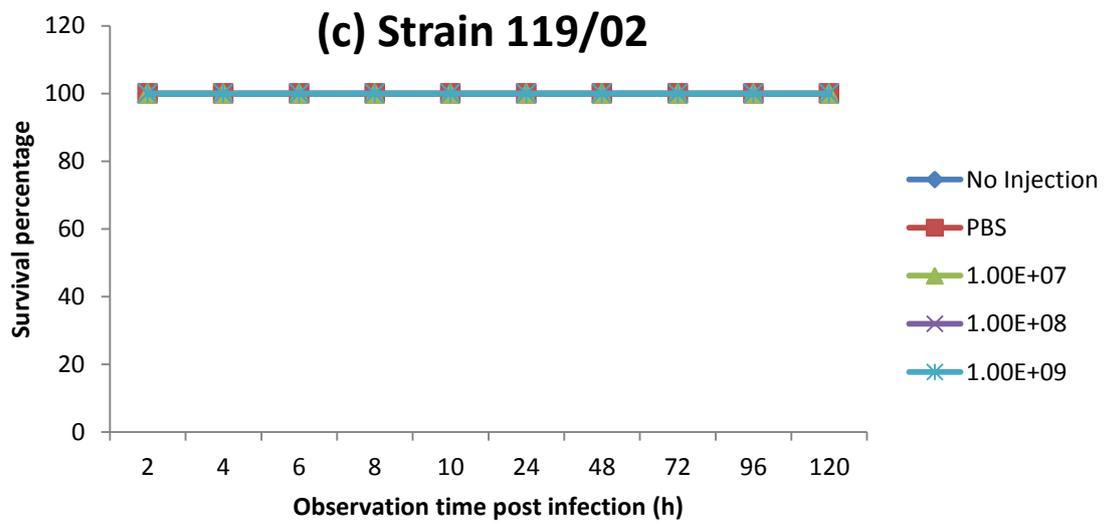
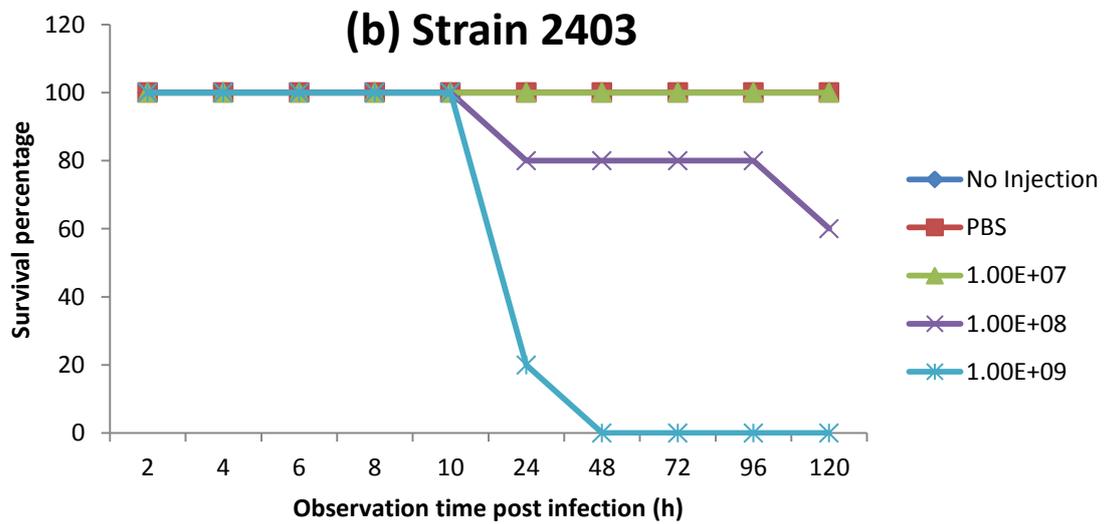
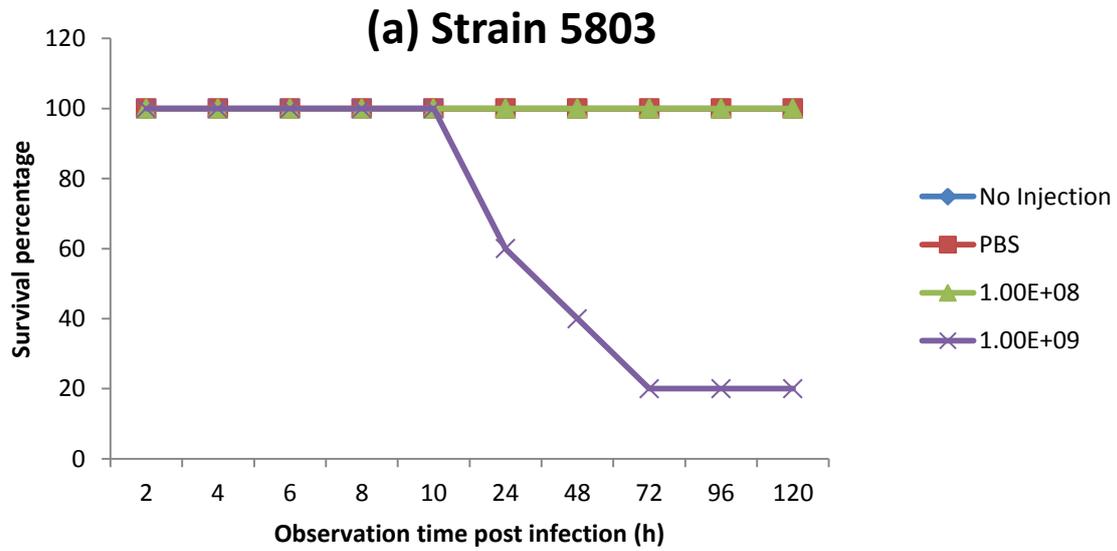


Figure 4.4: Survival curves of *Galleria* infected with BT 1A strains pre-grown at 25°C of 37°C incubated *Galleria*. Figure 4.4 includes five sub figures (4.4a, 4.4b, 4.4c, 4.4d & 4.4e) representing the percentage of survival rates of the *Galleria mellonella* larvae at 37°C incubation temperature infected with the 25°C pre-grown *Y. enterocolitica* strains belonging to the non-pathogenic biotype 1A. A number of 5 larvae were used for each dose. Figure 4.4a shows representative survival data of *Galleria* infected with strain 3403 Bio/serotype 1A/O:5. Figure 4.4b shows representative survival data of *Galleria* infected with strain 09/03 Bioserotype 1A/O:6,30. Figure 4.3c shows representative survival data of *Galleria* infected with strain 35/03 Biotype 1A/O? Figure 4.4d shows representative survival data of *Galleria* infected with strain NZ3 Biotype 1A/O? Figure 4.4e shows representative survival data of *Galleria* infected with strain 09/02 Biotype 1A/O?. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

4.4.1.2.3 Survival curves of *Galleria* infected with Serotype O:9 strains

Part 4.4.1.2.3 shows the survival results of 5 *Y. enterocolitica* strains belong to serotype O:9. The first strain is the human isolate 5803 which belongs to bioserotype 3/O:9. This strain was lethal to *Galleria* larvae with only the 10^9 dose from which a total of 4 *Galleria* were killed (20% survival) within 72h post infection (Fig. 4.5a). The second strain belongs to serotype O:9 and is a human isolate strain 24/03 which belongs to bioserotype 3/O:9 (Fig. 4.5b). Strain 24/03 caused death to *Galleria* larvae with doses 10^9 and 10^8 . Dose 10^9 killed all the infected larvae at 48h post infection. Dose 10^8 killed only one larva out of the 5 (80% survival) within 24h. The third strain studied in the O:9 serotype group is strain 119/02 (Fig. 4.5c). Strain 119/02 belongs to bioserotype 2/O:9 and was isolated from sheep. Strain 119/02 was completely avirulent to the infected *Galleria* larvae and didn't cause any death to the *Galleria* larvae with all the injected doses. The fourth strain belongs to serotype O:9 part is strain 215/02 (Fig. 4.5d). Strain 215/02 belongs to bioserotype 3/O:9 and it was isolated from pigs. Doses 10^9 and 10^8 of strain 215/02 were able to kill *Galleria*. Dose 10^9 killed only 4 larvae of the 5 used (20% survival) after 72h post infection while dose 10^8 killed only one larva out of 5 (80% survival) at 72h (Fig. 4.5d). The fifth and last strain studied in this section is strain 218/02 belonging to bioserotype 3/O:9 and was isolated from pigs. Dose 10^9 of strain 218/02 killed all the *Galleria* larvae within 24 h post infection while dose 10^8 killed only one larva out of 5 (80% survival) within 24h post infection (Fig. 4.5e). Comparing the survival results of strains of bioserotype 3/O:9 to the reference strain 5603 (bioserotype 3/O:9) show that three strains are killing *Galleria* with the same doses of the reference strain while only one strain kills *Galleria* with one dose less. Moreover, the reference strain 21202 of bioserotype 2/O:9 was able to kill *Galleria* with doses 10^9 and 10^8 while the strain 119/02 (bioserotype 2/O:9) was completely avirulent to *Galleria*.

- Survival curves of *Galleria* infected with Serotype O:9 strains



- Survival curves of *Galleria* infected with Serotype O:9 strains

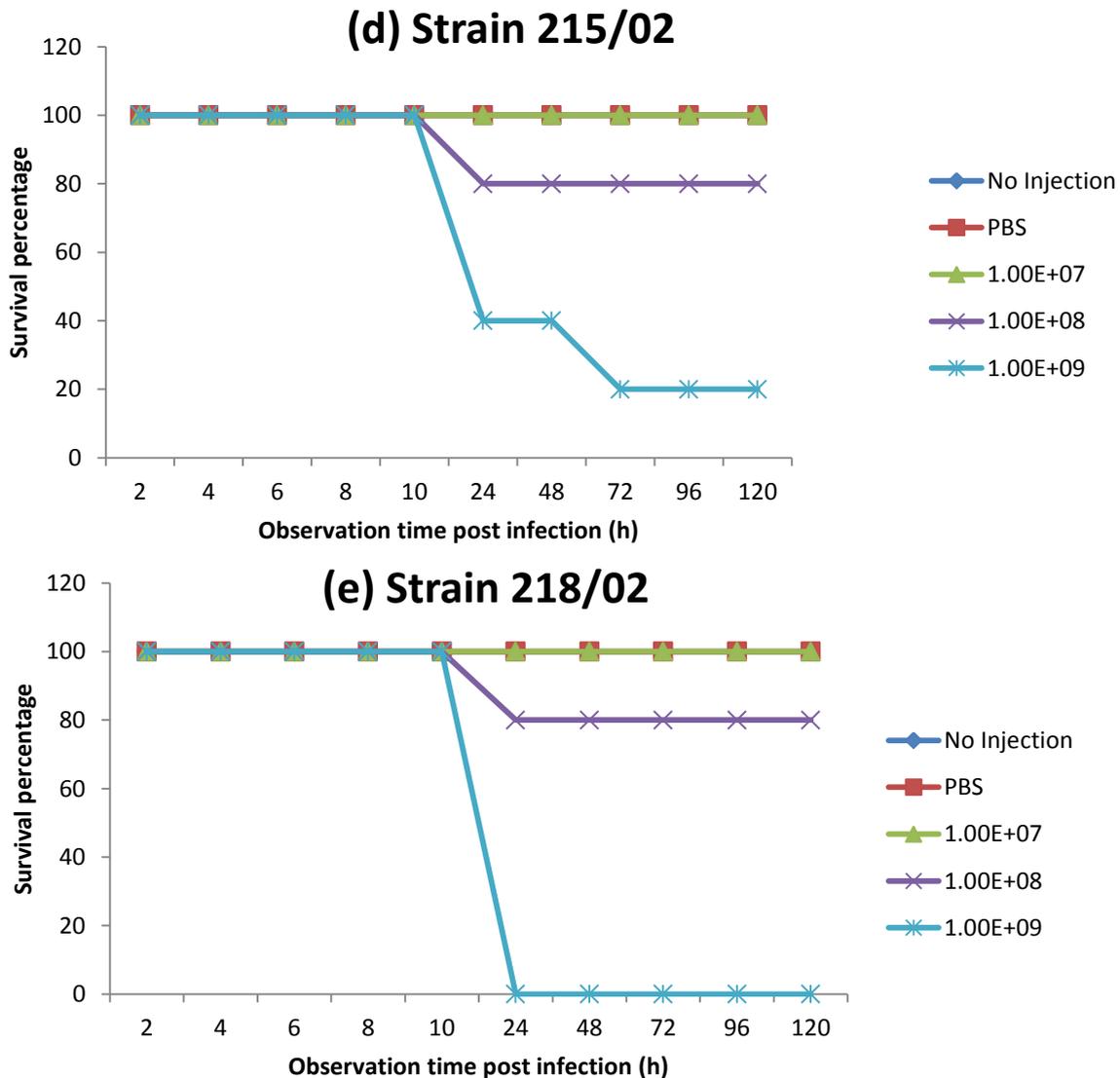
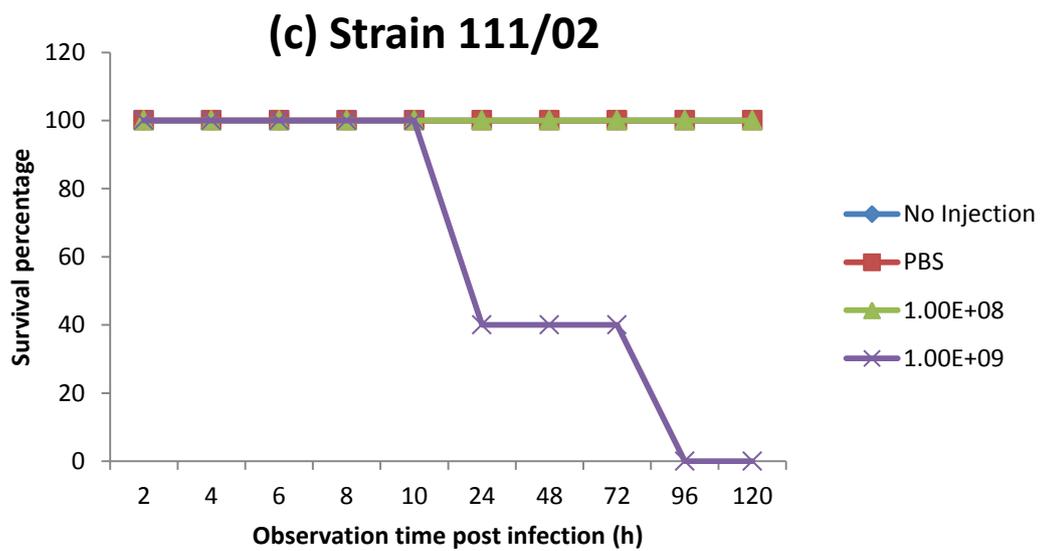
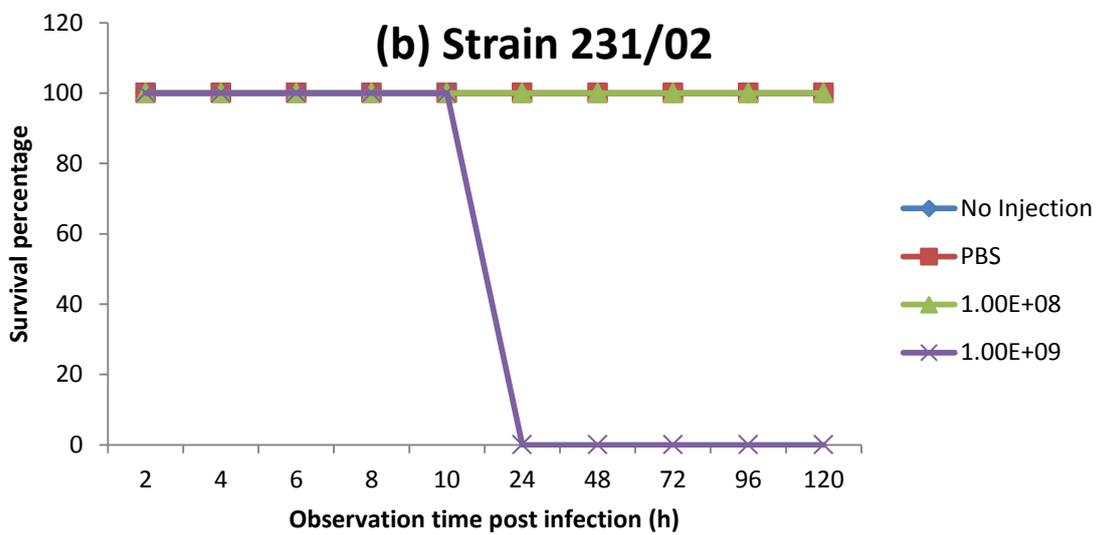
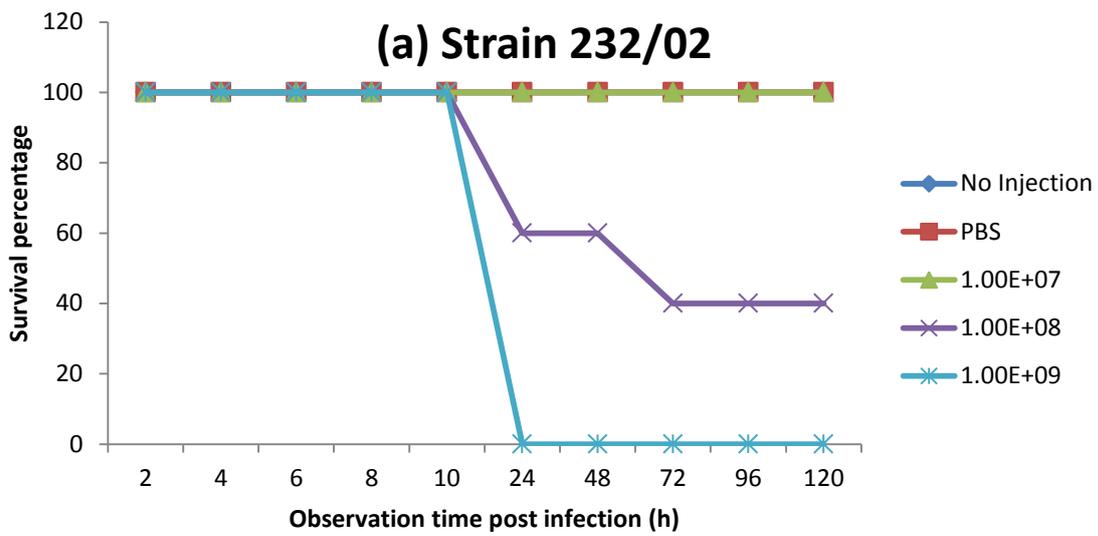


Figure 4.5: Survival curves of *Galleria* infected with serotype O:9 strains pre-grown at 25°C of 37°C incubated *Galleria*. Figure 4.5 includes five sub figures (4.5a, 4.5b, 4.5c, 4.5d & 4.5e) representing the percentage of survival rates of *Galleria* larvae at 37°C incubation temperature infected with the 25°C pre-grown *Y. enterocolitica* strains belonging to the non-pathogenic serotype O:9. A number of 5 larvae were used for each dose. Figure 4.5a shows representative survival data of *Galleria* infected with strain 5803 Bioserotype 3/O:9. Figure 4.5b shows representative survival data of *Galleria* infected with strain 2403 Bioserotype 3/O:9. Figure 4.5c shows representative survival data of *Galleria* infected with strain 119/02 Biotype 2/O:9. Figure 4.5d shows representative survival data of *Galleria* infected with strain 215/02 Bioserotype 3/O:9. Figure 4.5e shows representative survival data of *Galleria* infected with strain 218/02 Bioserotype 3/O:9. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

4.4.1.2.4 Survival curves of *Galleria* infected with Serotype 3/O:5,27 strains:

Section 4.4.1.2.4 includes the survival results of 5 strains of the low pathogenic bioserotype 3/O:5,27 which were isolated from different animal origins. The 5 strains are 232/02 (isolated from pigs); (Fig. 4.6a), strain 231/02 (isolated from pigs); (Fig. 4.6b), strain 111/02 (isolated from sheep); (Fig. 4.6c), strain 153/02 (isolated from cattle); (Fig. 4.6d) and strain 226/02 (isolated from pigs); (Fig. 4.6e). All the 5 strains caused death to the infected *Galleria* larvae. Dose 10^9 of strain 232/02 killed all the *Galleria* larvae at 24h post infection and the 10^8 dose killed 3 out of 5 larvae (40% survival) within 72h post infection (Fig. 4.6a). Strain 231/02 also caused death to the infected *Galleria*. Strain 231/02 killed all the *Galleria* larvae with only the dose 10^9 by which killing was observed after 24h post infection (Fig. 4.6b). Furthermore, strain 111/02 caused death to *Galleria* after the injection with the dose 10^9 only by which all the infected *Galleria* larvae were killed at 96h post infection (Fig. 4.6c). Moreover, strains 153/02 and 226/02 caused death with doses 10^9 and 10^8 from each strain. Dose 10^9 from both strains killed all the *Galleria* larvae at 24h post infection. Dose 10^8 from each strain killed 1 larva out of 5 (80% survival) after 24h post infection in both strains (Fig. 4.6d & 4.6e). In comparison to the reference strain 14902 (bioserotype 3/O:5,27), it can be observed that three of the five bioserotype 3/O:5,27 tested strains were killing infected *Galleria* larvae with the same doses 10^9 and 10^8 while only two strains were killing the *Galleria* larvae with only the 10^9 dose.

- Survival curves of *Galleria* infected with Serotype 3/O:5,27 strains



- Survival curves of *Galleria* infected with Serotype 3/O:5,27 strains

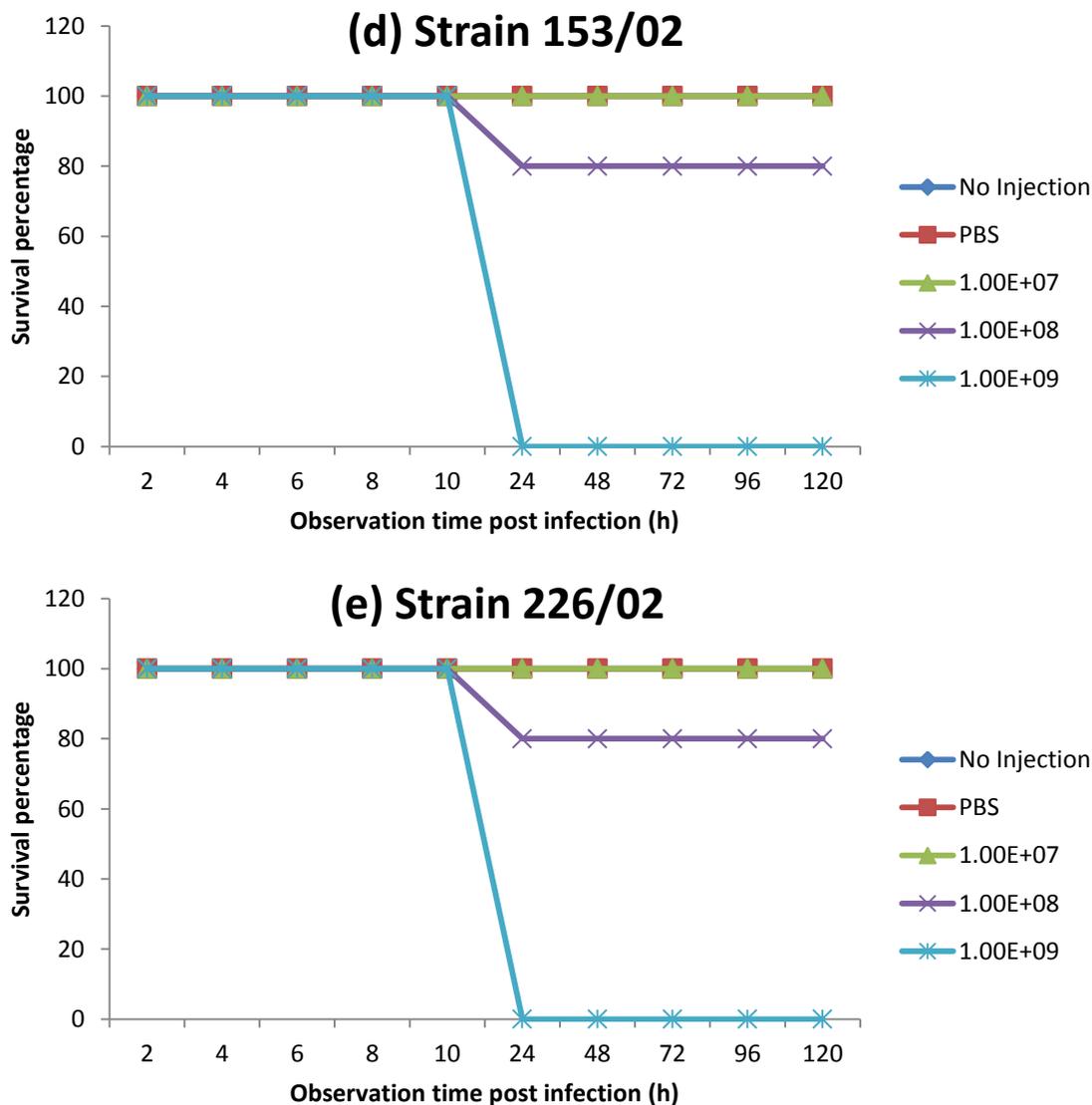


Figure 4.6: Survival curves of *Galleria* infected with BT 3/O:5,27 strains pre-grown at 25°C of 37°C incubated *Galleria*. Figure 4.6 includes five sub figures (4.6a, 4.6b, 4.6c, 4.6d & 4.6e) representing the percentage of survival rates of the *Galleria mellonella* larvae at 37°C incubation temperature infected with the 25°C pre-grown *Y. enterocolitica* strains belonging to the non-pathogenic serotype 3/O:5,27. A number of 5 larvae were used for each dose. Figure 4.6a shows representative survival data of *Galleria* infected with strain 232/02. Figure 4.6b shows representative survival data of *Galleria* infected with strain 232/01. Figure 4.6c shows representative survival data of *Galleria* infected with strain 111/02. Figure 4.6d shows representative survival data of *Galleria* infected with strain 153/02. Figure 4.6e shows representative survival data of *Galleria* infected with strain 226/02. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

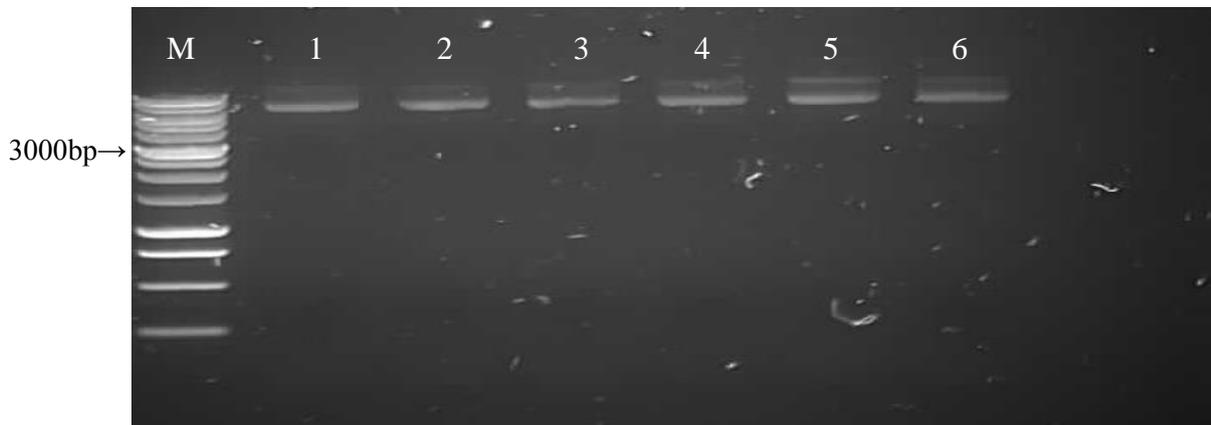
4.4.2 Heat Killed *Y. enterocolitica* (HKY):

Heat killed *Y. enterocolitica* strains were injected in *Galleria* larvae to observe any lethal effect from *Y. enterocolitica* dead cells. *Y. enterocolitica* strains 8081, 1203, 14902, 5603 & 5303 were heat killed at 60°C for 1h and different doses from each strain were injected into the *Galleria* larvae. All the heat killed *Y. enterocolitica* strains were harmless to the infected *Galleria* larvae and didn't induce any killing. All the injected doses were avirulent to the infected larvae. There was no growth for any viable cells on the LB agar plates which indicates a successful heat killing procedure.

4.4.3 Electroporation results:

The pAJD434 plasmid transformation was checked by plasmid extraction from all the electro-transformed *Y. enterocolitica* strains 8081, 1203, 14902, 21202 & 5303 and from the *E. coli* DH5 α control strain followed by visualisation on agarose gel electrophoresis and PCR amplification. Gel electrophoresis images shows bands of DNA content which initially displays a successful transformation and extraction of the pAJD434 plasmid DNA in all the *Y. enterocolitica* and the *E. coli* DH5 α control strain (Fig. 4.7a). To confirm that the transformed plasmid is the pAJD434 plasmid, PCR targeting the trimethoprim gene located on the pAJD434 plasmid was done for all the extracted plasmid samples. PCR product was visualized on agarose gel electrophoresis and the gel image shows bands which indicates a successful amplification of the PCR trimethoprim target gene and hence confirms the successful transformation of the pAJD434 plasmid (Fig. 4.7b).

a- Gel electrophoresis image for the pAJD434 plasmid extraction



b- Gel electrophoresis image for the Trimethoprim gene PCR amplification

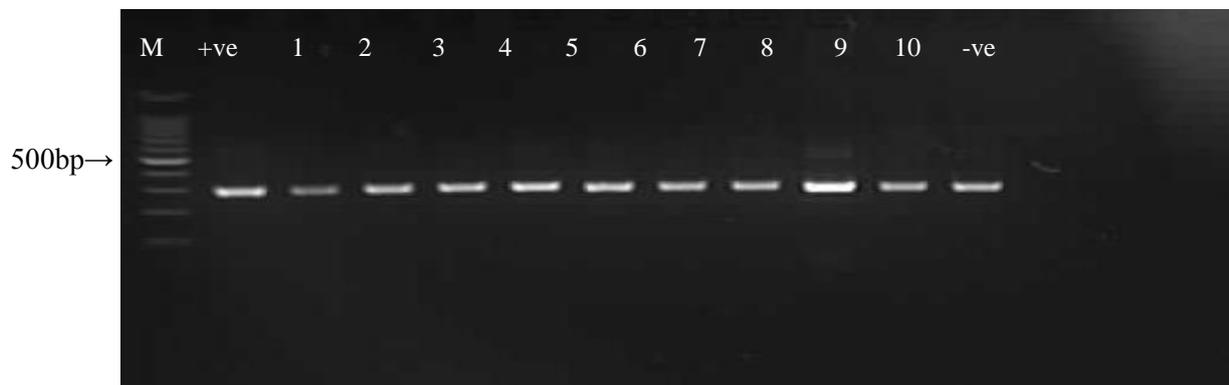


Figure 4.7: Gel electrophoresis results of checking the pAJD434 plasmid transformation. Figure 4.7 contains two subfigures 4.7a and 4.7b. Figure 4.7a represents a gel image of extracting the pAJD434 plasmid from the electro-transformed *Y. enterocolitica* strains (1-5) and from the control strain *E. coli* DH5 α . Figure 4.7b represents the gel image of detecting the trimethoprim gene located on the pAJD434 plasmid by PCR amplification. The trimethoprim gene was from all *Y. enterocolitica* electro-transformed strains (1-10) and from the *E. coli* DH5 α strain which is used as a positive control (+ve). M= the 1kbp DNA marker.

4.4.4 Infection Dynamics Results

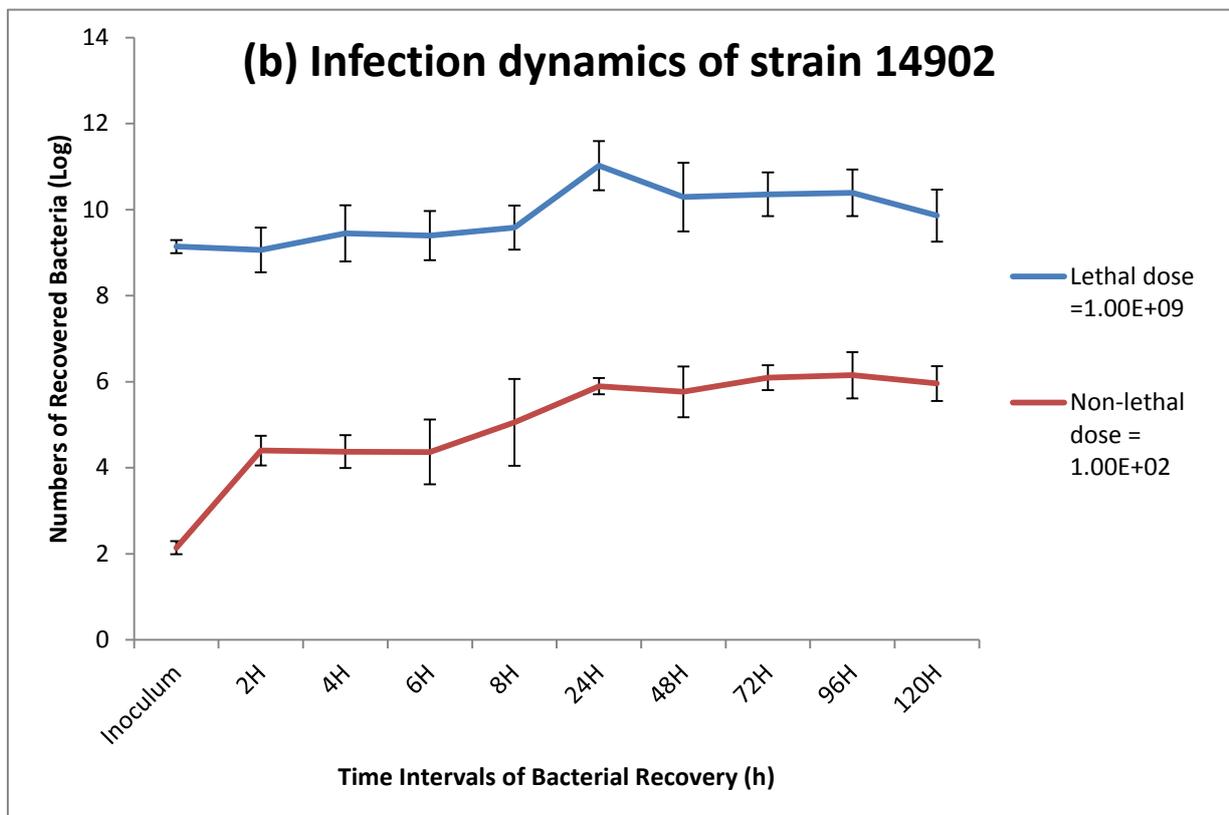
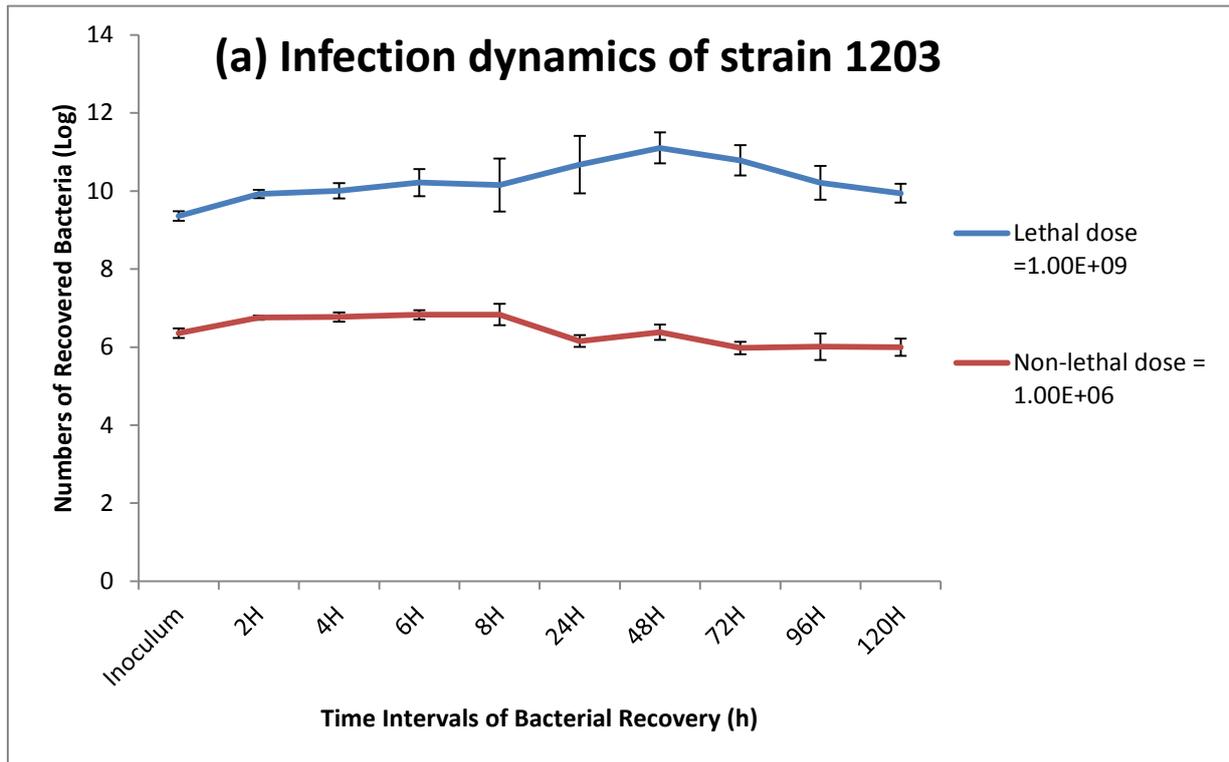
Here, five *Y. enterocolitica* strains were examined to study the infection dynamics inside the *Galleria* alternative infection model. The 5 *Y. enterocolitica* strains were transformed with pAJD434 plasmid which makes them trimethoprim-resistant in order to enable the recovery of these strains on a selective media containing trimethoprim antibiotic. The strains tested are the non-pathogenic strain 5303/pAJD434 (Bio serotype 1A/O:5), the high pathogenic strain 8081/pAJD434 (Bio serotype 1B/O:8), and the low pathogenic strains 1203/pAJD434 (Bio serotype 4/O:3), 14902/pAJD434 (Bio serotype 3/O:5,27) and 21202/pAJD434 (Bio serotype 3/O:9). The infection dynamics results are shown in different parts. Part **4.4.4.1** include the infection dynamics of the low pathogenic strains (1203/pAJD434, 14902/pAJD434 & 21202/pAJD434). Part **4.4.4.2** shows the infection dynamics of the high pathogenic strain 8081/pAJD434 and part **4.4.4.3** shows the infection dynamics of the non-pathogenic strain 5303/pAJD434. The infection dynamics of each strain is represented in a separate graph. Each graph represents the mean of 3 independent experiments.

4.4.4.1 Infection dynamics of the low pathogenic biotypes *Y. enterocolitica* strains:

This section describes the *in vivo* infection dynamics of the low pathogenic strains 1203 (Bio serotype 4/O:3), 14902 (Bio serotype 3/O:5,27) and 21202 (Bio serotype 2/O:9). All the low pathogenic strains were pre-grown at 25°C and the infected *Galleria* larvae with these strains were incubated at 25°C. Lethal doses of all the low pathogenic strains have killed all the injected larvae at the 24h time point while all larvae injected with the non-lethal doses remained alive. For strain 1203, the selected lethal dose was 9.35 Log₁₀ c.f.u/ml (10⁹) and the non-lethal dose was 6.35 Log₁₀c.f.u/ ml (10⁶); (Fig. 4.8a). Bacterial numbers of the lethal dose started to increase gradually during the incubation time reaching a bacterial maximum *in vivo* growth at the 48h time point with a recovered bacterial numbers that equal 11.1Log₁₀

c.f.u/ml. This increase was statistically significant with a p -value = 0.0002. The bacterial cells then started to decrease from the 48h time point and ending with a final bacterial number equal to 9.94 Log₁₀ c.f.u/ml at 120h time point. This decrease from the 48h time point to the 120h time point is showing a statistical significance of a p -value = 0.0001. On the other hand, bacterial numbers of the non-lethal dose were fluctuating by which the starting inoculum was 6.35 Log₁₀ c.f.u/ml and the last recovered bacterial number was 5.99 Log₁₀ c.f.u/ml. This fluctuation in recovered bacterial numbers is statistically insignificant (p -value = 0.060). In strain 14902, the chosen lethal dose was 9.14 Log₁₀ c.f.u/ml (10^9) and the non-lethal dose was 2.14 Log₁₀ c.f.u/ml (10^2) (Fig. 4.8b). The bacterial numbers started to increase gradually during the first 8 hours of incubation. At the 24h time point, the bacterial numbers reached a maximum growth of 11.02 Log₁₀ c.f.u/ml and this increase was statistically significant giving a p -value = 0.0002. Then the bacterial numbers started to decrease slowly by which the final recovered bacterial number recovered at the last time point (120h) was 9.86 Log₁₀ c.f.u/ml. This decrease in bacterial numbers from the 24h time point to the 120h time point was statistically significant with a p -value = 0.0005. Moreover, bacterial numbers of the non-lethal dose showed a remarkable increase. The non-lethal starting dose was 2.14 Log₁₀ c.f.u/ml then the bacterial numbers increased sharply after only 2 hours post infection reaching a numbers of recovered cells equals 4.4 Log₁₀ c.f.u/ml with more than 2 Log₁₀ units difference giving a statistical significance of p -value = 0.0006. After the 2h time point, the bacterial numbers started to increase reaching a maximum growth at 72h with a bacterial numbers equals 6.09 Log₁₀ c.f.u/ml and giving a statistical significance of a p -value = 0.0008. Then the growth monitoring ended with a 5.95 Log₁₀ c.f.u/ml at the last 120h time point with no statistical significance from the 72h time point (p -value = 0.053). For strain 21202, the selected lethal dose is 9.17 Log₁₀ c.f.u/ml (10^9) and the non-lethal dose is 5.17 Log₁₀c.f.u/ml (10^5); (Fig. 4.8c). In the lethal dose, the bacterial numbers started to decrease slightly and

constantly in the first 8h by which the starting inoculum was 9.17 Log₁₀ c.f.u/ml and the recovered bacterial numbers at the 8h time point was 8.66 Log₁₀c.f.u/ml with statistical insignificance (p -value = 0.08). At the 24h time point, the bacterial numbers showed a sudden increase with the maximum growth equals 10.45 Log₁₀ c.f.u/ml. This increase in bacterial number was statistically significant with a p -value = 0.0005. Then the bacterial cells started to decrease until they reached a final growth number of 9.35 Log₁₀ c.f.u/ml at the last 120h recovery time point and this decrease was statistically significant showing a p -value = 0.0029. On the other hand, bacterial growth of the non-lethal dose was showing fluctuating decrease and increase during the 5 days incubation period. The starting inoculum was 5.17 Log₁₀ c.f.u/ml, the maximum growth was indicated at the 72h time point with a bacterial number equals 5.86 Log₁₀ c.f.u/ml and the final bacterial numbers recovered at the last 120h time point were 5.29 Log₁₀ c.f.u/ml. Statistical analysis shows that there is no statistical significance difference in the recovered bacterial numbers between the starting inoculum and the 72h time points (p -value = 0.06) and no statistical significance between the 72h time point and the last 120h time point (p -value = 0.081).



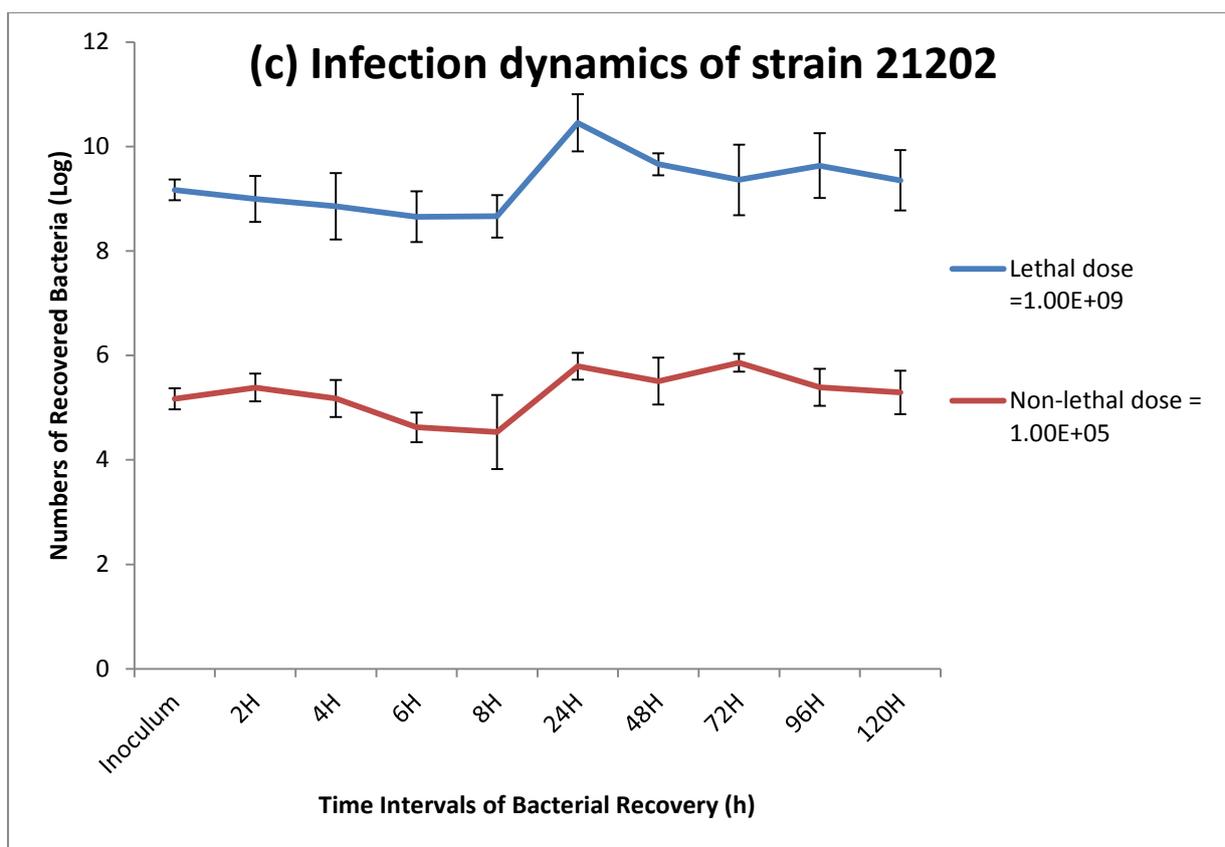


Figure 4.8: Infection dynamics results of *Y. enterocolitica* low pathogenic biotypes strains. Figure 4.8 contains three sub figures 4.8a, 4.8b & 4.8c. Figure 4.8a represents the infection dynamics of the low pathogenic 1203 strain Bioserotype 4/O:3. Figure 4.8b represents the infection dynamics of the low pathogenic strain 14902 Bioserotype 3/O:5,27. Figure 4.8c represents the infection dynamics of the low pathogenic strain 21202 Bioserotype 2/O:9. Strains 1203, 14902 & 21202 were pre-grown at 25°C and the infected *Galleria* with each strain were incubated at 25°C. *Galleria* larvae were injected with the highest lethal dose and the highest non-lethal dose from each strain. Values in each graph shows the mean of 3 independent experiments. Error bars were plotted after calculating the standard deviation of the mean.

4.4.4.2 Infection dynamics results of the high pathogenic strain 8081 (Bioserotype 1B/O:8):

The high pathogenic 8081 strain is used here to represent the infection dynamics of the high pathogenic bioserotype 1B/O:8 (Fig. 4.9). The high pathogenic strain was pre-grown at 25°C prior the infection of the *Galleria* larvae. The infected *Galleria* larvae were incubated at both 25°C and 37°C. *Galleria* larvae incubated at 25°C were infected with the lethal dose 9.45 Log₁₀ c.f.u/ml (10⁹) and the non-lethal dose 5.45 Log₁₀ c.f.u/ml (10⁵). *Galleria* larvae incubated at 37°C were infected only with the highest dose 9.45 Log₁₀ c.f.u/ml (10⁹). The lethal dose injected into the 25°C incubated *Galleria* larvae has killed all infected larvae at 24h post infection. The bacterial numbers of the lethal dose started to increase regularly till they reached a maximum growth at 24h time point with a total number of recovered bacteria that equals 11.06 Log₁₀ c.f.u/ml with a statistical significance difference of p -value = 0.0004. Following that, the bacterial numbers started to decrease slightly till they reached a final number of bacterial cells of 10.72 Log₁₀ c.f.u/ml isolated at the final time point (120h) and were statistically insignificant (p -value = 0.17). On the other hand, all the *Galleria* larvae, incubated at 25°C, and infected with the non-lethal dose 5.45 Log₁₀ c.f.u/ml (10⁵) have survived. The bacterial growth of the non-lethal dose was fluctuating by which the highest bacterial number was recovered after only 8h post infection (bacterial cells = 6 Log₁₀ c.f.u/ml), while the lowest recovered number was 4.74 Log₁₀ c.f.u/ml which was recovered at the 72h time point with a statistical significance of a p -value = 0.0006. The final recovered bacterial cells at the final 120h time point was 5.39 Log₁₀ c.f.u/ml and it still showing a significant difference from the recovered bacterial numbers at 72h time point giving a p -value = 0.03. Furthermore, the *Galleria* larvae incubated at 37°C was infected with the highest dose 9.45 Log₁₀ c.f.u/ml (10⁹) didn't cause any killing to the *Galleria* larvae. The *in vivo* bacterial growth started to decrease drastically until it reached a minimum bacterial

growth at 72h time point by which the recovered bacterial number was 4.86 Log₁₀ c.f.u/ml. The bacterial numbers had decreased dramatically within 72h by which the growth difference between the starting inoculum and the 72h bacterial growth was 4.5 Log₁₀ units approximately and was statistically very significant showing a p -value < 0.00001. The growth then jumped approximately 1.20 Log₁₀ units difference during the last 48h monitoring time and the last recovered bacterial number was 6.05 Log₁₀c.f.u/ml and still showing a significant difference with a p -value = 0.0014.

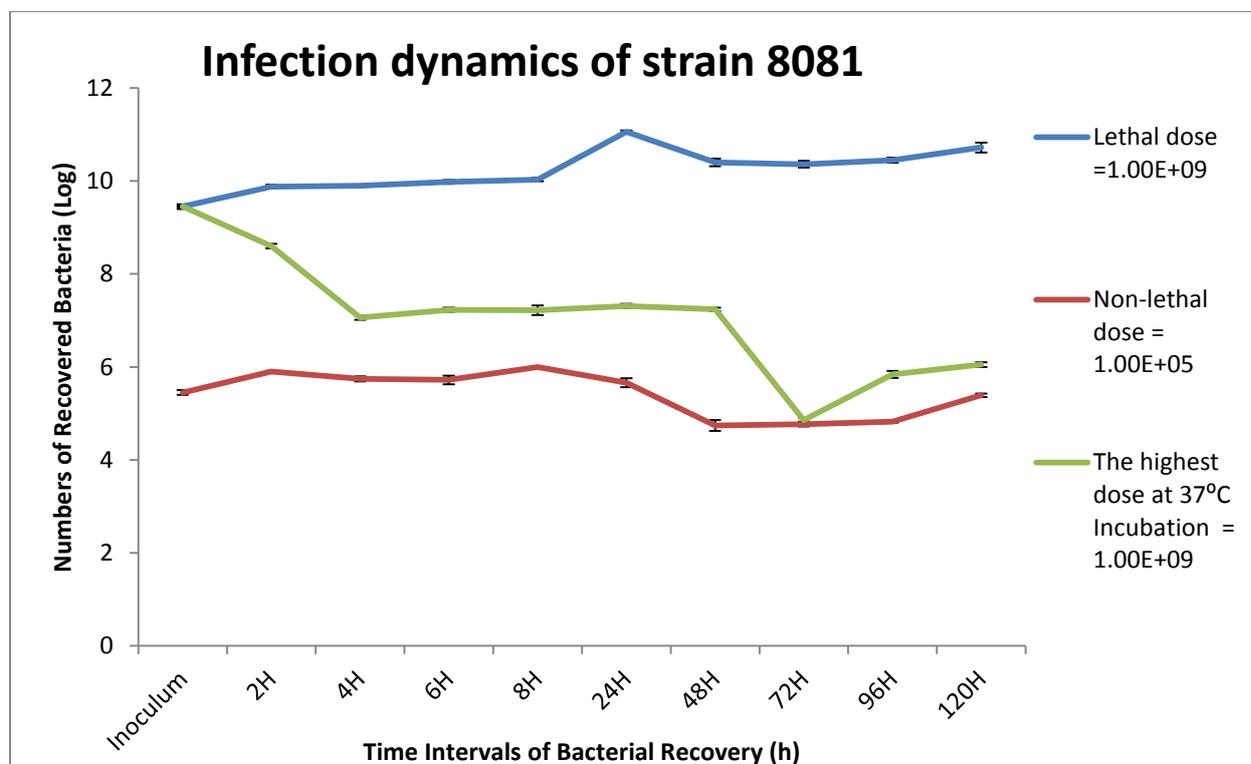


Figure 4.9: Infection dynamics results of the high pathogenic 8081 strain of bioserotype 1B/O:8. Strain 8081 was pre-grown at 25°C and the infected *Galleria* were incubated at 25°C and 37°C. *Galleria* larvae incubated at 25°C were injected with both of the highest lethal dose and the highest non-lethal dose. *Galleria* larvae incubated at 37°C were injected with only the highest dose. Values in the graph shows the mean of 3 independent experiments. Error bars were plotted after calculating the standard deviation of the mean.

4.4.4.3 Infection dynamics of the non-pathogenic strain 5303 (Bioserotype 1A/O:5):

The non-pathogenic 5303 strain has been applied here to study the infection dynamics of the non-pathogenic biotype 1A (Fig. 4.10). The non-pathogenic strain was pre-grown at 25°C prior the infection of the *Galleria* larvae. The infected *Galleria* larvae were incubated at 37°C. *Galleria* larvae incubated at 25°C were infected with the lethal dose 9.33 Log₁₀ c.f.u/ml (10⁹) and the non-lethal dose 4.33 Log₁₀ c.f.u/ml (10⁴). The lethal dose has killed all the infected *Galleria* larvae at the 24h post infection while the non-lethal dose didn't kill any of the infected larvae. Bacterial cells of the injected lethal dose were drastically killed during the 5 days incubation period. Within 24 hours, the *in vivo* growth of the non-pathogenic 5303 strain was hugely dropped and reached a bacterial cell number of 5.85 Log₁₀ c.f.u/ml with a 3.48 Log₁₀ units difference showing a statistical significance difference from the starting inoculum with a *p*-value = 0.00017. The bacterial cell growth commenced to decrease slightly in the remaining 96h incubation period by which the final bacterial number recovered at the last time point (120h) was 5.46 Log₁₀c.f.u/ml which was showing no statistical significance difference (*p*-value = 0.1074). On the other hand, the bacterial cells of the non-lethal dose were rapidly eliminated *in vivo* in 2 hours' time post infection. The recovered bacterial cells from the non-lethal dose of non-pathogenic 5303 strain at the 2h time point was zero giving a huge statistical significant difference of *p*-value < 0.00001. The recovery of the bacterial cells was commenced to 5 days to recover any possible viable bacterial cells. No bacterial growth was observed after the 2h recovering time point.

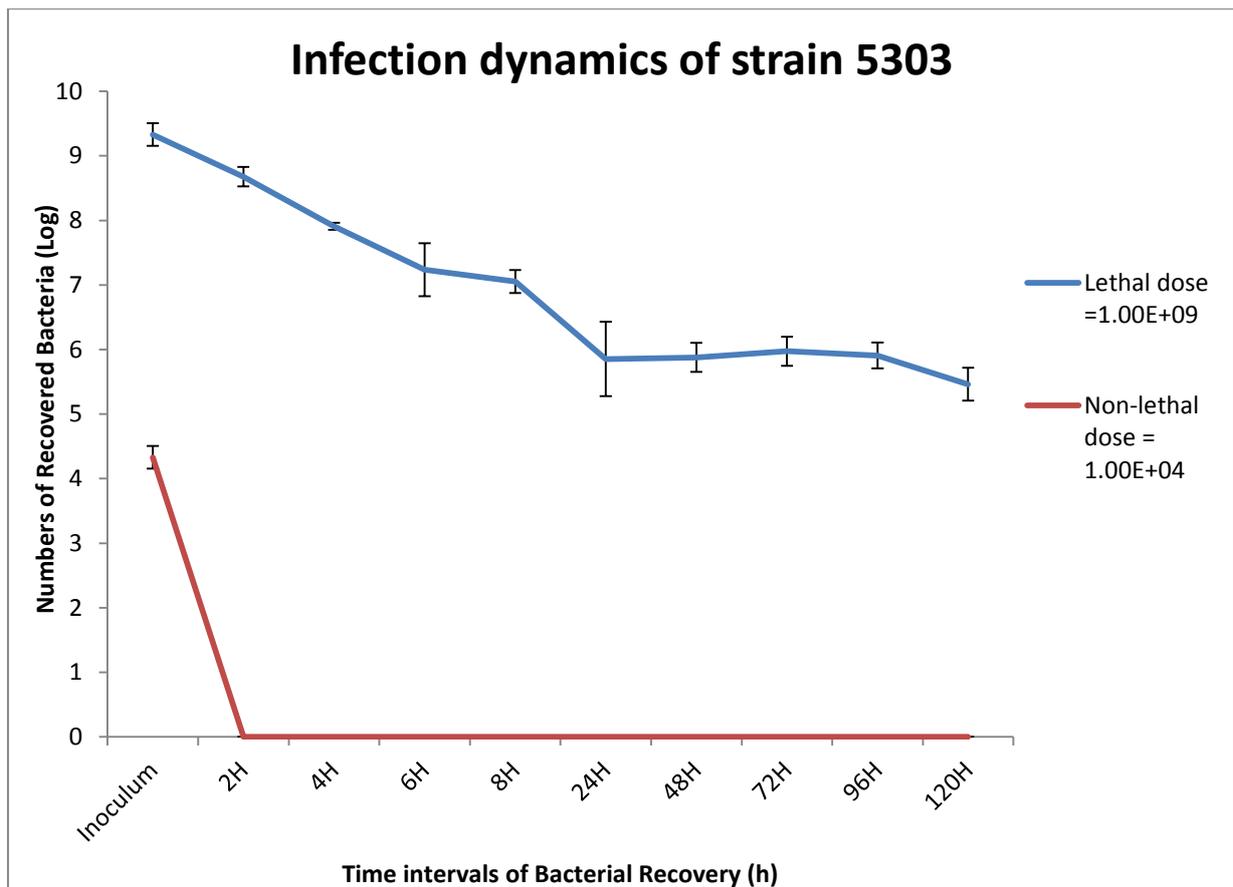


Figure 4.10: Infection dynamics results of the non-pathogenic 5303 strain of bioserotype 1A/O:5. Strain 5303 was pre-grown at 25°C and the infected *Galleria* were incubated at 37°C. *Galleria* larvae incubated at 37°C were injected with both of the highest lethal dose and the highest non-lethal dose. Values in the graph shows the mean of 3 independent experiments. Error bars were plotted after calculating the standard deviation of the mean.

4.5 Discussion

Y. enterocolitica species contains heterogeneous strains which belong to six different biotypes and many serotypes (Reuter *et al.*, 2014). Some literature has documented that *Y. enterocolitica* contains more than 50 serotypes (Virdi and Sachdeva, 2005) while more recent publications describe more than 70 serotypes (Garzetti *et al.*, 2013; Skurnik and Toivonen, 2011). This wide diversity of the *Y. enterocolitica* species increases the need to study the pathogenicity of a more diverse set of *Y. enterocolitica* strains belonging to different biotypes and serotypes in the *Galleria* infection model. Adding a larger number of *Y. enterocolitica* strains to this study allows to investigate the pathogenic potential of *Y. enterocolitica* biotypes and serotypes in more detail and in a wider range rather than depending only on the 10 representative strains that have been extensively studied in Chapter three.

Twenty three different *Y. enterocolitica* strains which belong to different biotypes and serotypes were tested in this chapter. These 23 strains have been isolated from human and animal origins including pigs, sheep and cattle. These strains also include isolates from unknown origin. All the 23 *Y. enterocolitica* strains were pre-grown at 25°C prior their injection into the *Galleria* larvae. The infected *Galleria* larvae with all the 23 *Y. enterocolitica* strains were incubated at 37°C post infection. This condition, of pre-growing *Y. enterocolitica* diverse strains at the environmental 25°C temperature and incubating the infected *Galleria* at 37°C, was chosen since *Y. enterocolitica* pathogenic strains induce their virulence in human bodies by up shifting from environmental temperatures (25°C-28°C) to the 37°C human optimal body temperature (Fàbrega and Vila., 2012). The chosen 23 *Y. enterocolitica* strains were organized under 4 biotypes and serotypes which are the low pathogenic bioserotype 4/O:3, the low pathogenic bioserotype

3/O:5,27, serotype O:9 and the non-pathogenic biotype 1A. Five strains for each of the bioserotype 3/O:5,27, serotype O:9 and biotype 1A were tested.

Eight strains from bioserotype 4/O:3 were studied. Moreover, the selected 8 strains of the bioserotype 4/O:3 were further subdivided into two sub groups by which each sub group contains 4 strains. The first bioserotype 4/O:3 sub group contains 4 strains of human origin. The second bioserotype 4/O:3 sub group contains 4 non-human origin strains by which these strains were isolated from animal and unknown origins. However, bioserotype 4/O:3 have been described as a predominant bioserotype by which bioserotype 4/O:3 clinical strains were frequently isolated from humans worldwide (Fredriksson-Ahoma 2000; Pham *et al.*, 1995). Seven out of the 8 bioserotype 4/O:3 tested strains were virulent to *Galleria* larvae. Strain NZ15 was the only avirulent in *Galleria* infection model among the rest bioserotype 4/O:3 strains by which strain NZ15 was isolated from an unknown origin. The percentage of the bioserotype 4/O:3 virulent strains is 87.5%.

Y. enterocolitica strains belongs to serotype O:9 are pathogenic to humans and they are frequently isolated in Europe and Scandinavia in addition to other parts of the world (Skurnik and Toivonen, 2011). We studied 5 *Y. enterocolitica* strains that belong to serotype O:9 (biotypes 2 and 3) by which 4 strains belong to bioserotype 3/O:9 and 1 strain was from bioserotype 2/O:9. All the 4 strains of the bioserotype 3/O:9 were lethal to the infected *Galleria* larvae. Strain 119/02 bioserotype 2/O:9 was the only serotype O:9 strain that didn't cause any killing to *Galleria*. The percentage of the virulent serotype O:9 strains is 80.0%.

The non-pathogenic biotype 1A is serologically the most heterogeneous biotype between all the 6 *Y. enterocolitica* biotypes (Tennant *et al.*, 2003). The pathogenic potential of 5 strains

of the non-pathogenic biotype 1A was tested in the *Galleria* host infection model. These strains belong to different serotypes and were isolated from human and different animal origins. All the 5 tested biotype 1A strains were lethal to the infected *Galleria* larvae. These biotype 1A strains belong to serotypes O:5, O:6,30 in addition to the O-non typable serotype. Biotype 1A strains that belong to the O-non typable, O:5 and O:6,30 serotypes are the most isolated strains from human cases worldwide (Tennant *et al.*, 2003). The percentage of virulent biotype 1A strains is 100%.

Finally the human pathogenic potential of the low pathogenic bioserotype 3/O:5,27 was studied using 5 strains. All the bioserotype 3/O:5,27 tested strains were virulent to *Galleria* and caused killing to the infected larvae. These strains have been isolated from several animal origins including pigs, sheep and cattle. The bioserotype 3/O:5,27 strains have been isolated from animals in the UK and never associated with human disease (McNally *et al.*, 2011). The percentage of the virulent bioserotype 3/O:5,27 strains is 100%. In conclusion, *Galleria* results shows that 21 out of the 23 diverse strains tested here were lethal and caused death to the *Galleria* larvae. This means that 91.3% of the selected *Y. enterocolitica* diverse strains were pathogenic to the *Galleria* alternative infection model. More importantly, the results of the reference strains were validated against this more diverse strain collection suggesting that the lineage specific results observed in the *Galleria* model are not strain specific but truly reflect the pathogenic potential of each lineage.

Since all previous results were observed from a live *Y. enterocolitica* strains, the pathogenic potential of the Heat Killed *Y. enterocolitica* (HKY) cells in the *Galleria* host model was investigated. Examining if the killing caused by *Y. enterocolitica* strains in the *Galleria* infection model is resulting from an interaction of an active live virulence mechanism with

Galleria larvae and not resulting from a larval passive cellular response for a foreign antigen or from blocking the larvae hemocoel with a large number of bacterial cells is necessary. Previous studies have used HKY in mouse infection model to test the immune response between the HKY and the live *Y. enterocolitica* strains (Autenrieth *et al.*, 1994; Ruiz-Bravo *et al.*, 2003). HKY were found to be immunomodulatory in the mouse infection model immune system but to a limited extent compared to the live *Y. enterocolitica* cells (Ruiz-Bravo *et al.*, 2003). Five HKY *Y. enterocolitica* strains belonging to different bioserotypes were tested to detect any biotype or serotype specific differences. *Y. enterocolitica* strains 8081, 1203, 14902, 5603 & 5303 were heat killed at 60°C for 1 hour in order to avoid cellular damage and therefore avoid damaging the surface antigens. *Y. enterocolitica* LPS are an example of surface antigens and they are able to stimulate the murine model immune system (Ruiz-Bravo *et al.*, 2003). However, the HKY results show that all the heat killed *Y. enterocolitica* strains didn't cause any death to the infected *Galleria* larvae. As a result, it can be observed that only the *Y. enterocolitica* living cells that can cause killing to the *Galleria* larvae. These results guided me to investigate the fate (dead or alive) of the *Y. enterocolitica* cells inside the infected *Galleria* larvae and how do they behave inside the infected larvae.

Next, the 8081, 1203, 14902, 21202 and 5303 *Y. enterocolitica* strains transformed with trimethoprim pAJD434 resistance plasmid, were tested for their *in vivo* behaviour in the alternative infection model *G. mellonella*. This was done to measure the bacterial growth rates within *Galleria* at different time points. All strains were pre-grown at 25°C optimal growth temperature. *Galleria* larvae were infected with a chosen lethal dose and a non-lethal dose from each strain to compare their *in vivo* behaviour. I chose to incubate the infected *Galleria* larvae with the low pathogenic biotype strains at the 25°C temperature since the

25°C incubation temperature shows more virulence in the infected *Galleria* larvae and hence enable an easy selection of a lethal dose. The previous work in chapter three, which involved infecting *Galleria* larvae with the high pathogenic 8081 strain bioserotype 1B/O:8, revealed that the infected *Galleria* larvae incubated at 25°C were susceptible to killing while the incubated larvae at the 37°C were completely resistant to killing and remained alive. For this reason, an extra group of infected *Galleria* larvae at 37°C was incubated in addition to the group incubated at 25°C. This non-lethal effect at the 37°C incubation temperature is unique for the high pathogenic 8081 strain and it is important to investigate the bacterial behaviour at this temperature. Since the *Galleria* larvae infected with the high pathogenic strain 8081 don't die at the 37°C incubation temperature, thus there will be no lethal dose that can be selected. As a result, I chose only one dose to infect the *Galleria* larvae and I decided to select the highest dose 10^9 since this dose is also selected to infect the *Galleria* larvae at the 25°C incubation temperature. This will provide a similar comparison of the same bacterial number but at two different incubation temperatures. Selecting the highest dose 10^9 to infect the *Galleria* larvae provides enough bacterial numbers that can remain and recovered through the 5 days incubation period. I named this non killing 10^9 high dose as the highest injected dose but not "as non-lethal" to avoid confusing with the non-lethal doses in the rest incubation conditions. The infected *Galleria* larvae with the non-pathogenic strain 5303 were incubated at 37°C but not at 25°C as were done previously. This is because all doses of the non-pathogenic 5303 strain can kill the *Galleria* larvae at the larval 25°C incubation temperature and as a result there are no any possible non-lethal doses that can be selected. Alternatively, *Galleria* larvae infected with the non-pathogenic strain 5303 and incubated at 37°C contains both lethal and non-lethal doses which allows selecting both types of doses. All the injected doses were delivered in 10µl volumes in the *Galleria* larvae. This volume contains cell number 100 times less than the actual stock suspension. As a result, to provide

an accurate bacterial numbers compared to the actual stock, every recovered bacterial number was multiplied in 100 (x100) to obtain the actual bacterial cells number. *G. mellonella* have been frequently used to study the *in vivo* infection dynamics of many human pathogens comprising *Listeria monocytogenes* (Joyce *et al.*, 2010), *Campylobacter jejuni* (Senior *et al.*, 2011), Group A Streptococcus (GAS) (Olsen *et al.*, 2011), *Yersinia pseudotuberculosis* (Champion *et al.*, 2009) and *Yersinia pestis* (Erickson *et al.*, 2011). These studies show that species manage to grow and persist in the *G. mellonella* insect infection model. The recent experimental work shows that the high pathogenic biotype and low pathogenic biotypes strains were growing and persist in both dead and alive infected *Galleria* larvae during the 5 day incubation period. However, the non-pathogenic biotype strain showed a marked reduction in growth in the lethal dose and a complete elimination of bacterial cells in the non-lethal dose but also the remaining live bacterial cells of the lethal dose stayed alive till the end of the incubation period. Death in *G. mellonella* larvae infected with lethal doses of both the high and low pathogenic biotypes was accompanied with an *in vivo* bacterial growth with a peak of maximum growth at 24h (and at 48h for only the low pathogenic strain 1203 bioserotype 4/O:3). *Galleria* death resulted from the lethal dose of the non-pathogenic 5303 strain and was accompanied by bacterial cell death with a maximum growth drop occurring at 24h post infection. On the other hand, all the tested *Y. enterocolitica* strains were starting to cause death in *Galleria* larvae at the 24h time point. This indicates that the maximum host-pathogen interaction occurs between 8h and 24h time points post infection. Moreover, the statistical analysis of the recovered numbers of bacterial cells from *Galleria* is showing that (*p*) values of the early and middle stages of *Galleria* infection, from the starting inoculum to the 72h time point recovery, were always lower than (*p*) values at the late *Galleria* infection stages that occur at the last 48h hours of the 5 days incubation period. This indicates that the

maximum *Y. enterocolitica in vivo* interaction with *Galleria* occurs during the first 72h post infection whether this interaction was an increase or decrease in bacterial numbers.

4.6 Conclusion

In conclusion, *Galleria* insect infection model was susceptible to killing by a heterogeneous collection of *Y. enterocolitica* diverse strains. The heat-killed *Y. enterocolitica* strains were completely avirulent in *Galleria* larvae. The pathogenic *Y. enterocolitica* biotypes bacterial cells were growing inside *Galleria* while the non-pathogenic biotype cells were dying. This proves that there are lineage specific differences in the virulence of *Y. enterocolitica* to *Galleria*. This also shows that the extreme virulence shown by Biotype 1A strains to *Galleria* is not as a result of surface associated factors, but rather is the result of interplay between the *Galleria* and the bacteria, which results in a reduction in bacterial cell numbers, but ultimately death to the *Galleria*.

Chapter 5

Investigating the role of pYV in modulating the lack of virulence of the pathogenic biotypes in *Galleria* host model

5.1 Introduction

Y. pestis, *Y. pseudotuberculosis* and *Y. enterocolitica* are the human pathogenic species of the genus *Yersinia*. These three species share a conserved 70-kb pYV (Plasmid of *Yersinia* Virulence) plasmid which has been shown to be fundamental for their virulence (Bhaduri and Smith, 2011; Hammer *et al.*, 2007). The loss of the pYV plasmid correlates with a loss of virulence in the pathogenic *Yersinia* species (Hammer *et al.*, 2007). pYV is the name most commonly used to describe the virulence plasmid when referring to *Y. enterocolitica* but it's also called pCD1 in *Y. pestis* and pIB1 in *Y. pseudotuberculosis* (Mulder *et al.*, 1989). These names derive from the first genetically mapped virulence plasmids: pYV227 which was mapped from the low pathogenic *Y. enterocolitica* W22703 strain bioserotype 2/O:9; pCD1 from *Y. pestis* KIM of biovar *medievalis*; and pIB1 from *Y. pseudotuberculosis* strain YPIII (Cornelis *et al.*, 1998). *Y. enterocolitica* pathogenic strains harbour a pYV plasmid with a size ranging from 64- to 75-kb (Bottone, 1997). Recent phylogeny of genome sequenced *Y. enterocolitica* pYV plasmids revealed that the *Y. enterocolitica* pathogenic biotypes carry different copies of the pYV plasmid (Reuter *et al.*, 2014). According to the phylogenetic tree analysis, the pYV plasmid copies of the low-pathogenic biotypes are closely related while the pYV plasmid of the high-pathogenic biotype forms a distant lineage with a unique replication origin (Reuter *et al.*, 2014). The pYV plasmid is found in the *Y. enterocolitica* high pathogenic biotype 1B strains and the low pathogenic 2-5 biotypes strains but absent from the non-pathogenic biotype 1A strains (Howard *et al.*, 2006). *Y. enterocolitica* pYV plasmid encodes for the major virulence factors of these organisms, the Ysc type three secretion system, the secreted Yops (*Yersinia* outer membrane proteins) and the YadA adhesin protein. The Ysc system enables *Y. enterocolitica* to avoid phagocytosis by injected the cytotoxic Yop proteins into the cytosol of

Macrophages. The injected Yops inhibit the phagocytic mechanisms and cause apoptosis to Macrophages (Cornelis *et al.*, 1998). The injected effector Yop proteins (YopH , YopE, ,YopO , YopM, YopP and YopT) into the cytosol of the eukaryotic host cell proteins are a set of virulence factors that interrupt the cytoskeletal dynamics of macrophages and polymorphonuclear leukocytes which inhibit the phagocytosis process (Perez-Gutierrez *et al.*, 2007) and hence enable *Y. enterocolitica* to avoid the immune system (Sing *et al.*, 2002). In addition to the Yop effector proteins, *Y. enterocolitica* Ysc secretes another set of Yop proteins called the translocator proteins (LcrV, YopB and YopD) required to translocate the Yop effector proteins (Sorg *et al.*, 2006). Moreover, YadA binds to collagen, fibronectin and laminin extracellular proteins and secures *Y. enterocolitica* from lysis by complement system and from phagocytosis (Schaake *et al.*, 2013).

5.2 Aim:

To Investigate the role of pYV in modulating the lack of virulence of the *Y. enterocolitica* pathogenic biotypes in *Galleria* host model.

5.3 Objectives:

A) To study the pathogenic potential of pYV-cured high pathogenic and low pathogenic *Y. enterocolitica* strains in the *Galleria* infection model.

B) To investigate the stability of the pYV plasmid (pYV plasmid dynamics) during the infection of the *Galleria* larvae with strains of high pathogenic and low pathogenic *Y. enterocolitica* biotypes.

5.4 Materials and Methods

5.4.1 Bacterial strains list

All bacterial strains tested in chapter 5 are listed in table 5.1.

Table 5.1: Bacterial strains tested in chapter 5. *: *Y. enterocolitica* strains carrying the virulence plasmid pYV. **: *Y. enterocolitica* strains cured from (lack) the virulence plasmid pYV.

| No. | Strain Name | Bio/Serotype | Biological Origin | Source |
|-----|-------------|--------------|-------------------|------------------------------|
| 1 | 8081* | 1B/O:8 | Human | Thomson <i>et al.</i> (2006) |
| 2 | 21202* | 2/O:9 | Pig | McNally, UK |
| 3 | 14902* | 3/O:5,27 | Sheep | McNally, UK |
| 4 | 1203* | 4/O:3 | Human | McNally, UK |
| 5 | 8081c** | 1B/O:8 | This Study | Thomson <i>et al.</i> (2006) |
| 6 | 14902c** | 3/O:5,27 | This Study | McNally, UK |
| 7 | 1203c** | 4/O:3 | This Study | McNally, UK |

5.4.2 PCR Primers

Table 5.2: PCR primers

| Primer Name | Nucleotide Sequence | Primer Function | Annealing Temperature (°C) | Riplicon size (bp) |
|---------------|-----------------------------------|--|----------------------------|--------------------|
| Yent1_F (16S) | 5'-AAT ACC GCA TAA CGT CTT CG-3' | Control for <i>Y. enterocolitica</i> 16S rDNA gene amplification | 57 | ~300 |
| Yent2_R (16S) | 5'-CTT CTT CTG CGA GTA ACG TC-3' | | | |
| YscP1_F | 5'-ATT AGA ACC TGA GTA TCA ACC-3' | Retention of virulence plasmid pYV. <i>yscP</i> gene amplification | 52 | ~500 |
| YscP2_R | 5'-AAC AAA TAA CTC ATC ATG TCC-3' | | | |

5.4.3 pYV Plasmid curing procedure:

The pYV plasmid was knocked out of the high pathogenic biotype 1B/O:8 strain 8081, the low pathogenic biotype 4/O:3 strain 1203 and the low pathogenic biotype 3/O:5,27 strain 14902. The pYV curing procedure involves subsequent sub culturing on LB agar plates at 37°C. All wild type strains pYV⁺ were grown on LB agar plates aerobically at 37°C in static incubator and incubated overnight for 24 hours. For each strain, three similar colonies were individually picked up from each overnight culture and sub cultured on separate LB agar plate. After every 2 passages on LB agar plates the third passage was cultured on CRMOX agar using the same last sub culturing procedure. The cultured CRMOX agar plates were incubated aerobically in static incubator at 37°C for 18-24 hours. Each experiment was repeated individually three times. The presence of the pYV plasmid was checked by PCR by targeting the *yscP* gene with YscP1 and YscP2 primers pair described in table 5.2. The non-pathogenic biotype 1A/O:5 strain 5303 was used as a pYV⁻ negative control since this strain naturally lacks the pYV plasmid. All pYV⁻ cured strains were stocked in glycerol freezing medium and stored in -80°C freezer. To ensure the plasmid cured colonies were not contaminants the primers pair Yent1 and Yent2 (described in table 5.2) were used to detect the *Y. enterocolitica* 16S rDNA gene.

5.4.4 pYV plasmid Stability:

Four wild type *Y. enterocolitica* pYV⁺ strains were used to study the stability of pYV: the high pathogenic biotype 1B/O:8 strain 8081, the low pathogenic biotype 4/O:3 strain 1203, the low pathogenic biotype 2/O:9 strain 21202 & the low pathogenic biotype 3/O:5,27 strain 14902. *Y. enterocolitica* strains were injected in *Galleria* larvae to study the stability of the pYV plasmid during the infection of the *Galleria* larvae. The *Y. enterocolitica* strains were

pre-grown on 25°C as described in chapter two section 2.1. The lethal dose in addition to the non-lethal dose was selected for each strain as previously described in chapter four. Each dose was injected in 50 *Galleria* larvae (total of 100 *Galleria* per strain). All the infected *Galleria* larvae with all tested strains were incubated at 25°C for 5 days. Every 24H, bacterial cells were recovered from a pool of 5 larvae for each lethal and non-lethal dose for each injected strain. The recovering procedure was carried out as previously described in chapter four. The bacterial cells were recovered from each larva and was drained and diluted as described in chapter four. All recovered bacterial cells were plated on CIN agar and incubated at 37°C for 24h. Bulls-eye forming colonies were picked up and the presence of the plasmid was checked by PCR by detecting the presence of the *yscP* gene. Primers pair Yent1 and Yent2 described in table 5.2 were used to detect the *16S rDNA* gene confirm recovering testing *Y. enterocolitica* strains.

5.4.5 Statistical analysis

The statistical significance difference between the recovered bacterial numbers for each strain in the infection dynamics assay was determined by the one sample T-test. The statistical significance difference of LD₅₀ values between the *Y. enterocolitica* wild type strains and their pYV-cured derivative tested strains were determined by one sample T-test. The statistical difference between the 25°C and 37°C *Galleria* incubation temperatures were determined by the T-test of two independent samples.

5.5 Results

5.5.1 pYV curing

The pYV plasmid was cured from three *Y. enterocolitica* pathogenic strains; the high pathogenic bioserotype 1B/O:8 strain 8081, the low pathogenic bioserotype 4/O:3 strain 1203 and the low pathogenic bioserotype 3/O:5,27 strain 14902. pYV plasmid loss was checked by PCR targeting the *yscP* gene followed by visualizing the PCR product on gel electrophoresis (Fig.5.1a). The *Y. enterocolitica 16S rDNA* gene was detected by PCR (Fig.5.1b) to ensure the isolated plasmid minus mutants were *Y. enterocolitica*. The pathogenicity of the *Y. enterocolitica* cured strains in the *Galleria* infection model was tested by calculating the median lethal doses (LD₅₀) and the survival curves for each strain. All the *Y. enterocolitica* pYV cured strains were pre-grown at 25°C and the infected galleria with each strain were incubated at 25°C and 37°C. The median lethal doses (LD₅₀) and survival results of the pYV- cured derivatives strains were compared to the median lethal doses (LD₅₀) and survival results of the wild type references strains at the same *Y. enterocolitica* pre-growing temperatures and the injected *Galleria* larvae incubation condition.

5.5.1.1 Gel electrophoresis image of pYV curing results

a- Gel of *yscP* gene amplification



b- Gel of 16S rDNA gene amplification

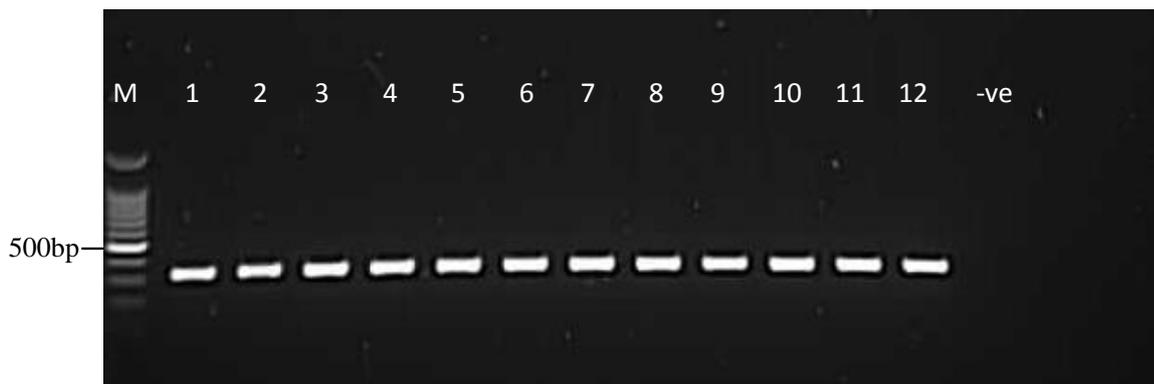


Figure 5.1: Gel electrophoresis image of pYV curing results. Figure 5.1 contains two subfigures 5.1a and 5.1b. Figure 5.1a represents the gel image of checking the pYV plasmid curing by detecting the *Y. enterocolitica yscP* gene of the 3 pYV-cured *Y. enterocolitica* strains YE8081c, YE1203c and YE14902c. Figure 5.1b represents the gel image of detecting the *Y. enterocolitica 16S rDNA* gene of the 3 pYV-cured *Y. enterocolitica* strains YE8081c, YE1203c and YE14902c. The lanes of both figure 5.1a and figure 5.1b gel images are labelled with numbers from 1-12 and each number represents a different sample. In figure 5.1a, 1= *yscP* gene detection of the wild type YE8081 strain which is used as a positive control (+ve) to insure that the *yscP* gene amplification primers are working.

5.5.1.2 The LD₅₀ values of *Y. enterocolitica* pYV-cured strains

The LD₅₀ results show that the LD₅₀ value of 8081c derivative strain was 9.56log₁₀ c.f.u/ml at 37°C incubation temperature and 7.6log₁₀ c.f.u/ml at 25°C incubation temperature (Fig. 5.2). The LD₅₀ of the 8081c derivative strain at the 37°C incubation temperature had slightly decreased from the 8081 wild type strain at the same incubation temperature by which the LD₅₀ value was 9.6log₁₀ c.f.u/ml. Therefore strain 8081c has the same LD₅₀ value as the wild type 8081 strain at the 25°C incubation temperature which was 7.6log₁₀ c.f.u/ml in both. The 1203c derivative strain had LD₅₀ values of 8.5log₁₀ c.f.u/ml and 7.3log₁₀ c.f.u/ml at 37°C and 25°C incubation temperatures respectively. The 1203c derivative strain also showed a decreased LD₅₀ value compared to the wild strain 1203 at both incubation temperatures. Wild type 1203 strain have LD₅₀ values of 8.9log₁₀ c.f.u/ml and 7.8log₁₀ c.f.u/ml at 37°C and 25°C incubation temperatures respectively which are higher than the LD₅₀ values of the 1203c derivative strain. The last tested pYV-cured strain in this part is the 14902c. The LD₅₀ values of the derivative 14902c strain at both the 25°C and 37°C incubation temperatures were noticeably lower than the 14902 wild type strain LD₅₀ values. The 14902 LD₅₀ values were 7.6log₁₀ c.f.u/ml and 3.8log₁₀ c.f.u/ml at 37°C and 25°C incubation temperatures respectively while LD₅₀ values of the 14902 wild type strain were 8.8log₁₀ c.f.u/ml (at 37°C incubation temperature) and 3.8log₁₀ c.f.u/ml (at 25°C incubation temperature). The *Y. enterocolitica* pYV-cured strains are showing a significance difference between the 25°C and 37°C incubation temperatures ($p= 0.001898$). This shows that the *Y. enterocolitica* pYV-cured strains still respond differently to different incubation temperature and still more virulent at the 25°C incubation temperature where the LD₅₀ values are lower from those at the 37°C. The *Y. enterocolitica* pYV-cured strains were then compared to the *Y. enterocolitica* wild strains (pYV⁺) at the same pre-growing and incubation temperatures and we found that

there was no significance difference between the *Y. enterocolitica* (pYV⁻) and *Y. enterocolitica* (pYV⁺) strains (p-values= 0.055175 and 0.237761 respectively).

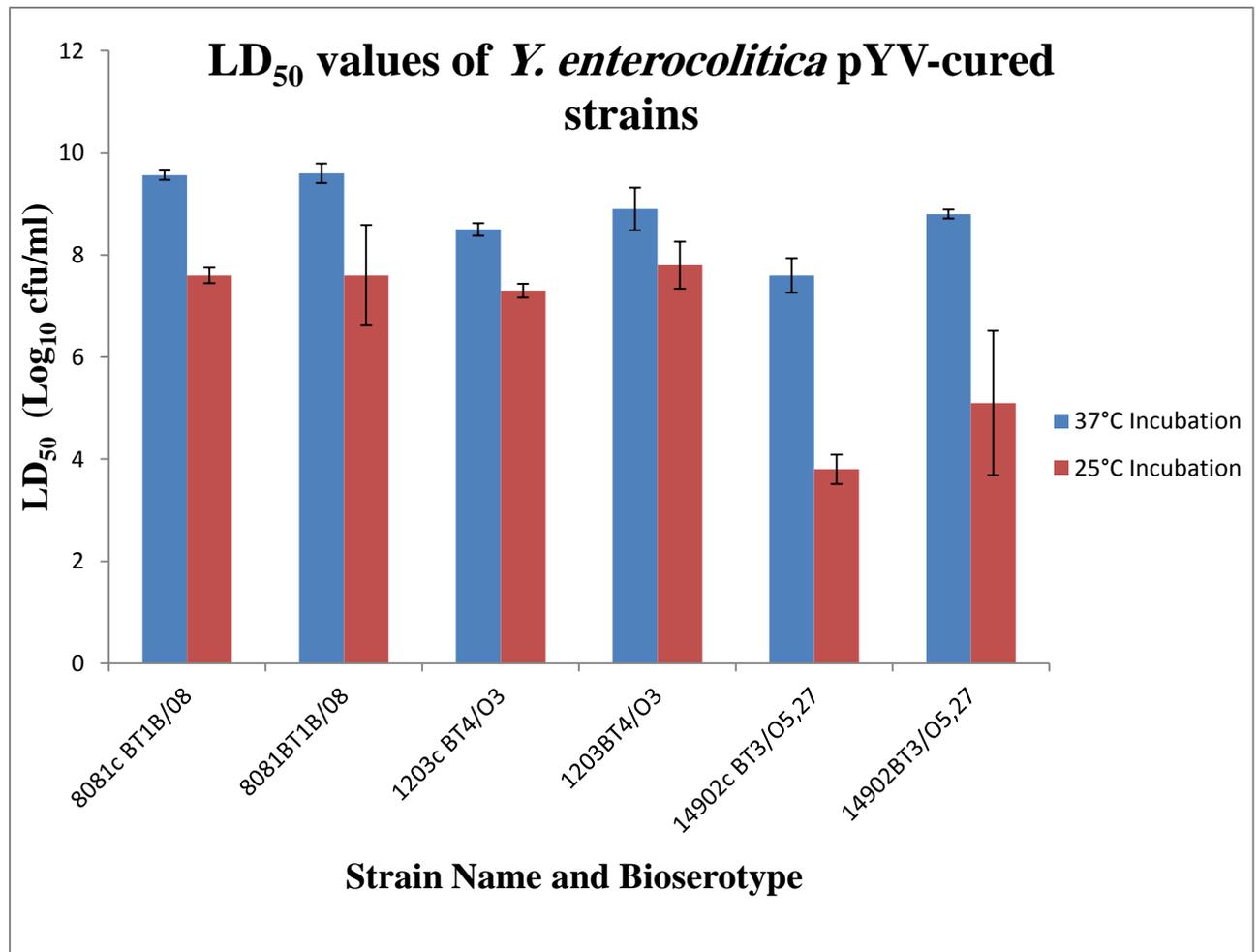


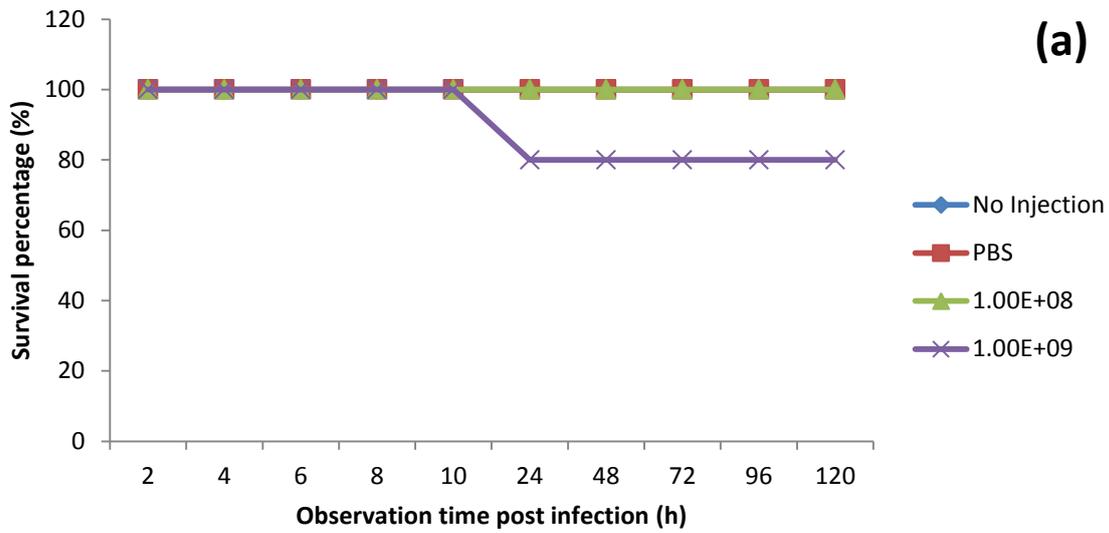
Figure 5.2: LD₅₀ values of *Y. enterocolitica* pYV-cured strains 8081 (Bioserotype 1B/O:8), 1203 (Bioserotype 4/O:3) and 14902 (Bioserotype 3/O:5,27). The graph shows the mean LD₅₀ of independent triplicate experiments for each individual strains. The LD₅₀ values of the wild type pYV⁺ and the cured pYV⁻ derivative for each strain are represented in columns by which each strain is represented in 2 columns. The x-axis represents the strain name and the y-axis represents the LD₅₀ values in log₁₀ c.f.u/ml. The blue bars represent the LD₅₀ values of *Y. enterocolitica* strains incubated at 37°C. The red bars represent the LD₅₀ values of *Y. enterocolitica* strains incubated at 25°C. Error bars were plotted after calculating the standard deviation of the mean.

5.5.1.3 Survival Curves

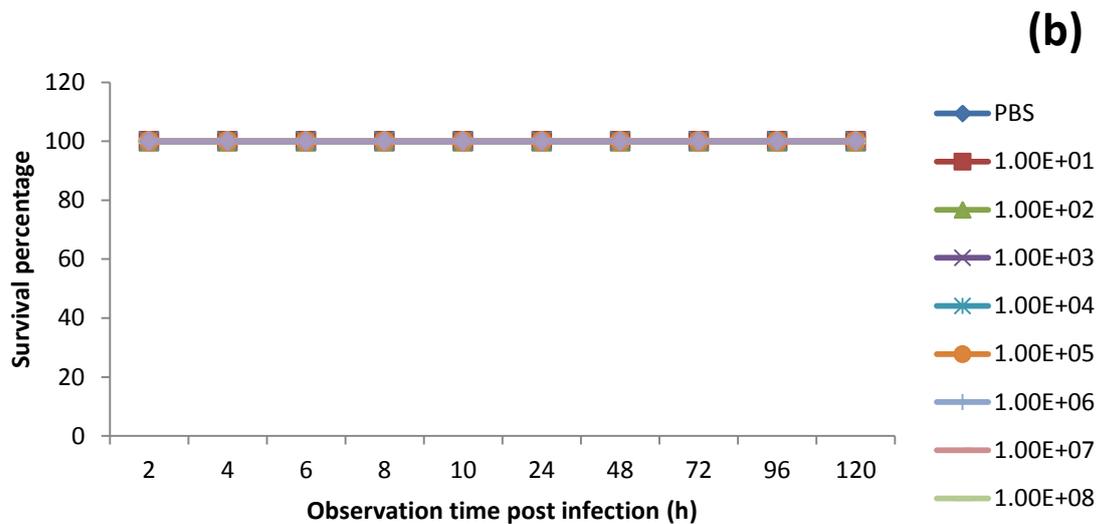
5.5.1.3.1 The survival curves of *Galleria* infected with the derivative strain 8081c

Strain 8081c was pre-grown on 25°C and it was virulent to *Galleria* larvae. The infected *Galleria* larvae with the 8081c strain were incubated at both 25°C and 37°C incubation temperatures. At the 37°C incubation temperature, dose 10^9 was the only dose caused killing to the infected *Galleria* larvae by which only one larva was killed (80% survival) at 24h p.i. (Fig. 5.3a). This is the first result of 8081 strain was able to kill the injected larvae at this condition by which the wild type 8081 strain was completely avirulent at the 37°C incubation (Fig. 5.3b). At the 25°C incubation temperature, 10^9 and 10^8 doses killed all the infected *Galleria* larvae by which the 10^9 dose killed all the infected *Galleria* larvae after 48h p.i. while the 10^8 dose killed all the infected larvae after 72h p.i. (Fig. 5.3c). No difference in survival was observed at this condition compared to the survival result of the 8081 wild type reference strain in the same conditions (Fig. 5.3d).

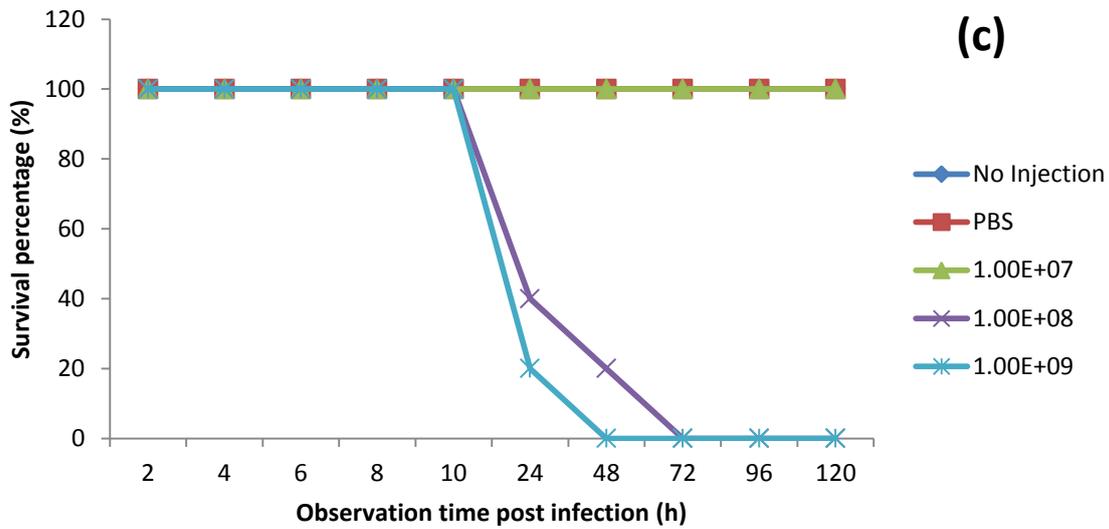
a- Survival curve of *Galleria* incubated at 37°C and infected with the pYV-cured derivative strain 8081c



b- Figure 5.3b: Survival curve of *Galleria* incubated at 37°C and infected with the pYV⁺ wild type strain 8081



c- Survival curve of *Galleria* incubated at 25°C and infected with of the pYV-cured derivative strain 8081c



d- Survival curve of *Galleria* incubated at 25°C and infected with of the pYV⁺ wild type strain 8081

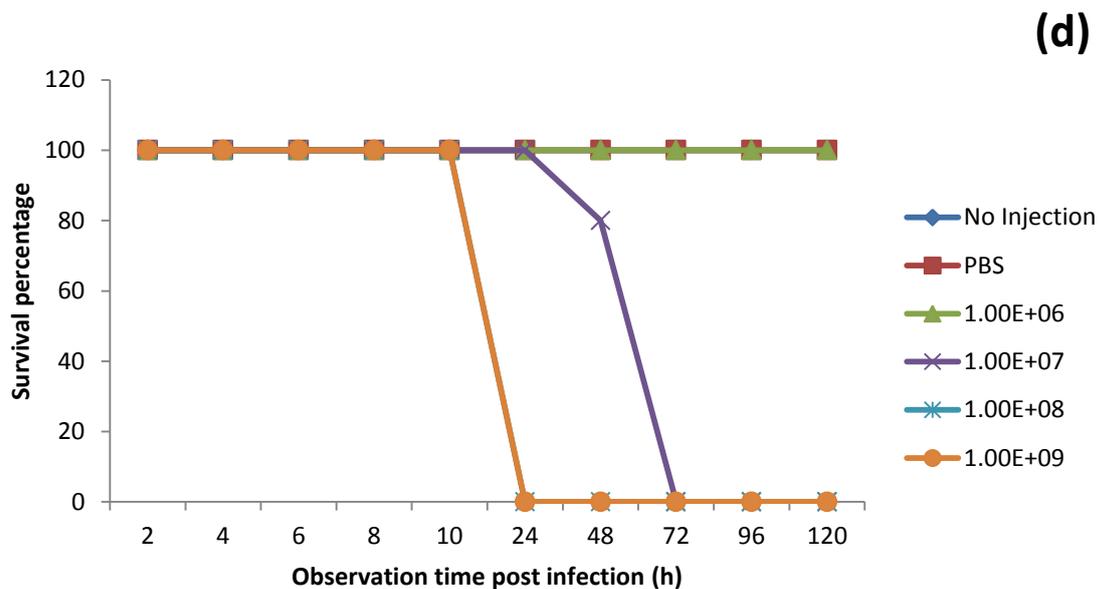
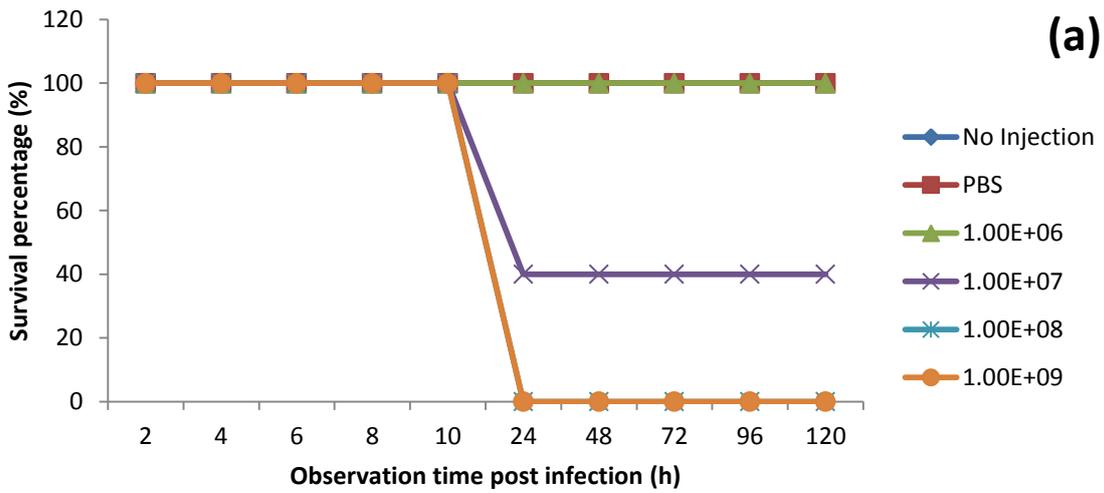


Figure 5.3: Survival curves results of *Galleria* infected with the derivative strain 8081c & the wild type 8081 strain. Figure 5.3 includes four sub figures (5.3a,5.3b,5.3c & 5.3d). Figures 5.3a & 5.3b represent the survival curves results of the pYV⁻ derivative strain 8081c and the wild type pYV⁺ 8081 strain respectively at the 37°C incubated *Galleria*. Figures 5.3c & 5.3d represent the survival curves results of the pYV⁻ derivative strain 8081c and the wild type pYV⁺ 8081 strain respectively at the 25°C incubated *Galleria*. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

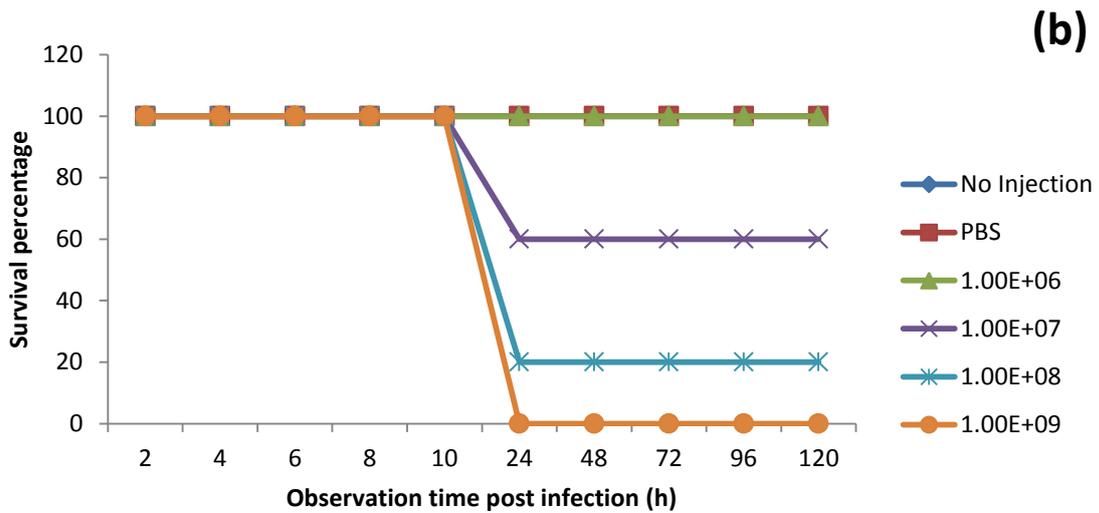
5.5.1.3.2 The survival curves of *Galleria* infected with the derivative strain 1203c

Strain 1203c was pre-grown at 25°C and it was also virulent to *Galleria* larvae. The infected *Galleria* larvae with the 1203c strain were incubated at both 25°C and 37°C incubation temperatures. At the 37°C incubation temperature, dose 10^7 , 10^8 and 10^9 produced killing to the infected *Galleria* larvae. Doses 10^9 and 10^8 killed all the infected larvae at 24h p.i. while doses 10^7 killed 3 larvae (40% survival) also at the 24h p.i. time point (Fig. 5.4a). At the 25°C incubation temperature, 10^9 and 10^8 doses caused death to the *Galleria* larvae where the 10^9 dose killed all the infected *Galleria* larvae after 24h p.i. while the 10^8 dose killed 2 larvae (60% survival) after 48h p.i. (Fig. 5.4c). Compared to the wild type strain 1203 at the same condition of the 1203c derivative strain, it can be observed that same doses of 1203c strain were able to kill the *Galleria* larvae compared to the wild type reference strain 1203 (Fig. 5.4b & 5.4d). However, the number of killed larvae injected with the doses of the 1203c derivative strain have slightly increased compared to the 1203 wild type strain.

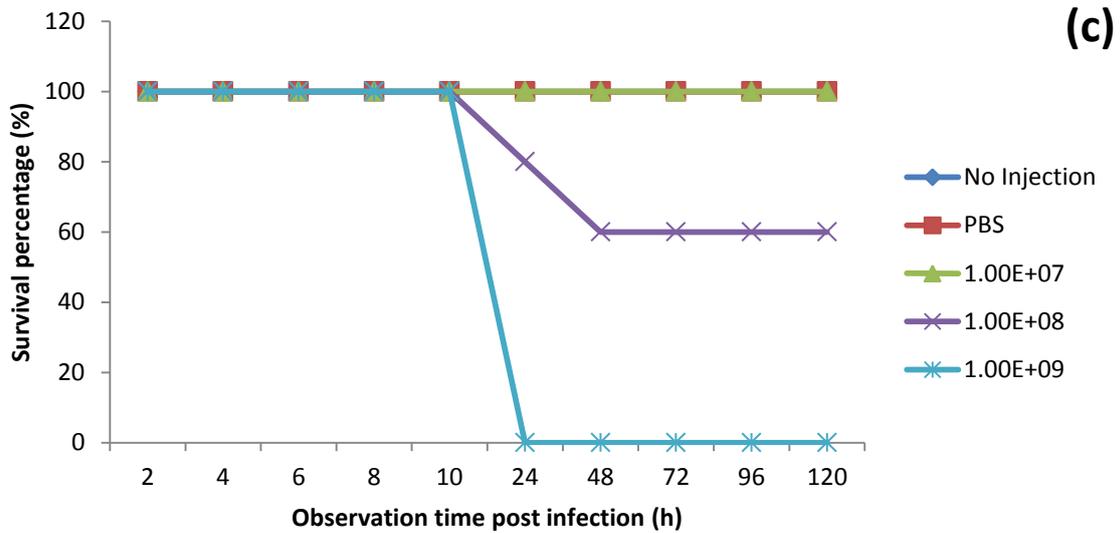
a- Survival curve of *Galleria* incubated at 37°C and infected with of the pYV-cured derivative strain 1203c



b- Survival curve of *Galleria* incubated at 37°C and infected with the pYV⁺ wild type strain 1203



c- Survival curve of *Galleria* incubated at 25°C and infected with the pYV-cured derivative strain 1203c



d- Survival curve of *Galleria* incubated at 25°C and infected with of the pYV⁺ wild type strain 1203

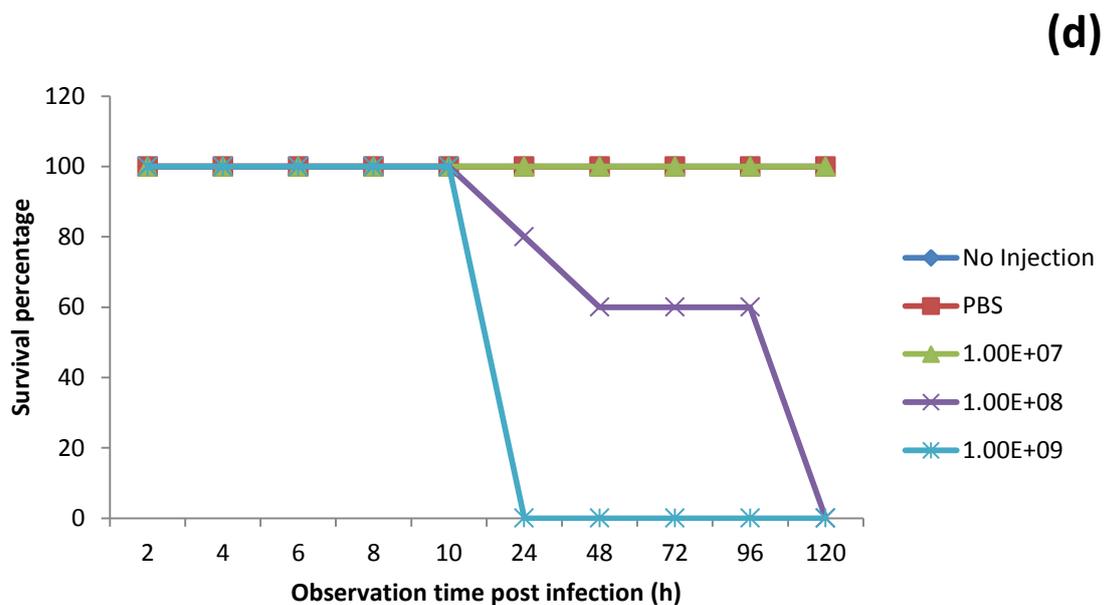
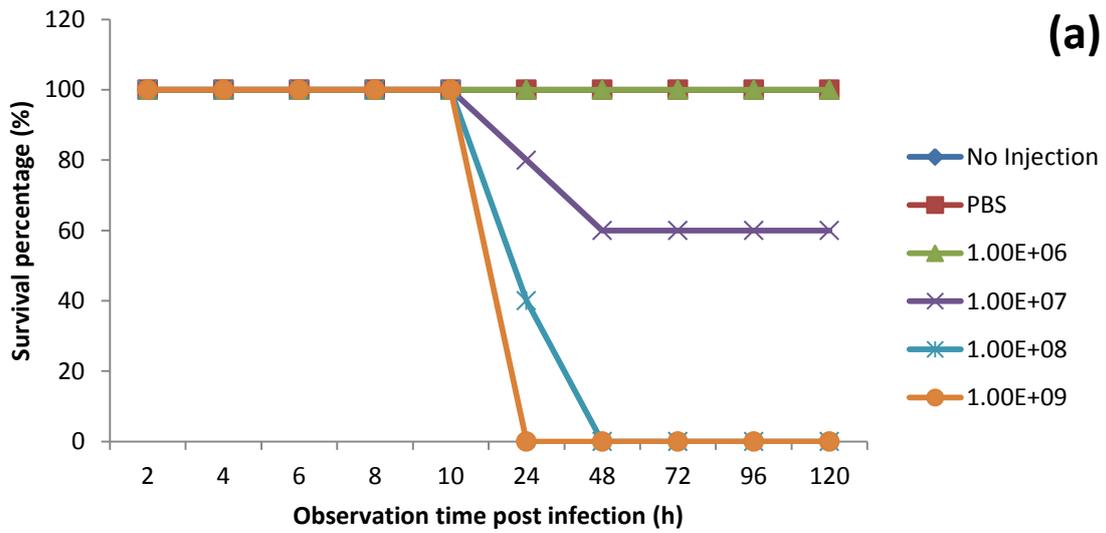


Figure 5.4: Survival curves results of *Galleria* infected with the derivative strain 1203c & the wild type 1203 strain. Figure 5.4 includes four sub figures (5.4a,5.4b,5.4c & 5.4d). Figures 5.4a & 5.4b represent the survival curves results of the pYV⁻ derivative strain 1203c and the wild type pYV⁺ 1203 strain respectively at the 37°C incubated *Galleria*. Figures 5.4c & 5.4d represent the survival curves results of the pYV⁻ derivative strain 1203c and the wild type pYV⁺ 1203 strain respectively at the 25°C incubated *Galleria*. Survival graphs of the *Galleria* larvae were constructed by plotting the time of observation post infection in hours (x-axis) against the percentage of the larvae survival (y-axis). The coloured lines represent the different doses used to infect *Galleria* addition to the controls used. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

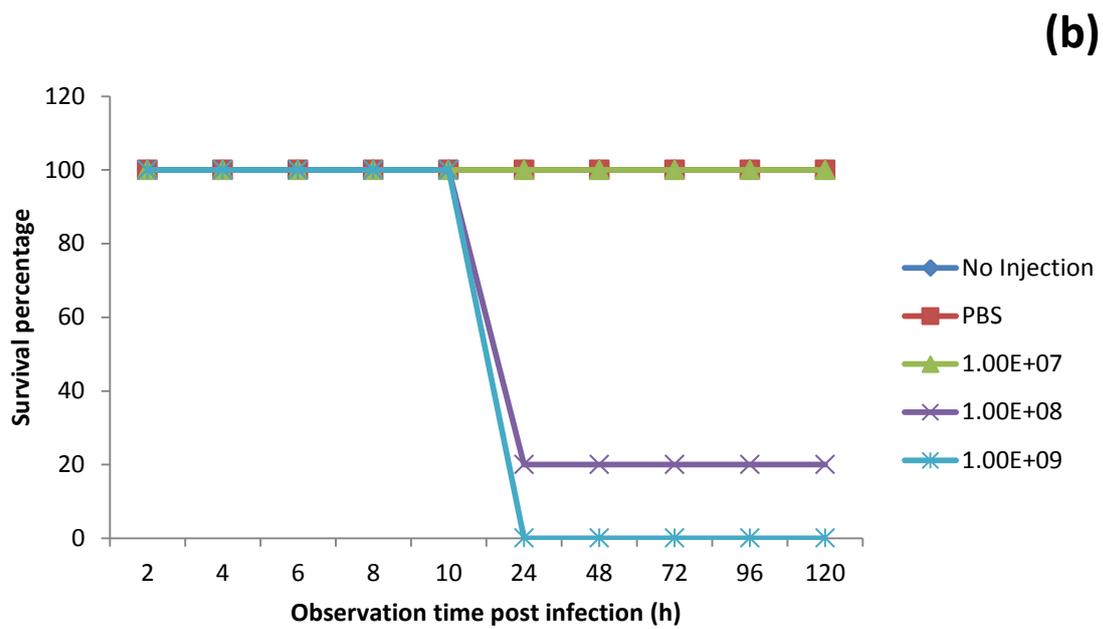
5.5.1.3.3 The survival curves of *Galleria* infected with the derivative strain 14902c:

The pYV-cured 14902 strain (14902c) was pre-grown at 25°C. Strain 14902c was injected into the *Galleria* larvae and the infected larvae were incubated at both 25°C and 37°C. At both incubation temperatures, strain 14902c caused death to the infected *Galleria* larvae. At the 37°C incubation temperature, doses 10^9 , 10^8 and 10^7 were virulent to the *Galleria* larvae. Both the 10^9 and 10^8 doses killed all the infected larvae at 24h p.i. and 48h p.i. respectively for each dose. In addition, doses of 10^7 killed 2 larvae (60%) at the 48h p.i. (Fig. 5.5a). At the 25°C incubation condition, 8 different doses of the 14902c strain killed the infected larvae and these doses are 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 and 10^9 . All the *Galleria* larvae groups infected with doses 10^9 , 10^8 , 10^7 and 10^6 were killed. Doses 10^9 and 10^8 killed all the infected larvae at 24h p.i. while doses 10^7 and 10^6 killed all the infected larvae at 48h p.i. In addition, dose 10^5 killed 4 larvae (20% survival) after 48h p.i. and the dose 10^4 killed 3 larvae (60% survival) after 96h p.i. On the other hand, the 10^3 dose killed 2 larvae (60%) at 96h p.i. while the 10^2 dose killed only one larva (80% survival) at 96h p.i. (Fig. 5.5c). Comparing the survival results of the 14902c derivative strain to the survival results of the wild type 14902 reference strain, at the same conditions, it can be observed that the 14902c derivative strain was able to kill *Galleria* larvae with one more dose at each incubation temperature while the wild type pYV⁺ strains kills *Galleria* with one less dose at each incubation temperature (Fig. 5.5b & 5.5d).

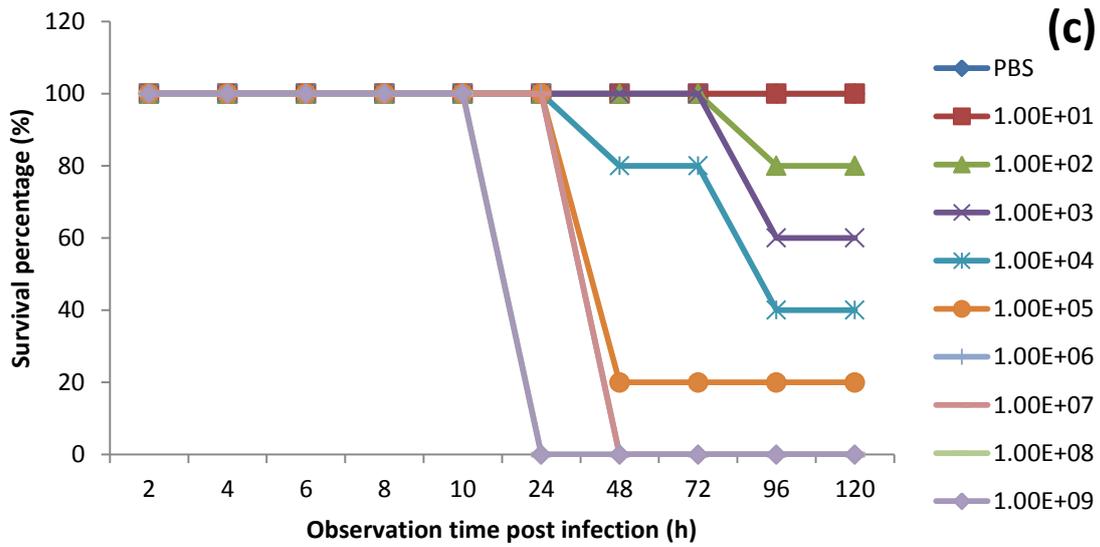
a- Survival curve of *Galleria* incubated at 37°C and infected with the pYV-cured derivative strain 14902c



b- Survival curve of *Galleria* incubated at 37°C and infected with the pYV⁺ wild type strain 14902



c- Survival curve of *Galleria* incubated at 25°C and infected with the pYV-cured derivative strain 14902c



d- Survival curve of *Galleria* incubated at 25°C and infected with the pYV⁺ wild type strain 14902

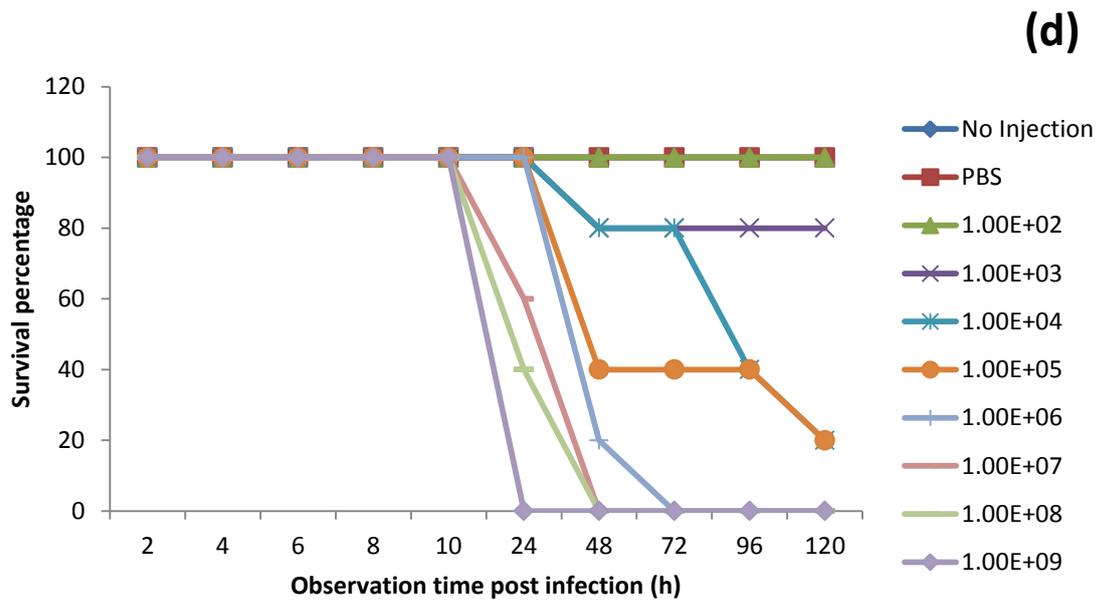


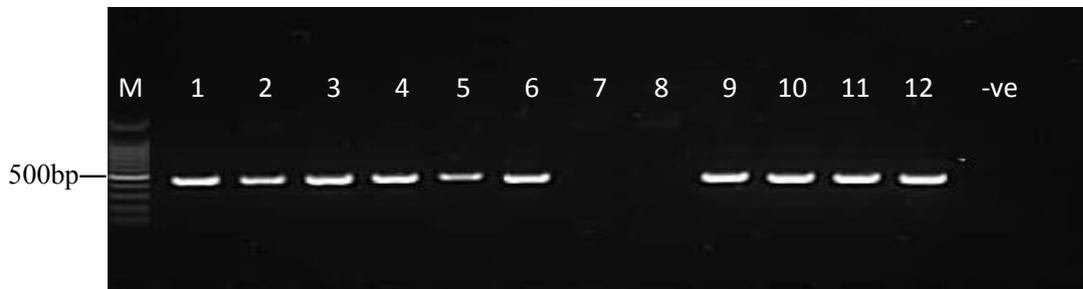
Figure 5.5: Survival curves results of *Galleria* infected with the derivative strain 14902c & the wild type 14902 strain. Figure 5.5 includes four sub figures (5.5a,5.5b,5.5c & 5.5d). Figures 5.5a & 5.5b represent the survival curves results of the pYV derivative strain 14902c and the wild type pYV⁺ 14902 strain respectively at the 37°C incubated *Galleria*. Figures 5.5c & 5.5d represent the survival curves results of the pYV⁻ derivative strain 14902c and the wild type pYV⁺ 14902 strain respectively at the 25°C incubated *Galleria*. Values shown in each survival graph are representative values obtained from performing the experiment in triplicate.

5.5.2 Stability of pYV dynamics during *Galleria* infection

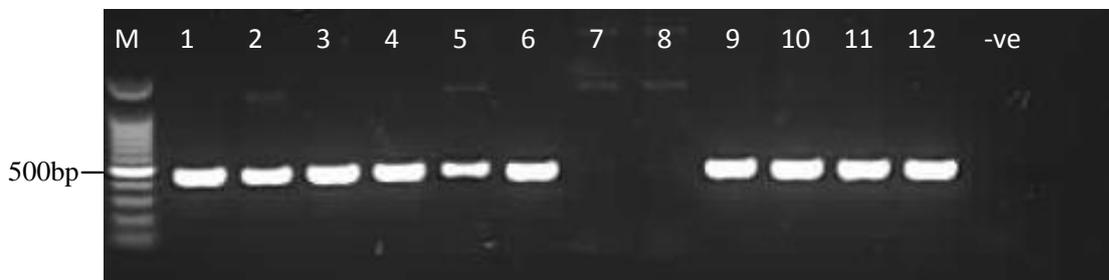
The plasmid occurrence of 4 *Y. enterocolitica* wild type strains was tested during their infection in the *Galleria* larvae. The selected lethal and non-lethal doses, of each strain, were chosen depending on previous survival curves described in chapter three. The stability of the pYV plasmid was checked by PCR targeting the *yscP* gene at time points 24h, 72h and 120h post infection. The *Y. enterocolitica 16S rDNA* gene was detected by PCR to ensure the isolation of the *Y. enterocolitica* strains only. The PCR detection of the *yscP* gene revealed that *Y. enterocolitica* strains YE8081, YE1203 and YE21202 have retained the pYV plasmid during the *in vivo* *Galleria* infection while only the YE14902 *Y. enterocolitica* strains had lost the pYV plasmid during the *in vivo* infection (Fig. 5.6). The low pathogenic bioserotype 3/O:5,27 strain YE14902 have lost the pYV plasmid from the first day of infection (24h p.i.) (Fig.5.6a) in both of the lethal and non-lethal doses. The pYV plasmid presence in the 14902 strain was also checked at 72h and 120h time points (Fig. 5.6b & 5.6c) to ensure if there are any of the 14902 strain cells that may still retain the pYV plasmid in the other infected *Galleria* larvae. Strain 14902 has lost the pYV plasmid in all time points during the 5 days of *Galleria* infection period. Moreover, *Y. enterocolitica* strains YE8081, YE1203 and YE21202 have retained the pYV plasmid by both of the injected lethal and non-lethal doses during the 5 days period of the *Galleria* infection (Fig. 5.6). Figures 5.6a, 5.6b and 5.6c are showing the presence of pYV plasmid at 24h, 72h and 120h post infection respectively of *Y. enterocolitica* YE8081, YE1203 and YE21202 strains. The *Y. enterocolitica 16S rDNA* gene was detected by PCR at 24h (Fig. 5.7a), 72h (Fig. 5.7b) and 120h (Fig. 5.7c) time points. The *Y. enterocolitica 16S rDNA* gene is confirming that these recovered bacteria are actually *Y. enterocolitica*.

5.5.2.1 Gel electrophoresis results of checking the stability of the pYV plasmid

a- Gel image after 24h:



b- Gel Images after 72h:



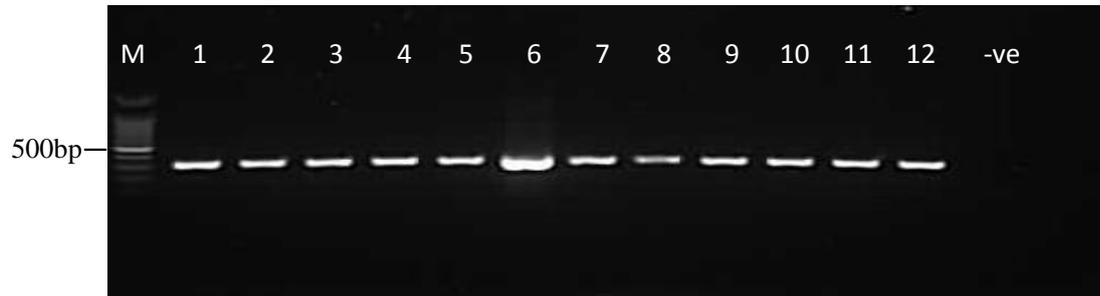
c- Gel Image after 120h:



Figure 5.6: Gel electrophoresis results of checking the stability of the *yscP* gene. Figure 5.6 contains the three subfigures 5.6a, 5.6b and 5.6c. Each sub figure represents a gel image of detecting the *Y. enterocolitica yscP* gene of the 4 tested *Y. enterocolitica* strains YE8081, YE1203, YE14902 and YE21202. Subfigures 5.6a, 5.6b and 5.6c represents gel images of detecting the *Y. enterocolitica yscP* gene of the 4 tested *Y. enterocolitica* strains after 24h, 72h and 120h of *Galleria* post infection respectively. The lanes of each gel are labelled with numbers from 1-12 and each number represents a different. (-ve)= the PCR DNA template-less negative control. M= the 100bp DNA marker.

5.5.2.2 Gel electrophoresis results for detecting the *16S rDNA* gene:

a- Gel image after 24h:



b- Gel Images after 72h:



c- Gel Image after 120h:

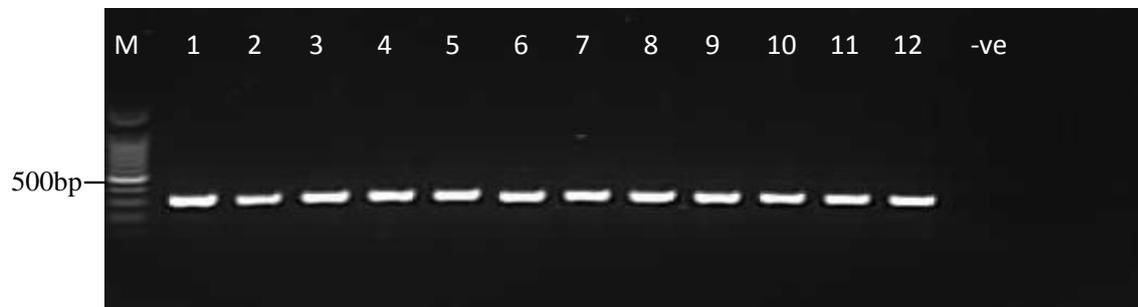


Figure 5.7: Gel electrophoresis results for detecting the *16S rDNA* gene. Figure 5.7 contains the three subfigures 5.7a, 5.7b and 5.7c. Each sub figure represents a gel image of detecting the *Y. enterocolitica 16SrDNA* gene of the 4 tested *Y. enterocolitica* strains YE8081, YE1203, YE14902 and YE21202. Subfigures 5.7a, 5.7b and 5.7c represent gel images of detecting the *Y. enterocolitica 16SrDNA* gene of the 4 tested *Y. enterocolitica* strains after 24h, 72h and 120h of *Galleria* post infection respectively. The lanes of each gel are labelled with numbers from 1-12 and each number represents a different sample. The broth cultures are used as positive controls of detecting the *16SrDNA* gene in all tested strains. (-ve)= the PCR DNA template-less negative control. M= the 100bp DNA marker.

5.7 Discussion

In this chapter, the role of *Y. enterocolitica* pYV plasmid in modifying the *Y. enterocolitica* pathogenic biotypes was investigated in the insect infection model *Galleria mellonella*. The main difference between the pathogenic biotypes and the non-pathogenic biotype is the presence of pYV in all pathogenic biotypes and absent from the non-pathogenic biotype (Reuter *et al.*, 2012). pYV plasmid is known as the major virulence factor in pathogenic *Y. enterocolitica* biotypes which encodes for the Ysc type three secretion system and Yop proteins (Hammer *et al.*, 2007) and responsible in host immunomodulation (Ruiz-Bravo *et al.*, 2003). From previous results in this project it was observed that all pathogenic biotypes were showing less virulence than the non-pathogenic biotype in *Galleria* larvae. As a result, the pathogenic potential of three *Y. enterocolitica* derivative strains lacking the pYV plasmid (pYV⁻) was investigated to observe any difference in the pathogenic phenotype. The tested strains are the high pathogenic bioserotype 1B/O:8 strain 8081c, the low pathogenic bioserotype 4/O:3 strain 1203c and the low pathogenic bioserotype 3/O:5,27 strain 14902c. The pYV plasmid was removed from these strains by the continuous sub culturing on LB agar plates at the 37°C growing temperature in the absence of Ca²⁺. We have checked on the loss of plasmid by sub culturing on the CRMox agar (Bhaduri and Smith., 2011; Riley and Toma., 1989) and by targeting the *yscP* chromosomal gene which encodes for the YscP protein responsible for determining the needle length of the Ysc injectisome (Agrain *et al.*, 2005; Wood *et al.*, 2008). From the LD₅₀ data of the pYV cured strains, it was found that all the pYV⁻ *Y. enterocolitica* tested strains are showing an increase in virulence in the *Galleria* infected larvae at both incubation temperatures. The pYV⁻ high pathogenic derivative strain 8081c has shown the minimum and a very slight increase in virulence only at the 37°C incubation temperature compared to the 8081 wild type pYV⁺ strain. The 8081c derivative strain managed to kill only one larva with the highest 10⁹ dose at the 37°C incubation

temperature. No difference in virulence was found at the 25°C incubation temperature by which the LD₅₀ value was 7.6log₁₀c.f.u/ml for both strains. Previous work of this project has shown that the 8081 wild strain was completely avirulent to the *Galleria* larvae incubated at the 37°C incubation temperature. The pYV⁻ 1203c derivative strain has shown a small increase in virulence in the infected *Galleria* larvae at both 25°C and 37°C incubation temperatures but this increase was higher and more observable from the high pathogenic pYV⁻ 8081c derivative strain. The 1203c derivative strain has shown a decrease in the LD₅₀ values with a difference of 0.4log₁₀ c.f.u/ml at 37°C incubation temperature and a difference of 0.5log₁₀ c.f.u/ml at 25°C incubation temperature compared to the 1203 wild type strain LD₅₀ values. This decrease in LD₅₀ values indicates a higher virulence profile of the 1203c derivative strain. Furthermore, the pYV⁻ low pathogenic 14902c derivative strain is showing a manifest high virulence ability compared to all the other tested pYV-cured strains. Although the wild type 14902 strain was the most virulent pathogenic strain in the *Galleria* larvae, the 14902c derivative strain shows an additional virulence ability to the infected *Galleria* larvae at both 25°C and 37°C incubation temperatures compared to the pYV⁺ wild type 14902 strain. The LD₅₀ value of the 14902c derivative strain was decreased 1.2log₁₀ c.f.u/ml and 1.3log₁₀ c.f.u/ml at 25°C and 37°C incubation temperatures respectively compared to the wild type 14902 strain. However, this experimentally noticed slight increase in virulence of *Y. enterocolitica* pYV-cured strains did not show significant statistical increase in virulence compared to the wild type strains. This provides an evidence that *Y. enterocolitica* virulence doesn't depend only on the pYV plasmid but it utilizes a chromosomally encoded virulence factors as well for causing disease.

The second part of this work included the investigation of the pYV plasmid stability during the life infection of the *Galleria* larvae. The *in vivo* stability of the pYV plasmid during the life infection of *Galleria* larvae have been investigated with four pathogenic wild type strains.

These strains are the high pathogenic bioserotype 1B/O:8 strain 8081 and three low pathogenic strains 1203 (BT 4/O:3), 14902 (BT 3/O:5,27) and 21202 (BT 2/O:9). The *Galleria* larvae were infected with lethal and non-lethal doses from each of the tested *Y. enterocolitica* wild type strains. The injected strains were recovered from the infected *Galleria* larvae every 24 hours for 5 days. The presence or the absence of the pYV plasmid was checked by PCR through targeting the *yscP* gene (Agrain *et al.*, 2005; Wood *et al.*, 2008). It has been observed that the 14902 had lost the pYV plasmid from the first day of infection while the other remaining strains retained the pYV plasmid. This may indicate that the 14902 strain pathogenicity in infected *Galleria* depends on chromosomal virulence determinants rather than the virulence of the pYV plasmid. On the other hand, the other strains kept the pYV plasmid during the 5 days incubation period which may indicate that these strains are using the pYV plasmid in addition to the chromosomal virulence factor for their pathogenesis in the infected *Galleria* larvae. It also can be concluded that the pYV absence in 14902 may play some role in increasing the pathogenicity of the low pathogenic 14902 strain in the *Galleria* alternative infection model.

5.8 Conclusion

This work shows that the pYV⁻ *Y. enterocolitica* strains are showing similar virulence in the *Galleria* infected larvae. However there is a slight increase in pathogenicity, depending on the bioserotype of the *Y. enterocolitica* pYV⁻ tested strain, observed experimentally but statistically it is not significant. It is well known that the pYV loss in *Y. enterocolitica* pathogenic strains leads to loss of virulence (Hammer *et al.*, 2007). However, the *Galleria* results are showing that loss of plasmid has increased the virulence of the tested *Y. enterocolitica* bioserotype 3/O:5,27 strain lacking the pYV plasmid. Studies have provided evidence that *Y. enterocolitica* pYV-cured strains were capable of invading the mice infection model intestinal mucosa and were able to disseminate to the mesenteric lymph nodes. These studies also show that *Y. enterocolitica* pYV-cured strains were recovered from the mesenteric lymph nodes, the liver and the spleen of the infected gnotobiotic piglets animal model (Lian *et al.*, 1987). These results may support the pathogenic profile of *Y. enterocolitica* pYV-cured strains in the infected *Galleria* larvae. The present results of monitoring the presence or the absence of the pYV plasmid during the *Y. enterocolitica* life infection in *Galleria* larvae have shown that *Y. enterocolitica* pathogenic strains maintain the pYV plasmid during *Galleria* infection with one exception, the bioserotype 3/O:5,27 strain 14902. The low pathogenic 14902 strain and the non-pathogenic 5303 strain were the most lethal strains to *Galleria* larvae. As a result it can be concluded that the low pathogenic 14902 strain, similar to the non-pathogenic 5303 strain, causes pathogenesis in *Galleria* using chromosomal virulence determinants, but that a loss of pYV may help to exacerbate this effect.

Chapter 6
Project Final Conclusion

6.1 Project Final Conclusion

Y. enterocolitica is a zoonotic, food-borne pathogen associated with human infections and outbreaks worldwide. The main aim of this project is to provide a comparative study of the pathogenesis of *Y. enterocolitica* high-, low- and non-pathogenic biotypes. The human epithelial HEP-2 cell line and the emerging *G. mellonella* alternative insect infection model are used in this project to study the pathogenic potential of *Y. enterocolitica* high-, low- and non-pathogenic biotypes. Here, all the *Y. enterocolitica* high pathogenic, low pathogenic and non-pathogenic biotypes strains were able to invade the HEP-2 human epithelial cell line. The High pathogenic and low pathogenic *Y. enterocolitica* biotypes strains were efficiently able to invade HEP-2 epithelial cells to identical levels. The non-pathogenic biotype 1A strain has also shown invasive potential in this cell culture model but it was the least invasive biotype compared to the other *Y. enterocolitica* pathogenic biotypes. Moreover, *Y. enterocolitica* strains were pre-grown at 25°C and 37°C and at both pre-growing temperatures all the *Y. enterocolitica* strains were able to invade HEP-2 cell line *in vitro*.

A diverse population of *Y. enterocolitica* strains encompassing different biotypes and serotypes and isolated from different biological origins, were able to kill the *Galleria* insect model. Killing of *Galleria* larvae was temperature-dependent and dose-dependent. The median lethal doses (LD₅₀) and the survival results showed that all *Y. enterocolitica* biotypes were more virulent to *Galleria* larvae incubated at 25°C and less virulent at the 37°C incubation. Here *Y. enterocolitica* is showing a novel pathogenic potential in *Galleria* alternative infection by the high pathogenic biotype and the non-pathogenic biotype. The non-pathogenic biotype strains were the most lethal biotype in *Galleria* at all incubation conditions. The high pathogenic biotype strain was completely avirulent to *Galleria* at 37°C incubation temperature and weakly pathogenic at 25°C incubation temperature. Moreover,

the low pathogenic bioserotype 3/O:5,27 strain 14902 was the most virulent to *Galleria* between the pathogenic biotypes by which this serotype is always associated with animal infections and never isolated with human clinical cases. Both of the 25°C and 37°C pre-grown *Y. enterocolitica* representative strains were very efficient to infect and kill the *Galleria* larvae. Furthermore, the heat killed *Y. enterocolitica* (HKY) biotypes strains were completely avirulent to the *Galleria* larvae. This result assumes that *Galleria* larvae were dying from living *Y. enterocolitica* bacterial cells and not from the dead ones. Infection dynamics results revealed that all the tested low pathogenic biotypes strains show static growth kinetics in *Galleria*. On the other hand, the non-pathogenic strain shows a big reduction in numbers during the *Galleria* infection. This indicates that non-pathogenic biotype strain appears to trigger an immune response in the infected *Galleria* larvae. After that, the pYV role in modulating the lack of virulence of the pathogenic biotypes was investigated in *Galleria* host model. Curing the pYV plasmid from the pathogenic *Y. enterocolitica* showed little to no effect on virulence in *Galleria* larvae. Moreover, the pYV plasmid stability of the pathogenic biotypes wild type strains (pYV⁺) in the infected *Galleria* larvae was investigated. During infection of *Galleria* larvae, pathogenic wild type strains retained their pYV plasmid during the infection period. However, the low pathogenic bioserotype 3/O:5,27 strain 14902 lost the pYV plasmid during the infection period by which this strain was the most virulent strain between the pathogenic strains.

Finally, from the experimental work of this project it can be concluded that all *Y. enterocolitica* biotypes show pathogenic potential to the infection models used. In the mouse infection model, the high pathogenic biotype is able to kill mice, low pathogenic biotypes cause only a mild disease while the non-pathogenic biotype are completely avirulent. However, the high pathogenic biotypes are rarely associated with human clinical cases while low pathogenic biotypes are the most isolated. Moreover, the non-pathogenic

biotype is frequently isolated from healthy and infected humans but the pathogenicity of the non-pathogenic still confusing. Here, all *Y. enterocolitica* biotypes were able to infect *Galleria* larvae insect infection model. The non-pathogenic biotype was the most lethal to *Galleria* infection model followed by the other pathogenic biotypes. So it can be observed that all *Y. enterocolitica* biotypes are capable of causing disease, and that the severity of that disease is lineage specific dependant on the different hosts studied. Therefore it is should not refer to any of the *Y. enterocolitica* biotypes as a non-pathogenic biotype.

6.2 Future Work

In this project an extensive phenotypic work have been provided to investigate the pathogenic potential of all the *Y. enterocolitica* biotypes. In addition, an experimental work at the molecular level was also provided to investigate the *in vivo* infection dynamics of *Y. enterocolitica* strains in *Galleria*. The molecular work also investigated the role of pYV plasmid in modulating the lack of virulence in the pathogenic biotypes because the pYV is the main virulence factor of *Yersinia*. However, the virulence mechanisms causing the pathogenicity of *Y. enterocolitica* biotype 1A in *Galleria* remains ambiguous. I would like to suggest that future work should investigate Biotype 1A unique, putative virulence-associated genes for their role during the infection of the *Galleria* insect host model. Many techniques are provided in the research field to investigate gene functions and here I would like to propose an example of two of them that may be useful in the future work:

RNA sequencing (RNA-seq)

One way to investigate the *Y. enterocolitica* virulence genes implicated in *Galleria* infection is to use the functional genomics approach which enables the study of global gene expression during host infection. RNA-seq technology is a technique used to sequence large transcriptomics content rapidly. RNA sequencing technique includes the next-generation sequencing techniques to provide a rapid sequencing output (Ozsolak and Milos, 2010). Since this project is providing a new pathogenicity phenotypes and potentials of *Y. enterocolitica* biotypes that no previous studies have encountered before, there is a need for a technique to study global gene expression for a large amount of genomes of different biotypes to reveal which genes are causing this new novel pathogenicity. RNA-seq will allow the accurate identifications of all genes expressed by all the biotypes during live infection.

Transposon directed insertion-site sequencing method (TraDIS)

The other suggested approach is to assess a the function of wide range of genes by generating a large number of mutants using the transposon directed insertion-site sequencing method (TraDIS) (Langridge *et al.*, 2009). TraDIS include the Signature Tag transposon Mutagenesis (STM) and the modern Illumina sequencing method making it a high-throughput technology. Knocked out genes that going to show an attenuated virulence strains may be suggested as the genes responsible for *Y. enterocolitica* pathogenesis.

Chapter 7

References

7 References

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